DEVELOPMENT OF THE ARK ASSAY FOR QUANTITATING DNA-PROTEIN CROSSLINK ACCUMULATION AND FANCONI ANEMIA PATHWAY INVOLVEMENT IN THE REPAIR PROCESS

Naeh Klages-Mundt

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DEVELOPMENT OF THE ARK ASSAY FOR QUANTITATING DNA-PROTEIN CROSSLINK ACCUMULATION AND FANCONI ANEMIA PATHWAY INVOLVEMENT IN THE REPAIR PROCESS

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DEVELOPMENT OF THE ARK ASSAY FOR QUANTITATING DNA-PROTEIN CROSSLINK ACCUMULATION AND FANCONI ANEMIA PATHWAY INVOLVEMENT IN THE REPAIR PROCESS

DISSErTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by
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Houston, Texas
February 2022
Copyright Addendum

Sections of this dissertation are based on my previously published research. Any experimental work from such publications that has been completed by colleagues other than myself will be noted accordingly.

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Acknowledgements

First, I would like to thank my advisors, Dr. Lei Li and Dr. Bin Wang. Their support – both intellectually and motivationally – have been crucial in developing my skills, mindset, and acumen as a scientist. The critical thinking skills and scientific knowledge I have garnered from my advisors since 2015 have been instrumental in helping me to accomplish and share my work with the scientific community.

I also thank the members of my advisory committee – Dr. Bin Wang, Dr. Lei Li, Dr. Junjie Chen, Dr. Boyi Gan, Dr. Xuetong (Snow) Shen, Dr. Katharina Schlacher, and Dr. John Tainer – for their helpful guidance and expert-level advice over the past several years. Both my individual encounters and committee meetings with each one of them have inspired my scientific and intellectual curiosity, for which I will forever be grateful.

I also express my deepest thanks to my fellow lab members – both from the Li lab and the Wang lab – for all of the assistance and scientific advice I’ve received from them. From the Li lab, Dr. Xi (Sherman) Shen, Dr. Yanyan Tian, Dr. Yin Ye, Dr. Rui Wang, Dr. Hyeung Kim, Dr. Qianghua Hu, and Erica Lynn were all key in helping me to learn research methodologies – from cell culture to an assortment of bench work. Dr. Qianghua Hu was also the co-first author of a research paper we published together, and our collaboration on this effort was truly ameliorative. I also want to express another sincere thanks to Erica Lynn, who provided not only technical help on certain research projects and endeavors, but also helped me learn the basics of human cell culture while I was a rotation student.
I also thank the members of the Wang lab – Dr. Shengfeng (Ella) Xu, Dr. Xiao Wu, Dr. Shichang Liu, Dr. Longqiang Wang, Erin Atkinson, and Ahmed Emam. Their knowledge and support have been immensely helpful since I joined their lab in January 2020.

In addition, I also want to thank my other rotation advisors during my first year – I learned a lot about different model systems and a diverse range of methodologies throughout my first year. I thank Dr. Lei Li, Dr. Nayun Kim, Dr. Katharina Schlacher, and Dr. Xuetong (Snow) Shen for their guidance.

I also would like to thank Dr. Susan Wallace (emeritus professor of the University of Vermont) for her helpful recommendations on my research objectives and trajectory.

Dr. Orlando Schärer (Stony Brook University, Ulsan National Institute of Science and Technology) also offered me helpful advice at the beginning stages of my graduate school research.

I thank the graduate school and their administrative staff here at MD Anderson for their continued support and nurturing environment for graduate students like myself.

I thank my friend Dr. Alex Kosanovich, a college colleague of mine, for astutely helping edit certain portions of my dissertation writing.

I also express sincere gratitude to my family and friends for their support and guidance during my years in graduate school. They have been a guiding force for me every step of the way on this explorative journey, and I am thankful for that.
DNA-protein crosslinks (DPCs) are a common DNA lesion naturally arising in cells, wherein protein becomes covalently and irreversibly bound to the DNA. Given their excessive size, these adducts present a significant challenge to replication and transcription, thus requiring timely and efficient repair. However, the precise mechanisms involved with processing DPC removal remain unclear. Moreover, current methodologies to quantitate DPC accumulation and removal are restrained by a range of limitations. Here, we describe and discuss a new DPC detection assay – the ARK assay – capable of overcoming the limitations incurred by prior assays. The design, which uses dual chaotropic lysis and anionic denaturation to remove excessive background signal, is premised upon isolating and measuring DPC-associated DNA and free, soluble DNA fragments. We show that the ARK assay effectively detects DPCs induced by a range of agents, and that
DPC-defective models exhibit increased DPC accumulation. Functionally, we observe that Fanconi anemia pathway-inactivated cells incur increased DPC accumulation and delayed repair, suggesting a role for the Fanconi anemia pathway in the processing of these deleterious lesions.
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Abbreviations

5-azaC – 5-azacytidine
5-azadC – 5-aza-2’deoxycytidine
ARK – Advanced Recovery of K [potassium]-SDS precipitates
CPT – camptothecin
DpC – DNA-peptide crosslink
DPC – DNA-protein crosslink
EBNA – Epstein-Barr nuclear antigen-1
eChIP – episomal chromatin immunoprecipitation
FA – Fanconi anemia
HR – homologous recombination
ICL – interstrand crosslink
NER – nucleotide excision repair
RADAR – Rapid Approach to DNA Adduct Recovery
Tdp1 – Tyrosyl-DNA-phosphodiesterase 1
Tdp2 – Tyrosyl-DNA-phosphodiesterase 2
Top1cc – Topoisomerase 1 cleavage complex
Top2cc – Topoisomerase 2 cleavage complex
Introduction

(Sections of this chapter are based upon (Klages-Mundt and Li, 2017), with permission granted by Science China Life Science).

The integrity of cellular DNA is constantly challenged by a wide variety of genotoxic agents, both environmentally and endogenously, leading to an array of mutational or deleterious lesions in the genome. To maintain genomic stability, cells must continually repair such damage in a timely manner [Lindahl, 1993]. Common forms of DNA damage include oxidation and alkylation of DNA bases, ultraviolet light-induced dimerization of pyrimidines, base mismatches, and single- and double-strand breaks. The structure and repair mechanisms for these forms of DNA damage have all been studied extensively. However, one category of DNA lesions that remains poorly understood is DNA crosslinks, of which there are two general types: DNA-DNA crosslinks and DNA-protein crosslinks. A DNA-DNA crosslink lesion entails a covalent bond linking either the same strand of DNA (intrastrand crosslinks) or opposing strands of DNA (interstrand crosslinks, ICLs). DNA-protein crosslinks (DPCs) are formed when a nucleotide residue on DNA forms a covalent bond with a protein (or a peptide, to form a DNA-peptide crosslink, DpC). Crosslinks are particularly hazardous, as they can effectively block gene transcription and DNA replication. Repair of crosslinks appears to involve complex mechanisms which encompass a large number of protein factors.
I. Formation of DPCs

DPCs can form through a variety of different means, facilitated by naturally occurring and synthetic compounds. Such compounds can broadly be categorized as environmentally-induced, therapeutically-induced, and endogenously-induced (Figure 1).

![Diagram of DPC formation sources](image)

**Figure 1. DPCs arise from a variety of sources.**

A variety of sources induce DPCs in cells. DPCs can be formed through a variety of different means, including from environmental, therapeutic, and endogenously-generated agents. DPCs arrest transcription and replication, resulting in mutagenesis or cell death.
a. Environmentally-Induced DPCs

Several different DPC-inducing agents exist in our environment. DPCs can be induced upon exposure to ionizing radiation, ultraviolet light, and various transition metal ions, including chromium and nickel [Swenberg et al., 2011]. A number of other carcinogens are also known to induce DPCs, including bifunctional alkylating agents such as 1,3-butadiene, diepoxynbutane, acrolein, and crotonaldehyde [Costa et al., 1997; Kurtz and Lloyd, 2003; Loeber et al., 2006; Minko et al., 2008].

b. Therapy-Induced DPCs

Ionizing radiation and chemical compounds that induce interstrand crosslinking or DNA-protein crosslinking are routinely used in chemotherapy regimens, either independently or in conjunction with other regimens [Huang and Li, 2013]. Each gray of ionizing radiation is thought to produce about 150 DPCs in the genome per cell [Barker et al., 2005]. Several cancer drugs induce DPCs, including nitrogen mustards, 5-aza-2′-deoxycytidine (5-azadC, also clinically known as decitabine), and platinum-based agents such as cisplatin- and transplatin-derivatives [Santi et al., 1983; Barker et al., 2005; Loeber et al., 2009; Ide et al., 2011; Stingele and Jentsch, 2015]. The chemotherapeutic drugs camptothecin (CPT) and etoposide are used clinically to trap topoisomerase complexes onto the DNA. Thus, although detrimental to healthy cells, deliberate and targeted DPC induction can ameliorate certain cancer therapeutic regimens.

c. Endogenously-Induced DPCs
Endogenously-induced DPCs can be derived through enzymatic and non-enzymatic means. Certain DNA-interacting enzymes that otherwise form transient covalent complexes with DNA can become entrapped onto the DNA, forming DPCs. There are also multitudes of endogenously-produced metabolites and other species that lead to the formation of DPCs in cells through non-enzymatic means. Reactive aldehydes are a well-known class of DPC-inducing agents occurring endogenously in cells.

i. Non-Enzymatic

Assortments of reactive aldehydes are present in cells [Swenberg et al., 2011]. Reactive aldehydes are often generated as a result of various metabolic and regulatory processes in cells, such as amino acid metabolism and oxidative demethylation [Swenberg et al., 2011]. When the carbonyl carbon of an aldehyde is sufficiently electrophilic, it can react with nucleophiles in the surrounding environment [Dellarco, 1988]. When such an aldehyde is in the vicinity of chromatin, it can react with the primary amine of a DNA base, producing a methylol adduct; following a dehydration reaction to form a Schiff base, this intermediate adduct can then further react with a primary amine of another nearby DNA base (forming either an ICL or intrastrand crosslink) or, alternatively, form a stable amide bond with a lysine or arginine residue of a nearby protein (forming a DPC). This mechanism of DPC formation has been validated experimentally, as NEIL1 has been shown to form this Schiff base intermediate with DNA [Bandaru et al., 2002].
The best-studied DPC-inducing aldehyde is formaldehyde. DPCs are the principal DNA lesion induced by formaldehyde in cells [Conaway et al., 1996; Quievryn and Zhitkovich, 2000]. For this reason, formaldehyde has commonly been used as a DPC-inducing agent in many studies in the field thus far. However, it is important to recognize that formaldehyde generates an assortment of other lesions in the DNA as well, including ICLs [Chaw et al., 1980] and monoadducts. Such heterogeneity of lesions can complicate readouts of experiments, such as clonogenic survival and cell viability assays as it is difficult to distinguish whether the readouts are a consequence of formaldehyde-induced DPCs versus other lesions such as ICLs.

Formaldehyde is particularly genotoxic, given that it is released as a byproduct during histone demethylation at nucleosomes [Walport et al., 2012]. Formaldehyde is also produced as an intermediate during the enzymatic removal of methyl groups from DNA [Trewick et al., 2002]. To help mitigate the problem of having such a toxic and reactive chemical species being produced in such close proximity to DNA, cells utilize a class of enzymes called aldehyde dehydrogenases. These enzymes metabolize various aldehydes such as formaldehyde and acetaldehyde, effectively detoxifying them [Vasiliou et al., 2004], while also serving as key components of other metabolic pathways. Formaldehyde dehydrogenase converts formaldehyde to formic acid, thereby reducing formaldehyde levels and likely diminishing the abundance of DPCs in the genome. However, evidence suggests that the formaldehyde concentration in human blood typically ranges between 2–3 mg/L (66–100 micromolar) [Zhang et al., 2009], and so DPC lesions
still may be expected to have a steady-state presence. Whether other cellular and metabolic sources also contribute to the steady-state levels of circulatory formaldehyde is unclear. Genes associated with DNA repair, DNA damage tolerance, and chromatin remodeling have been identified as notable contributors to ameliorating formaldehyde sensitivity, according to genetic evidence in budding yeast [de Graaf et al., 2009].

ii. Enzymatic

DPCs can arise from normal enzymatic transactions with DNA. Various enzymes form transient complexes covalently linked to DNA as a reaction intermediate. Sometimes these transient covalent complexes can become trapped onto the DNA, giving rise to an enzymatically-derived DPC.

The most frequent occurrence is with topoisomerase upon inhibitor treatment [Pommier et al., 2006]. Abasic sites can misalign the DNA strands, preventing DNA religation and trapping intermediate Topoisomerase 1 cleavage complexes (Top1ccs) onto the DNA [Pourquier et al., 1997]. These complexes are commonly formed in cells while topoisomerases interact with DNA to relieve torsional stress, but they are typically only transient intermediates rather than persisting structures. Several commonly used chemotherapeutic agents, such as camptothecin and etoposide, specifically trap these Top1ccs or Topoisomerase 2 cleavage complexes (Top2ccs), respectively, onto the DNA by inhibiting the religation reaction, thereby preventing replication and transcription [Mao et al 2001; Pommier et al., 2006].
II. Cytotoxicity of DPCs

DPCs are highly deleterious to living cells, constituting steric blockades to the DNA replication and RNA transcription machineries, as well as interfering with accessibility of DNA repair and chromatin remodeling factors [Barker et al., 2005]. If not repaired in a timely manner, these lesions can lead to deleterious mutations and cell death [Barker et al., 2005; Fu et al., 2011; Nakano et al., 2012; Nakano et al., 2013]. DNA and RNA polymerases are unable to extend past DPCs and some DpCs in vitro [Chvalova et al., 2007; Yeo et al., 2014]. DPCs also prevent strand separation during replication and repair. In E. coli, a 16 kDa DPC prevented UvrD (DNA helicase II) from separating DNA strands [Kumari et al., 2010]. DPCs less than 14.1 kDa could be cleared by several helicases [Nakano et al., 2013]. Replication fork stalling by DPCs is readily observed in E. coli [Kuo et al., 2007]. Treatment with 5-azadC or its nucleoside analog 5-azacytidine (5-azaC) leads to γ-H2AX and 53BP1 foci, indicating the presence of DNA damage and double-strand breaks [Palii et al., 2008; Orta et al., 2013].

Failure to repair DPCs leads to a variety of genotoxic consequences, such as chromatid breaks, chromosomal aberrations, and mutations [Stingele and Jentsch, 2015]. It was observed several decades ago that acetaldehyde exposure increases the incidence of mutations, sister chromatid exchanges, micronuclei, and aneuploidy in mammalian cells [Dellarco, 1988]. Replication fork collapse is known to occur from acetaldehyde treatment in fission yeast [Noguchi et al., 2017]. 5-azadC also leads to replication fork collapse in mammalian cells [Orta et al., 2013]. Much like mitomycin
C, DPC-inducing aldehydes also cause chromosomal aberrations [Speit et al., 2000; Mechilli et al., 2008; Lorenti Garcia et al., 2009]. It has also been observed that DPCs do not induce sister chromatid exchanges to the same extent as mitomycin C-induced DNA ICLs [Lorenti Garcia et al., 2009]. This observation suggests that chromosomal aberrations observed upon DPC induction may be the result of a DPC repair mechanism less favorable for homologous recombination (HR) [Lorenti Garcia et al., 2009]. Even the generally less deleterious DpC adducts have demonstrated mutagenicity. For instance, glutathione DNA-peptide crosslinks and a 10-mer Myc peptide incur mutagenic consequences in human fibroblasts and HEK 293T cells, respectively, particularly generating single base substitutions [Voitkun et al., 1998; Pande et al., 2017]. Translesion synthesis polymerases are important in bypassing smaller DpC lesions, thereby alleviating the replication block [Duxin et al., 2014; Yeo et al., 2014; Wickramaratne et al., 2016; Pande et al., 2017]. It is likely that proteolytic processing of larger DPCs and DpCs enables bypass by translesion synthesis polymerases as well.

III. Proteolytic Strategies in DPC Repair

The protein moiety of a DPC poses a major steric hindrance to DNA repair factors, which require direct access to the affected bases. As a result, the general strategy for removing DPCs likely involves proteolytic size reduction of the protein adduct. This can be accomplished in one of two ways. Proteolytic cleavage by metalloproteases – including Wss1 (in S. cerevisiae), Dvc-1 (in C. elegans), and SPRTN (in metazoans) – degrades DPCs. Some evidence also suggests that
proteasome-mediated degradation can achieve proteolytic size reduction of DPCs [Quievryn and Zhitkovich, 2000; Reardon and Sancar, 2006; Reardon et al., 2006; Baker et al., 2007; de Graaf et al., 2009] (Figure 2). Proteolytic size reduction is expected to produce a peptide remnant covalently linked to the DNA strand, allowing repair factors to access the damaged base to complete repair and restore the DNA to its original state. Alternatively, the small peptide remnant may allow lesion bypass polymerases to synthesize over the adducted base, alleviating replication fork blockage.
Figure 2. Schematics illustrating possible NER- and FA-based DPC repair mechanisms.

DPCs are hypothesized to be repaired by various mechanisms, including proteolytic cleavage of the protein adduct, followed by subsequent nucleotide excision repair of the remnant adduct; as well as Fanconi anemia pathway-based incisions, leading to removal via an ICL-repair-like pathway.

a. Proteolytic Digestion of DPCs

Proteolytic cleavage of DPCs seems to be a highly conserved strategy to begin repair of these lesions, with DPC-specific proteases discovered in budding yeast, *Xenopus*, *C. elegans*, and mammalian systems. Processing of DPCs into smaller DpCs likely serves as an initial intermediate during the repair process [Quievryn and Zhitkovich, 2000].

Several studies have recently identified the metalloprotease SPRTN as a DPC-cleaving protease in humans [Lopez-Mosqueda et al., 2016; Stingele et al., 2016; Vaz et al., 2016]. SPRTN harbors a ubiquitin-binding domain, a PCNA-interacting peptide box that enables its recruitment to chromatin upon DNA damage [Centore et al., 2012], and an SHP-box domain that associates with the AAA ATPase VCP/p97/Cdc48, a ubiquitin- and SUMO-dependent segregase that plays several key roles in regulating the DNA damage response [Davis et al., 2012;]
Mosbech et al., 2012; Stingele et al., 2014; Balakirev et al., 2015]. It is conceivable that VCP/p97/Cdc48 might assist the proteolysis of DPC intermediates.

In mammalian cells, knockdown of SPRTN with siRNA resulted in hypersensitivity toward formaldehyde and the persistence of DPCs on DNA [Lopez-Mosqueda et al., 2016; Stingele et al., 2016; Vaz et al., 2016]. These results suggest that SPRTN is involved in DPC repair.

b. Proteasome-Mediated Degradation of DPCs

Proteasome-mediated degradation may serve as either an alternative or complementary means of achieving DPC size reduction, thereby enabling nucleotide excision repair to remove the residual peptide adduct [Quievryn and Zhitkovich, 2000; Reardon and Sancar, 2006; Reardon et al., 2006; Baker et al., 2007; de Graaf et al., 2009]. The protein portions of various enzymatic DPCs are suggested to be targets for degradation by the proteasome. For example, Top1ccs and Top2ccs are targeted for proteasomal degradation [Desai et al., 1997; Mao et al., 2001]. Following proteasome-mediated degradation, a covalently-bound DNA-peptide adduct remains. This peptide adduct is then processed by the enzyme tyrosyl-DNA-phosphodiesterase 1 or 2 (Tdp1, Tdp2), which hydrolyzes the covalent bond linked to the DNA and cleaves the remaining moiety of the topoisomerase [Pouliot et al., 1999; Pommier et al., 2006].

Consistently, several reports showed that proteasome inhibition incurs sensitivity to and hinders the repair of assorted non-enzymatic DPCs. In mammalian cells, formaldehyde-induced DPCs were less efficiently repaired following
proteasome inhibition [Quievryn and Zhitkovich, 2000; Baker et al., 2007]. Chromium(VI)-mediated DPC repair seemed to require proteasome function [Zecevic et al., 2010]. Proteasome inhibition increased sensitivity of human lung cells toward formaldehyde, suggesting a possible role for the proteasome in repairing formaldehyde-mediated DNA adducts [Ortega-Atienza et al., 2015].

However, a number of other studies reported that proteasome inhibition does not significantly affect non-enzymatic DPC repair efficiency [Nakano et al., 2009; Duxin et al., 2014]. For instance, two different proteasome inhibitors added to Xenopus egg extracts did not affect DPC repair in vitro. However, the investigators did observe that depletion of ubiquitin blocked effective DPC repair, indicating that ubiquitination per se, but not proteasome-dependent degradation, is critical for DPC repair [Duxin et al., 2014]. Ubiquitin depletion affects a host of cellular processes; whether the coinciding inhibition of DPC repair is a direct or indirect consequence is not clear. The precise roles of ubiquitination in DPC repair have yet to be elucidated. The role of the proteasome in DPC repair necessitates combined genetic and biochemical studies.

IV. Nucleotide Excision Repair and DPCs

While many studies suggest that nucleotide excision repair (NER) is an important pathway in the repair of DPCs, this mechanism of DPC removal may be influenced strongly by the model organism and experimental approaches used. Using defined DPC substrates, the bacterial NER system was capable of repairing DPCs below 12–14 kDa [Nakano et al., 2007; Ide et al., 2011]. Reconstituted
UvrABC, the bacterial NER complex, excised a site-specific 16 kDa protein covalently attached to DNA; however, this incision was observed at diminished kinetics relative to incision of smaller protein adducts [Minko et al., 2002; Minko et al., 2005]. Reconstituted mammalian NER proteins excised these same crosslinks at greater efficiency, wherein, on a smaller scale, DpCs were removed with high efficiency but repair of a 16 kDa protein adduct seemed unattainable [Reardon and Sancar, 2006]. Further in vitro experiments demonstrate that mammalian NER is capable of removing small DPCs wherein the protein size is below 8 kDa [Nakano et al., 2009]. Proteins above this approximate threshold were not effectively removed [Nakano et al., 2009]. These studies suggest that adduct size plays a critical role in determining the efficiency with which NER will be able to excise a DPC or DpC in vitro.

However, studies involving NER-deficient mammalian cells have yielded conflicting results with regards to the NER pathway’s involvement in DPC repair in vivo [Fornace and Seres, 1982; Quievryn and Zhitkovich, 2000; Speit et al., 2000; Nakano et al., 2009]. While NER-deficient human cells were shown to be capable of removing formaldehyde-induced DPCs [Quievryn and Zhitkovich, 2000; Speit et al., 2000; Zecevic et al., 2010], NER-deficient fibroblasts were defective in repairing crosslinks induced by the DPC-inducing compound transplatin, suggesting a role for NER in repairing specific forms of DPC lesions [Fornace and Seres, 1982]. Furthermore, mammalian NER was unable to excise even small formaldehyde-induced DPCs of 7.4 or 8.0 kDa in vivo, conflicting with the in vitro experiments
using mammalian NER, and also in contrast with the DPC sizes excised in bacterial 
\textit{in vivo} experiments [Nakano et al., 2007; Nakano et al., 2009].

Taken together, the steric hindrance of protein adducts necessitates that 
proteolytic cleavage provide a means to achieve the necessary size reduction to 
process the lesion into intermediates that can (possibly) be effective NER 
substrates. Thus, we hypothesize that a combined proteasome/protease-NER 
mechanism is a viable mechanism for the removal of DPCs. When the protein 
moiety of a DPC exceeds the size threshold with which NER can directly excise the 
lesion, we predict that the predominant mechanism to repair the adduct will entail 
proteolysis followed by NER; thus, we expect to see defective DPC repair following 
NER inactivation, unless alternative or redundant mechanisms can compensate for 
the loss of NER.

\textbf{V. Fanconi Anemia Pathway and DPC Repair}

Fanconi anemia (FA) proteins are involved in the DNA damage response 
upon recognition of ICLs [Kottemann and Smogorzewska, 2013]. While the precise 
roles of FA proteins in recognizing and facilitating the repair of ICLs has been 
heavily pursued, it remains unclear whether these proteins have a direct role in 
repairing DPC lesions [Duxin and Walter, 2015].

Several studies implicate various FA proteins in the repair of DPCs [Ridpath 
et al., 2007; Langevin et al., 2011; Rosado et al., 2011]. FA-deficient chicken and 
mammalian cell lines exhibited hypersensitivity toward formaldehyde and 5-azadC, 
respectively [Ridpath et al., 2007; Rosado et al., 2011; Orta et al., 2013]. CHO cells
depleted in FANCG were unable to engage in HR to repair DNMT DPCs incurred by 5-azadC, resulting in increased chromatid breaks and radial fusions [Orta et al., 2013]. This same FANCG-depleted CHO cell line exhibited high sensitivity toward acetaldehyde [Mechilli et al., 2008], though formaldehyde treatment did not incur the same level of sensitivity [Lorenti Garcia et al., 2009]. FANCD2-depleted cells, however, did exhibit sensitivity toward formaldehyde [Karanja et al., 2014; Vaz et al., 2016]. Intake of acetaldehyde in human cells resulted in FANCD2 monoubiquitination [Marietta et al., 2009; Abraham et al., 2011]. Upon acetaldehyde exposure, cells depleted of FANCQ/XPFF and FANCG also had an elevated incidence of chromosomal aberrations [Mechilli et al., 2008; Lorenti Garcia et al., 2009]. Moreover, FA-deficient blood progenitor cells undergoing differentiation exhibit a significant increase in formaldehyde-induced DPCs relative to wild-type cells, indicating that the FA pathway is instrumental in removing DPCs arising from the surge of nuclear formaldehyde during transcriptional reprogramming [Shen et al., 2020]. Collectively, these studies suggest roles for FA proteins in repairing lesions incurred by reactive aldehydes and 5-azadC.

Other studies have found that FA proteins are not required for repairing DPC lesions. Immunodepletion of FANCI-FANCD2 from *Xenopus* egg extracts only inhibited ICL repair, but not DPC repair/bypass [Duxin et al., 2014]. FANCD2 depletion in *C. elegans* larvae did not affect formaldehyde sensitivity, even in the absence of Dvc-1 [Stingele et al., 2016]. Consistently, mouse embryonic fibroblasts deficient in FANCD2 were not defective in formaldehyde-induced DPC repair [Stingele et al., 2016]. FANCD2 depletion also had no effect on DPC accumulation
as measured by DPC detection from chromatin [Vaz et al., 2016]. While FANCD2-depleted cells did exhibit sensitivity toward formaldehyde in HeLa cells, camptothecin treatment had no such effect [Vaz et al., 2016], suggesting that sensitivity toward formaldehyde in these cells might be due primarily to the ratio of ICL lesions formaldehyde is known to induce [Chaw et al., 1980]. However, it is important to note that these results do not preclude the possibility that the FA pathway plays a role in DPC repair, given that Tdp1 can process the Top1ccs induced by camptothecin.

So far, results from different groups remain conflicting with regards to the precise role of the FA pathway in DPC repair. It is possible that DPCs encountered during replication are processed by the FA pathway, rendering its function as indirect. Perhaps some DPC structures require FA-mediated nucleolytic incisions. We hypothesize that, if a DPC or proteolytically processed DPC is too large for efficient NER removal, the FA pathway will repair the lesion, as its excessive size likely constitutes a replication block. We posit that such a mechanism could entail replication fork convergence on the DPC, detecting and processing the lesion in a manner similar to ICLs. Thus, depending on the extent of proteolytic cleavage, we expect that either NER and/or FA pathway inactivation will result in DPC repair deficiencies.

VI: Assay Systems for DPC Detection and Quantification

Several methods have been proposed and utilized in recent decades to measure and quantitate DPCs. These methods can broadly be categorized into
direct or indirect assays. Direct DPC assays are dependent on measuring the actual amount of proteins crosslinked to the DNA. This can be achieved in different ways. First, following removal of non-covalently bound proteins from a given sample, one can detect remaining covalently bound proteins directly via fluorescent labeling [Shoulkamy et al., 2012; Stingele et al., 2014; Mórocz et al., 2017; Nakano et al., 2017]. Second, following similar removal of non-covalently bound proteins, a particular DPC of interest can be quantitated via immunodetection with a corresponding antibody, using a technique called RADAR (Rapid Approach to DNA Adduct Recovery) [Kiianitsa and Maizels, 2013].

DPCs can also be assayed indirectly, typically contingent on isolating protein-bound DNA from soluble, free DNA, then measuring the DNA content of each fraction and calculating the proportion of protein-bound DNA over the total quantity of DNA collected [Zhitkovich and Costa et al., 1992; Costa et al., 1996; Stingele and Jentsch, 2015]. This assay, termed the K-SDS method, is typically done by dissolving cell samples in SDS, then extracting free DNA (in the soluble portion) upon potassium chloride precipitation of proteins and DNA-protein conjugates [Trask et al., 1984]. The precipitated protein-containing DNA can then be released by proteinase K digestion, collected, and measured alongside the free DNA. Comparing the DPC-associated DNA with the total DNA collected between the two fractions yields a calculation of the overall abundance of DPCs in the sample [Zhitkovich and Costa, 1992; Costa et al., 1996, Olin et al., 1996; Liu et al., 2006; de Graaf et al., 2009; Ye et al., 2013; Stingele et al., 2014; Stingele et al., 2016; Vaz et al., 2016; Mórocz et al., 2017].
Each of these primary methods of assaying DPC abundance – directly and indirectly – offers their own advantages, disadvantages, and caveats. For the direct DPC assay involving FITC-based fluorescent labeling, the technique is lengthy and by extension may lend itself to a higher rate of inconsistency. The direct DPC approach involving RADAR is limited to detecting only specific species of DPC, dependent upon the antibody (and its specificity) used during immunodetection, and therefore is unable to measure global DPC accumulation of unspecified DPC species. Moreover, the means of DNA isolation (exclusively via ethanol precipitation) lends another possible avenue for inconsistency, primarily due to a significantly higher abundance of residual free protein (i.e., not part of a DPC) in the sample used for subsequent immunodetection.

The indirect K-SDS assay, while being a more rapid technique, tends to incur high levels of background (incurred primarily by increased free protein and SDS addition), effectively disrupting positive DPC signal measurements [Olin et al., 1996; Liu et al., 2006; Ye et al., 2013; Mőrocz et al., 2017].

The primary goals in refining DPC detection protocols involve reducing background while maximizing true signal. Our objective was to achieve a sufficiently high signal:background ratio, while also limiting the number of individual steps and duration of the protocol, which otherwise could increase inconsistency.

Herein, we will discuss ways in which we have optimized a DPC detection protocol and defined parameters that lead to an increase in the signal:background ratio, effectively allowing us to more accurately and precisely analyze and interpret DPC accumulation under a range of experimental conditions. We term this new
assay system ARK (Advanced Recovery of K [potassium]-SDS precipitates). The ARK assay offers a robust, consistent, and sensitive means of detecting and quantitating DPCs – its readout range is consistently at least 5-fold more sensitive than those of pre-existing methods. We find that the ARK assay is also versatile, as it is capable of detecting DPCs induced by a range of DPC-inducing agents. Functionally, we find that cells inactive in the Fanconi anemia pathway exhibit significantly increased levels of DPC accumulation and retention, offering credence to the controversial notion that the Fanconi anemia pathway plays a key role in the DPC repair process.
Materials and Methods

(Sections of this chapter are based upon (Hu and Klages-Mundt, et al., 2020), with permission granted by Cell Reports).

Generation of CRISPR/Cas9-Mediated Single- and Double-Knockout XPA⁻/⁻ and FANCL⁻/⁻ Mutants in HeLa and 293A Cells

Our HeLa XPA⁻/⁻ knockout cell line was generated from a HeLa wild-type background. Our HeLa XPA⁻/⁻/FANCL⁻/⁻ double knockout cell line was generated from a FANCL⁻/⁻ background cell line previously established by our lab [Tian et al., 2017]. We targeted specific exon sequences for either XPA or FANCL, with the corresponding guide RNA-expressing sequence inserted into lentiGuide-Puro vectors (Addgene #52963), together with a lentiCas9-Blast vector (Addgene #52962). Guide RNAs for XPA targeted exon 1, while guide RNAs for FANCL targeted exon 2 (Table 1).

We also generated XPA⁻/⁻ and FANCL⁻/⁻ single- and double-knockout mutants in 293A cells, each mutant established from a wild-type background. We used a mix of guide RNA constructs to generate the FANCL⁻/⁻ single- and double-knockout, targeting exons 2, 4, and 5 (Table 1).
Table 1. Guide RNA sequences used to generate 293A and HeLa XPA\textsuperscript{−−} and FANCL\textsuperscript{−−} single- and double-knockout mutants.

<table>
<thead>
<tr>
<th>Knockout mutant</th>
<th>Guide RNAs against XPA exon 1</th>
<th>Guide RNAs against FANCL exon 2</th>
<th>Guide RNAs against FANCL exon 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>293 XPA\textsuperscript{−−}</td>
<td>#1 GGC GGCTTTAGAGCAACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#2 CGACGGGGCTTTGCGGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>293A FANCL\textsuperscript{−−}</td>
<td>TGAGGGATTCTCATCGGCTC</td>
<td>ATCATAAGCTCTAGATC</td>
<td></td>
</tr>
<tr>
<td>293A XPA\textsuperscript{−−}/FANCL\textsuperscript{−−}</td>
<td>#1 GGC GGCTTTAGAGCAACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#2 CGACGGGGCTTTGCGGAGG</td>
<td>TGAGGGATTCTCATCGGCTC</td>
<td>ATCATAAGCTCTAGATC</td>
</tr>
<tr>
<td>HeLa XPA\textsuperscript{−−}</td>
<td>#1 GGC GGCTTTAGAGCAACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#2 CGACGGGGCTTTGCGGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa FANCL\textsuperscript{−−}</td>
<td>GGAAGAGACCTCCACCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa XPA\textsuperscript{−−}/FANCL\textsuperscript{−−}</td>
<td>#1 GGC GGCTTTAGAGCAACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#2 CGACGGGGCTTTGCGGAGG</td>
<td>GGAAGAGACCTCCACCTT</td>
<td></td>
</tr>
</tbody>
</table>

Transfection of guide RNA constructs was facilitated by Mirus Bio TransIT-LT1 Transfection Reagent. Puromycin selection was used to isolate positive clones, which in turn were then allowed to grow into colonies from a single cell. Protein-null status was assessed for individual clones via Western blotting, using antibodies against either XPA or FANCL.

Clones that exhibited loss of expression of the corresponding gene were then genotyped. Primers flanking the corresponding CRISPR/Cas9 target sites were used to amplify DNA from XPA- and FANCL-knockout candidates via polymerase chain reaction. Following agarose gel electrophoresis, DNA bands were excised, purified and inserted into TA vectors, which were subsequently transformed into competent E. coli cells and later harvested for DNA sequencing. We sequenced at least ten E. coli clones (each containing a copy of our amplified sequence of interest) to confirm biallelic frameshifting mutations in the targeted exon sequences of XPA or FANCL.
Biallelic frameshifting mutations were extrapolated to amino acid sequence, showing premature truncation of the sequence within the targeted exon. Analysis of Sanger sequencing results for each genotype, as well as confirmation of protein-null status of the knockout gene via Western blot, are shown in Figure 3 and Figure 4.

**Figure 3.** Genotypes of CRISPR/Cas9-mediated single- and double-knockout mutants in HeLa cells.

Biallelic frameshifting mutations and protein-null status are depicted.
Figure 4. Genotypes of CRISPR/Cas9-mediated single- and double-knockout mutants in 293A cells.

Biallelic frameshifting mutations and protein-null status are depicted.

Drug Treatments

Unless otherwise indicated, formaldehyde treatment was for 2 hours, and camptothecin/etoposide treatments were for 1 hour. 293A and HeLa cells were grown to approximately 60-70% confluency in 6-well plates prior to treatment, while TK6 cells grew to approximately 0.5 x 10^6 cells/mL in 100mM dishes. Following treatment, cells were either immediately harvested for lysis or washed and allowed a given recovery interval in fresh media, then collected at later time points.

Clonogenic Survival Assay

Cells were counted and seeded in triplicate onto 6-well plates, approximately 12 hours before treatment. For formaldehyde treatments, a given dosage was prepared in serum-free media and applied to wells for 2 hours. For mitomycin C treatments, dosages were prepared in serum-free media, then applied to wells for 1 hour. 5-aza-2’-deoxytidine treatments were prepared in DMEM containing 10% FBS and applied to wells for 48 hours. UVC was administered using a UVC crosslinker at the respective energy setting. After treatments, the media was removed, wells were washed, then replaced with fresh, 10% FBS-containing media. Colonies were allowed to grow for 10-15 days, after which they were stained with 0.5% crystal
violet (w/v) in methanol for visualization and quantification. A colony was defined by clusters determined to contain greater than 50 cells.

**K-SDS Assay to Isolate and Quantify DPCs**

The K-SDS assay we used was originally detailed in earlier literature [Zhitkovich and Costa, 1992; Costa et al., 1996; Liu et al., 2006; Ye et al., 2013]. Briefly, following formaldehyde treatment 8 \times 10^5 cells were trypsinized, washed with PBS, then lysed with a 2% SDS solution. Following lysis, DNA was sheared via passage through a 22-gauge needle six times. An equal volume of a KCl buffer (200mM KCl, 20mM Tris-HCl (pH 7.5)) was added to the SDS lysate and incubated at 4 degrees C for 6 minutes, thereby precipitating SDS-bound DPC-associated DNA. Supernatants were collected following centrifugation at 6,000 x g for 5 minutes at 4 degrees C, which constituted free DNA. The precipitated pellets were washed at 65 degrees C for 10 minutes with another KCl buffer (100mM KCl, 20mM Tris-HCl (pH 7.5)), incubated on ice for 6 minutes, and centrifuged again at 6,000 x g for 5 minutes at 4 degrees to isolate the supernatant, which contained remaining free DNA. This wash cycle was done three times, and supernatants following each repetition were combined for subsequent analysis of free DNA concentration. The remaining pellets were then digested with proteinase K, and 80μl of BSA (5mg/mL) was added to each sample. Following digestion and subsequent centrifugation, the supernatant was collected, which contained DNA that had been DPC-associated. The free DNA and DPC-associated DNA fractions were quantified by PicoGreen analysis.
RADAR Assay for Immunodetection of DPCs

The RADAR assay was performed as originally described [Kiianitsa and Maizels, 2013]. Briefly, cells were seeded onto a 6-well plate in triplicate and treated with 2.5-10μM camptothecin for 1 hour. Cells were then lysed in 800μL GTC lysis buffer (6M GTC, 10mM Tris-HCl (pH 6.5), 20mM EDTA, 4% Triton X-100, 1% Sarkosyl, and 1% dithiothreitol). 400μL of 100% ethanol was added to each sample, then incubated at -20 degrees C for 5 minutes before centrifugation at maximum speed for 15 minutes. The pellet was washed twice with 75% ethanol, then re-suspended in 200μL of freshly prepared 8mM NaOH. DNA concentrations were quantified using Nanodrop measurements, then diluted in Tris-buffered saline (10mM Tris (pH 7.5), 150mM NaCl) for a final volume of 200μL and loaded onto a nitrocellulose membrane via a slot blot (Bio-Rad BioDot SF) for subsequent immunodetection using a rabbit anti-Topoisomerase 1 antibody (Bethyl, 1:2000) or a mouse anti-double-stranded DNA antibody (Abcam, 1:2000). Band intensities were quantified using ImageJ software and normalized to the corresponding double-stranded DNA loading for each sample.

For the enhanced RADAR assay, the following modifications were made, in accordance with our ARK assay optimization. Prior to lysis, the GTC lysis buffer was incubated at 55 degrees C for 5-10 minutes. Upon direct lysis in the culture wells (in the case of 293A and HeLa cells), lysates were collected by scraping and mild shearing with 6 passages through a 1mL pipet tip. Upon collection, samples were centrifuged briefly to eliminate bubbles, then sheared with a 22-gauge needle 6
times. Once the DNA sample was later collected and solubilized in 8mM NaOH, the sample was incubated in a 37 degrees C water bath for 1 hour, prior to loading onto the slot blot for subsequent immunodetection.

**ARK Assay to Isolate and Quantify DPCs**

Cells were seeded in 6-well plates in triplicate, then treated with given dosages of DPC-inducing agents (formaldehyde, camptothecin, etoposide, or 5-aza-2'-deoxycytidine). In dose-dependent studies, cells were harvested for lysis immediately following treatment. In time-course-dependent experiments, media containing the DPC-inducing agent was removed at the appropriate timepoint, the wells were washed with TD buffer and then replaced with fresh media for a series of recovery intervals. Following treatment or recovery, 950μL GTC lysis buffer (5.6M GTC, 10mM Tris-HCl (pH 6.5), 20mM EDTA, 4% Triton X-100, 1% Sarkosyl, and 1% dithiothreitol) (pre-warmed at 55 degrees C for 5-10 minutes) was either added directly to wells (for 293A and HeLa adherent cells) or cells were collected and pelleted (centrifugation at 300 x g for 5 minutes) prior to adding 950μL pre-warmed GTC lysis buffer (for TK6 suspension cells). For adherent cells, lysates were collected by scraping and mild shearing with 6 passages through a 1mL pipet tip. Upon collection, samples were centrifuged briefly to eliminate bubbles, then sheared with a 22-gauge needle 6 times. 100% ethanol (pre-chilled at -20 degrees C) was added in equal volume to each sample, which were then centrifuged at maximum speed at 4 degrees C for 20 minutes to precipitate free and DPC-containing DNA. The DNA pellet was washed with a DPC wash buffer (20mM Tris-HCl (pH 6.5), 150
mM NaCl, and 50% ethanol) at 4 degrees. The resulting DNA pellet was then stored at -80 degrees C until further processing (while avoiding air drying).

The frozen DNA pellet was then dissolved in 500μL of pre-warmed SDS buffer solution (1% SDS, 20mM Tris-HCl (pH 7.5)) and incubated for 6 minutes at 42 degrees C. Samples were briefly centrifuged (3500 x g for 30 seconds), then further fragmented by shearing through a 25-gauge needle 5 times. Samples were then centrifuged again for 45 seconds to minimize the presence of bubbles.

To precipitate SDS-bound DPC-associated DNA, we added 500μL of KCl precipitation buffer (200mM KCl, 20mM Tris-HCl (pH 7.5)). We incubated samples on ice for 6 minutes, then pelleted the precipitate with centrifugation at maximum speed for 5 minutes at 4 degrees C. The supernatant, which represents free DNA, was collected and set aside. We then washed the remaining pellet in 1.5mL of KCl wash buffer (100mM KCl, 20mM Tris-HCl (pH 7.5)) by incubating samples at 55 degrees C for 10 minutes, followed by 6 minutes on ice and subsequent centrifugation at 20,000 x g for 5 minutes at 4 degrees C. We again collected the supernatant, which we combined with the corresponding previously collected supernatant. We repeated this wash procedure an additional time – again combining the corresponding supernatants – before dissolving the DPC precipitate pellet in 1mL of proteinase K buffer (100mM KCl, 20mM Tris-HCl (pH 7.5), 10mM EDTA). Proteinase K was then added to constitute a concentration of 0.2mg/mL for each sample, which we incubated at 55 degrees C for 45 minutes. After this digestion, we placed each sample on ice for 6 minutes, followed by centrifugation at 20,000 x g for 10 minutes at 4 degrees C. We then isolated the supernatant, which comprised the
DPC-associated DNA. The isolated free DNA samples and DPC-associated samples were then used for Picogreen analysis, to calculate the DPC coefficient (representing the percentage of DNA from DPCs over the total DNA in each sample).

**PicoGreen Assay for Double-Stranded DNA Quantification**

To measure DNA concentrations of free and DPC DNA, samples were loaded onto a 96-well plate for PicoGreen analysis. 10μL were used for each 4mL sample of free DNA, while 62.5μL were used for each 1mL supernatant corresponding to the DPC-associated DNA. These samples were diluted in TE buffer for a total volume of 125μL/well. DNA standards were prepared at concentrations of 8ng, 80ng, 400ng, 800ng, 1400ng, and 2000ng/mL, also diluted in TE buffer and applied to the wells. We also loaded a TE buffer control well without DNA for use as a blank following fluorescence readings. Equal volume (125μL) of diluted PicoGreen stock (1:200) was added to each well and thoroughly mixed. The 96-well plate was placed on a Synergy2 plate reader (BioTek) for fluorescence detection. Fluorescent comparison with the standard curve yielded extrapolations of DNA amounts in each sample. We calculated the amount of DPC-associated DNA divided by the total DNA in each sample, which we expressed as a percentage termed the “DPC coefficient.”

**Statistical Analyses**

Statistical analysis was performed using built-in functions in GraphPad Prism Version 8. For experiments involving three or more groups but only one variable,
one-way ANOVA analysis was conducted to broadly determine if the means of all
groups are all the same through an F-test with $p < 0.05$ being considered significant.
If significant, a further Tukey-Cramer test was performed for comparisons between
any two groups. Analysis involving only two groups was carried out using Student’s
t-test with $p < 0.05$ being considered statistically significant. Symbols for different
significance levels are assigned as the following: * for $p < 0.05$; ** for $p < 0.01$; *** for
$p < 0.001$; **** for $p < 0.0001$; NS for not significant. All data for measured variables
were expressed as means ± SD as indicated. Sample size was $n ≥ 3$ containing both
biological and experimental replicas unless otherwise indicated.
Results

(Sections of this chapter are based upon (Hu and Klages-Mundt, et al., 2020), with permission granted by Cell Reports).

Development and Optimization of the ARK Assay

Given the unmet need of an effective and robust quantitative assay to detect DNA-protein crosslinks in cells, we developed and optimized a methodology in the initial stages of our research, which we termed “Advanced Recovery of K (potassium)-SDS precipitates” (ARK) assay (Figure 5). While the full procedure is described in the Methodology chapter, the ARK assay is premised upon a combination of pre-existing methods as well as an optimization of conditions to minimize background DPC reading noise and maximize the readout efficiency of positive signals. Briefly, following the respective treatment regimen, cells are lysed with a guanidine thiocyanate-based buffer, which ensures the maximum disruption of noncovalent interactions between proteins and DNA that likely constitute a major source of background noise in other DPC assays. The mixture of protein-free and DPC-associated DNA was recovered via ethanol precipitation. This DNA pellet is then dissolved in an SDS buffer, which serves to denature the proteins and further remove any remaining non-covalently bound proteins from our sample. Next, we add a KCl buffer to precipitate SDS-bound proteins, leaving free DNA soluble. A series of centrifugation steps and washes allow us to isolate the total free DNA fraction for normalization purposes. The precipitated DPC-associated DNA is then digested with
proteinase K, allowing us to recover this DNA fraction. The proportion of free DNA and DPC-associated DNA is then assessed with PicoGreen quantification, allowing us to calculate the DPC coefficient (i.e., the percentage of DPC-associated DNA divided by the total DNA isolated).

Figure 5. Schematic depicting the ARK assay and recovery of DPC-containing DNA and protein-free DNA.
The combination of chaotrope- and detergent-based lysis in our methodology offered a means of minimizing background; subsequently, we wanted to enhance our assay in other ways as well to maximize readout. We first tested several modifications to our general protocol, aimed to (1) minimize background levels attributing to the DPC coefficient, and (2) enhance the sensitivity of the assay.

The first modifications entailed increasing the lysis temperature from room temperature to 55 degrees C by pre-warming the lysis buffer and shearing the DNA. As seen in Figure 6a, this pre-warming step incurred a significant reduction in the DPC coefficient relative to room temperature lysis in untreated TK6 and 293A cells. Moreover, when we combined the lysis buffer pre-warming with a subsequent shearing step to fragment the DNA, the DPC coefficient decreased even further. Background readings were decreased by ~61% +/- 5.7% in 293A cells and ~67% +/- 5.5% in TK6 cells. Thus, these two steps helped optimize our methodology by reducing our DPC coefficient background levels.

The next modification assessed how removing RNA from the DNA sample following proteinase K digestion affected the DPC coefficient. As shown in Figure 6b, addition of a combination of RNase A and RNase T1 did not have any significant effect on reducing background DPC levels in HeLa and TK6 cells. This suggests that the presence of RNA in our DNA samples has a negligible effect on impacting the resulting DPC coefficient.

The original SDS/KCl assay [Zhitkovich and Costa, 1992; Costa et al., 1996] entails adding 0.4mg/mL BSA to mitigate potential interference of free SDS in the DNA samples and their respective fluorescence readings following PicoGreen.
addition. However, it is conceivable that the presence of BSA in the sample may in turn interfere with the fluorometric readout via the PicoGreen assay. Thus, we sought to determine how the addition of two concentrations of BSA (0.2mg/mL and 0.4mg/mL) would affect PicoGreen fluorescence readings over a range of DNA quantities that correspond with those typically observed in our experiments. As demonstrated in Figure 6c, when either concentration of BSA is added to the proteinase K buffer solution, the resulting fluorescence readings decline significantly, indicating that the addition of BSA hinders the sensitivity of the assay. Thus, the presence of BSA appears to have a more significant role in abrogating PicoGreen fluorescence than any free SDS that may be present in the DPC-associated DNA sample. As further confirmation that our DNA preparations were sufficiently pure, we measured the 260:280nm absorbance ratio of our samples, which consistently read near 1.9. In turn, we eliminated BSA addition from our final ARK assay protocol.

We also made a comparison of the relative fold induction of DPCs following either the conventional K-SDS protocol or our ARK assay in 293A, HeLa, and TK6 cells. While background DPC coefficient readings ranged between 8-14% following the conventional K-SDS protocol [Liu et al., 2006], use of the ARK assay minimized background to approximately 1%. Following 400μM formaldehyde treatment for 2 hours, we showed that the percentage of DPC-associated DNA increases significantly following use of the ARK assay relative to the conventional K-SDS method, as shown in Figure 6d. Fold inductions for 293A, HeLa, and TK6 cells were 5.3 +/- 0.69, 6.4 +/- 0.28, and 5.1 +/- 0.28 times higher, respectively, with the ARK assay relative to the conventional K-SDS method. The data here demonstrate that
modifications made to the ARK assay dramatically improve sensitivity of the readout, while also eliminating high degrees of background signal.

Figure 6. ARK assay optimization yielded significantly reduced background and increased sensitivity.

(A) Reduction of DPC coefficient background by pre-warming lysis buffer to 55°C (versus room temperature [RT]) and by syringe shearing after DPC precipitate is dissolved in 1% SDS buffer. The background DPC levels before these optimizations were set to 100% for comparison purposes.

(B) Impact of RNA removal on DPC fold induction reading from HeLa and TK6 cells exposed to 200 μM FA (2 h). DNA samples recovered after
proteinase K digestion were treated and mock-treated with RNase A-T1 mix and subsequently measured by PicoGreen quantification.

(C) Effect of BSA in PicoGreen DNA measurement. Recovered DNA samples after proteinase K digestion were subjected to PicoGreen quantification in the presence or absence of the indicated amount of BSA.

(D) Parallel comparison of assay readout between the K-SDS and ARK methods. Cells were treated with 400 μM FA for 2 h. DPC levels are represented by fold induction compared to mock-treated cells.

Number of biological repeats: n = 6 for (A), n = 3 for (B)–(D). The error bars depict standard deviations.

Experiments for this figure were completed by Qianghua Hu.

Detection of Nonenzymatic DPCs using the ARK Assay

As discussed in the Introduction, DPCs can be formed in various ways, and can be described by two overarching categories: enzymatic and non-enzymatic. We wanted to establish the efficacy with which our ARK assay would detect DPCs of both natures.

In order to detect nonenzymatic DPCs, formaldehyde was used as a DPC-inducing agent. Treatment with formaldehyde is expected to result in non-enzymatic DPCs, covalently linking nearby proteins to the DNA base via an amide bond. Using a range of formaldehyde dosages, we used the ARK assay to measure the DPC coefficient following treatment for 2 hours in 293A, HeLa, and TK6 cells. As expected, DPC induction is relatively linearly dose-dependent for all three cell lines.
As an assessment of the ARK assay’s ability to account for the kinetics of DPC repair, we also measured DPC abundance in a time-course study, such that cells were treated with 400μM formaldehyde for 2 hours, then allowed a recovery interval (ranging from 3 to 24 hours) for repair. As expected, compared to the baseline levels immediately following 2 hours treatment, DPC levels diminished progressively over the course of 24 hours post-treatment in all three cell lines, returning to baseline levels in 293A and HeLa cells, while retaining approximately 30% of DPCs in TK6 cells after 24 hours of recovery (Figure 7b).

Figure 7. Detection of nonenzymatic DPCs by the ARK assay.

(A) Dose response of 293A, HeLa, and TK6 cells exposed to the indicated concentrations of FA for 2 h. DPC fold inductions were calculated by normalizing DPC-associated DNA to that of mock treatment.
(B) DPC repair time course in 293A, HeLa, and TK6 cells exposed to 400 μM FA treatment for 2 h. DPC coefficient of each cell line after treatment is set as 100%.

n = 4 for (A) and n = 6 for (B). The error bars depict standard deviations. Experiments for this figure were completed by Qianghua Hu.

**Detection of Enzymatic DPCs using the ARK Assay**

Next, we tested whether enzymatic DPCs could be detected effectively via the ARK assay. To do this, we treated 293A, HeLa, and TK6 cells with either camptothecin or etoposide. As discussed previously, camptothecin covalently traps topoisomerase 1 onto the DNA, whereas etoposide covalently traps topoisomerase 2 onto the DNA; these agents account for a significant portion of cancer therapeutic regimens. We found that DPC levels increased in a dose-dependent manner following either camptothecin or etoposide 1-hour treatments (Figure 8a, 8b). With camptothecin treatment, DPC induction plateaued around 20μM, likely indicating a saturation point for trapped topoisomerase 1 complexes.
Figure 8. Detection of enzymatic DPCs induced by topoisomerase inhibitors with the ARK assay.

(A) 293A, HeLa, and TK6 cells were exposed to various doses of CPT for 1 h and analyzed by the ARK assay to generate DPC fold induction by normalizing the DPC coefficient of each sample against that of the mock-treated control.

(B) HeLa, 293A, and TK6 cells were exposed to various doses of etoposide for 1 h and analyzed by the ARK assay to generate the DPC fold induction by normalizing the DPC coefficient of each sample against that of the mock-treated control.

Each data point was generated from no less than 5 biological repeats with triplication. The error bars depict standard deviations.

Experiments for this figure were completed by Qianghua Hu.

Together, these results demonstrate that the ARK assay can be employed to measure a range of DPC species, both enzymatic and non-enzymatic. Importantly,
drugs inducing select forms of DPCs are readily detected by the ARK assay, without reliance on immunodetection-based DPC assay systems.

**DPC Repair-Deficient Genetic Models Validate the ARK Assay**

While the precise mechanism(s) underlying DPC repair are still unclear, the metalloprotease SPRTN has been shown in recent years to proteolytically digest proteins adducted to the DNA, thereby facilitating downstream repair pathways [Duxin et al., 2014; Stingele et al., 2014; Vaz et al., 2016; Larsen et al., 2019]. As a known DPC repair factor in higher eukaryotes, we wanted to determine whether deficiency in SPRTN would elevate the DPC coefficient. Therefore, we treated wild-type and two independent SPRTN knockout TK6 cells with 400μM formaldehyde for 2 hours, followed by 6- to 24-hour recovery intervals, and proceeded with ARK assay quantification of DPC levels. As shown in Figure 9a, DPC abundance was significantly elevated at each recovery interval (p<0.001) in both SPRTN knockout clones relative to wild-type TK6 cells. Following 24 hours of recovery, wild-type cells retained only 32.0 +/- 5.0% of DPCs compared to initial DPC induction, whereas the average knockout DPC retention after 24 hours recovery measured at 70.3 +/- 7.4%. This suggests that DPC repair is defective in cells devoid of SPRTN expression, in alignment with the notion that SPRTN is a critical DPC repair factor. We have demonstrated that the ARK assay is effective and robust in detecting the differential in DPC removal following knockout of a key DPC repair factor, SPRTN.

A similar pattern is observed following continuous low-dose formaldehyde treatment (50μM) for either 12 or 24 hours (Figure 9b). This dose falls within the
range detected endogenously in mammalian plasma [Ridpath et al., 2007]. TK6 SPRTN knockout cells had significantly increased DPC levels relative to wild-type cells following 12 hours of continuous low-dose formaldehyde exposure. Amongst the two knockout variants, DPCs accumulated by 3.2 +/- 0.5-fold (p=0.005) and 4.9 +/- 0.8-fold (p=0.0001) following 12 hours of continuous treatment, compared with untreated cells. This trend held true following 24 hours of formaldehyde exposure as well (2.5 +/- 0.6-fold (p=0.05) and 3.9 +/- 1.0-fold (p=0.04) increases in accumulation), although the DPC levels diminished in each cell line between the 12- and 24-hour timepoints, an event likely attributable to the diminishing potency of formaldehyde in the serum-enriched media at later timepoints. Wild-type TK6 cells, however, only incurred a slight increase in DPC accumulation following either time-point, suggesting that normal SPRTN expression enables cells to maintain DPC accumulation at close-to-baseline levels under physiological conditions.

A parallel means of repairing topoisomerase 1 cleavage complexes induced by camptothecin involves the protein TDP1, which cleaves the phosphotyrosyl linage between topoisomerase 1 and DNA [Zeng et al., 2012]. (Similarly, TDP2 cleaves the phosphotyrosyl linkage between TOP2ccs.) Loss of these two genes hinders efficient removal of Top1ccs and Top2ccs, respectively, leading to their accumulation [Hoa et al., 2016]. Using TK6 TDP1/2 knockout cells, we sought to determine how deficiency in TDP1 affected DPC levels following camptothecin treatment. As shown in Figure 9c, TDP1/2 knockout cells exhibited increased retention of DPCs relative to wild-type cells following 12- and 24-hour 75nM camptothecin exposure intervals. Camptothecin-induced DPCs accumulated at 12-
and 24-hour time intervals by 36.3% +/- 5.2% and 39.7% +/- 4.3%, respectively, relative to untreated wild-type cells. In contrast, TDP1/2 knockout cells incurred 129.9% +/- 11.1% and 160.7% +/- 9.8% increases, respectively, compared to untreated knockout cells. These results indicate that defective repair of camptothecin-induced DPCs can be readily detected by the ARK assay.

Figure 9. Analysis of DPC repair deficiency in SPRTN and TDP1/TDP2 knockout mutants with the ARK assay.

(A) Removal of DPCs in wild-type TK6 cells and 2 SPRTN knockout derivatives (KO1 and KO2). Cells were exposed to 400 μM FA for 2 h. DPC coefficients were determined for each cell line at the indicated time points and normalized against the 0 time point to arrive at the percentages of DPCs remaining.

(B) DPC accumulation in wild-type TK6 cells and 2 SPRTN knockout derivatives continuously exposed to low-dose FA (50 μM) for 12 and 24 h.

(C) DPC accumulation in wild-type TK6 cells and a TDP1/2−/− double-knockout derivative continuously exposed to CPT (75 nM) for 12 and 24 h.
Each data point in the plots and in the bar graphs was derived from no less than 5 biological repeats with duplicates or triplicates. One-way ANOVA analyses for the three time points 6, 9, and 24 h generated p < 0.0001 in (A) for the F-test and interested pairwise Tukey test results are indicated. Number of biological repeats with triplication = 3. The error bars depict standard deviations.

Experiments for this figure were completed by Qianghua Hu.

***Optimized Parameters of the ARK Assay incur Increased Sensitivity for DPC Immunodetection Compared to Traditional RADAR***

Having already shown that the ARK assay is more sensitive and reduces background to a significantly greater extent than the conventional K-SDS assay for DPC detection, we wanted to also compare the ARK assay methodology to a direct RADAR assay. The traditional RADAR assay, which involves guanidine thiocyanate-based lysis, ethanol precipitation of DNA, slot blotting, and subsequent antibody-based detection of proteins, has been used previously to detect Topoisomerase 1 DPCs induced by camptothecin [Kianitsa and Maizels, 2013]. The modifications we used in our sample preparation for the ARK assay, as described in Figure 5, involved pre-warming the lysis buffer to 55 degrees C and shearing of the DNA. Thus, we tested the RADAR approach either without these modifications (traditional RADAR) or with these ARK assay modifications (enhanced RADAR) following either 2.5\(\mu\)M or 10\(\mu\)M CPT treatment for 1 hour. Following ethanol precipitation, we
dissolved our DNA samples in 8mM NaOH and applied them to slot blotting and subsequent immunodetection using a Topoisomerase 1 antibody.

Figure 10a shows the accumulation of Topoisomerase 1 DPCs in 293A, HeLa, and TK6 wild-type cells following either control- or camptothecin-treatment and analyzed by traditional RADAR. Relative induction of Topoisomerase 1 DPCs is depicted in Figure 10b, following normalization of the anti-TOP1 signal to the corresponding anti-dsDNA signal from Figure 10a. Normalized signal induction following camptothecin treatment were 6.4 +/- 2.0, 4.1 +/- 0.9, and 10.0 +/- 3.6 times that of untreated cells in 293A, HeLa, and TK6 cells, respectively. After implementing the ARK assay modifications, detection of TOP1 signal increased significantly for each of the three wild-type cell lines (13.4 +/- 0.31, 12.2 +/- 0.6, and 14.7 +/- 2.1 times induction in 293A, HeLa, and TK6 cells, respectively, compared to untreated cells) (Figure 10c, 10d) relative to the traditional RADAR readout. This indicates that the ARK assay modifications significantly enhance DPC detection, as reflected by the immunoblotting quantification. Moreover, background CPT levels were substantially reduced following our enhanced RADAR method relative to traditional RADAR across all three wild-type cell lines (Figure 11a, 11b). Furthermore, treatment with 10uM CPT treatment incurred higher Topoisomerase 1 DPC levels relative to 2.5uM CPT treatment, consistent with both the traditional RADAR and enhanced RADAR assays (Figure 10e, 10f). These results clearly demonstrate that the ARK assay modifications discussed in Figure 5 – when employed in an immunodetection-based context – provide a much more sensitive assay to detect DPCs than the traditional RADAR method.
Figure 10. Parallel comparison of assay readout between the RADAR and ARK assays.

(A) RADAR assay of 293A, HeLa, and TK6 cells treated with 10 μM CPT for 1 h. Upper panel: representative slot blot of DPC samples visualized by an anti-TOP1 antibody as performed by the standard RADAR assay. Lower panel: slot blot (using anti-double-stranded DNA [dsDNA]) of DNA isolated from corresponding samples in the upper panel.

(B) Relative TOP1-DPC induction by normalizing the TOP1-DPC chemiluminescent signal to the corresponding DNA signal in (A). The background levels of the mock-treated sample (Ctrl) for each cell line were set to 1.

(C) ARK detection of TOP1-DPC from identical cell samples used in (A).

(D) DPC isolates were prepared by the ARK assay protocol from identical cell samples used in (A) and blotted with an anti-TOP1 antibody (upper panel). Lower panel: slot blotting of DNA isolated from corresponding samples in the upper panel.

(E) RADAR assay detection of DPCs from 3 indicated cell lines treated with 2.5 μM of CPT for 1 hour. Left panel: Relative Top1-DPC induction. The data were derived by normalizing the Top1- DPC band intensity against that of its corresponding dsDNA loading control. Right panel: a representative slot blot showing the TOP1 and dsDNA loading control bands. Background level of mock-treated sample (Ctrl) for each cell line was set to 1.

(F) ARK assay of identical cell samples used in panel E.
The number of biological repeats ≥3. The error bars depict standard deviations.

Figure 11. Parallel comparison of background DPC levels in untreated cells between the RADAR and ARK assays.

(A) TOP1cc in total DPC samples prepared from 293A, HeLa, and TK6 cell lines were assessed by slot blotting using an anti-TOP1 antibody. Total cellular DNA from each sample was used as a loading control and visualized by anti-dsDNA.

(B) Quantification of (A).

Relative DPC background of each cell line was derived from normalizing the TOP1 band intensity against that of its corresponding DNA loading control. Number of biological repeats with duplication = 3. Error bars depict standard deviation.
Fanconi Anemia Pathway-Inactivated Cells exhibit Increased DPC Accumulation and Retention

Having demonstrated that the ARK assay is a versatile method that increases DPC sensitivity, reduces background levels of non-specific signal, and detects a range of DPC species, we next assessed functional involvement of both the Fanconi anemia and nucleotide excision repair pathways in DPC removal by using the ARK assay. In accordance with our original hypothesis, NER may be able to excise DPCs below a certain size threshold, including those sufficiently proteolytically degraded. Repair of small DPCs and proteolytically cleaved DPCs by NER has been shown experimentally in prior literature [Minko et al., 2002; Reardon and Sancar, 2006; Nakano et al., 2009]. While controversial, some evidence suggests that the Fanconi anemia pathway may have roles in resolving DPCs as well [Pontel et al., 2015]. FA-deficient cells exhibit hypersensitivity toward DPC-inducing agents [Rosado et al., 2011], although it is important to note that several such agents induce a range of other genotoxic and cytotoxic effects as well. Synthetic phenotypes are also observed between FA gene knockouts and aldehyde dehydrogenases in mice, implicating a potential role in DPC repair [Langevin et al., 2011; Rosado et al., 2011; Garaycoechea et al., 2012].

Using CRISPR-Cas9, we generated HeLa single-knockout mutants for XPA and FANCL and a double-knockout mutant of both XPA and FANCL, as described in the Methods chapter. Loss of XPA expression functionally inactivates the NER pathway (both global NER and transcription-coupled NER), while loss of FANCL
expression functionally inactivates the Fanconi anemia pathway (abrogating FANCD2 monoubiquitination).

We initially tested clonogenic survival of these single- and double-knockout mutants in response to formaldehyde treatment. As shown in Figure 12a, loss of FANCL alone dramatically hypersensitized cells to formaldehyde, whereas loss of XPA alone incurred marginal sensitization. The XPA\(^{-/-}\)/FANCL\(^{-/-}\) double knockout cells exhibited added sensitivity to formaldehyde relative to the FANCL\(^{-/-}\) single knockout cells. These results indicate that the toxicity induced by formaldehyde is most severe in cells lacking an active FA pathway, while is only modest in cells lacking an active NER pathway. As formaldehyde induces a range of DNA lesions (amid other cytotoxic effects), we cannot specifically attribute the sensitivity to DPC induction, however.

To assess how effectively XPA\(^{-/-}\) and FANCL\(^{-/-}\) HeLa mutants removed formaldehyde-induced DPCs relative to wild-type cells, we quantitated DPC levels using the ARK assay following various recovery intervals post-treatment (500\(\mu\)M formaldehyde for 2 hours). As shown in Figure 12b, DNA from FANCL\(^{-/-}\) single-knockout cells and XPA\(^{-/-}\)/FANCL\(^{-/-}\) double-knockout cells retained a higher percentage of DPCs relative to wild-type cells and XPA\(^{-/-}\) single-knockout cells following 9- and 18-hour recovery intervals. This data is consistent with the trends observed from our clonogenic survival results following formaldehyde treatment. Importantly, this data suggests that inactivation of the Fanconi anemia pathway abrogates efficient DPC repair.
We also tested how low-dose formaldehyde (75μM, coinciding with a normal physiological range [Ridpath et al., 2007]) affects DPC accumulation in XPA and FANCL mutant cells. Consistently, the $FANCL^{+/+}$ single-knockout and $XPA^{+/+}$/FANCL$^{-/-}$ double-knockout exhibited increased DPC accumulation following continuous exposure to 75μM formaldehyde for both 6 and 12 hours, relative to wild-type and $XPA^{-/-}$ single-knockout cells (Figure 12c). Similar to what we observed with the TK6 SPRTN$^{-/-}$ knockout cells, at longer durations of formaldehyde exposure the DPC coefficient began to diminish from its initial peak at shorter exposures — an event we suspect is likely attributable to the diminishing potency of formaldehyde in cells and the culture medium over time.
Figure 12. Removal of DPCs in Fanconi anemia and nucleotide excision repair mutants.

(A) Clonogenic survival of HeLa wild-type, XPA\(^{-/-}\), FANCL\(^{-/-}\), XPA\(^{-/-}\)/FANCL\(^{-/-}\) cells treated with FA.

(B) Removal of DPCs in wild-type HeLa cells and other indicated knockout derivatives. Cells were exposed to 500 \(\mu\)M FA for 2 h. DPC coefficients were determined for each cell line at indicated time points and normalized against the 0 time point to arrive at the percentages of DPC remaining.

(C) Left panel: DPC accumulation in wild-type HeLa cells and other indicated knockout derivatives continuously exposed to low-dose FA (75 \(\mu\)M) for 6 and 12 h; right panel: analysis of FA-induced DPC accumulation among HeLa cells examined in the left panel after formaldehyde treatment for 6 and 12 h, respectively. Accumulated DPCs are calculated from the DPC coefficient at the selected time point with a deduction of background level (time 0) for corresponding HeLa cell lines.

Number of biological repeats with duplication: \(n = 2\) for (A), \(n = 4\) for (B) and (C). The error bars depict standard deviation. One-way ANOVA analyses for the indicated time points (9 and 18 hr in B; 6 and 12 h in C) generated \(p < 0.0002\) for the F-test, and interested pain/vise Tukey test results are displayed.

Work on this figure was done in tandem with Qianghua Hu.
Given that FANCL is the E3 ubiquitin ligase responsible for monoubiquitinating FANCD2, enabling its recruitment to damage sites to initiate downstream events in the FA pathway, we wanted to assess FANCD2 foci formation following formaldehyde treatment (500μM for 6 hours). As expected, FANCL−/− cells were unable to form FANCD2 foci in either untreated or treated conditions. However, wild-type cells did form FANCD2 foci after formaldehyde treatment, indicating that treatment triggered the initiation of the FA pathway (Figure 13).

**Figure 13.** FANCD2 foci formation in wild-type and mutant HeLa cells following formaldehyde treatment.

After 500 μM formaldehyde treatment for 2 hours (A) or 75 μM formaldehyde treatment continuously for up to 12 hours (B), cells were collected and viability determined using automatic Trypan Blue exclusion counter at indicated time points. Viability of Mock-treated (Ctrl) cells was set as 100%.
Number of biological repeats with duplication: \( n = 2 \) for each genotype. Error bars depict standard deviations.

(C) Immunostaining of FANCD2 foci in wild type and \( \text{FANCL}^{-/-} \) HeLa cells treated with indicated concentrations of formaldehyde for 6 hours. Scale bar = 10 \( \mu \)m

Experiments for this figure were completed by Rui Wang.

Cumulatively, these results demonstrate that the Fanconi anemia pathway has a crucial role in the efficient removal of formaldehyde-induced DPC lesions from the genome. Importantly, the ARK assay can readily detect the disparity in DPC induction in wild-type cells versus cells inactive in critical DNA repair pathways relevant for DPC processing.

**Clonogenic Survival of NER and FA Mutants to DNA Damaging Agents and DPC-Inducing Agents**

To determine the sensitivity of NER and FA mutants to DPC-inducing agents, we performed several clonogenic survival assays in both \( \text{XPA}^{-/-} \) and \( \text{FANCL}^{-/-} \) single- and double-knockout mutants in both HeLa and 293A cells. We used an assortment of DNA damaging agents, targeting either the NER or FA pathways, as well as DPC-inducing agents.

In this context, we decided to use mitomycin C (known to sensitize FA-deficient cells) and UVC (known to sensitize NER-deficient cells) to validate the protein-null (and thus pathway-deficient) status of the respective FA and NER
pathways. We then used 5-aza-2’-deoxycytidine and formaldehyde in our clonogenic survival assays – both agents that induce DPC formation.

In both HeLa and 293A cells, loss of FANCL hypersensitized cells to mitomycin C (Figure 14). This is expected, given that mitomycin C induces interstrand crosslinks – a lesion processed by the Fanconi anemia pathway. The $XPA^{+/}/FANCL^{-/-}$ double-knockout did not incur added sensitivity in either HeLa or 293A. However, the single $XPA^{+/}$ knockout in HeLa exhibited modest sensitivity relative to wild-type cells after mitomycin C treatment. Importantly, these results functionally confirm that our FANCL-deficient cells indeed exhibit substantial hypersensitivity to ICL-inducing agents, as expected for cells inactivated in the Fanconi anemia pathway.

![Figure 14. FA mutants exhibit mitomycin C sensitivity.](image)
Both HeLa and 293A cells exhibit sensitivity when exposed to mitomycin C for 1 hour. The error bars depict standard deviations amongst triplicates.

We next performed a UVC sensitivity assay using our $XPA^{-/-}$ and $FANCL^{-/-}$ single- and double-knockout mutants in both HeLa and 293A cells (Figure 15). Our $XPA^{+/}$ single- and double-knockouts exhibited hypersensitivity to UVC, as expected; however, the HeLa $XPA^{-/-}$ single-knockout exhibited higher hypersensitivity relative to the HeLa $XPA^{+/} / FANCL^{-/-}$ double-knockout, for reasons we have not yet been able to establish. In 293A cells, both the $XPA^{-/-}$ single- and double-knockout cells exhibited similar levels of hypersensitivity to UVC relative to wild-type cells, as expected.

**Figure 15. NER mutants exhibit UVC sensitivity.**

As expected, cells deficient in only FANCL do not exhibit UVC sensitivity. Cells deficient in XPA exhibit hypersensitivity to UVC exposure. In HeLa cells, as mentioned in the text, the $XPA^{+/} / FANCL^{-/-}$ double knockout consistently
exhibits less sensitivity to UVC than the single $\textit{XPA}^{+/−}$ knockout mutant. The error bars depict standard deviations amongst triplicates.

Figure 12a shows the hypersensitivity of HeLa FANCL-deficient cells following formaldehyde treatment. We also showed that FANCL-deficient 293A cells were hypersensitive to formaldehyde (Figure 16). However, unlike in HeLa cells, loss of XPA in 293A cells did not incur any added sensitivity relative to wild-type cells, suggesting that the NER pathway may be less critical in repairing formaldehyde-induced damage in 293A cells than HeLa cells.

**Figure 16. FA mutants exhibit drastic formaldehyde sensitivity.**

HeLa and 293A cells deficient in FANCL exhibit hypersensitivity to formaldehyde. XPA-deficient HeLa cells exhibit a smaller degree of
formaldehyde sensitivity. The error bars depict standard deviations amongst triplicates.

Given that formaldehyde induces a mix of DNA lesions (including DPCs, ICLs, and an assortment of monoadducts), as well as cytotoxic damage, we wanted to investigate how a DPC-specific inducing agent would affect sensitivity in our XPA and FANCL mutants. We used 5-aza-2'-deoxycytidine, a cytosine analog that covalently traps DNMTs onto its DNA base, thereby inducing a DPC [Santi et al., 1983]. Our data demonstrate that, similar to formaldehyde treatment, FANCL deficiency results in hypersensitivity to 5-aza-2'-deoxycytidine in both HeLa and 293A cells (Figure 17), suggesting that the Fanconi anemia pathway plays an important role in processing damage induced by this analog.

Cell survival following 5-aza-2'-deoxycytidine treatment is also most severe (i.e., incurring the steepest decline in survival post-treatment) in the 0-2μM range with FANCL deficiency (Figure 17). This perhaps suggests a saturation point at which this analog incurs damage, possibly as a result of a limiting factor such as incorporation into the DNA or DNMT expression/availability.
Figure 17. FA mutants exhibit 5-aza-2’-deoxycytidine sensitivity.
(A) FANCL-deficient HeLa and 293A cells exhibit increased sensitivity to 5-azadC relative to wild-type cells. The error bars depict standard deviations amongst triplicates.

(B) FANCL-deficient HeLa cells exhibit increased sensitivity to 5-azadC relative to wild-type cells. Sensitivity appears to be most dramatic for treatments below ~2μM. The error bars depict standard deviations amongst triplicates.

We also measured DPC accumulation following 5-aza-2’-deoxycytidine treatment using the ARK assay in 293A wild-type cells (Figure 18). As the only species of DPC that accumulate following 5-azadC treatment are DNMTs, detection using the ARK assay should be considerably lower than detection of formaldehyde-induced global DPCs. Indeed, DPC accumulation was only 1.3x baseline levels following 24 hours exposure to 100μM 5-azadC in 293A wild-type cells.
Figure 18. Induction of DPCs by 5-azadC using the ARK assay.

Pilot experiments demonstrate that DNMT DPC induction can be detected in 293A cells using the ARK assay, increasing from baseline levels in a concentration-dependent manner.

Used with permission from Qianghua Hu, premised upon our pilot experiments.

**Histone H3 DPCs Accumulate following Formaldehyde Treatment**

We also performed some preliminary experiments using our enhanced RADAR immunodetection method to detect histone DPCs following formaldehyde treatment. Histones are predicted to comprise a major source of DPC species following formaldehyde treatment, given their close vicinity to the DNA. Prior literature has demonstrated that the traditional RADAR method could be used to detect camptothecin-induced Topoisomerase 1 DPCs and 5-azadC-induced DNMT1
DPCs [Kiianitsa and Maizels, 2013]; however, to our knowledge, detection of formaldehyde-induced H3 DPCs has not yet been investigated.

Our initial pilot experiment demonstrated that histone H3 DPC detection increased in a dose-dependent manner following formaldehyde treatment (Figure 19). Formaldehyde-induced histone H3 DPCs increased following 0.5mM and 5mM treatments in HeLa wild-type, XPA<sup>-/-</sup>, FANCL<sup>-/-</sup>, and XPA<sup>-/-</sup>/FANCL<sup>-/-</sup> cells, though biological replicates are necessary to establish the relative statistical significance in such increase amongst the four cell lines.

**Figure 19.** Histone H3 DPCs accumulate following formaldehyde treatment.

Pilot experiments (n=1) demonstrate a concentration-correlated accumulation of Histone H3 DPCs following formaldehyde treatments. Further experimentation is needed to establish statistical significance amongst each mutant at respective treatment regimens.
We also performed a time-course pilot experiment aimed to establish the efficiency with which $XPA^{−/−}$ and $FANCL^{−/−}$ mutant cells repair formaldehyde-induced histone H3 DPCs. We treated 293A wild-type, $XPA^{−/−}$, $FANCL^{−/−}$, and $XPA^{−/−}/FANCL^{−/−}$ cells with 500μM formaldehyde for 2 hours, followed by recovery intervals in fresh media for 4 or 8 hours. In this preliminary experiment, accumulation of DPCs following treatment was between 3.1-4.0x relative to untreated cells, which diminished to some extent in each cell line other than the $XPA^{−/−}/FANCL^{−/−}$ double mutant. Biological replicates are necessary to establish a discernable pattern between the initial DPC accumulation as well as the recovery rate in each mutant, as well as for each cell line generated for our research.

Taken together, our results demonstrate that immunodetection-based RADAR quantification of DPCs is both more inconsistent and less sensitive than the readout provided by the ARK assay. Indeed, the ARK assay provides a more sensitive, robust, and versatile means of quantitating global DPC accumulation than prior established methods.
Discussion and Future Studies

DNA-protein crosslinks are among the most deleterious forms of DNA damage, given their propensity to effectively block essential nuclear functions, such as transcription and replication. DPCs are commonly induced as a result of formaldehyde release following various nuclear metabolic processes, such as oxidative demethylation [Swenberg et al., 2011]. Detection of DPCs can be achieved either directly (via immunodetection of particular proteins adducted to the DNA) or indirectly. These comprise the two main strategies utilized in the field thus far to detect DPCs. One can use direct SDS lysis, KCl precipitation, and proteinase K digestion to isolate free DNA and DPC-associated DNA fractions, allowing for a global detection of DPCs in the genome – this is the K-SDS method [Zhitkovich and Costa, 1992; Costa et al., 1996]. Alternatively, immunodetection of prominent DPC species, such as topoisomerase cleavage complexes, can be applied to observe a single form of DPC – this is the RADAR approach [Kiianitsa and Maizels, 2013]. However, each of these methodologies has its own limitations. The K-SDS approach incurs high background levels of DPCs, indicating a lack of sufficient specificity in the assay. The RADAR approach only allows observation of particular DPC species rather than global accumulation of DPCs; moreover, quantification of immunodetection bands lends itself to inconsistency, as undersaturated and oversaturated bands often reside outside of a linear range of measurement accuracy.
Thus, our objective was to develop a more sensitive and versatile approach to quantitating global DPC abundance, while utilizing certain aspects in the two pre-existing methods described above. Our assay – termed the ARK assay – was premised on optimizing a much more stringent method of isolating DPCs, thereby diminishing background levels caused by non-covalent protein-DNA interactions and other factors. Following treatment, similar to the RADAR procedure, we used a guanidine thiocyanate-based buffer for a chaotropic lysis, which strongly disrupts the hydrogen binding network in the sample, effectively stripping most non-covalent interactions between proteins and DNA. Following ethanol precipitation to isolate DNA, we then used a second SDS-based denaturation step to further abolish non-covalent DNA-protein interactions. The use of these two separate denaturation steps diminished background readings and significantly enhanced measurement sensitivity relative to the original K-SDS assay, which uses only SDS-based denaturation.

As additional means of optimizing our DPC readout with the ARK assay, we found that pre-warming the guanidine thiocyanate-based lysis buffer at 55 degrees C prior to lysis significantly decreased background levels for our DPC coefficient. We believe that this is likely attributable to the warmer temperature ameliorating denaturation and dissociation of non-covalent interactions in our samples. Moreover, we found that shearing of the DNA led to a similar degree of background minimization as lysis buffer pre-warming, effectively decreasing background levels in our readout by nearly half. Importantly, when combining both the lysis pre-warming and shearing steps, background levels were decreased even further. Fragmentation of the DNA by our two shearing steps (immediately following lysis, then after
dissolving the DNA pellet in 1% SDS) likely minimizes the probability of a DPC being present on a given fragment of DNA, thereby increasing the proportion of free DNA we isolated from each individual sample, in turn decreasing DPC background readings and boosting readout sensitivity.

Our studies have also demonstrated that the ARK assay is versatile and can be applied to detection of DPCs induced by a range of DPC-inducing agents. Indeed, we showed that formaldehyde-induced non-enzymatic DPCs are readily detected in a dose-dependent manner by the ARK assay. Additionally, camptothecin- and etoposide-induced enzymatic DPCs are readily detected as well, also in a dose-dependent manner. We have demonstrated that these results are generally consistent with three distinct human cell lines – 293A, HeLa, and TK6.

As further validation of the accuracy of the ARK assay, we showed that TK6 SPRTN knockout cells had significantly higher DPC retention post-formaldehyde exposure, a result we expected given SPRTN's known roles in promoting DPC repair. SPRTN-dependent proteolysis plays an important role in cleaving the protein moiety of at least some portion of DPC species. Without SPRTN-dependent cleavage, downstream repair pathways are unlikely to be able to continue processing the remnant damage.

Furthermore, TK6 cells deficient in TDP1/TDP2 also incurred increased DPC accumulation after increasing durations of camptothecin exposure. As TDP1 cleaves the phosphotyrosyl bond linking topoisomerase 1 to the DNA, loss of TDP1 expression would expectedly increase DPC abundance, as we show. It would be interesting to see whether loss of both TDP1 and SPRTN would result in an even
further degree of DPC accumulation and retention; any epistatic relationship between these two repair mechanisms remains unknown.

Endonucleolytic activity of Mre11 provides an alternative means of excising camptothecin-induced Top1ccs, which are flanked by a single-strand break [Stingele and Jentsch, 2015]. Using both the ARK assay and clonogenic survival assays, it may be a fruitful endeavor to assess how inhibition of Mre11 endonuclease (using an Mre11 endonuclease-specific inhibitor such as PFM039 [Shibata et al., 2014]) affects DPC accumulation. Such experiments could be completed using wild-type and the TDP1\textsuperscript{-/-}/TPD2\textsuperscript{-/-} knockout TK6 cells, with or without inhibitor treatment. If Mre11 endonuclease activity at least partially rescues loss of TDP1 (as we would expect), then knockout of TDP1 combined with inhibitor treatment may significantly increase camptothecin-induced Top1cc accumulation relative to TDP1 deficiency alone. Abrogating alternative repair mechanisms would allow us to more definitively assess the full impact of TDP1 in mediating Top1cc repair.

In recent years, several studies have characterized new DPC repair proteins. While the precise mechanisms underlying DPC repair are still being unraveled, our studies offer credence to the hypothesis that the Fanconi anemia pathway plays a key role in resolving these lesions. Using the ARK assay with FANCL\textsuperscript{-/-} mutants following formaldehyde-induced DPC induction, we found that both FANCL\textsuperscript{-/-} and XPA\textsuperscript{-/-}/FANCL\textsuperscript{-/-} HeLa mutants exhibited increased DPC accumulation following formaldehyde exposure, as well as diminished DPC repair following recovery intervals post-formaldehyde treatment, relative to wild-type cells. These results provide a line of evidence suggesting that the Fanconi anemia pathway plays a
critical role in removing formaldehyde-induced DPC damage. Importantly, the ARK assay provides a separate means by which to confirm these formaldehyde clonogenic survival results, as the ARK assay detects DPCs directly (as opposed to readout being affected by other genotoxic and cytotoxic events, as in the case of formaldehyde).

Mechanistically, how Fanconi anemia proteins mitigate DPC accumulation is still open to conjecture. However, our model posits that DPCs may be recognized in a manner akin to ICLs, perhaps following replication fork convergence upon the lesion. This process may or may not be directly dependent on SPRTN-mediated recognition and proteolysis of the DPC. If so, genetic studies involving combined SPRTN deficiency and Fanconi anemia protein knockouts would help delineate such a pathway. Incisions flanking the DPC – in conjunction with significant proteolysis of the adducted protein – may allow for a combined double-strand break repair and translesion synthesis past the remnant, cleaved peptide adduct.

To further identify the physiological implications of FA pathway inactivation in DPC repair, we believe it is important to follow up with several additional assays. First, we would like to assess how FANCL-deficient cells contribute to genomic instability following DPC induction. Specifically, we can quantify levels of micronuclei using immunofluorescence microscopy and conduct metaphase spreads to visualize chromosomal aberrations, such as potential chromatid breaks and radial chromosome formation. Conceivably, inactivation of the FA pathway may result in error-prone DPC repair pathways becoming dominant; thus, genomic instability
could be a consequence of either unrepaired DPCs or mutagenic activity of alternative pathways.

Now that we have established that FANCL deficiency incurs elevated formaldehyde-induced DPC accumulation and retention, it would also be salient to assess the extent to which the FA pathway effectively removes DPC lesions of varying sizes \textit{in vivo}. Per a technique developed in the Li lab, we can prepare site-defined monovalent streptavidin adducts (mimicking DPCs) on oligonucleotide duplexes containing a biotin-labeled thymine base. Our lab has also prepared single- and triple-FLAG-conjugated oligonucleotide duplexes. Each of these protein-conjugated duplexes can then be ligated into expression vectors, placed between the promoter and the luciferase coding region, thereby allowing luciferase activity to serve as an indicator of successful DPC removal. These reporter substrates (and their respective negative controls) would then be transfected into wild-type and FANCL-deficient cells and incubated to allow time for repair. The extent of luciferase activity would then be assessed and compared to control experiments using expression vectors without a protein adduct. Luciferase expression would indicate that the protein adduct has been successfully removed. Thus, in comparison to wild-type cells, the level of luciferase expression in mutant cells would indicate whether the FA pathway is required for removal of DPCs of varying size ranges.

Evidence of direct interaction of FA proteins with DPC lesions would further support our hypothesis that the FA pathway is involved in the repair process. To address this possibility, we could use an episomal chromatin immunoprecipitation (eChIP) assay, a method previously developed in the Li lab. This method involves
ligating a duplex oligonucleotide containing a single defined lesion (in this case, either a monovalent streptavidin adduct or a single- or triple-FLAG-conjugated DPC) into a vector downstream of the EBV viral replication origin. This vector would then be transfected into HeLa-EBNA (Epstein-Barr nuclear antigen-1) cells, where the substrate can be replicated until the fork becomes stalled at the adduct, upon which we could then measure the enrichment of certain FA proteins recruited to the damage site by ChIP. In tandem, these adduct-containing eChIP vectors would also be transfected into HeLa and 293A cells (without EBNA). Lack of EBNA prevents replication initiation of this vector in these cells, allowing the same enrichment experiment outside of a replication context to observe if similar FA repair factors are recruited. Key FA proteins that could be assessed for enrichment include FANCA, FANCD2, FANCM, and SLX4. If we were only to observe upstream FA proteins enriched at the damage site (e.g., FANCM), this could imply that these proteins are signaling other repair processes rather than initiating repair directly via downstream FA proteins. We would also expect to recover SPRTN when the eChIP is performed in the replication context. At least one piece of evidence also suggests that SPRTN may have a replication-independent function, as the protein appears to be recruited to chromatin outside of S phase as well [Delabaere et al., 2014]; therefore, it is conceivable that SPRTN may also be recovered when the eChIP is performed without replication.

Our studies were also the first to directly show that histone DPCs accumulate following formaldehyde treatment, as demonstrated by our enhanced RADAR assay using histone H3 immunodetection. While our initial results on this front clearly
demonstrated a dose-dependent increase in histone H3 DPCs following formaldehyde treatment (in line with our original hypothesis), we were unable to ascertain a consistent pattern for relative H3 DPC accumulation amongst our sets of NER and FA mutants across several biological replicates. As we demonstrated with sets of 293A wild-type cells, biological replicates were considerably less consistent with the RADAR assay relative to our ARK assay, following formaldehyde treatment. Another intrinsic problem with using an immunodetection-based assay to quantitate specific types of DPCs is that it relies on antibody detection of the epitope, which may or may not be present if the protein moiety is proteolytically cleaved to achieve size reduction before harvesting/lysing cells. As such, this assay might not be detecting histone (or other) DPCs that have already been sufficiently processed prior to downstream repair events. This caveat may or may not be contributing to the lack of consistency we have observed across biological replicates for formaldehyde-induced histone H3 DPC detection. As a whole, global detection of DPCs using the ARK assay offers a much more consistent and reliable means of quantitating DPC accumulation compared to the protein-specific RADAR immunodetection assay.

Lack of consistency amongst biological replicates has been corroborated by other labs using the RADAR assay (unpublished). Therefore, we believe that the ARK assay provides the most meaningful and robust readout of DPC detection and should be applied to future studies to assess global DPC accumulation. Indeed, there are several lines of experimentation we would like to pursue on this front.

First, to further extend the resourcefulness of the ARK assay in expounding upon DPC repair mechanisms, we would like to investigate the interaction between
the NER and FA pathways and potential proteolytic mechanisms facilitating DPC cleavage. While we know that SPRTN plays a key role in degrading at least some portion of DPCs, it remains unclear the functional relationship between SPRTN and other potential repair pathways, such as FA. Ideally, we would like to generate double- and triple-knockout 293A and HeLa mutants in SPRTN and SPRTN/FANCL to study their genetic interactions and potential epistatic relationships. Presently, we are limited in our capacity to generate and validate such a set of knockouts, given that we lack access to a functional SPRTN antibody. We would be able to carry out genotyping of CRISPR/Cas9-mediated SPRTN knockouts; however, validation of protein-null status – an essential follow-up step – is not currently feasible in our hands. Similarly, lack of a SPRTN antibody makes siRNA-mediated SPRTN knockdown impractical as well. An alternative approach, as was done with the TK6 SPRTN-deficient cells we received for our studies, would be to utilize reverse transcription PCR as a means of determining SPRTN transcript levels following CRISPR-Cas9; this technique indirectly validates lack of protein expression.

While SPRTN is known to play a definitive role in DPC repair, whether other proteolytic strategies may also be utilized remains unclear. Several studies have offered evidence suggesting that the proteasome is involved in a DPC repair pathway [Quievryn and Zhitkovich, 2000; Reardon and Sancar, 2006; Reardon et al., 2006; Baker et al., 2007; de Graaf et al., 2009], though this notion remains somewhat controversial in the field. Future studies should address whether the proteasome might serve as either an additive or alternative approach to SPRTN-mediated proteolytic degradation in processing DPCs, as well as whether the
proteasome works together with the FA pathway in mediating DPC repair. Preliminary experiments aimed at ascertaining whether proteasome function contributes to DPC repair would entail treating cells with MG132 (a competitive inhibitor of the proteasome) prior to DPC induction with either formaldehyde, 5-azadC, or camptothecin, then proceeding with the ARK assay. Increased DPC levels in MG132-treated cells relative to proteasome-proficient cells would suggest a role – either direct or indirect – for the proteasome in DPC repair. If MG132 treatment increases DPC accumulation or retention, we would then want to assess whether that effect is compounded in FANCL knockout cells relative to wild-type cells. (Given that MG132 is a pleiotropic agent that affects a wide variety of cellular processes, we cannot ascertain whether the data we generate following its administration is a direct consequence of its effect on DPC proteolysis. It is conceivable that proteasome inhibition – and/or ubiquitin pool reduction – may indirectly hinder DPC repair-related processes. The scope of these experiments would instead assess the general requirement for the proteasome in enabling DPC repair, rather than specific degradation of the protein moiety of the DPC.)

To provide an understanding of whether the proteasome plays a role in addition to SPRTN in processing DPC intermediates, wild-type and SPRTN-knockout TK6 cells would be treated with or without MG132 prior to DPC induction and DPC coefficients would be assessed following the ARK assay. If cells that are deficient in both SPRTN and proteasome activity have increased DPC accumulation relative to cells that are only deficient in SPRTN, this would suggest that the
proteasome might provide an additional pathway for repairing at least some portion of DPCs.

As an assessment of ubiquitination in DPC repair, wild-type, FANCL knockout, and SPRTN knockout cells could be treated with ubiquitin-vinyl-sulfone, a deubiquitylating enzyme inhibitor that disables ubiquitin recycling, depleting the ubiquitin pool. If treatment with this inhibitor increases DPC levels, this would suggest a role for either direct ubiquitination of the protein moiety or perhaps reliance on ubiquitin signaling in promoting DPC repair.

In a broader context, it is also important to note that our experiments using XPA\textsuperscript{−/−} and FANCL\textsuperscript{−/−} mutants were carried out with single knockout clones. An important internal control to further validate our findings and utilize in subsequent experiments would entail use of additional knockout clones for each single- and double-knockout mutant, possessing alternative biallelic frameshifting mutation genotypes.

In summary, the ARK assay offers a robust, sensitive, and versatile method of detecting global DPCs induced by a variety of agents, and we have shown that it can be applied to demonstrate the relative importance of various repair proteins and pathways. Critically, our studies provide key evidence that the Fanconi anemia pathway plays a role in DPC repair. Future studies will enhance our understanding of precisely how the Fanconi anemia pathway mitigates this potentially lethal form of genotoxic stress.
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