GENOME INSTABILITY: OLD PROBLEM, NEW SOLUTIONS

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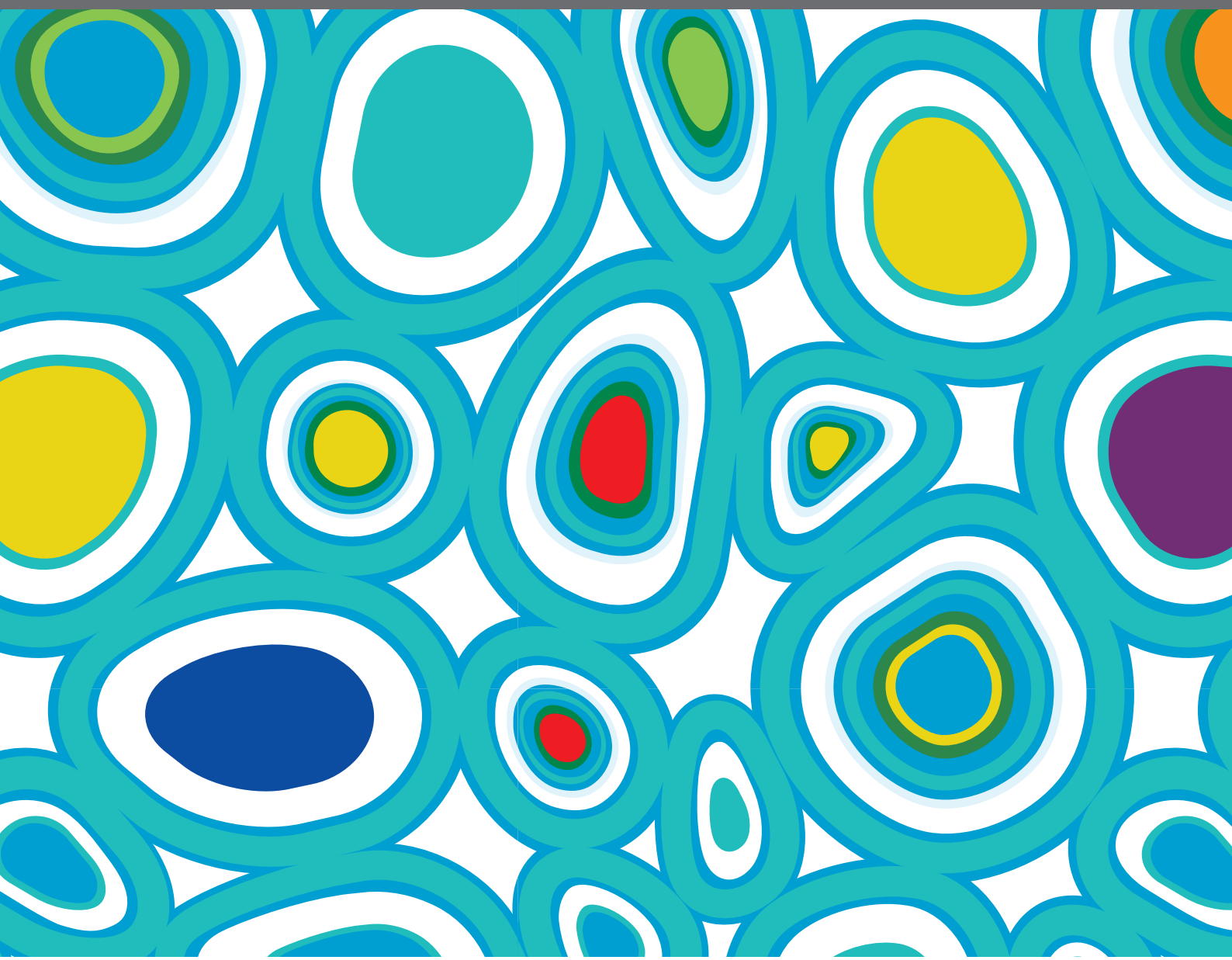

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GENOME INSTABILITY: OLD PROBLEM, NEW SOLUTIONS

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EDITORIAL
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Editorial: Genome Instability: Old Problem, New Solutions

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Keywords: Genomic Instability, DNA Repair, DNA Damage, Emerging methods, DNA Replication

Editorial on the Research Topic

Genome Instability: Old Problem, New Solutions

Cells must accurately preserve the genetic information contained in their DNA and faithfully pass that information on to the next generation. However, DNA is not an inert molecule, so a number of different DNA lesions must be detected, signalled, and repaired by the DNA damage response (DDR) machinery to avoid the genomic instability that contributes to ageing, neurodegeneration, and oncogenic processes.

Research interest in the mechanisms of DNA damage signalling and repair, and their relationship to DNA replication, telomere maintenance and other cellular signalling pathways has increased dramatically in recent years, facilitated by a number of technological and conceptual advances. Our goal in this special issue was to highlight how new and emerging methods and concepts are helping us to solve this old problem.

When cells suffer damage to their DNA, it is important to signal the occurrence of these lesions, both to recruit the DNA repair machinery and to coordinate DNA repair with other cellular processes. DNA damage signalling relies heavily on posttranslational modification of proteins, such as phosphorylation by the kinases ATM, ATR, and DNA-PK. However, it is becoming increasingly clear that other modifications such as ubiquitination and ADP-ribosylation also play a central role. Foster et al. review recent progress in developing proteomic, biochemical, and structural techniques to understand the mechanisms by which ubiquitination regulates DNA repair. The authors discuss CRISPR screening, chromatin mass spectrometry, nascent chromatin capture, cryo-EM, and more specific tools to study ubiquitin signalling, including the development of TUBEs and bispecific antibodies, ubiquitin chain quantification, and UbiChem mass spectrometry (Foster et al.). Meanwhile, Schutzenhofer et al. reviewed the mechanisms by which PARP1 and PARP2 catalyse ADP-ribosylation at DNA damage sites and highlighted the role of the auxiliary factor HPF1 in ADP-ribosylation of serine residues and of the (ADP-ribosyl)hydrolase ARH3, which is required for removal of this signal. They propose HPF1 and ARH3 as new potential cancer biomarkers and drug targets, while deficiency of ARH3 may be a novel mechanism for resistance to PARP1 inhibitors (Schutzenhofer et al.).

One of the most important roles of DNA damage signalling pathways is to suppress genomic instability induced by DNA replication stress, both by reducing encounters between unrepaired DNA lesions and the replication machinery and by regulating the response to stalled and/or collapsed replication forks. Fagundes and Teixeira provided a comprehensive overview of the consequences of oncogenic hyperactivation of the cyclin E/CDK2 complex, which triggers DNA replication stress via impaired replication origin firing, insufficient nucleotide biosynthesis and transcription-replication collisions. Zhang et al. review an important recent conceptual advance in our understanding of DNA replication stress in human cells. Using DNA fibre assays to visualise

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replisome encounters with DNA interstrand crosslinks (ICLs), the authors observed a restart of DNA synthesis on the distal side of ICLs, which were previously considered an absolute block to replisome progression. Furthermore, the authors suggested that different factors are required for this process depending on the chromatin status of the replicating locus (Zhang et al.). It is understandable that these complex cellular responses to DNA replication stress may vary between different cell types. Matos-Rodrigues and Martins present a review of tissue-specific studies to understand responses to replication stress during eye development, thus highlighting the high heterogeneity of these pathways, particularly in progenitor cells.

Important technical advances in imaging and microscopy-based approaches have revolutionised the field in recent years, proving that "seeing is believing". Zentout et al. review state-of-the-art microscopy approaches for spatiotemporal analysis of DNA repair factor behaviour in living cells, including tools for inducing localised DNA damage, analysing repair factor recruitment to the site of damage, and protein turnover during repair. They also emphasise the need for mathematical models to ensure appropriate interpretation of experimental data. Kong and Greene discuss advanced single-molecule imaging methods used to study DNA-protein interactions, and how their application provides mechanistic understanding of double-strand break repair by homologous recombination (HR) and non-homologous end joining (NHEJ). Their in-depth overview of presynaptic filament formation, homology search, and DNA synapses highlights the remarkable mechanistic insights offered by single-molecule approaches that will continue to impact DDR research in the years to come (Kong and Greene).

Another topic of growing interest is the role of DNA repair factors at specific genomic structures, in the crosstalk between different DNA repair pathways and between DNA repair and other signalling cascades. In this context, De Rosa et al. discuss the role of base excision repair and, in particular, the 8-oxoguanine DNA repair system, in protecting telomeres from oxidative stress, highlighting the guanine oxidation system (GO) and its key players OGG1, MUTYH, and MTH1. Busatto et al. offer new mechanistic insights into DNA lesion repair induced by topoisomerase 2 inhibitors: the transcription-coupled nucleotide excision repair protein CSB interacts with TOP2A/B and stimulates TOP2-mediated DNA cleavage. The authors suggest that CSB deficiency leads to a delay in TOP2-mediated R-loop resolution and thus an increase in genomic instability. Cinat et al. examine inflammatory responses triggered by DNA damage-induced cytoplasmic DNA and secretion of senescence-associated cytokines, both of which affect the adult stem cell microenvironment, with important implications for self-renewal, and thus degenerative states caused by stem cell exhaustion. Meanwhile, Oliveira et al. show that chemical inhibition of APE1/Ref-1 reveals a partial overlap of the redox and DNA repair functions of APE1 in modulating transcriptional responses during LPS-induced inflammation and identifies transcriptional master regulators mediating these activities.

We would like to thank all authors and reviewers for accepting our invitation to contribute to this special issue and hope that these articles can serve as valuable resources for the community.

AUTHOR CONTRIBUTIONS

VK, MP, and NH contributed equally for the editorial process of the special issue and for the writing of this editorial.

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Replication of the Mammalian Genome by Replisomes Specific for Euchromatin and Heterochromatin

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Replisomes follow a schedule in which replication of DNA in euchromatin is early in S phase while sequences in heterochromatin replicate late. Impediments to DNA replication, referred to as replication stress, can stall replication forks triggering activation of the ATR kinase and downstream pathways. While there is substantial literature on the local consequences of replisome stalling–double strand breaks, reversed forks, or genomic rearrangements–there is limited understanding of the determinants of replisome stalling vs. continued progression. Although many proteins are recruited to stalled replisomes, current models assume a single species of "stressed" replisome, independent of genomic location. Here we describe our approach to visualizing replication fork encounters with the potent block imposed by a DNA interstrand crosslink (ICL) and our discovery of an unexpected pathway of replication restart (traverse) past an intact ICL. Additionally, we found two biochemically distinct replisomes distinguished by activity in different stages of S phase and chromatin environment. Each contains different proteins that contribute to ICL traverse.

Keywords: replication stress, replisome, CMG, FANCM, DONSON, GINS

INTRODUCTION

The replication machinery consists of a helicase to unwind parental strands and DNA polymerases and primase to synthesize daughter strands (Li et al., 2020). Replisomes also contain accessory factors that stabilize the association of the polymerases with DNA, contribute to the superstructure of the complex, and are important for initiation of replication (Bai et al., 2017; Douglas et al., 2018; Baretić et al., 2020; Li et al., 2020). The helicase contains a six subunit off-set open ring structure formed by the MCM (M) proteins and is loaded on duplex DNA, only in G1 phase, at sites that

may become origins of replication (origin licensing) (Deegan and Diffley, 2016). In S phase MCM complexes accumulate additional proteins, including CDC45 (C) and the four GINS (G) proteins. This association is accompanied by localized DNA melting, locking of the MCM ring around the template strand for leading strand synthesis, and activation of the CMG helicase (origin firing). While the locked ring confers resistance to detachment it would seem to pose insurmountable problems when the replisome encounters large impediments (**Figures 1A,B**).

Replication stress is imposed by blocking either the CMG or the DNA polymerases. Most experiments target the polymerases taking advantage of drugs that are direct inhibitors or suppress nucleotide triphosphate synthesis (Schwab et al., 2010; Whalen and Freudenreich, 2020). However, this strategy cannot report the consequences of replisome encounters with helicase blocks. To model these events, we developed an experimental approach based on interstrand crosslinks (ICLs), always considered impassable blocks to replication (Marmur and Grossman, 1961) and potent inducers of replication stress (Vesela et al., 2017; Renaudin and Rosselli, 2020). Crosslinking agents are highly toxic to growing cells and are frequently used in cancer chemotherapy (Rycenga and Long, 2018).

Understanding replication dependent ICL removal in mammalian cells was a considerable challenge for decades. Most models described stalling of a replisome at an ICL followed by unlinking of the duplex strands (unhooking) after which the replication fork could be rebuilt to allow resumption of synthesis (Kuraoka et al., 2000; Muniandy et al., 2010). Although genes were identified as being important for repair, notably those linked to Fanconi Anemia, there was little insight regarding events following fork encounters with ICLs. This changed with the development by the Walter group of a Xenopus egg extract system which supported replication of a plasmid with a site-specific crosslink. They observed that replication was completed on either side of the ICL before unhooking (Raschle et al., 2008) and that repair occurred after replication on both sides of the ICL was concluded (Zhang et al., 2015). Their observations have been very influential and their model has replaced earlier depictions of ICL repair.

Although the Xenopus extract system is very powerful, the extent to which it recapitulates replication fork encounters with genomic ICLs in living mammalian cells is unclear. To address this, we designed a strategy based on DNA fiber technology (Schwab and Niedzwiedz, 2011). Although this technology has been applied to studies investigating the influence of DNA damaging agents on DNA replication (Merrick et al., 2004; Elvers et al., 2011; Li et al., 2018), it was not possible to distinguish between a global response to stress vs. local effects due to fork encounters with a DNA adduct. To overcome this limitation we exploited the properties of psoralens, which are photoactive crosslinking compounds (Hearst et al., 1984). Psoralens form a high frequency of ICLs, more than 90% with the trimethyl psoralen (TMP) used in our experiments (Lai et al., 2008; Muniandy et al., 2010), and can be conjugated to an antigen tag without altering the crosslink: monoadduct ratio (Huang et al., 2013).

RESULTS AND DISCUSSION

Replication Tract Encounters With Digoxigenin Tagged Trimethyl Psoralen

To visualize ICLs we linked TMP to digoxigenin, frequently used as an immunotag (**Figures 1C,D**; Thazhathveetil et al., 2007). Cells were incubated with Digoxigenin Tagged Trimethyl Psoralen (Dig-TMP), exposed to long wave UV (UVA), and pulsed successively with nucleoside analogs to label newly synthesized DNA. Replication tracts were displayed on DNA fibers by immunofluorescence against the analogs. The ICLs were visualized by immunoquantum dot detection (Simons et al., 2015; Kong et al., 2016). Less than 10% of tracts had an encounter, and, as anticipated, we observed both single and double fork stalling events at ICLs (Raschle et al., 2008). Notably, however, a major outcome of our analysis, one that we termed replication traverse, was the restart of DNA synthesis past intact ICLs (**Figures 1E,F**; Huang et al., 2013). While replication restart past monoadduct blocks has been known for many years (Rupp and Howard-Flanders, 1968; Heller and Marians, 2006; Lehmann and Fuchs, 2006; Taylor and Yeeles, 2018; Guilliam and Yeeles, 2020), our observations were contrary to over 50 years of conventional wisdom (Marmur and Grossman, 1961). However, ICL traverse has been confirmed by recent work from other laboratories (Mutreja et al., 2018; González-Acosta et al., 2021).

Comparison of the lengths of tracts with or without ICL encounters indicated that traverse required only a few minutes. We also found that ICLs embedded in replication tracts were unhooked (first repair step) over a period of several hours. Although the time required for unhooking an individual ICL is not known, it is apparent that resolving the population of replication associated ICLs occurs over a much longer time than traverse (Huang et al., 2019).

The Walter group showed that the immediate product of double fork collisions on either side of an ICL was an "X" structure. This is also the product of ICL traverse once Okazaki fragment ligation has occurred (Huang et al., 2013; Zhang and Walter, 2014; **Figure 1F**). Consequently, the traverse pathway and the less frequent double fork collisions provide options for completing replication on the distal side of a block. Relative to a stalled single fork, the much greater frequency of these two options points to an evolutionary cost benefit analysis that favors the completion of S phase over removal of the impediment. We have proposed the term "replication imperative" to characterize the priority of replication over lesion repair (Yang et al., 2019).

ATR and FANCM Are Important for Replication Traverse of ICLs

Replication stress activates the damage responsive kinase, ataxia telangiectasia and Rad3-related (ATR), which has hundreds of substrates, including MCM proteins and those involved in restarting stalled forks (Cortez et al., 2004; Matsuoka et al., 2007). The embryonic lethality of ATR knockout mice (O'Driscoll, 2009) emphasizes the importance of the response pathways to cell and organismal viability. Inhibition of ATR completely suppressed

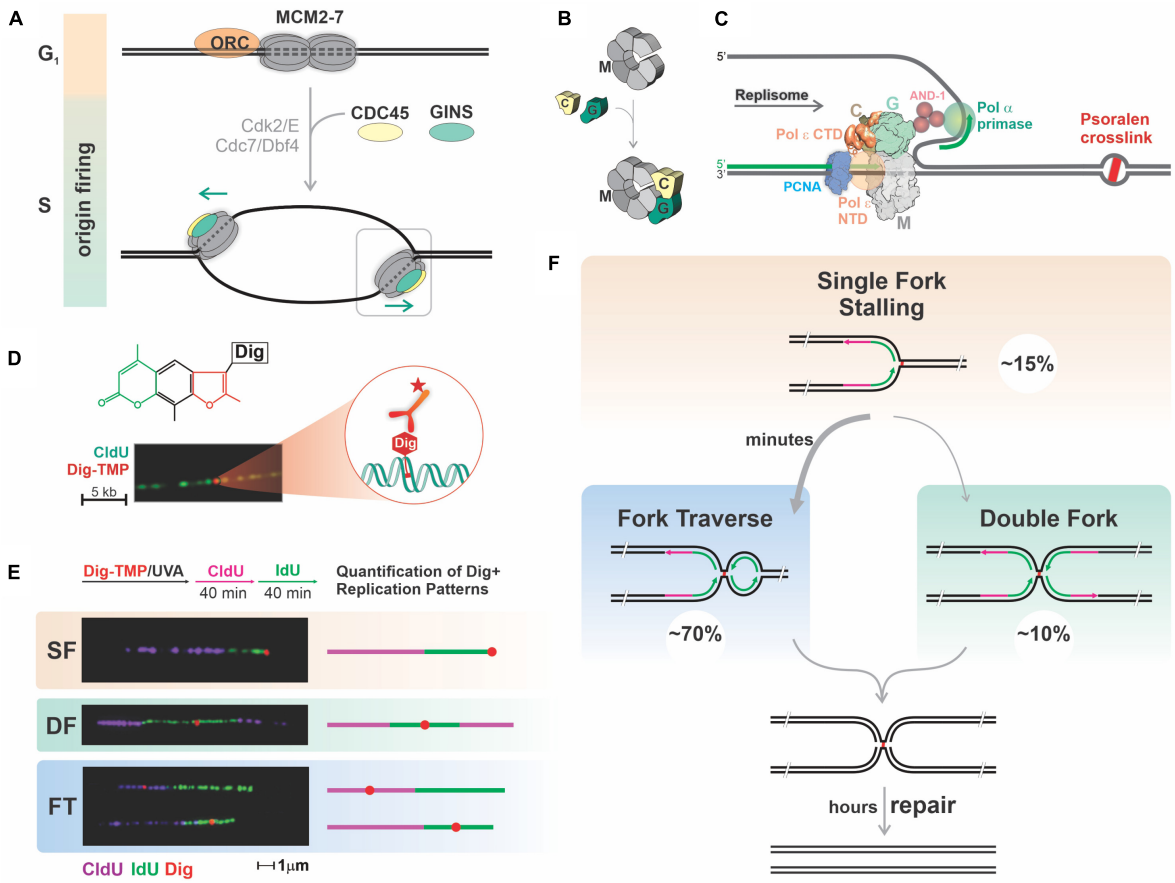

FIGURE 1 | Mammalian replication forks faced with potent blocks restart DNA synthesis past the block, prioritizing replication over repair. **(A,B)** Several mechanisms ensure proper regulation of replication origin firing to prevent re-replication of chromosomal DNA. Origin licensing, the process of loading double hexamers of MCM2-7 (M) rings onto dsDNA at many potential origins, occurs exclusively in G1 phase under conditions that prevent initiation. Upon transition into S phase, some of these pre-replicative complexes are activated by association with CDC45 (C) and GINS (G) accompanied by melting of duplex DNA and locking of the MCM ring around the leading strand template. The temporal uncoupling of origin licensing and origin firing restricts replication to once per cell cycle. **(C)** DNA lesions covalently linking the two DNA strands pose a block to the CMG helicase pulling through the leading strand template. The replisome includes several proteins interacting with the CMG helicase, such as DNA Polymerases α, δ and ε (Pol ε Carboxy and N terminal domains are depicted), PCNA and CTF4. **(D)** Tagging of TMP with Digoxigenin (Dig-TMP) permits detection of a single ICL on a DNA fiber with Quantum-dot conjugated antibodies against the digoxigenin tag. **(E,F)** Quantification of replication patterns in the vicinity of ICLs: cells are treated with Dig-TMP/UVA and labeled with pulses of CldU and IdU, followed by DNA spreading, immunostaining, imaging, and quantification. Representative images and schemes of the replication patterns observed and their corresponding percentage. SF, single fork stalling; DF, converging double forks; FT, fork traverse. About 70% of the patterns correspond to fork traverse, which takes 5–6 min to complete. Fork traverse and double fork conversion would result in the same structure. Unhooking of the population of ICLs takes hours.

ICL traverse indicating that it was a component of the ATR response to replication stress.

The traverse pathway was partially dependent on the activity of the DNA translocase FANCM, a substrate of ATR (Huang et al., 2013). Expression of a phospho-resistant, or a translocase inactive, form of FANCM in a FANCM knockout cell, reduced traverse frequencies to levels equivalent to those displayed by the knockout cells (Huang et al., 2019). Thus, the traverse option was dependent on a translocase activity under ATR control. It should be noted that the CMG helicase has no translocase activity while FANCM has no helicase activity (Meetei et al., 2005).

Chromatin Immunoprecipitation (ChIP) against FANCM from cells exposed to TMP/UVA demonstrated an interaction with replisome proteins. Also recovered was MCM2 phosphorylated at Serine 108, a site of ATR-dependent phosphorylation and a marker of a "stressed" replisome (Cortez et al., 2004). Importantly, incubation of cells with an ATR inhibitor eliminated pMCM2S108 and abolished the interaction between FANCM and the replisome (Huang et al., 2019).

Loss of the GINS in ICL Proximal Replisomes

The locked ring structure of the active replisome and the prohibition on replisome loading during S phase raised questions about replisome composition following collisions with ICLs. We identified a replisome complex in TMP/UVA treated cells containing FANCM, pMCM2S108, but not the GINS. Notably,

the loss of the GINS complex was not affected by translocase defective FANCM. Thus, it was possible to split the role of FANCM into two stages: the displacement of the GINS requiring ATR dependent association with the stressed replisome; the restart of replication, dependent on the translocase function (Huang et al., 2019).

Proximity Ligation Assays (PLA) (Koos et al., 2014) reported the interaction of MCM2 or pMCM2 and the Dig tag on the ICLs, while the PLA between the GINS proteins and the tag remained at background levels. Furthermore, as expected, in cells treated with an ATR inhibitor there was an increase in PLA signal between MCM2 and the ICL and a greatly increased frequency of GINS proximal to ICLs.

These results demonstrated that ICL proximal replisomes, marked by pMCM2, lacked the GINS complex. In addition, the increased proximity of GINS containing replisomes to ICLs following the inhibition ATR is indicative of the accumulation of GINS associated replisomes stalled at ICLs, implying the loss of an ATR-dependent mechanism to release the structural constraints of the CMG. These observations were consistent with a model in which, upon encounters with ICLs, replisomes lose the GINS complex, thus unlocking the CMG ring during the few minutes required for traverse. In the absence of ATR, FANCM is not recruited, there is no traverse, and the GINS complex is retained on replisomes that accumulate at the ICLs (**Figure 2**, lower right panel).

DONSON Contributes to Replication Traverse of ICLs

Our finding that while traverse events were entirely dependent on ATR but only approximately 50% of these depended on FANCM suggested that cells contained another pathway to restart replication. After testing of several candidate proteins we found that traverse frequencies were reduced in cells deficient in DONSON (downstream neighbor of Son) protein, a constitutive replisome component (Evrony et al., 2017; Reynolds et al., 2017). Double knockdown of both DONSON and FANCM

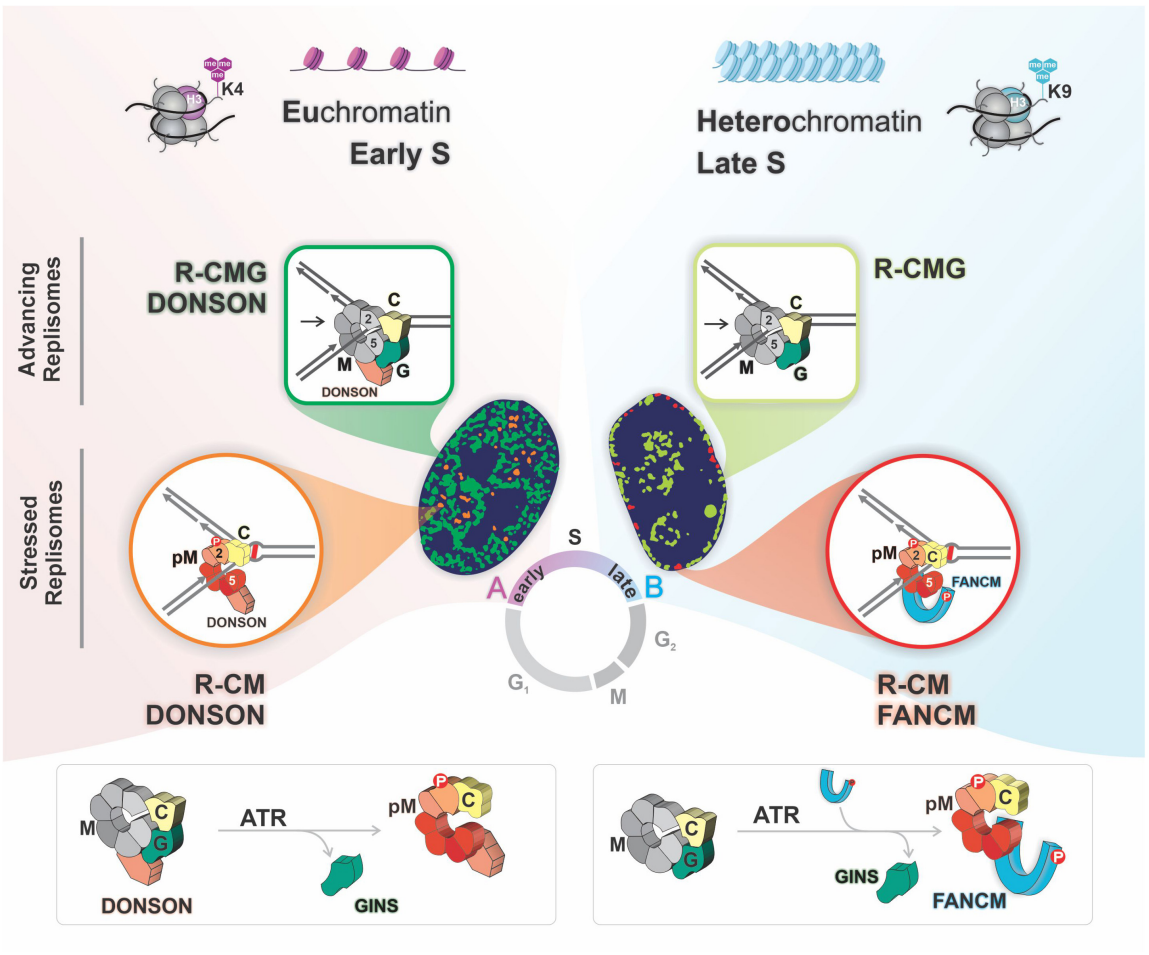

FIGURE 2 | Replisomes with alternative compositions are active in the two chromatin compartments. There are two advancing replisomes, one with DONSON (R-CMG DONSON), biased toward early replicating euchromatin, and one without (R-CMG), preferentially localized to late replicating heterochromatin. In euchromatin the encounter with an ICL triggers MCM2 phosphorylation on serine 108 by ATR, and eviction of the GINS proteins yielding R-CM DONSON. In heterochromatin there is an ATR dependent recruitment of FANCM which is required for the loss of the GINS and the formation of R-CM FANCM.

revealed a decline greater in ICL traverse than with either individual deficiency, indicating that they functioned in separate pathways. PLA analysis indicated that DONSON was proximal to ICLs and the signal frequency rose upon ATR inhibition. These results were consistent with DONSON being retained on replisomes transiently proximal to ICLs, unlike the GINS proteins. Furthermore, following ATR inhibition there was an accumulation of DONSON containing replisomes stalled at ICLs (Zhang et al., 2020).

The presence of replisomes containing DONSON and/or FANCM raised the question of whether they resided within the same or separate replisome complexes. To address this, we prepared chromatin from cells exposed to TMP/UVA. After digestion of the DNA, the solution was cleared of "non-stressed" replisomes by immunoprecipitation against a GINS protein. Then DONSON bound complexes were recovered from the supernatant, after which FANCM associated complexes were captured from the residual supernatant. DONSON was present in both GINS positive and negative replisomes, the latter marked as stressed replisomes by pMCM2S108. FANCM coprecipitated with replisomes that also contained pMCM2S108, but not DONSON. These results were confirmed by PLA. Thus, there were separate and distinguishable stressed replisomes containing either DONSON or FANCM but not both. Furthermore, DONSON clearly had a different role than FANCM because it was associated with both stressed and unstressed replisomes while FANCM was associated only with stressed replisomes (Zhang et al., 2020). This argued against the assumption of a single species of stressed replisome and raised the question: Do these different replisome complexes exist in the same cell at the same time?

To answer this, we performed a sequential PLA experiment in cells exposed to TMP/UVA. After PLA between DONSON and pMCM2S108 the cells were imaged, stripped, and PLA between FANCM and pMCM2S108 performed. Alignment of the first and second images of the same cell demonstrated that the complexes could reside within the same cell at the same time but not at the same place. The frequency of DONSON: pMCM2S108 was biased toward early S phase while FANCM: pMCM2S108 strongly favored late S phase.

Analysis of the DNA sequences associated with the two stressed replisomes supported this conclusion. Alu sequences replicate in early S phase and were found in the DONSON fraction, while Satellite 3 sequences replicate late and were captured in the FANCM fraction. ChIP and PLA analyses of DONSON: H3K4me3 (a euchromatin marker) and FANCM: H3K9me3 (heterochromatin marker) confirmed the localization of the DONSON stressed replisome to predominantly euchromatin while the FANCM-containing stressed replisome was more frequently localized within heterochromatin.

DONSON was originally described as a replisome component in unstressed cells (Reynolds et al., 2017). Consequently, it was of interest to ask about the distribution of DONSON replisomes in cells without treatment with a DNA reactive agent. We again found the same bias toward euchromatin and early S phase as above. FANCM associated replisomes were heterochromatic and were more active in late S phase. The FANCM signal frequency was much lower than in cells with ICL induced replication stress and was likely due to "spontaneous" replisome impediments. DNA fragments bound by the DONSON complex were preferentially located in early replicating regions and in euchromatin, while the FANCM associated sequences were strongly biased toward late replicating regions and heterochromatin.

Outstanding Questions

Why Two Replisomes?

We suggest that the answer lies in the differences between eu- and heterochromatin. Replisomes in euchromatin are more likely to encounter DNA damage (Takata et al., 2013), transcription complexes, and R loops (Hamperl et al., 2017). Deficiencies in DONSON would be expected to adversely influence the response to replication stress in these areas of the genome. DONSON is a member of a group of replication associated proteins, mutations in which result in microcephaly and dwarfism (Bicknell et al., 2011; Evrony et al., 2017; Reynolds et al., 2017; Van Esch et al., 2019; Cicconi et al., 2020; Matos-Rodrigues et al., 2020; Starokadomskyy et al., 2021). Compromised replication through genomic areas with active transcription could have a negative impact on completing S phase and consequently, cell number, resulting in smaller brain and body size. Additional pathology may be derived from stalled replication forks that can activate inflammatory responses through the elaboration of DNA fragments that enter the cytoplasm and stimulate interferon pathways (Ardeljan et al., 2020).

In contrast to DONSON, FANCM does not appear to be a constitutive replisome component. Instead, it is preferentially recruited to replisomes stalled in heterochromatin, most likely at "difficult to replicate" sequences during late S phase (Janssen et al., 2018). FANCM has homologs in archaea (Meetei et al., 2005), and may have evolved, in part, to assist replisomes duplicating sequences with an inclination to block replication. In disorders with mutant FANCM (Bogliolo et al., 2018; Catucci et al., 2018) we would predict an exacerbation of replication stress in regions of heterochromatin (Nikolov and Taddei, 2016).

What Is the Mechanism of Traverse?

Our proposal of restart of replication past ICLs is based on an interpretation of the pattern of nucleoside analog incorporation in DNA fibers. However, these patterns cannot distinguish between multiple explanations for the incorporation. The identification of the molecular machinery responsible for replication traverse of the ICLs is a key question awaiting answer. Some relevant considerations are:

(1) Parental strand replacement synthesis. Standard fiber patterns cannot distinguish between synthesis of daughter DNA strands or replacement synthesis of a parental strand ("nick translation" of the strand). However, in experiments in which parental strands were differentially marked, we have not observed any replacement synthesis (Huang et al., 2013, 2019).

(2) Extension synthesis primed by RNA in an R loop has been described in *Escherichia coli* (Camps and Loeb, 2005). Treatment of cells with RNA polymerase inhibitors blocks

R loop formation (García-Muse and Aguilera, 2019) but had no effect on traverse frequencies. Furthermore, deficiencies in FANCM increase the frequency of R loops (Schwab et al., 2015), but we found that traverse frequencies declined in FANCM mutant or knockout cells.

(3) Is the restart synthesis due to a CMG replisome? Replication traverse of ICLs is inconsistent with an irreversibly locked CMG. We do not know if a CMG that encounters an ICL drives DNA synthesis on the distal side. If so, a gate must transiently open and close. Recent work implies reversible gates in replisomes (Yardimci et al., 2012; Gao et al., 2019) suggesting a mechanism to permit passage across large impediments. There may be more than one gate as the MCM2-MCM5 gate (closed by GINS and CDC45 and used for origin licensing) was not opened in the recent analysis of the CMG gate involved in transitions between single and double strand DNA binding (Wasserman et al., 2019). Furthermore, the GINS were not lost in the Walter group's characterization of replisome movement past a bulky protein adduct (Sparks et al., 2019). The relationship between these results and events in a live cell in which a stalled replisome activates an ATR cascade remains to be determined.

(4) Restart of replication would require priming downstream of the ICL. Recently the Mendez lab described the requirement of the PrimPol primase for about 50% of traverse events (González-Acosta et al., 2021). While these results identify PrimPol as important for traverse they also argue that there are other factors that support repriming downstream of an ICL.

(5) The Lopes group has suggested a requirement for replication fork reversal prior to ICL traverse (Mutreja et al., 2018). Reversal of a replication fork after an encounter would restore duplex DNA to the proximal as well as distal side of an ICL (Kondratick et al., 2021). One of the rationales for fork reversal is that it allows for resolution of the impediment. However, the ICLs were intact at the time of traverse. Consequently, while ICLs might provoke fork reversal it is not clear what contribution this would make to the restart process. One way to assess the relevance of fork reversal to traverse would be to perform the fiber assay in cells deficient in key reversal factors such as RAD51, ZRANB3, and SMARCAL1. These experiments are underway.

Finally, we note the difficulty of addressing many of the mechanistic questions raised by the traverse phenomenon. While the powerful system developed by the Walter group would seem ideal for this inquiry, the restart pathway does not occur in Xenopus egg extracts. Early stage replication in frog embryos is very rapid as a result of many origins with short distances between them. This would favor double fork convergence at ICLs and there may be no need for the traverse option (Semlow and Walter, 2021). Elucidation of the effectors of the molecular steps of traverse will require an assay system that can distinguish fork proximal and distal sides of an ICL. The resolution of current fiber assays is far from adequate and new assays will need to be developed to satisfactorily address these questions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JZ, MAB, RCJ, JH, DP, JG, and HG performed the experiments and generated the concepts derived from their experimental work. MAB prepared the figures. MAB, GS, and MMS wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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New Methodologies to Study DNA Repair Processes in Space and Time Within Living Cells

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DNA repair requires a coordinated effort from an array of factors that play different roles in the DNA damage response from recognizing and signaling the presence of a break, creating a repair competent environment, and physically repairing the lesion. Due to the rapid nature of many of these events, live-cell microscopy has become an invaluable method to study this process. In this review we outline commonly used tools to induce DNA damage under the microscope and discuss spatio-temporal analysis tools that can bring added information regarding protein dynamics at sites of damage. In particular, we show how to go beyond the classical analysis of protein recruitment curves to be able to assess the dynamic association of the repair factors with the DNA lesions as well as the target-search strategies used to efficiently find these lesions. Finally, we discuss how the use of mathematical models, combined with experimental evidence, can be used to better interpret the complex dynamics of repair proteins at DNA lesions.

Keywords: DNA damage, live cell imaging, fluorescence fluctuation analysis, spatio-temporal analysis, kinetic modeling

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DNA REPAIR: A MULTISTEP PROCESS COORDINATED IN SPACE AND TIME

Throughout the lifetime of a cell, its genome is continuously challenged by a variety of stresses which can originate from the cell itself, including metabolic byproducts, or from external sources such as environmental mutagens or radiations (reviewed in Chatterjee and Walker, 2017). These genomic stresses can result in a variety of lesions ranging from base modifications to single- and double-strand breaks (SSBs and DSBs) (Carusillo and Mussolino, 2020). To detect and restore the genomic integrity, cells use highly sophisticated mechanisms, often gathered within the generic term of DNA damage response (DDR). To preserve the genome and avoid accumulation of mutations deleterious for the cell or promoting tumorigenesis, the DDR must achieve two objectives: (i) it has to be highly efficient, meaning that the lesions need to be detected both rapidly and exhaustively, and (ii) it must be accurate, restoring genomic integrity not only at the DNA level, but also at higher scales of the genome organization such as the chromatin folding or the epigenetic marks (Dabin et al., 2016). To fulfill these two criteria, the DDR is organized in a multistep process which will be described in the following (**Figure 1**).

The Initiation Step: Navigating the Crowded Nucleus to Efficiently Detect DNA Lesions

In each human nucleus, about two meters of DNA is wrapped around histone proteins to form a chromatin fiber which itself needs to be folded to fit within a nucleus with a diameter of about 10 μm (Pombo and Dillon, 2015; Ou et al., 2017). It is this dense and complex structure that needs to be constantly scanned by the DNA repair machinery to detect the presence of DNA lesions. This detection is performed by proteins that can sense specific DNA lesions, such as the DNA-glycosylase OGG1 which detects the oxidized form of guanine (D'Augustin et al., 2020) or the Ku complex which binds to DNA ends consecutive to DSBs (Ma et al., 2020). Nevertheless, each sensor faces a paradox: it needs to scan the DNA quickly, to allow a rapid detection of rare lesions, but also needs to be highly specific, which requires a careful and potentially lengthy inspection of the DNA to avoid missing a lesion or initiating illegitimate repair. The strategies developed by the sensors of DNA lesions to resolve this speed/specificity paradox remains the subject of intense research. A common trait shared by many of these sensors is that they explore the nuclear volume by alternating phases of 3D diffusion within the nucleoplasm with transient aspecific binding onto the DNA, which may itself involve short diffusive scans along the double helix (Woringer and Darzacq, 2018). This complex dynamic, often referred as facilitated diffusion, is strongly impacted by the local architecture displayed by the chromatin. It seems obvious that compacted chromatin domains may partially hinder lesion detection but more complex effects of the spatial topology of the chromatin/nucleoplasm interface have also been reported (Baum et al., 2014). Indeed, theoretical and experimental work predict that the smoothness of this interface may impact how exhaustive the search process will be (Condamin et al., 2008; Bancaud et al., 2009).

The Amplification Step: Signaling the Presence of the Lesions

Because the search step might be tedious, once a lesion has been detected, its localization needs to be clearly highlighted to facilitate further repair steps. This highlighting is ensured by multiple signaling pathways that mark the chromatin with specific post-translational modifications (PTMs). A typical example is poly(ADP-ribosyl)ation (PARylation) signaling, which is essential at early steps of the SSB repair and also important for resolving other types of damage. Upon binding to a DNA lesion, the poly(ADP-ribose) polymerase PARP1 will catalyze negatively charged PAR chains on itself and on surrounding proteins in particular histones located nearby the lesion (Kamaletdinova et al., 2019). These PAR chains are recognized by several effector proteins, which promotes their accumulation at the sites of damage. Similar processes occur at DSBs, where the initial complexes detecting these lesions contain the kinases ATM, ATR, or DNA-PKcs, which are responsible for marking the nearby chromatin, among many other regulatory functions (Her and Bunting, 2018; Wright et al., 2018). Therefore, this signaling step, combined to specific protein/protein interactions, amplifies the initial trigger emanating from the sensor proteins. This allows for the local concentration of repair actors, often leading to the formation of so-called repair foci, which is a classical strategy used by the cell to accelerate biochemical reactions.

The Structuring Step: Establishing a Repair Competent Environment

Signaling the presence of the DNA lesion not only promotes the recruitment of later repair actors, but is also crucial to establish an environment favorable to efficient repair (Yasuhara and Zou, 2021). In particular, this involves complex chromatin restructuring processes aimed at facilitating the access to DNA lesions as well as their processing (Smith et al., 2019). These chromatin remodeling processes are controlled by several post-translational modifications targeting histones as well as chromatin remodelers and histone chaperones (Piquet et al., 2018; Rother et al., 2020). This structuring step not only involves changes in the chromatin architecture, but it likely also promotes the establishment of properly organized repair foci. 53BP1 (Mirman and de Lange, 2020) is recruited to DBSs in response to a combination of signaling cues involving histone ubiquitination and methylation and contributes to the formation of repair foci by establishing a recruitment platform for multiple other repair factors (Mirza-Aghazadeh-Attari et al., 2019; Lou et al., 2020). More recently, 53BP1 was also shown to promote liquid-liquid unmixing, a process that could help accumulate factors within repair foci without the need for specific protein/protein interactions (Kilic et al., 2019; Ghodke et al., 2021). Importantly, these 53BP1 foci were also proposed to locally hold the chromatin architecture, to keep it in a configuration favorable for repair (Ochs et al., 2019). Therefore, altogether, the different actors involved in this structuring step, although not directly participating to the resolution of the DNA lesion, can improve the efficiency of the repair and also potentially dictate the pathway that will be chosen for restoring the genome (Xu and Xu, 2020). Indeed, while the early chromatin "breathing" triggered by the joint activities of CHD7 and HDAC1/2 promotes DSB repair by non-homologous end-joining (NHEJ) (Rother et al., 2020), chromatin remodeling via CHD4 rather seems to favor DSB repair by homologous recombination (Qi et al., 2016; Smith et al., 2018).

The Processing Step: Restoring the Genome Integrity

All the steps mentioned so far were important to initiate the restoration of the genome integrity but none of them directly participate in the processing of the DNA lesions. This key step is ensured by sets of actors that each fulfill a specific function. For example, in the context of base excision repair, the damaged base is first excised by a dedicated glycosylase (D'Augustin et al., 2020). This leaves an abasic site that is itself processed by the endonuclease APE1, generating a single-strand break that is then resolved by the combined action of specific DNA polymerases and ligases (Abbotts and Wilson, 2017). Obviously, the choice of the actors involved in lesion resolution depends on the initial detection event but, as described in the previous section, is

also controlled by later steps of the DDR that integrate several sources of information: the type of lesion, the local chromatin landscape, as well as the cell-cycle stage (Hustedt and Durocher, 2017; Her and Bunting, 2018; Schep et al., 2021). Importantly, restoring genome integrity is not restricted to the recovery of the original DNA sequence, it also involves the reestablishment of the chromatin landscape. The activity of several histone chaperones is needed at late stage of the repair process (Chen et al., 2008). These chaperones probably participate in depositing specific histones such as the H3.3 variant, a process that is needed to shut down DNA damage signaling and allow transcription recovery at the damage locus (Kim and Haber, 2009; Adam et al., 2013).

This brief introduction regarding the key steps of the DDR demonstrates that this process is a spatio-temporal orchestra involving a large number of instruments. The studies performed over the last decades have allowed the specific function of many of the repair factors to be uncovered but the current challenge in the field is now to identify the bandmasters able to coordinate all these factors to get them playing in tune and allow efficient repair. Addressing this difficult question relies in particular on the use of quantitative tools able to assess at high spatio-temporal resolution the dynamics of the different repair factors at the sites of damage, but also within the rest of the nucleus. In the following, we will review the tools deriving from fluorescence imaging that are currently available to monitor in living cells the multiple steps of the DDR.

TOOLS TO ASSESS RECRUITMENT KINETICS AT SITES OF DAMAGE

Expressing the Needs: The Right Damage in the Right Place at the Right Time

As described in the previous section, there is a need for a better description of the spatio-temporal dynamics of the repair actors within the cell nucleus after DNA damage induction in addition to the biochemical characterization of the repair machinery. Live-cell fluorescence microscopy is the method of choice to address this question. Using classical single-beam scanning or spinning-disk confocal imaging, one can follow protein dynamics at timescales ranging from tens of milliseconds to hours, with a spatial resolution of few hundreds of nanometers within the 3D space of the nucleus of individual cells (Aleksandrov et al., 2018). Higher spatial resolutions can be achieved by using methods such as stimulated emission depletion or structured illumination microscopy, although this is usually at the expense of the speed of acquisition (Ochs et al., 2019). Ultimately, single-molecule imaging methods allow the behavior of individual repair proteins to be monitored (see below section "Single-Molecule Approaches to Assess Protein Turnover at Sites of Damage" for more details). They remain, however, difficult to use for non-experts and therefore have not yet been applied extensively in the DNA repair field despite having the potential to provide highly valuable information about protein dynamics (Miné-Hattab et al., 2021).

While all the fluorescence microscopy methods mentioned above have been used to study the dynamics of multiple intracellular processes, a specificity of the DNA repair field is that these imaging techniques need to be combined with a way to inflict DNA lesions to be able to follow the cellular response. Ideally, the DNA damaging method should allow a single type of lesion to be induced at a predefined location in the genome and at a time point that can be precisely estimated. It is only under such circumstances that it will be possible to precisely assess the sequence of events associated with the repair of a given type of lesion in the context of a particular chromatin landscape. Unfortunately, to date, such an ideal DNA damaging method does not exist. In the following we will review the methods that are currently available to induce DNA damage and to follow the DNA damage response in living cells using microscopy. We will show how each of these methods only fulfills some of the three criteria mentioned above, making them more or less suitable depending on the question of interest.

Genotoxic Agents, Nucleases, Irradiation: Different Ways to Induce DNA Damage to Answer Different Questions

Three main approaches are currently in use to induce DNA damage in the context of live cell imaging: genotoxic drugs, endonuclease targeting and irradiation using various sources (**Table 1**). Genotoxic agents have been used for many years to induce DNA lesions, with the advantage that some of them are used in the clinic as anticancer agents. These agents display two modes of action. They can either directly alter the DNA or inhibit the activity of some cellular factors, ultimately leading to DNA damage. A well-known example of the first category of genotoxic agent is cisplatin, which induces intra- or inter-strand crosslinks (Cohen and Lippard, 2001; Hu et al., 2016). Inhibitors of the topoisomerases are a prominent family of molecules within the second category of genotoxic agents (Xu and Her, 2015). These different types of molecules have been used extensively within the DNA repair field. However, as they tend to induce multiple types of damage relatively evenly within the genome and since the time of damage induction cannot be precisely estimated, these genotoxic agents are often not compatible with a precise spatio-temporal characterization of the DNA damage response.

To be able to induce a specific type of lesion at a given locus, several approaches have been developed over the last years based on DNA endonucleases. The expression of I-SceI in cells whose genome integrates the 18-bp recognition site of this nuclease (Rouet et al., 1994) or the use of a construct associating the nuclease domain of the *Fok*I enzymes with the Lac repressor/Lac operator assay (Shanbhag et al., 2010) allows for the induction of DSBs at one or few known locations in the genome. The restriction enzyme *Asi*SI, which recognizes about 150 endogenous sequences along the genome, can also generate multiple DSBs within a given nucleus (Iacovoni et al., 2010). More recently, programmable endonucleases such as Zinc-finger nucleases or CRISPR-Cas9 have been used to induce either single or multiple DSBs at different genomic loci (Morton et al., 2006; Wang et al., 2019; Emmanouilidis et al., 2021, 9).

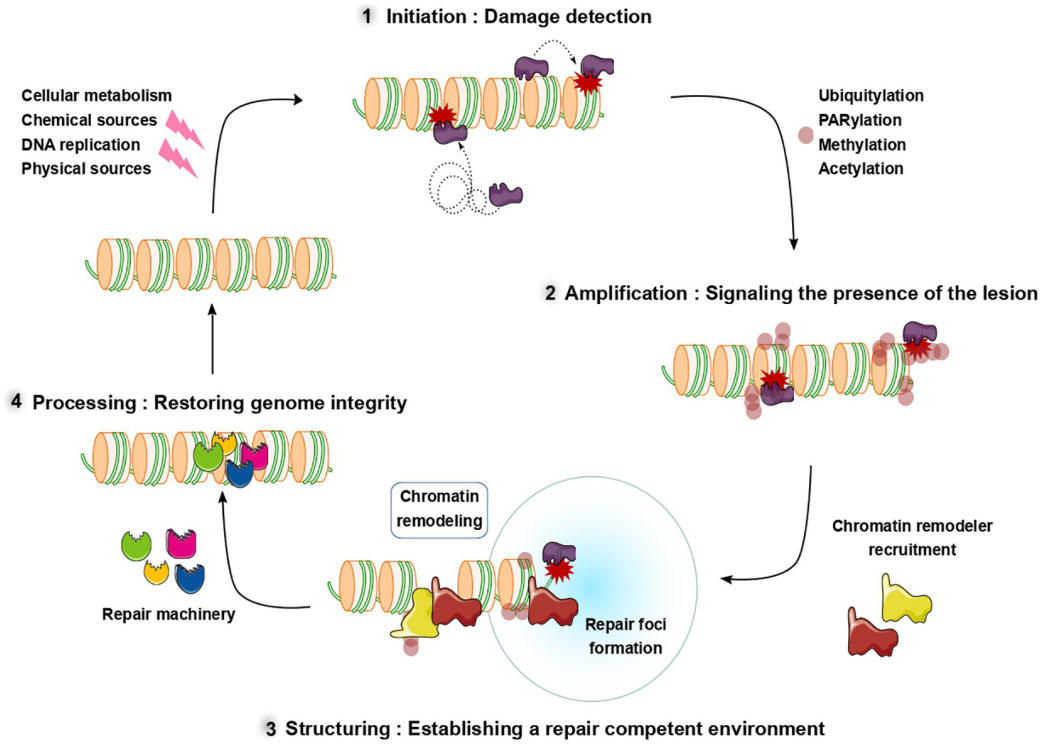

FIGURE 1 | Schematic overview of the multiple steps of the DNA repair process. After damage induction, sensor proteins recognize and recruit to DNA lesions (1) initiating the DNA damage response. In the second phase (2), post-translational modifications signal the presence of the damage and promote the recruitment of downstream repair factors including chromatin remodelers. These downstream factors restructure chromatin to establish a repair competent environment (3) allowing efficient repair. After the DNA lesions are repaired, chromatin is restored to a pre-damaged conformation (4).

TABLE 1 | Comparison between the different methods used to induce DNA lesions.

Tools	Induction of a single type of DNA lesion	Ability to choose the genomic location	Synchronization of damage induction	Characterization of early steps of DNA repair
Genotoxic drugs	+	−	−	−
Endonuclease targeting	+++	+++	+	+
Microirradiation	−	+	+++	+++

Interestingly, some of these enzymes have been mutated to switch from a nuclease to a nickase activity, allowing to generate SSBs (Davis and Maizels, 2014). This strategy is nevertheless inherently limited to the study of DNA breaks. Another limitation of this approach is the poor resolution regarding the timing of damage induction which precludes a precise analysis of the sequence of events composing the DDR. To circumvent this limitation, inducible systems have been developed by fusing the nucleases to nuclear receptors to allow the relocalization of these fusion proteins upon addition of the receptor agonist (Soutoglou et al., 2007; Caron et al., 2012, 2015). More recently, a strategy based on light-inducible uncaging of the guide RNA has also been proposed to trigger damage induction with Cas9 at a specific timepoint (Liu et al., 2020). With these inducible methods, it is possible to reach a precision of a few minutes in terms of the timing of damage induction. While sufficient to analyze repair processes displaying characteristic timescales of tens of minutes or hours, this time resolution is not suitable to monitor the early fast steps of the DDR. The last limitation of the nuclease strategy is the risk of recurrent damage since these enzymes have the potential to reinitiate cleavage as soon as the break is resolved. These breaks may be recognized as unrepairable, leading to the activation of specific pathways (Oza et al., 2009). To limit this problem, some authors have proposed the use of auxin-inducible degron to degrade the nuclease within a timeframe of approximately half an hour and therefore stop damage induction (Aymard et al., 2014).

The third method to induce DNA lesions under the microscope is based on irradiation. This approach allows DNA lesions to be induced locally within the nucleus, with the extent of the damage area depending on the size of the irradiation beam. With this approach, the precise timing of irradiation is known, making this DNA damaging approach particularly suitable for characterizing the initial steps of the

DDR (Gassman and Wilson, 2015; Aleksandrov et al., 2018). Nevertheless, the main drawback of irradiation is that it usually does not lead to the formation of a single type of DNA lesion but rather creates a mixture of damage which are clustered within the irradiation area (Schipler and Iliakis, 2013). The induction of such complex array of DNA damage types are not only problematic for the study of specific repair pathways, potentially leading to seemingly contradictory results depending on the irradiation method, but also represent a major challenge for the cell that may experience difficulties to efficiently resolve these genomic alterations (Asaithamby et al., 2011). In the following, we will briefly review the different irradiation methods that are currently available and describe the different types of DNA lesions that they induce.

Various Modalities of Irradiations for Different Types of DNA Damage

DNA damage induced by irradiation gathers a large number of approaches that differs not only from the type of irradiation sources but also the design of the irradiation scheme in space and time as well as the potential use of chemical sensitizers. Ionizing radiations such as γ-rays, X-rays, or ion beams have been used extensively to generate DNA lesions around which form the so-called ionizing radiation-induced foci (IRIF), where different repair factors accumulate (van Veelen et al., 2005; Jakob et al., 2009; Costes et al., 2010). Depending on the ionizing radiation, the type of lesions that are created can be mainly SSBs and DSBs but more complex types of damage are also observed (Ward, 1988; Datta et al., 2005). One motivation for the use of these ionizing radiation is that they are similar to those used in anticancer radiotherapies and therefore, the analysis of the cellular response improves our understanding of the molecular mechanisms underlying this therapeutic strategy (Mohamad et al., 2017). Nevertheless, the access to some of these radiation sources might be limited. Moreover, directly coupling these sources with fluorescence imaging setups, which are required to monitor the DDR in living cells from its early stages, remains challenging (Hable et al., 2012; Jakob et al., 2020).

The most common sources of irradiation used in the context of the study of the DDR by live cell imaging are the lasers that are either already present in the common set of lines used to excite fluorescence, or that can easily be coupled to the microscope (Holton et al., 2017). Irradiation lasers are divided in two main categories, continuous and pulsed lasers. Continuous 405 nm lasers are available on most confocal setups and therefore represent a widely used method to induce DNA lesions, provided that the cells have been pretreated with sensitizing agents such as the DNA intercalating agent Hoechst 33342 or the nucleotide analog BrdU (Singh et al., 2004; Suzuki et al., 2010; Vítor et al., 2020). Pulsed lasers induce DNA lesions without the need for such photosensitizers. They are often in the near UV (∼350 nm) or infrared ranges (∼800 nm), sparing the visible window for fluorescence imaging. Shorter UV wavelengths are also available although they require the use of specific lenses (Kong et al., 2009; Gassman and Wilson, 2015). Besides their emission wavelengths, these lasers also differ significantly by the duration of the pulses, which ranges from about 150 femtoseconds to few nanoseconds, as well as the pulse rates, which cover six orders of magnitudes. Irradiation with these different laser sources generate multiple types of DNA lesions: base oxidation, crosslinks, SSBs, or DSBs. Although the relative abundance of these types of damage within the irradiated area differs depending on the laser, it is probably difficult to find irradiation conditions that induce only a single type of lesion (Dinant et al., 2007; Kong et al., 2009). With regards to pulsed lasers, nanosurgery data (Colombelli et al., 2007) suggests that shorter pulses induce more local and potentially cleaner cuts, but it remains unclear whether this also holds true for irradiation aiming at inducing DNA lesions.

An interesting new approach is the combination of laser irradiation with targetable photosensitizers. These photosensitizers can be genetically encoded fluorophores such as the Killer-red that tends to generate reactive oxygen species (ROS) upon illumination (Lan et al., 2014), or fluorogen-activating proteins (FAP) that bind a photosensitizer ligand (He et al., 2016). These combined methods might be more specific than simple laser irradiation to induce a particular type of damage such as base oxidation. Moreover, fusing Killer-red or the FAP to domains that localize to specific genomic loci would allow DNA lesions to be introduced within predefined chromatin regions, something that is not easily manageable with simple laser irradiation. Such an example of this targetable DNA damage approach was recently described in a report by Fouquerel et al., in which FAP was fused to TRF1 to allow inducing base oxidation specifically at telomeres (Fouquerel et al., 2019).

Despite the limitations described above, laser irradiation currently remains the method of choice to assess the sequence of processes occurring during the DDR with a well-defined time origin corresponding to the irradiation event. In particular, multicolor live-cell imaging allows easy monitoring of recruitment kinetics of several fluorescently tagged proteins in parallel to the sites of damage (Garbrecht et al., 2018). While the crosstalk between the spectrum of the different fluorophores makes it difficult to assess simultaneously more than three repair factors (Tie and Lu, 2020), a quantitative analysis of the recruitment kinetics of proteins expressed in separated cells still allows for a detailed picture of the complex choreography taking place at DNA lesions to be drawn. In the following section, we will describe tools currently available for such quantification as well as the parameters that can be extracted from this analysis.

Extracting Accurate and Quantitative Information From the Recruitment Data

Current imaging setups allow the accumulation and release of fluorescently labeled repair factors from sites of laser irradiation to be recorded at timescales ranging from tens of milliseconds to hours (Aleksandrov et al., 2018). Classically, a tagged version of the protein of interest is expressed in living cells by transient transfection or by establishing stably expressing clones. However, this results in protein overexpression which can create an imbalance between the different actors of the DDR, potentially leading to artifacts. To overcome this problem, the tagging of the endogenous protein can be achieved by CRISPR/Cas9

based genome editing (Stewart-Ornstein and Lahav, 2016) or via the use of fluorescently labeled nanobodies raised against the repair proteins of interest (Buchfellner et al., 2016; de Beer and Giepmans, 2020). Importantly, the association between the nanobody and its target must be tight enough to ensure that the dynamics of the fluorescence distribution adequately represents the one of the repair protein, but it should also have no impact the function of this protein (Buchfellner et al., 2016). Image sequences of cells expressing the fluorescently tagged repair factors can then be recorded by timelapse imaging after laser irradiation. To be able to precisely assess the recruitment kinetics of these factors at DNA lesions, the first step consists in the quantification of the fluorescence signal within the irradiated area. Manually delineating such region of interest (ROI) on the image sequences can easily be performed using any image-processing tool. However, this manual approach may introduce some user-bias and may also be tedious if the ROI needs to be updated due for example to cell movement during the timecourse (Lebeaupin et al., 2017). Alternatively, automatic segmentation tools can be used to identify the irradiation ROI based on the spatial distribution of the repair protein of interest. The drawback of such approach is that the segmented area may differ from one repair protein to another and may also evolve along with the recruitment kinetic since strong protein accumulation will lead to the segmentation of larger ROIs than for fainter ones. A more appropriate strategy is to include an additional marker that is independent from the repair factors but identifies the irradiated region. This can be achieved by the use of photoactivatable proteins, whose fluorescence is activated by the laser used for irradiation, fused to core histones (Sellou et al., 2016). The irradiated area is then highlighted with good signal-to-noise ratio, allowing an easy automatic segmentation that does not depend on the repair protein of interest. Fluorescent proteins such as PA-GFP (photoactivatable GFP) or PA-TagRFP can be easily converted with continuous 405 nm or pulsed near-infrared lasers (Testa et al., 2008; Lebeaupin et al., 2017). When using other lasers to induce damage, one possibility is to combine them with a 405 nm illumination. Indeed, provided that the cells are not presensitized, the level of 405 nm illumination needed to induce photoactivation is too low to induce significant DNA lesions (Lebeaupin et al., 2017).

Based on the segmentation of the irradiation area as well as the one of a whole nucleus, it is then possible to estimate the overaccumulation of repair proteins at sites of damage relative to the rest of the nucleus and to monitor the temporal evolution of this overaccumulation. Several classical parameters can then be readily extracted from these recruitment curves such as, for example, the time and amplitude of the recruitment peak or the time needed for dissipating half of the peak accumulation (Mistrik et al., 2016; Prokhorova et al., 2021). Alternatively, when focusing specifically on the accumulation phase of the curve, phenomenological models such as first or second order response to a step change can be used to extract characteristic rising times (Bekker-Jensen et al., 2005). These different parameters are useful to compare the relative kinetics of different repair proteins and cluster them based on their dynamic behavior at DNA lesions (Kochan et al., 2017; Garbrecht et al., 2018) but they do not bring much information regarding the molecular mechanisms underlying the accumulation and release of the repair factors. To go one step further, the individual recruitment curves can be fitted with analytical models assuming different scenarios including one or multiple step reactions for the accumulation and the dissipation phases as well as characteristic residency times at DNA lesion. Recently, the Stoynov team analyzed the recruitment kinetics of 70 proteins using such models (Aleksandrov et al., 2018). By clustering the repair proteins based on their characteristic accumulation and dissipation times, they were able to propose some coordination mechanisms between factors in charge of repairing different DNA lesions at sites of irradiation containing multiple types of damages.

A simplification common to all these analytical models is to consider that the diffusion of the repair proteins within the nuclear volume is instantaneous and therefore, that the recruitment kinetics are governed solely by reactions rates associated with accumulation and dissipation at the DNA lesions. Nevertheless, it has been shown that multiple chromatin-interacting proteins display diffusion-limited dynamics in the nucleus (Beaudouin et al., 2006), indicating that protein displacement within the nuclear space needs to be taken into account in addition to the binding and unbinding events onto the chromatin. Unfortunately, differential equations that are derived from models that take both the reaction and diffusion components into account usually cannot be solved analytically, thus precluding a simple fit of the experimental recruitment data with a predefined mathematical expression. Therefore, more elaborated approaches involving the fitting of the data with numerically solved reaction-diffusion models need to be implemented. A clear demonstration of the interest to develop such strategies has been recently highlighted by work from the Luger lab (Mahadevan et al., 2019; Bowerman et al., 2021). Using a Monte-Carlo based model that assumes the repair factors can either diffuse by pure Brownian motion or bind to the DNA lesions, Mahadevan et al. were able to simulate their accumulation kinetics at DNA lesions and adjust these simulations to the experimental data. They show that the nuclear shape has a strong influence on the recruitment curves, highlighting the fact that space matters for repair factors that explore the nucleus searching for DNA lesions. This work opens the way for more complex numerical models describing not only the rising of the recruitment curves but also their decline (see section "The Need for a Quantitative Model to Integrate the Data Obtained From Different Tools" below).

Refined models of the reaction-diffusion processes occurring at sites of damage necessarily come with more unknown parameters. Getting a precise estimation of these parameters requires an increase in data sample size to better catch the cell-to-cell variability. This is achievable by combining regular irradiation patterns designed to hit tens of cells within the microscope field of view simultaneously (Mistrik et al., 2016), with automated analysis pipelines (Oeck et al., 2019). But besides acquiring more data, a better description of the behavior of the repair factors at sites of damage also requires to extend beyond the analysis of recruitment curves. In the next section, we will show how the characterization of the turnover of the

repair factors at sites of damage can bring crucial information to improve our understanding of the mechanisms regulating the successive steps of the DDR.

GOING BEYOND RECRUITMENT KINETICS: THE TOOLS TO ASSESS PROTEIN TURNOVER AT SITES OF DNA DAMAGE

Reasons for Investigating the Turnover of Repair Proteins at Sites of Damage

The development of fluorescence microscopy methods over the last decades have shown that, while the analysis of the steady-state localization of proteins in fixed or live tissues brings valuable information about the function of these proteins, analyzing their dynamics is also essential to understand how this localization is regulated and therefore better describe the molecular mechanisms underlying these functions (Lippincott-Schwartz et al., 2001, 2003). The DNA repair field appears relatively unexplored regarding these questions of protein dynamics compared to related topics such as transcription control (Kimura et al., 2002; Mueller et al., 2008, 2010). Indeed, while the recruitment kinetics of repair proteins at DNA lesions have been studied extensively as described in the previous section, only a limited number of studies addressed the question of the local turnover of these factors within the damaged area.

Yet, such analysis could potentially dramatically change our perception of some of the molecular processes at work in the vicinity of the DNA lesions. A relevant example is the one regarding PARP1 trapping at DNA lesions. As described above, PARP1 recruits rapidly at sites of damage where it triggers the grafting of ADP-ribose chains on nearby targets. The main target of PARylation is PARP1 itself and this process is essential for the dissipation of the protein from DNA lesions, although the precise underlying mechanism remains unclear (Juhász et al., 2020; Prokhorova et al., 2021). The inhibition of the catalytic activity of PARP1 via small-molecule inhibitors leads to a sustained accumulation of this protein at DNA lesions, a process referred to as PARP1 trapping (Murai et al., 2012). A precise definition of this trapping mechanism is essential since it is at the basis of the cytotoxicity of the PARP inhibitors used in the clinic to treat BRCA-deficient tumors. Nevertheless, while chromatin fractionation assays suggest that inhibited PARP1 is stably bound to chromatin (Murai et al., 2012), Fluorescence Recovery After Photobleaching (FRAP) experiments demonstrate that there is a rapid turnover of inhibited PARP1 at DNA lesions (Shao et al., 2020), thus challenging the classical trapping model. This example illustrates the importance of analyzing protein turnover and we will describe below the approaches that are currently available to study this question (**Figure 2**).

Population Approaches to Assess Protein Turnover at Sites of Damage

Fluorescence Recovery After Photoperturbation

One of the most commonly available techniques to assess protein turnover is FRAP as well as its closely related derivatives based on fluorophore photoactivation or photoconversion instead of photobleaching (Bancaud et al., 2010). In the following, we shall refer to all these methods by using the generic FRAP acronym, in which the last letter refers to photoperturbation instead of photobleaching. The basic principle of FRAP is to locally perturb the steady-state spatial distribution of the fluorescence in a cell expressing a protein of interest tagged with a fluorophore. Analyzing how fluorescence redistributes in space and time after this initial perturbation gives access to the local dynamics of the protein. Therefore, performing FRAP at the sites of DNA damage, which could have been induced by laser irradiation but also other approaches such as nucleases, allows for the assessment of the dynamics of the interaction between repair factors and the DNA lesions (Mortusewicz et al., 2007; Kleppa et al., 2012; Campalans et al., 2013).

A classical way to analyze FRAP recovery curves is to estimate the time needed to recover half of the fluorescence lost upon photobleaching (Bancaud et al., 2010). The visual inspection of the recovery curves may also allow the identification of different populations of molecules differing by the stability of their association with DNA lesions. While this semi-quantitative analysis is useful to compare the behavior of different repair factors or the impact of a given cell treatment on protein turnover (Kimura and Cook, 2001), it does not allow for the assessment of the core components regulating this turnover. To go further, one needs to fit the FRAP recovery curves with appropriate models that include the three parameters that can affect this recovery, the diffusion of the protein, assessed by its diffusion coefficient D, as well as its binding and unbinding rates (k_{on} and k_{off}) to DNA lesions (Sprague et al., 2004). Depending on the relative values of these three parameters, protein dynamics follow different regimes. If the characteristic time spent bound to the lesions is long compared to the time spent to move from one binding sites to the next, the proteins are within a so-called reaction-limited regime (Sprague and McNally, 2005). In the opposite situation, the protein dynamics are considered as diffusion-limited. Then, a mixed regime corresponds to the intermediate scenarios. Defining which regime better describes the behavior of the protein of interest is essential for fitting the FRAP data with the appropriate model (McNally, 2008). Furthermore, depending on the reaction-diffusion regime, it might not be possible to properly estimate the three parameters mentioned above. For example, in the diffusion-limited, only a ratio of k_{on} and k_{off} can be estimated (Beaudouin et al., 2006). Noteworthy, while the fitting of the FRAP recovery curves potentially allows for D and k_{off} to be estimated, it does not directly give access to the k_{on} but rather to a pseudo first-order binding rate k'_{on} that correspond to the product between the actual k_{on} and the local concentration of binding sites, which could be DNA breaks or other substrate depending on the studied protein.

Therefore, in combination with the analysis of the recruitment kinetics, the FRAP data can provide relevant information about the mechanisms regulating protein accumulation at sites of damage. Estimating the k_{on} and k_{off} rates would allow one to assess whether, for example, the reduced recruitment of a repair protein A upon knock-down of a co-factor B, is due either to a decrease in the k_{on}, meaning defective association at the DNA lesions, or an increase in the k_{off}, that would correspond

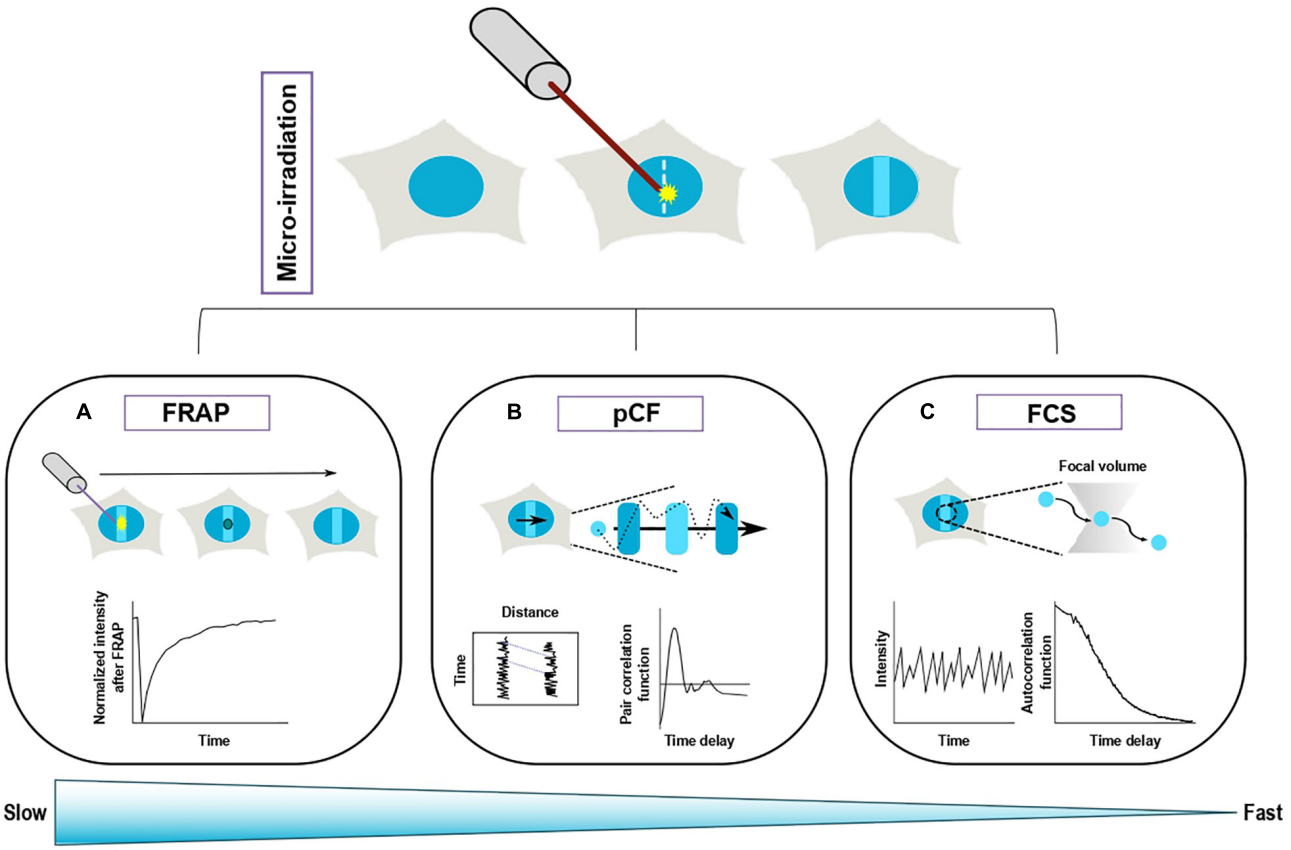

FIGURE 2 | Complementary fluorescence-based methods to study the turnover of repair proteins at sites of DNA damage. Top panel: Recruitment of a fluorescently tagged protein at sites of DNA damage induced by laser irradiation. Bottom panel: Three different methods allow to monitor protein turnover at sites of damage. **(A)** Fluorescence recovery after photobleaching (FRAP) is based on the photobleaching of a sub-region (solid circle) of the area of DNA damage. The fluorescence recovery within the bleached area gives access to protein turnover at DNA lesions. **(B)** Pair correlation function (pCF) assesses the movements of the proteins within a line-scan across the damage region. The fluorescence signals from two different pixels along the acquisition line are cross-correlated, which allows to estimate the characteristic transit time between these two pixels. **(C)** Fluorescence Correlation spectroscopy (FCS) collects the fluctuations arising from the movement of fluorescently tagged proteins in and out of a confocal volume located within the DNA damage region. The characteristics of the protein turnover at sites of damage are derived from the analysis of the autocorrelation of the fluorescence fluctuations. FRAP is more appropriate to assess slow turonver while FCS allows to study fast protein exchange.

to an impaired retention within the repair focus (Smith et al., 2019). Distinguishing between these two hypotheses dramatically impacts the interpretation of the role of the co-factor B in the regulation of the accumulation of protein A at sites of damage. Nevertheless, such fitting approach requires good quality data, which is not always achievable due to the limited time resolution of the FRAP assay. To be able to monitor proteins displaying very fast turnover, it is necessary to switch to fluorescence correlation spectroscopy, that will be described in the next section.

Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) relies on the analysis of fluorescence fluctuations arising from the displacements of fluorescently tagged proteins entering or exiting the parked confocal spot of a laser-scanning setup (Schwille et al., 1997). Focusing the laser beam at the site of damage allow the assessment of protein dynamics within this area (Jeyasekharan et al., 2010; Smith et al., 2019). To quantitatively characterize the dynamics of the proteins, an auto-correlation curve is derived from the fluctuation traces. Similar to half-recovery time derived from the FRAP curves, semi-quantitative estimates such as the characteristic residency time within the focal volume can be obtained from the FCS data. However, based on the same original model as the one used for FRAP, it is also possible to estimate the main parameters controlling protein dynamics at sites of damage, the diffusion coefficient D as well as the k_{on} and k_{off} rates (Michelman-Ribeiro et al., 2009).

Because FCS characterizes protein dynamics at higher sampling rates than FRAP, it can be used to assess faster turnover and also allow a better characterization of the diffusive component. In particular, it can distinguish pure Brownian motion from an anomalous sub-diffusive behavior that would arise from motion hindering by the high level of crowding in the nuclear space (Bancaud et al., 2009). FCS acquisitions with variable sizes of the probed volume allow the characterization of the diffusional behavior of proteins to be pushed even further

(Wawrezinieck et al., 2005; Abdisalaam et al., 2014). In particular, White et al. used such approach to demonstrate that transcription factors explore the nuclear environment by alternating 3D diffusion in the nucleoplasm with transient association with DNA potentially involving 1D diffusive sliding (White et al., 2016). This same approach could be applied to the analysis of DNA repair factors in determining whether they follow the same strategy of nuclear exploration when searching for DNA lesions.

As mentioned above, FCS is more appropriate than FRAP to assess fast protein turnover. Conversely, because it requires that the proteins move within the focal volume to generate signal, FCS is blind to slow turnover. In the following, we will describe a third method that aims at filling the gap in accessible timescales between FCS and FRAP.

Analysis by Pair Correlation Functions

Pair correlation function (pCF) is based on the analysis of fluorescence fluctuations measured along a confocal line scan that arise from the movements of individual fluorescently tagged proteins (Digman and Gratton, 2009). The acquisition of fluorescent signal during a line scan brings an added spatial dimension to fluctuation-based analysis compared to the static FCS. During analysis, the fluorescence signals from two different pixels along the acquisition line are cross-correlated, allowing the characteristic transit time of a given molecule between these two pixels to be estimated. Therefore, pCF has the ability to assess protein dynamics slower than those accessible by FCS and still remains faster than FRAP since it only requires scanning a single line. While this technique has been primarily used to describe the movement of proteins across different cells or cellular compartments (Clark et al., 2016; Hinde et al., 2016), it has recently been applied to the characterization of the turnover of the repair factor of 53BP1 at DNA repair foci (Lou et al., 2020). Using a two-color version of pair correlation analysis, the authors showed that 53BP1 binds to the repair foci as dimer but dissociates from these foci as monomer. This first application of pair correlation to the DNA repair field demonstrates that this technique has the potential to bring unique information about the dynamics of repair factors at sites of damage in the future.

Single-Molecule Approaches to Assess Protein Turnover at Sites of Damage

Recruitment data or fluorescence recovery curves acquired by live cell fluorescence imaging characterize the dynamics of repair proteins at the population level. Fluorescence correlation spectroscopy or pair correlation monitor fluctuations arising from single molecules, but these fluctuations are averaged over time and therefore these methods only give access to a mean behavior of the proteins. Thus, there is a need for an approach to monitor the behavior of repair factors at the single molecule level. Indeed, reaching such resolution would bring invaluable information in particular regarding the way the repair proteins navigate within the nucleus to find the DNA lesions and associate with these lesions. Several super-resolution methods have been proposed to break the diffraction limit and gain access to structural details below ∼150 nm. In the DNA repair field, the gain in spatial resolution brought by these approaches contributed to uncover new functions of proteins participating to the DDR. For example, the characterization of repair foci by super-resolution imaging highlighted the importance of 53BP1 in the maintenance of the local chromatin conformation in the vicinity of the sites of DNA damage (Ochs et al., 2019). Colocalization at the scale of few tens of nanometers helped to prove that BRCA2 contributes to the recruitment of RNASEH2A and control the levels of DNA:RNA hybrids at DSBs (D'Alessandro et al., 2018).

Among these super-resolution methods, single molecule light microscopy (SMLM) has shown to be useful not only in bringing structural insights but also to characterize protein dynamic. When applied in living cells, SMLM gives access to the trajectories of individual proteins (Izeddin et al., 2014). Key features of the initial steps of the DDR can be extracted from the quantitative analysis of these trajectories. Uphoff and coworkers used SMLM to determine the dynamics of polymerase I and ligase molecules searching for DNA gaps and nicks in live *Escherichia coli* cells and estimated that these two factors need about 10 s to find their substrate within the cells and 2 s to resolve it (Uphoff et al., 2013). This finding asks the question of the strategies used by the repair factors to find their target and reconcile the two opposite requirements of an efficient search process: being fast and specific. Multiple *in vitro* data on naked DNA demonstrate that repair proteins perform facilitated diffusion to optimize this search process, alternating between 3D exploration phases and 1D diffusion along the DNA (D'Augustin et al., 2020). Nevertheless, it is unclear whether this also holds true when the DNA is wrapped around nucleosomes and folds in the complex multiscale architecture observed in cell nuclei. Only few publications report 1D diffusion along the DNA in living cells (Hammar et al., 2012; Esadze et al., 2017). Instead, a common feature of multiple nuclear proteins looking for rare targets along the chromatin is their propensity to alternate stretches of 3D diffusion with short unspecific chromatin binding events (Reuter et al., 2014; Normanno et al., 2015; Jones et al., 2017). Whether these transient associations with chromatin underly 1D diffusion along the DNA or not remains unclear but theoretical models indicate that a fine regulation of the switch between DNA-bound and diffusive phases is essential to ensure a rapid finding of the target (Bénichou et al., 2011).

Besides the analysis of these search mechanisms, SMLM approaches also allow the behaviour of the repair proteins to be followed at later stages of the DDR in order to investigate how the local environment established nearby the DNA lesions influences the dynamic behavior of the repair factors. In a recent report, Miné-Hattab et al. analyzed the individual trajectories of Rad52 (the functional analog of human BRCA2) and RFA1 (a member of the RPA complex) in *Saccharomyces cerevisiae* (Miné-Hattab et al., 2021). They demonstrate that RFA1 displays subdiffusive motions similar to those reported for the break itself, while Rad52 shows Brownian motion within the repair foci. Therefore, while both factors accumulate at the repair foci, this accumulation is triggered by two different mechanisms: RFA1 binds strongly to DSBs, in agreement with its role in the protection of single-stranded DNA (Wold, 1997), in contrast to Rad52, which could be confined within the repair foci due to liquid-liquid phase

separation mechanisms (Oshidari et al., 2020). Such detailed analysis comes from the unique ability of SMLM to analyze the trajectories of single molecules, demonstrating its high potential to address complex questions in the DNA repair field.

THE NEED FOR A QUANTITATIVE MODEL TO INTEGRATE THE DATA OBTAINED FROM DIFFERENT TOOLS

The Motivations for the Use of Mathematical Models to Analyze the Spatio-Temporal Dynamics of Repair Factors

Mathematical and computational models are increasingly used to help investigate biological systems in relation to a wide variety of experimental data. In cell biology, the focus has extensively been on cell signaling pathways (Aldridge et al., 2006), leading to the creation of hundreds of models, from a couple of interacting components to huge networks comprising many interacting molecules (Luijsterburg et al., 2010). The objective is to build a model based on reasonable assumptions regarding the behavior of the proteins at scales that are not readily accessible experimentally and to use this model to generate simulated outputs, such as recruitment curves at sites of DNA damage, that could be fitted to the real data (Lengert and Drossel, 2013; Tobias et al., 2013). This allows for the validation of whether the chosen model adequately catches the complexity of the studied biological process and, if so, for the estimation of the values of the unknown parameters of the model. Obviously, the better the initial knowledge about the process, the easier it is to build-up a meaningful model and then fit it to the data to get a precise estimation of the few remaining unknowns.

The Basic Principles Governing the Establishment of Reaction-Diffusion Models

To be more illustrative and explain how to build a model describing the behavior of repair factors at sites of damage, we shall take the specific example of PARP1. As discussed previously, PARP1 can be either in a PARylated or an unPARylated form and is either bound to the lesions, where it is catalytically active, or diffuse within the nucleus. Based on this description, one can build the simple model presented on **Figure 3**. This model is a simplified representation of reality and the conclusion that we will be able to draw when fitting it with the experimental data will be limited to the assumptions we made to build it. Here, for example, a critical assumption is that PARylated PARP1 cannot bind to DNA lesions (**Figure 3A**).

Once the elementary components of the model are established, its mathematical transcription can take two alternative forms: deterministic and stochastic (Cowan et al., 2012). Deterministic models are able to predict the spatio-temporal evolutions of concentrations of proteins according to a set of differential equations describing all the diffusion-reaction processes that impact these concentrations (**Figure 3B**). The stochastic models focus on the molecular scale. The state of each molecule is simulated in space and time and the choice between different elementary events (displacement due to diffusion, binding, etc.), is defined randomly at each time step based on probabilities derived from the reaction rates. By construction, these two types of models have different applications. While deterministic models are used to fit experimental outputs encompassing large number of molecules (recruitment curves, FRAP recovery), which mean behavior is estimated at the population level by their local concentration, the stochastic approach can catch the random characteristics associated with small number of molecules assessed by methods such as FCS or single molecule tracking (Axelrod et al., 1976; Furlan et al., 2019). Both deterministic and stochastic models can then be solved numerically using dedicated software such as Copasi, Virtual Cell, or Berkeley Madonna (Klipp et al., 2005; Cowan et al., 2012), allowing for the prediction of the spatio-temporal evolution of the protein distribution within the whole nucleus upon induction of DNA lesions (**Figure 3C**). Ultimately, it is then possible to generate outputs simulating those obtained experimentally (**Figure 3D**).

Integrating Data From Different Sources to Improve Model Predictions

Fitting the simulated outputs to the experimental data allows a first estimation of whether the model that was chosen is suitable for the studied process. Following the parsimony principle, one should start with the simplest possible model and progressively add extra components only when the simple model does not accurately represent the experimental data (Tyson and Novák, 2015). For example, in the context of the process described in **Figure 3**, one could build up model that is even simpler by assuming that the PARylated status of PARP1 does not impact its ability to interact with chromatin. Nevertheless, in such simplified situation, the simulated recruitment curves would only display an accumulation phase but no dissipation, showing that some key components of the biological process are missing in the model. Several quantitative tools based on the parsimony principle are available to guide the choice between two models of differential complexities, such as the Akaike or the Bayesian information criteria (Burnham and Anderson, 2002). Combined with biological knowledge about the studied process, they allow the definition of models that properly describe the experimental data with the least number of parameters.

Nevertheless, parsimony does not necessarily imply unicity, meaning that two models of similar complexity may equally fit the same experimental outputs. To choose between these two models, it is often necessary to provide additional knowledge to the system by including data from other assays or analyzing the response of the biological system to different perturbations. An interesting example in the context of protein recruitment to DNA lesions, is the report by Lengert et al. (2015). They performed FRAP experiments at the sites of damage at different timings of the recruitment kinetics. By adjusting these combined FRAP/recruitment data with different models that were equally fitting pure recruitment curves, they show

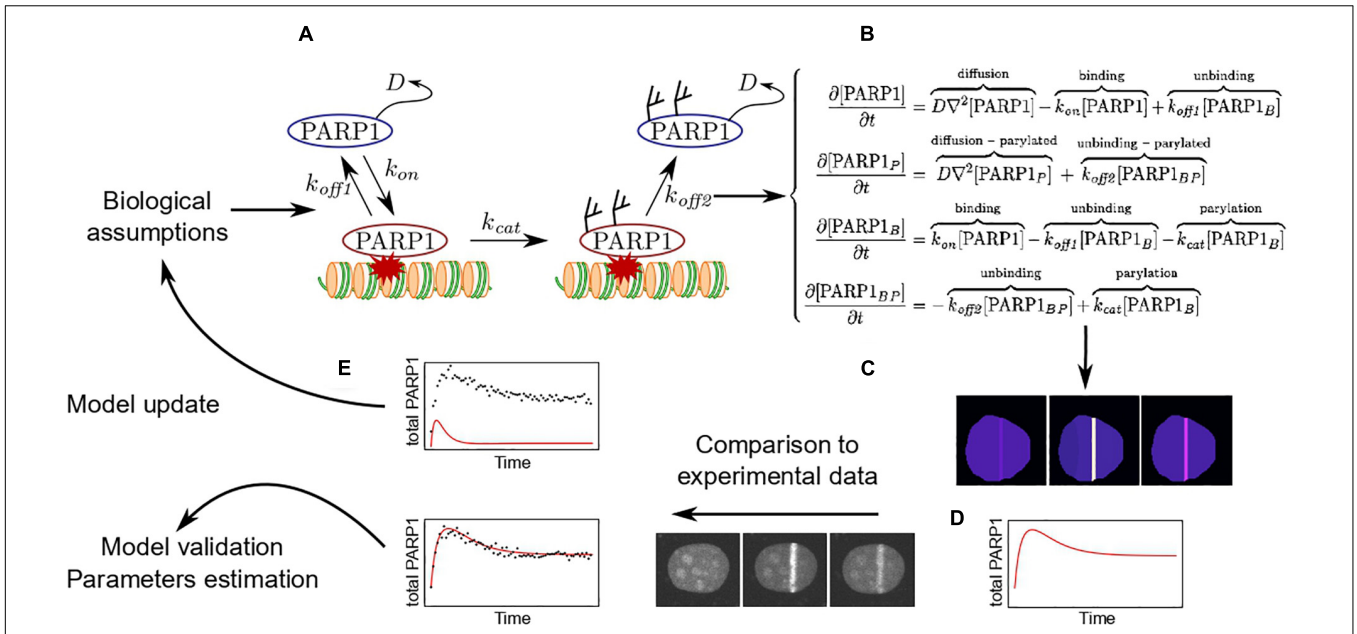

FIGURE 3 | Development of a mathematical model to assess PARP1 dynamics at DNA lesions. **(A)** Based on specific biological assumptions, it is possible to build a model describing the different states of PARP1 in and out the DNA damage area as well as the exchange rates between these different states. **(B)** The model can be transcribed in a set of reaction-diffusion differential equations. **(C)** Solving the set of equations allows to predict the evolution of the spatio-temporal distribution of PARP1 within the nucleus in response to induction of DNA damage. **(D)** Based on this simulation, it is possible to obtain predicted recruitment kinetic curves. **(E)** These simulated recruitment curves can be fitted to the experimental ones. If the model is unable to fit the experimental data, a new model needs to be rebuilt. In contrast, if the model allows to properly fit the data, it is possible to estimate the parameters included in the model.

that they are able to discriminate the most suitable model. Including additional information from other assays may also reduce the number of unknown parameters, thus facilitating the estimation of the remaining ones. The example model shown on **Figure 3** is composed of 5 unknown parameters. However, the diffusion coefficient of unbound PARP1 can be easily estimated by FCS or FRAP experiment in undamaged nuclei. Similarly, the binding and unbinding rates for the unPARylated PARP1 could probably be estimated by assessing the turnover of a catalytically inactive mutant of PARP1 at sites of damage, using FRAP or FCS. Therefore, ultimately, it would be possible to reduce the number of unknown parameters to only 2.

In summary, we showed in this section how mathematical models could be used to better interpret complex dynamics of repair proteins at DNA lesions. Plugging different experimental data into the model, assessing not only the local concentration of the repair factors at sites of damage but also its turnover, will help to establish complex robust models allowing to improve our understanding of the multiple steps of the DDR.

CONCLUSION

With the increasing number of quantitative live-cell microscopy techniques used within labs specialized in DNA repair, there comes the promise of future insights in the field due to the possibilities offered by imaging multiple aspects of protein dynamics. In particular, while protein recruitment curves at sites of laser irradiation have been analyzed extensively within the last years, only few reports have exploited methods such as FRAP, FCS, or pair correlation, to characterize the turnover of repair proteins at DNA lesions. Yet, this information is critical to better understand how these proteins interact with their substrate and accumulate within the repair foci. Furthermore, the recent progresses in SMLM methods for tracking the motions of single proteins within the nucleus now open a new avenue to investigate some aspects of the DDR that, so far, could only be addressed *in vitro* on naked DNA or reconstituted chromatin. This is particularly the case of the fascinating question of the search mechanisms employed by the initial repair factors to ensure the efficient detection of their target within the dense and highly complex nuclear space, a key event that is at the basis of the initiation of the whole DDR.

AUTHOR CONTRIBUTIONS

All authors contributed to the design, review, and proofreading of the manuscript. SZ and MJ prepared the figures.

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Chemical Inhibition of Apurinic-Apyrimidinic Endonuclease 1 Redox and DNA Repair Functions Affects the Inflammatory Response via Different but Overlapping Mechanisms

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The presence of oxidized DNA lesions, such as 7,8-dihydro-8-oxoguanine (8-oxoG) and apurinic/apyrimidinic sites (AP sites), has been described as epigenetic signals that are involved in gene expression control. In mammals, Apurinic-apyrimidinic endonuclease 1/Redox factor-1 (APE1/Ref-1) is the main AP endonuclease of the base excision repair (BER) pathway and is involved in active demethylation processes. In addition, APE1/Ref-1, through its redox function, regulates several transcriptional factors. However, the transcriptional control targets of each APE1 function are not completely known. In this study, a transcriptomic approach was used to investigate the effects of chemical inhibition of APE1/Ref-1 redox or DNA repair functions by E3330 or methoxyamine (MX) in an inflammatory cellular model. Under lipopolysaccharide (LPS) stimulation, both E3330 and MX reduced the expression of some cytokines and chemokines. Interestingly, E3330 treatment reduced cell viability after 48 h of the treatment. Genes related to inflammatory response and mitochondrial processes were downregulated in both treatments. In the E3330 treatment, RNA processing and ribosome biogenesis genes were downregulated, while they were upregulated in the MX treatment. Furthermore, in the E3330 treatment, the cellular stress response was the main upregulated process, while the cellular macromolecule metabolic process was observed in MX-upregulated genes. Nuclear respiratory factor 1 (NRF1) was predicted to be a master regulator of the downregulated genes in both treatments, while the ETS transcription factor ELK1 (ELK1) was predicted to be a master regulator only for E3330 treatment. Decreased expression of ELK1 and its target genes and a reduced 28S/18S ratio were observed, suggesting impaired rRNA processing. In addition, both redox

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and repair functions can affect the expression of NRF1 and GABPA target genes. The master regulators predicted for upregulated genes were YY1 and FLI1 for the E3330 and MX treatments, respectively. In summary, the chemical inhibition of APE1/Ref-1 affects gene expression regulated mainly by transcriptional factors of the ETS family, showing partial overlap of APE1 redox and DNA repair functions, suggesting that these activities are not entirely independent. This work provides a new perspective on the interaction between APE1 redox and DNA repair activity in inflammatory response modulation and transcription.

Keywords: apurinic/apyrimidinic endonuclease I (APE1), DNA repair, transcriptional control, inflammation, ETS transcription factor

INTRODUCTION

Apurinic-apyrimidinic endonuclease 1/Redox factor-1 (APE1/Ref-1) is a multifunctional protein involved in cell growth, transcriptional regulation, stress response, and genome stability. Two functionally distinct domains exert the biological activities of APE1/Ref-1. The N-terminal domain contains a nuclear localization signal. It is associated with the redox activity of APE1/Ref-1, while the C-terminal contains an endonuclease domain involved in the repair of abasic sites (or AP sites) in DNA (Xanthoudakis et al., 1994; Tell et al., 2010; Li and Wilson, 2014; Antoniali et al., 2017). The redox function activates transcription factors, such as AP-1 and NF-κB, which influence cellular processes such as stress responses, DNA repair, angiogenesis, and cell survival (Ando et al., 2008; Lee et al., 2009; Jedinak et al., 2011; Kelley et al., 2012). It also exhibits a redox-independent transcriptional regulatory function, acting as a transcriptional repressor by binding to negative Ca^{2+} response elements (nCaRE) (i.e., parathyroid hormone and *APEX1* promoters), which allows APE1/REF-1 to regulate its expression (Izumi et al., 1996; Kuninger, 2002). APE1/Ref-1 is also associated with transcription factors and other co-activators, which are required to assemble pre-initiation complexes and regulate transcription in a redox-independent manner (Fantini et al., 2008; Sengupta et al., 2012).

In addition to the role of APE1/Ref-1 in transcriptional regulation, the transcriptional role of base excision repair (BER) enzymes, including APE1/Ref-1, has recently emerged in both active demethylation processes mediated by ten-eleven translocation (TET) and thymine DNA glycosylase enzymes (Jin et al., 2015; Bochtler et al., 2016). The repair of 8-oxoguanine (8-oxoG) in promoter regions has also been described as an epigenetic mechanism (Ba et al., 2014; Fleming and Burrows, 2017). It has been demonstrated that the recruitment of APE1/Ref-1 and OGG1 (8-Oxoguanine DNA Glycosylase) to oxidized lesions generated by lysine-specific demethylase 1 (LSD1) activity on promoters, is required for further binding of transcription factors (TFs), such as c-Myc, and stabilization of the transcriptional complexes (Amente et al., 2010). In addition, the presence of DNA modifications, such as 8-oxoG and AP sites, in gene promoters has been related to increased gene expression (Pan et al., 2016; Fleming and Burrows, 2017). Recent studies have shown that the redox and repair activities of APE1/Ref-1 can affect the expression of the same genes (Li et al., 2019). Despite these vital functions, the targets and phenotypes associated with transcriptional control exercised by redox or DNA repair functions of APE1/Ref-1 are poorly understood.

Previously, we demonstrated the association between polymorphisms in *OGG1*, *PARP-1*, and *APEX1* with bacterial meningitis. In addition, the patient's carriers of *APEX1* 148 Glu allele presented reduced expression of cytokines and chemokines, such as IL-6, MCP-1, and IL-8, and an increase in DNA damage level, suggesting that APE1/Ref-1 repair activity is affected. Thus, these data indicate that DNA repair activity may be involved in these mechanisms (da Silva et al., 2011).

Deregulated APE1/Ref-1 is associated with various human pathologies, such as cancer, atherosclerosis, neurodegeneration, and infectious diseases, making it a potential therapeutic target (Thakur et al., 2014; Shah et al., 2017). Several research groups have collaborated to identify molecular inhibitors of APE1/Ref-1 activity. Quinone (E)-3-(2-[5,6-dimethoxy-3-methyl-1,4-benzoquinonyl])-2-nonyl propanoic acid (E3330) has therapeutic potential as a specific and direct redox inhibitor of APE1/Ref-1, as it acts like H_2O_2 by increasing Cys-65/93/99 oxidation (Kelley et al., 2011; Luo et al., 2012; Cesaratto et al., 2013; Zhang et al., 2013). Some studies have shown that E3330 decreases the expression of inflammatory modulators, such as tumor necrosis factor-α (TNF-α) and interleukin IL-8, and inhibits the growth and migration of cancer cells (Fishel et al., 2011; Kelley et al., 2011; Su et al., 2011; Li and Wilson, 2014; Ding et al., 2017).

The inhibition of APE1/Ref-1 DNA repair activity is associated with the sensitization of cancer cells to chemotherapy (Bobola et al., 2005; McNeill et al., 2009). Methoxyamine (MX), a synthetic molecule designed to inhibit BER, binds to high affinity to the aldehyde groups of AP sites, which are chemically refractory to APE1/Ref-1 endonuclease activity and resistant to BER processing (Rosa et al., 1991; Wilson and Simeonov, 2010). The inhibition of AP endonuclease activity of APE1/Ref-1 by MX has been studied in association with chemotherapeutic drugs, and positive results have been obtained (Liu et al., 2003; Guerreiro et al., 2013; Montaldi et al., 2015; Laev et al., 2017).

Although the APE1/Ref-1 redox function in transcriptional regulation of inflammatory mediators is known, the impact of E3330 and MX on the transcriptional regulation during inflammatory response is mediated by the inhibition of

APE1/Ref-1 activities is unknown, and it is exploited in this survey. The present study analyzed the cell transcriptome profile of a lymphoma-derived monocyte cell line (U937) stimulated with lipopolysaccharide (LPS) to investigate APE1/Ref-1 redox and repair activities on transcriptional regulation during the inflammatory response. We found that the expression of inflammatory modulators was reduced in this model after treatment with both APE1/Ref-1 activity inhibitors. Furthermore, comparative transcriptome analysis revealed that genes related to inflammatory responses and mitochondrial processes were downregulated in both treatments. However, the treatments differed in terms of rRNA processing and ribosome biogenesis. We also predicted master regulators to differentially expressed genes and identified NRF1, YY1, and the ETS family of TFs as the most likely APE1/Ref-1 partners in inflammatory signaling in monocytes.

MATERIALS AND METHODS

Cellular Model of Inflammation

U937 monocyte-like cells, derived from patients with histiocytic lymphoma (ATCC® CRL1593.2), were cultured in Gibco RPMI-1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, United States) supplemented with 44 mM sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, United States), Gibco 10% fetal bovine serum (Thermo Fisher Scientific Inc.), and 1% Penicillin-Streptomycin antibiotic solution (Sigma-Aldrich) in a humidified incubator at 37°C and a 5% CO_2 atmosphere unless stated otherwise. Cells (5×10^5) in 3 mL of medium were stimulated with 1 μg/mL LPS (Cat. No: L2654; Sigma-Aldrich) for 24 h, and then incubated with 100 μM E3330 (≥98% pure; Sigma-Aldrich) or 6 mM methoxyamine (MX) for 4 h. The cells were grouped as follows: unstimulated (control), LPS-stimulated (LPS), LPS + 100 μM E3330 (LPS + E3330), and LPS + 6 mM MX (LPS + MX). We ensured that the cell lines used in these experiments were free of mycoplasma infection.

Cell Viability Assays

For U937 cell viability measurement, 5×10^5 cells from each experimental group were incubated for 2, 4, 6, 24, and 48 h and subjected to trypan blue exclusion assay. In addition, the LPS + MX and LPS + E3330 groups were pre-treated with LPS for 1 h and co-incubated with MX and E3330. The cell suspension was mixed with Trypan blue dye 1:1 (v:v), and the cellular capacity to exclude the dye was analyzed using a hemocytometer and a CKX41 inverted microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Cell viability was calculated as the difference between the dead and total cell counts. The data were obtained in triplicate.

Cytokine and Chemokine Measurements

The proinflammatory cytokines and chemokines levels in U937 cells were measured using the Bio-Plex 200 suspension array system (Bio-Rad). The Human Cytokine/Chemokine Panel (MPXHCYTO-60k; EMD Millipore, Burlington, MA, United States) included the inflammatory modulators, TNF-α, IL-6, IL-10, MIP1α/CCL3, MIP-1β/CCL4, IL-8/CXCL8, and MCP1. Samples were processed and measured according to the manufacturer's instructions. Cytokine/chemokine expression was measured in duplicate. Results were determined based on a parametric logistic equation using Bio-Plex Manager 4.01 software (Bio-Rad) and are expressed as picograms per milliliter.

Apurinic/Apyrimidinic-Site Incision Assays

To determine if the 100 μM E3330 does not change the repair activity of APE1/Ref-1, we performed AP-site incision assays. An oligonucleotide gel-based APE1/Ref-1 endonuclease activity assay was performed as previously described by Silva-Portela et al. (2016) with modifications stated further in this section. The AP endonuclease activity of commercially available APE1 (NEB) was verified after treatment with E3330, based on the cleavage of a double-stranded DNA (dsDNA) substrate containing an abasic site at position 10 of the oligonucleotide 21-mer (5′-Cy5-CGGAATTAAAGXGCAAGACCT-3′ and 5′-AGGTCTTGCCCTTTAATTCCG-3′). This oligonucleotide was 5′-fluorescently labeled with Cy5. Standard reactions containing dsDNA (100 fmol), NEBuffer 4 (10×), and E3330, with or without APE1 (NEB), were incubated for 60 min at 37°C. The reactions were terminated by adding a "STOP" solution (98% formamide and 0.5 M EDTA) and heated to 95°C for 3 min. Samples (20 μL) were then run on a 20% polyacrylamide gel containing 8 M urea in 1×-TBE buffer at 300 V for 240 min. The reaction products were observed using a Chemidoc System (Bio-Rad).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using 10^6 cells stimulated according to our study model. DNA was cross-linked with 1% paraformaldehyde and sheared (average 200 bp) with five cycles of 10-s fragmentation using an ultrasonic bath (Ultra 800, Ciencor Scientific Ltd.). Further, DNA protein complexes were immunoprecipitated with ChIP quality Abs (APE1/Ref-1, sc-17774, Santa Cruz Biotechnology) using the Chromatin Immunoprecipitation Assay kit (Merck Millipore). The precipitates were washed three times, de-cross-linked, and subjected to PCR. TNF4 promoter primers were used: (−335 to −228 bp) F 5′AGGCAATAGGTTTTGAGGGCCAT3′ and R 5′TCCTCCCTGCTCCGATTCCG3′.

Immunofluorescence

We determined the subcellular localization of APE1 as follows. First, the cells were washed with phosphate-buffered saline (PBS), resuspended in 5 mL 3.7% paraformaldehyde in PBS for 15 min, washed with cold PBS, and seeded on coverslips that had been treated with poly-L-lysine for 30 min at room temperature. The cells were then incubated with Triton X-100 (0.5%) in PBS for 15 min and washed three times for 5 min each with Tween-20 (0.1%) in PBS (PBST). The cells were then incubated with anti-APE1 (sc-17774, Santa Cruz Biotechnology) for 1 h, washed three times with PBST, and incubated with FITC-conjugated

secondary antibody for 1 h. Finally, the sections were washed and mounted with Dako solution + DAPI (1.5 μg/mL), and stained cells were visualized using a CKX41 fluorescence microscope (Olympus) attached to a DP70 fluorescence camera (Olympus). All the mentioned methods were performed in the dark at room temperature.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from U937 cells using IllustraTM RNAspin Mini RNA Isolation Kits (GE Healthcare Little Chalfont, United Kingdom), as described by the manufacturer. Messenger RNA was obtained using an mRNA isolation kit (Roche Holdings AG, Basel, Switzerland). Briefly, the mRNA poly(A)+ tails were hybridized to a biotin-labeled oligo(dT) probe, and streptavidin-coated magnetic particles captured the biotinylated dT-A hybrids. A magnetic particle separator collected the magnetic particles, which were washed to remove contaminants, and then mRNA was eluted from the particles with water. The quantity of recovered mRNA was assessed using a Bioanalyzer 2100 (RNA 6000 Nano; Agilent Technologies GmbH., Waldbronn, Germany) and quantified using Nanovue (GE Healthcare). Total RNA for quantitative PCR was extracted using IllustraTM triplePrep Kit (GE Healthcare), as described by the manufacturer. Complementary DNA was prepared from the extracted total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems), as described by the manufacturer.

Transcriptome Analysis

Approximately 5 μg of complementary DNA from each group was sequenced using a 454 GS FLX Titanium, following the manufacturer's protocol (Roche Holdings AG). The sequenced data were aligned against the Ref-Seq database of human expressed sequences provided by the University of California Santa Cruz (UCSC[1]), accessed on January 30, 2014. Sequences were aligned using the BLAT (Kent, 2002). The results were filtered using the pslCDnaFilter tool,[2] with a minimum identity of 98%, minimum coverage of 90%, and only one alignment per sequence. All sequences that matched the ribosomal RNA (rRNA) were excluded. Gene expression was normalized as counts per million (CPM), calculated by counting reads per gene (Xi) and the total number of reads per sample (N): CPM = (Xi/N) × 10^7. Thus, the fold change in each gene was estimated between the samples. The Ensembl Gene ID from downregulated (≤−2-fold change) and upregulated (≥2-fold change) transcript lists were used for gene ontology (GO) enrichment analysis. GO term enrichment (biological process) was analyzed using the PANTHER tool (Mi et al., 2017). The list of differentially expressed genes was analyzed separately.

The lists of upregulated or downregulated transcripts (fold change ≥ 2 or ≤−2) were uploaded to the online software STRING 10.5[3] database and analyzed using default parameters without expansion. In addition, enrichment results for KEGG pathways, GO, and InterPro were also obtained from STRING, using the false discovery rate (FDR) for multiple testing correction ($p < 0.05$).

The obtained protein-protein interaction networks were downloaded and analyzed using Cytoscape 3.6.1 software (Shannon, 2003). The binary networks obtained from this screen were analyzed with the Molecular Complex Detection (MCODE) plugin to identify subnetworks with scores ≥ 2.5 (Bader and Hogue, 2003). Centrality parameters (node degree and betweenness) of each node were analyzed using the cytoHubba plugin (Chin et al., 2014). Biological process categories were generated by functional enrichment for a given cluster and category, with significance (p) assessed as a hypergeometric distribution. Multiple tests were also corrected using an FDR algorithm, which was fully implemented in the BiNGO software, with a significance level of $p < 0.05$ (Rivals et al., 2007).

The potential master regulators (TFs) of downregulated and upregulated networks were predicted using the iRegulon plugin version 1.3. The criteria for motif enrichment analysis were set as the identity between orthologous genes = 0.05, and a maximum FDR of motif similarity = 0.001. The consensus was searched in sequences up to 10 K in the promoter region using *Homo sapiens* as the reference. The motif with a normalized enrichment score (NES) of ≥4 was set as the threshold (Verfaillie et al., 2015).

Quantitative PCR

The qPCR reactions were prepared using 2Power SYBR Green PCR Master Mix (Life Technologies) and proceeded on an Applied Biosystems Real-Time PCR system. Briefly, PCR was performed using specific primers, 1 × Quantifast SYBR Green PCR master mix, and 10 ng of template cDNA in a 10 μL reaction volume. Reaction mixtures were initially denatured at 95°C for 5 min, followed by 45 cycles of 60°C for 1 min with a final melt at 45°C for real-time PCR analysis. Each assay was replicated using three independent biological samples. Cycle threshold (C_t) values were averaged for target genes and normalized against GAPDH (endogenous reference gene), and gene expression was quantified using the $2^{-\Delta\Delta CT}$ method. We validated the RNA-Seq data using the following equation: $\Delta\Delta C_t$ inhibitor = ΔC_t inhibitor – ΔC_t LPS. All primers were quality controlled to ensure that each set (forward and reverse) generated a specific PCR product. Primer's information has been provided in **Supplementary Table 1**.

Western Blotting

The expression of selected proteins in U937 cells was investigated by western blotting. Total protein was extracted using IllustratriplePrep Kits (GE Healthcare), as described by the manufacturer. Lysates (20 μg) were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to PVDF membranes, as described by Laemmli (1970). Membranes were incubated in a blocking buffer (5% dried milk, 0.5% Tween-20 in TBS, pH 7.2) for at least 40 min, then overnight at 4°C with the following primary antibodies: against APE1, NRF1, β-actin, and ELK1 (Santa Cruz Biotechnology) (1:1,000), MYC (Abcam) (1:1,000), and NF-κB p65 subunit (1:1,000; Millipore). Further, the blots were washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody

[1]http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/refMrna.fa.gz

[2]http://hgdownload.cse.ucsc.edu/admin/exe/

[3]https://string-db.org/

(1:1,000; Santa Cruz Biotechnology) for 1 h at room temperature. They were then immersed in Amersham ECL Prime western blotting Detection Reagent (GE Healthcare) before imaging on a Chemidoc System (Bio-Rad). Data are shown as those from three independent experiments. Expression levels were estimated using β-actin as the loading control in ImageLab software.

Microfluidic Gel Electrophoresis of Ribosomal RNA

The RNA integrity of U937 cells was analyzed using a chip-based microcapillary electrophoresis system (Agilent 2100 BioAnalyzer; Agilent Technology) and Agilent RNA 6000 Nano Chips. The reference was the RNA molecular weight ladder provided in the kit. The samples were resolved by electrophoresis, as described by the manufacturer. The molecular weight and integrity of rRNA were determined by the ratio of 28S/18S peaks using Agilent 2100 Expert Software.

Cell Cycle Analysis by Flow Cytometry

The DNA content and cell cycle distribution in U937 cells were determined by flow cytometry. The cells were harvested and pelleted by centrifugation, washed twice in phosphate-buffered saline (PBS, pH 7.2), and fixed with 70% cold absolute ethanol for at least 12 h at 4°C. Immediately before cell cycle determination, the cells were gently resuspended and stained with propidium iodide (PI; 20 μg/mL) and 10 μg/mL RNAse (Sigma-Aldrich) in PBS) and incubated at 37°C for 60 min. We acquired 10,000 events per sample using a flow cytometer (Becton Dickinson and Co., Franklin Lakes, NJ, United States) and a 488 nm argon laser. The data were analyzed using FlowJo 7.6.5 software (FlowJo LLC., Ashland, OR, United States).

Statistical Analysis

The normality of the data was assessed using the Kolmogorov-Smirnov test. Groups were evaluated using two-way analyses of variance (ANOVA), and individual groups were analyzed by Student's *t*-test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, United States). Data are expressed as the mean ± SE. Values with $p < 0.05$ were considered statistically significant.

RESULTS

Methoxyamine or E3330 Treatment Does Not Alter the Viability of U937 Cells After 24 h or the Expression of APE1/Ref-1 During Inflammation but Decreases the Expression of Proinflammatory Cytokines

Stimulation for 24 h with LPS (1 μg/mL) followed by incubation with E3330 (100 μM) or methoxyamine (MX) (6 mM) did not significantly affect the viability of U937 cells (**Figure 1A**). Similar data were obtained after stimulation for 1 h with LPS, followed by inhibitor addition. Until 24 h of exposure, no significant alterations in monocyte viability were observed compared to non-stimulated cells. However, after 48 h of treatment with LPS + E3330, we observed a significant decrease in the viability of U937 cells compared to that of LPS-stimulated cells (**Figure 1B**).

Inhibition of APE1/Ref-1 activities decreased the expression of proinflammatory cytokines and chemokines in response to LPS treatment, indicating that AP site repair and the redox function of APE1/Ref-1 are vital for expressing these genes (**Figure 1C**). Owing to its ability to self-regulate, we analyzed APE1/Ref-1 expression after treatment. We observed no changes in APE1/Ref-1 protein or mRNA levels (**Figure 1D**). In addition, using *in vitro* repair assays, we also observed that E3330 did not affect DNA repair endonuclease activity (**Figure 1E**).

Methoxyamine and E3330 Alter the Expression of Genes Related to Inflammatory Response, Mitochondrial Gene Expression, and rRNA Metabolism

Transcriptome analysis was used to investigate the profile of transcriptional changes in response to the inhibition of AP site repair or redox activity of APE1/Ref-1 and their involvement in inflammatory modulation. Comparative transcriptome analysis of U937 cells stimulated with LPS + E3330 and LPS-stimulated cells revealed 914 downregulated (fold change ≤ -2) and 2,222 upregulated genes (fold change ≥ 2). MX addition after LPS stimulation induced downregulation of 1,287 genes and upregulation of 1,362 genes (**Figure 2**). Sequencing results were validated using qPCR. For validation, six genes were chosen among the downregulated and upregulated genes in both treatments. The results shown in **Supplementary Figure 1** demonstrate that all genes evaluated presented similar expression patterns, both in RNA sequencing and qPCR analysis.

The list of upregulated and downregulated genes (fold change ≥ 2 or ≤ -2) was submitted for GO evaluation in Panther. The most enriched biological processes for each gene list are represented in **Figures 2C,D**. Regarding downregulated genes, we observed enrichment of genes related to mitochondrial gene expression and rRNA metabolic process after both treatments. Moreover, MX treatment also decreased gene expression related to the prostaglandin biosynthetic process (e.g., PTGES3, PTGES2, and PTGS1/COX-1) and MyD88-independent toll-like receptor signaling pathway (e.g., TRAF2, IKBKG, and TLR4). Together with the inhibition of cytokine expression, these results indicate a negative regulation of genes related to the inflammatory process after inhibition of AP site repair.

We constructed a Venn diagram with four sets of genes (**Figure 2E**). The results showed that 286 genes were negatively regulated, while 592 genes were positively regulated after both treatments.

E3330 Increases the Coupling of Apurinic-Apyrimidinic Endonuclease 1/Redox Factor-1 to the TNF4 Promoter and Decreases the mRNA Expression

Analyses of mRNA levels showed that both inhibitors act in the transcriptional regulation of cytokines such as TNF-α and

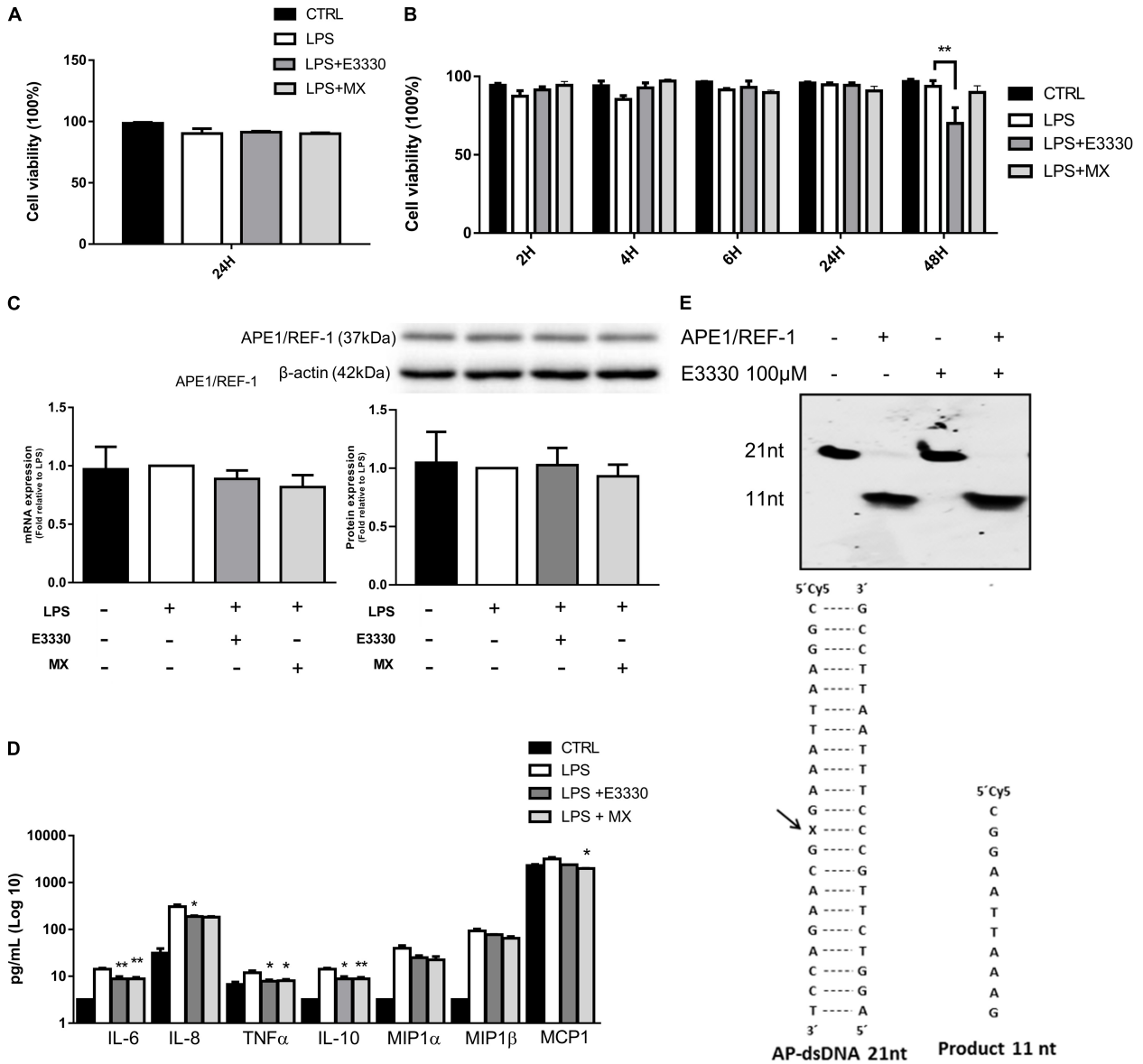


FIGURE 1 | Effect of E3330 and methoxyamine on apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) and inflammatory response in U937 cells. **(A)** Cell viability measurement after lipopolysaccharide (LPS) (1 μg/mL) stimulation for 24 h followed by treatment with E3330 (100 μM) or MX (6 mM) for 4 h. **(B)** Cell viability after 1 h of LPS stimulation and subsequent E3330 or MX treatment for 2, 4, 6, and 24 h. Two-way analyses of variance (ANOVA) was used to compare the treatment groups. **(C)** mRNA and APE1/Ref-1 protein expression after LPS stimulation for 24 h followed by treatment with E3330 or methoxyamine (MX) for 4 h. **(D)** Protein levels of cytokines and chemokines after LPS 24 h plus E3330 or MX for 4 h. Unpaired Student's *t*-test was performed for inflammatory response measurement. $p < 0.05$ was considered statistically significant. **(E)** Cleavage electrophoretic profiles of oligonucleotides (left) and double-stranded oligonucleotides containing an abasic site and cleavage product (right) E3330 did not alter APE1 endonuclease activity. $^{*}p < 0.05$, $^{**}p < 0.01$.

MCP1 (**Figure 3A**). To investigate the potential implication of APE1/Ref-1 repair activity in the inflammatory response, we evaluated whether the inhibition of AP sites by MX in the TNF4 promoter (of TNF-α) guanine-rich sequence would be different from that found in E3330. PCR amplification of the TNF4 promoter segment after APE1/Ref-1-Ab-ChIP DNA demonstrated that APE1/Ref-1 coupling was drastically reduced after LPS treatment compared to the control. However, the redox inhibition of APE1/Ref-1 increased the coupling of APE1/Ref-1 in the TNF promoter (**Figure 3B**). Interestingly, this result corroborates the findings of immunofluorescence, in which U937 cells without any treatment showed the APE1/Ref-1 protein located mainly in the nucleus, and after LPS stimulation, a marked increase in the cytoplasm was observed. In both E3330 and MX treatments, APE1/Ref-1 reduced its cytoplasmic location and was translocated to the nucleus (**Supplementary Figure 2**). To investigate the coupling of APE1/Ref-1 in the TNF4 promoter, we searched for transcription factor binding motifs in this region.

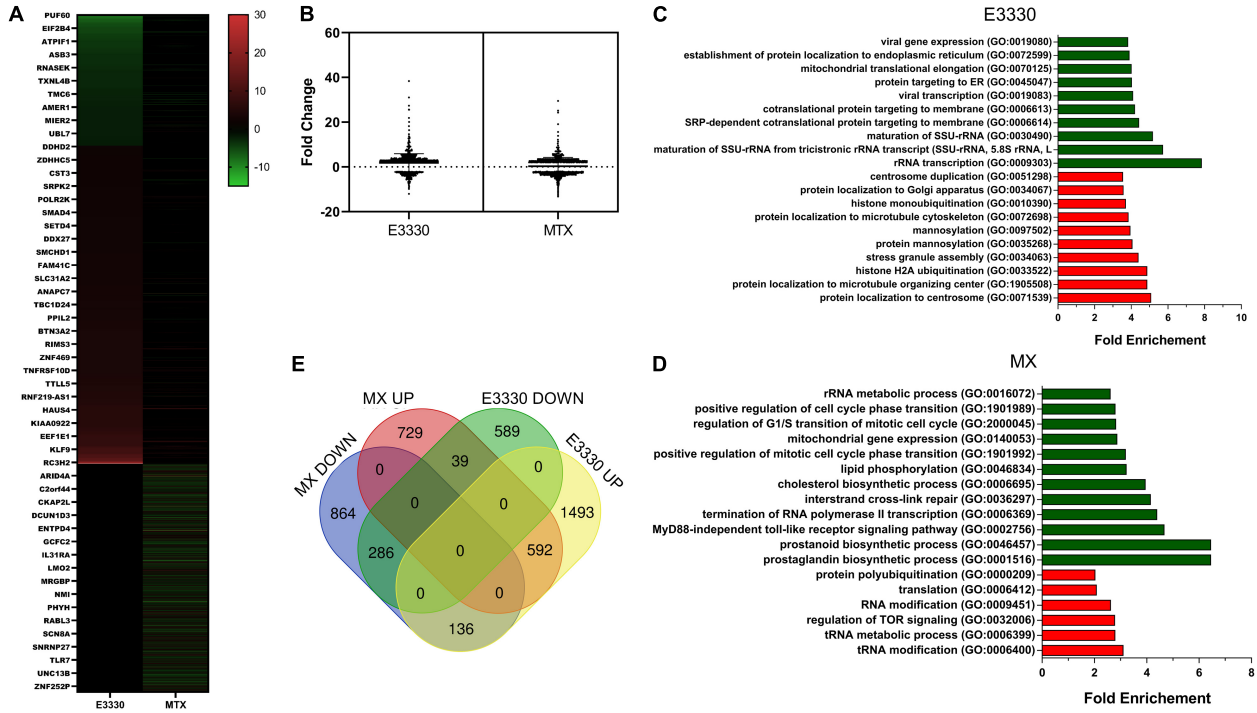


FIGURE 2 | RNA-seq data analysis. **(A)** Heatmap of differentially expressed genes after MX or E3330 treatment during inflammatory stimulation with LPS. **(B)** Distribution of differentially expressed genes according to the fold change. **(C)** Venn diagram describing the number of differentially expressed genes after MX and E3330 treatments. **(D)** Biological processes enriched between upregulated (red) and downregulated (green) genes after E3330 treatment. **(E)** Biological processes enriched between upregulated (red) and downregulated (green) genes after MX treatment. Gene ontology (GO) analysis was performed using the Panther classification system. All processes described were significant ($p < 0.05$).

Using TRANSFAC software, we identified motifs for ELK1, AP1, NRF2, and NF-κB (**Figure 3C**).

Nuclear Respiratory Factor 1 and the ETS Family of Transcription Factors Were the Master Regulators of the Negatively Expressed Genes

To identify the central genes and regulators of differentially expressed gene lists, we built a protein–protein interaction (PPI) network (**Figures 4A,B** and **Supplementary Figure 3**). All PPI networks were subjected to a centrality analysis (**Supplementary Figure 4**) and then analyzed by iRegulon to predict enriched motifs and their master regulators. After treatment with MX and E3330, iRegulon identified 34 TFs capable of binding to motifs represented in **Tables 1**, **2**, most of which belong to the ETS family. Fourteen TFs were not expressed in U937 cells according to the Protein Atlas database, and were not detected in our RNAseq. The others were considered transcription activators ($n = 9$), repressors ($n = 5$), or with both actions ($n = 3$) using the Protein Atlas (**Figure 4C**). From the downregulated network after E3330 treatment, ELK1 motifs were predicted to be the most significant. In contrast, ETV4 and GABPA were most likely in the MX-downregulated network.

Some differences in RNAseq expression were noted, such as the downregulation of some members of the TCF subfamily (ELK3 and ELk4) and SRF after treatment with MX (fold change = −4.02, −2.08, −2.62, respectively). On the other hand, the ERF repressor was downregulated (−4.37) after E3330 treatment and upregulation of several ETS such as GABPA (2.13), ELF1 (2.74), GABPAB1 (4.06), ELF4 (2.62), and ELF2 (2.19) (**Figure 4C**) was observed. Furthermore, enrichment of ETS binding motifs among MX-upregulated genes was also observed. FLI1 was the most likely transcription factor involved in the regulation of these genes. The list of likely TFs predicted by iRegulon is presented in **Tables 1**, **2**.

Similarly, 396 downregulated genes after MX treatment and 315 downregulated genes after E3330 showed binding motifs to the NRF1 transcription factor (**Tables 1**, **2**). These data indicate that the inhibition of APE1/Ref-1 can directly or indirectly regulate the expression of NRF1 targets. Also, YY1/YY2 motifs in genes negatively regulated for MX and positively regulated by E3330 were observed. We also submitted to iRegulon for each set of genes resulting from the Venn diagram. This result showed that NRF1, ELK1, and GABPA motifs-maintained enrichment among genes downregulated by both inhibitors (**Supplementary Figure 5**). The set of genes downregulated exclusively upon MX or E3330 treatment had binding motifs to RARG and ATF4, respectively. In addition, RARG (−3,74) expression decreased after MX treatment (**Figure 4D**), while YY1, ATF4, KAT2A, MEF2A, and POLR3G increased expression after E3330 treatment (**Figures 4D–F**).

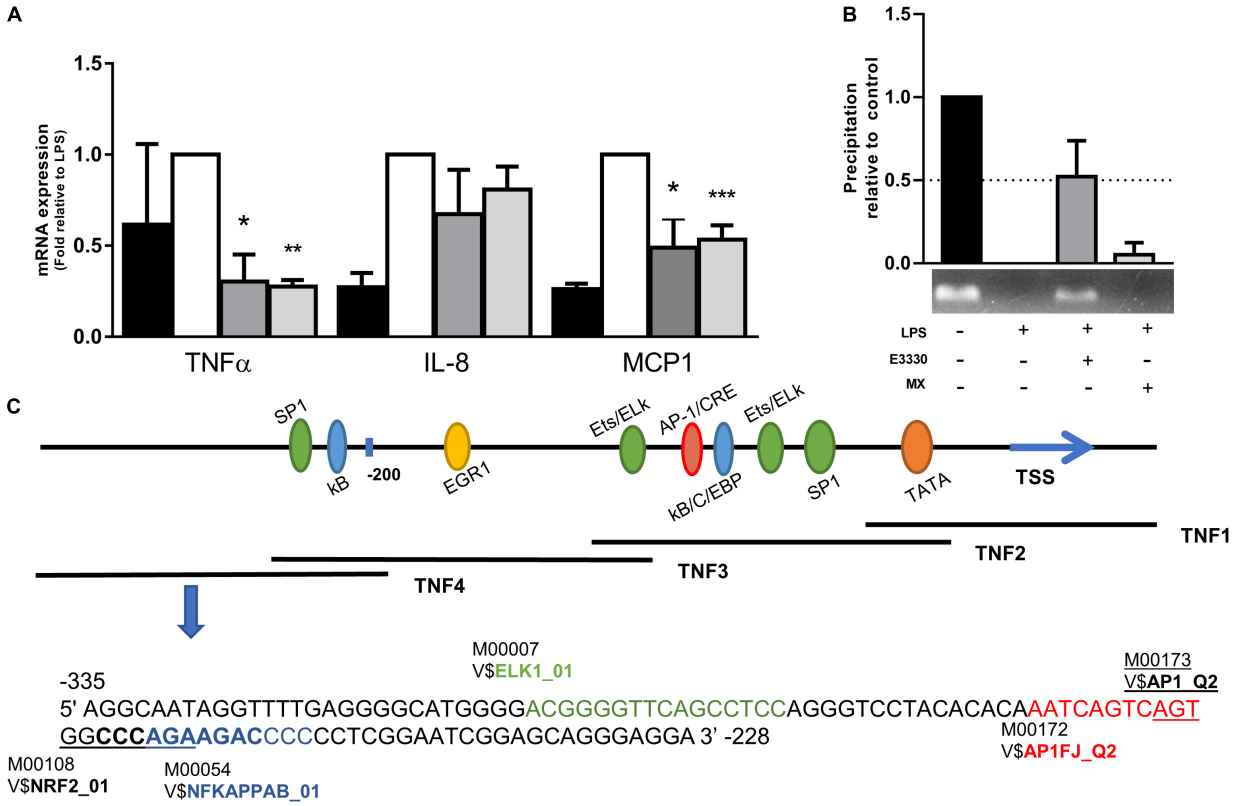


FIGURE 3 | E3330 treatment changes APE1/REF-1 DNA occupancy in TNF4 promoter. **(A)** mRNA levels of TNF-α and MCP1 were reduced by E3330 and MX treatment after LPS stimulation. Unpaired Student's t-test was performed for inflammatory response measurement. $p < 0.05$ was considered statistically significant. **(B)** PCR of TNF4 promoter after APE1/Ref-1-Ab-ChIP revealed that APE1/Ref-1 coupling is reduced after treatment with LPS compared to the control. E3330 increases APE1/REF-1 DNA occupancy in TNF4 promoter. **(C)** Representative scheme of the TNF-α promoter with the representation of transcription factors that bind to it. Highlighted region in TNF4 with binding motifs predicted by research in the Transfac database. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Methoxyamine and E3330 Decrease the Expression of Nuclear Respiratory Factor 1 Targets Related to Mitochondrial Organization

Nuclear Respiratory Factor 1 has many targets in both networks. The inhibition of APE1/Ref-1 redox activity downregulated 315 NRF1 targets related to several biological processes, such as regulation of transcription, mitochondrion organization, translation, and response to oxidative stress (**Figure 5A**, blue). In contrast, MX treatment downregulated 396 targets related to transcription, protein polyubiquitination, chromatin remodeling, and the cell cycle (**Figure 5A**). Some genes, such as *TFB2M* and *NCOA1*, are common to both treatments and are shown in **Figure 5B**. However, we observed that inhibitors did not significantly change NRF1 protein or mRNA expression (**Figure 5C**), indicating that APE1/Ref-1 regulates NRF1 activity and not its expression. We selected and analyzed, using qPCR, the expression of commons and exclusive genes downregulated by MX or E3330 and confirmed a decrease in the expression of NRF1 targets, corroborating the data obtained by iRegulon. Furthermore, AP repair inhibition promoted a significant reduction in mitochondrial gene expression, such as *TFAM*, *TFB2M*, *NDUFB5*, and *NDUFB9*. Similarly, the redox inhibition of APE1/Ref-1 also decreased the expression of the same genes (except for *TFB2M*), indicating that both inhibitors can act in the transcriptional regulation of these genes (**Figure 5D**).

Transcription Factors of the ETS Family Were Associated With the Expression of Genes Related to Transcription and rRNA Metabolism

Transcription factors belonging to the ETS family, such as ELK1, GABPA, and ETV4, were also identified by iRegulon as being responsible for the expression of most genes downregulated for APE1/Ref-1 inhibition; among them, the ELK1 motifs were enriched among the genes downregulated in the E3330 treatment. In comparison, ETV4 and GABPA were enriched among the genes downregulated in the MX treatment. Interestingly, ELK1 targets were related to rRNA processing and translation, mainly in the E3330 network (**Figure 6A**).

The ETS family has significant redundancy among its binding motifs; therefore, several targets were also common between ELK1 and GABPA (**Figure 6B**). Although some genes related to rRNA metabolism were also downregulated upon MX treatment, and ELK1 mRNA expression was decreased after both treatments (**Figure 6C**), the selected targets for

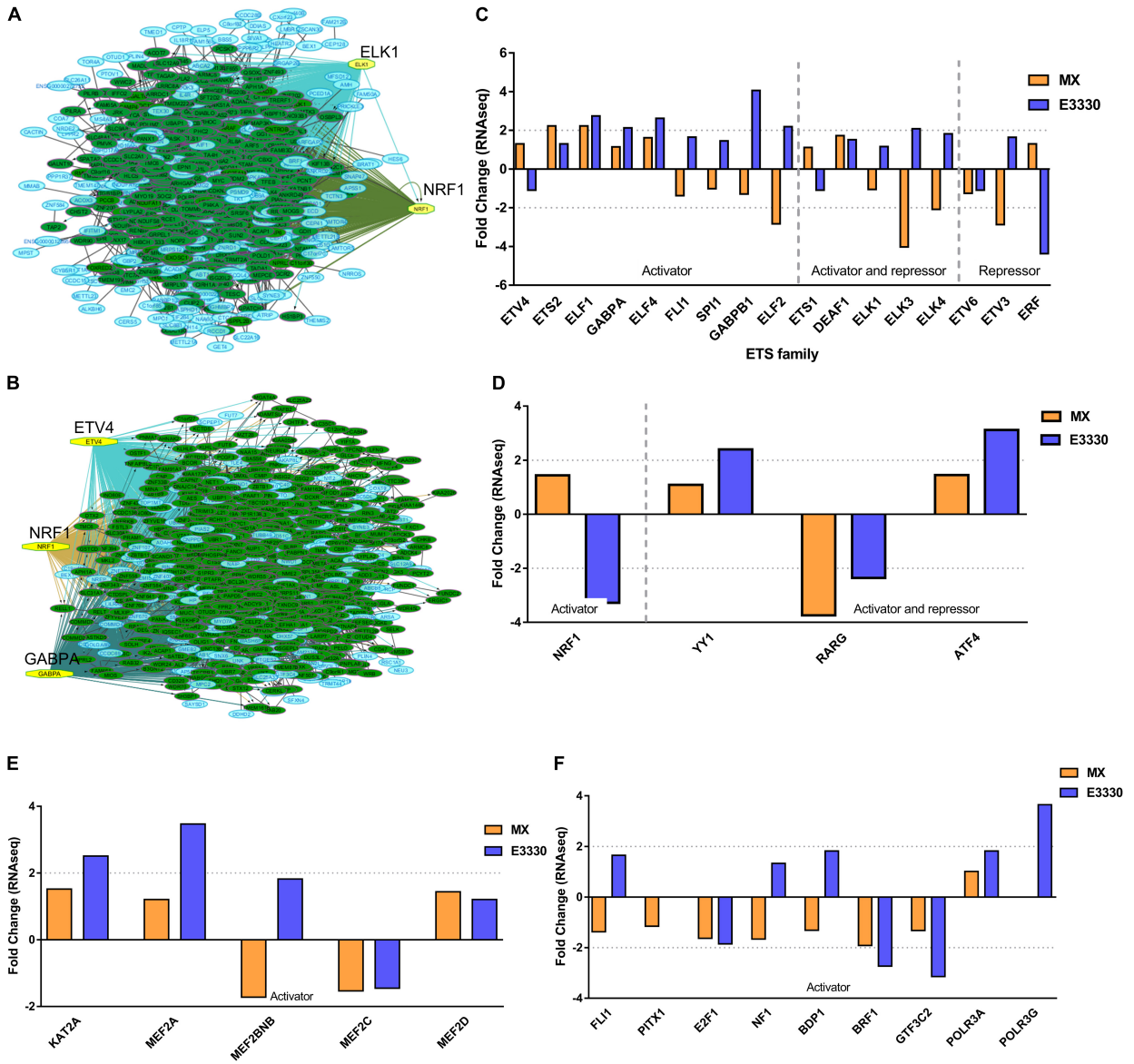


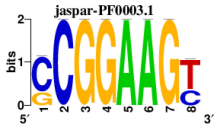
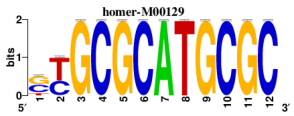
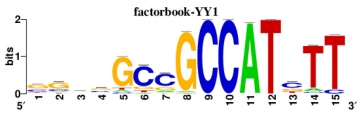
FIGURE 4 | RNAseq data of critical transcription factors predicted by iRegulon in response to exposure to inhibitors. Protein-protein interaction (PPI) networks generated by STRING formed by the products of differentially regulated genes were analyzed by Cytoscape and its master regulators predicted by the iRegulon plugin. Mater regulators predicted by iRegulon are highlighted in yellow, targets of the master regulators are shown in green. In blue, genes are not targets of the master regulators. **(A)** E3330 downregulated network. **(B)** MX downregulated network. **(C)** ETS family transcription factors found in RNAseq. TFs were divided into transcription activators and repressors or both, according to the Protein Atlas database. **(D)** NRF1, RARG, YY1, and ATF4 master regulators of at least one of the downregulated networks. **(E)** KAT2A and MEF2A master regulators of E3330 upregulated network compared to MX treatment. **(F)** FLI1, BDP1, and PITX1 master regulators of MX upregulated network compared to the E3330 treatment.

qPCR analysis showed different expression regulation among inhibitors. For example, E3330 treatment decreased the expression of *RPL35*, *MYC*, and *NIP7*. In contrast, repair inhibition of AP sites by MX increased the expression of some ribosomal proteins (RPS19 and RPL27) and decreased MYC protein and mRNA expression (**Figures 6D,E**). In addition, the expression of rRNA 47S was significantly reduced by MX, indicating that the repair of AP sites affects rRNA transcription (**Figure 6F**). On the other hand, the 28S/18S ratio decreased significantly after E3330 treatment, indicating that APE1/Ref-1 redox activity is essential for rRNA processing (**Figure 6G**).

Inhibition of Apyrimidinic Endonuclease 1/Redox Factor-1 Redox Function Affects the Control of Cell Growth and Stress Response

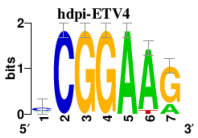
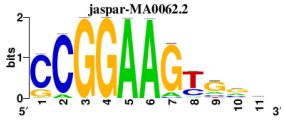
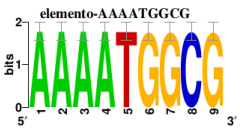
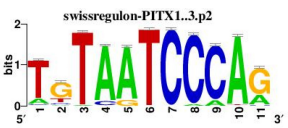
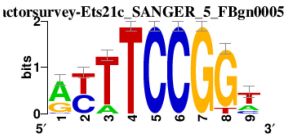
Cell viability was not affected after 24 h of LPS stimulation and treatment with E3330 or MX (**Figure 1B**). However,

TABLE 1 | Description of master regulators of differentially expressed genes in response to E3330 treatment predicted by iRegulon.

Transcription factors	Motifs	NES	Targets number
LPS + E3330 down-regulated network			
ELK1		5.802	457
NRF1		4.521	315
LPS + E3330 upregulated network			
YY1		3.672	221
KAT2A	*	3.438	82
MEF2A		3.241	630

**This motif could be shown by iRegulon, as it is part of TRANSFAC pro.*

TABLE 2 | Description of master regulators of differentially expressed genes in response to methoxyamine (MX) treatment predicted by iRegulon.

Transcription factors	Motifs	NES	Targets number
LPS + MX downregulated network			
ETV4		6.451	602
GABPA		5.893	629
NRF1		5.021	396
YY1		4.357	126
LPS + MX upregulated network			
CRX		5.125	386
FLI1		4.616	688
BDP1	*	4.033	259

**This motif could shown by iRegulon, as it is part of TRANSFAC pro.*

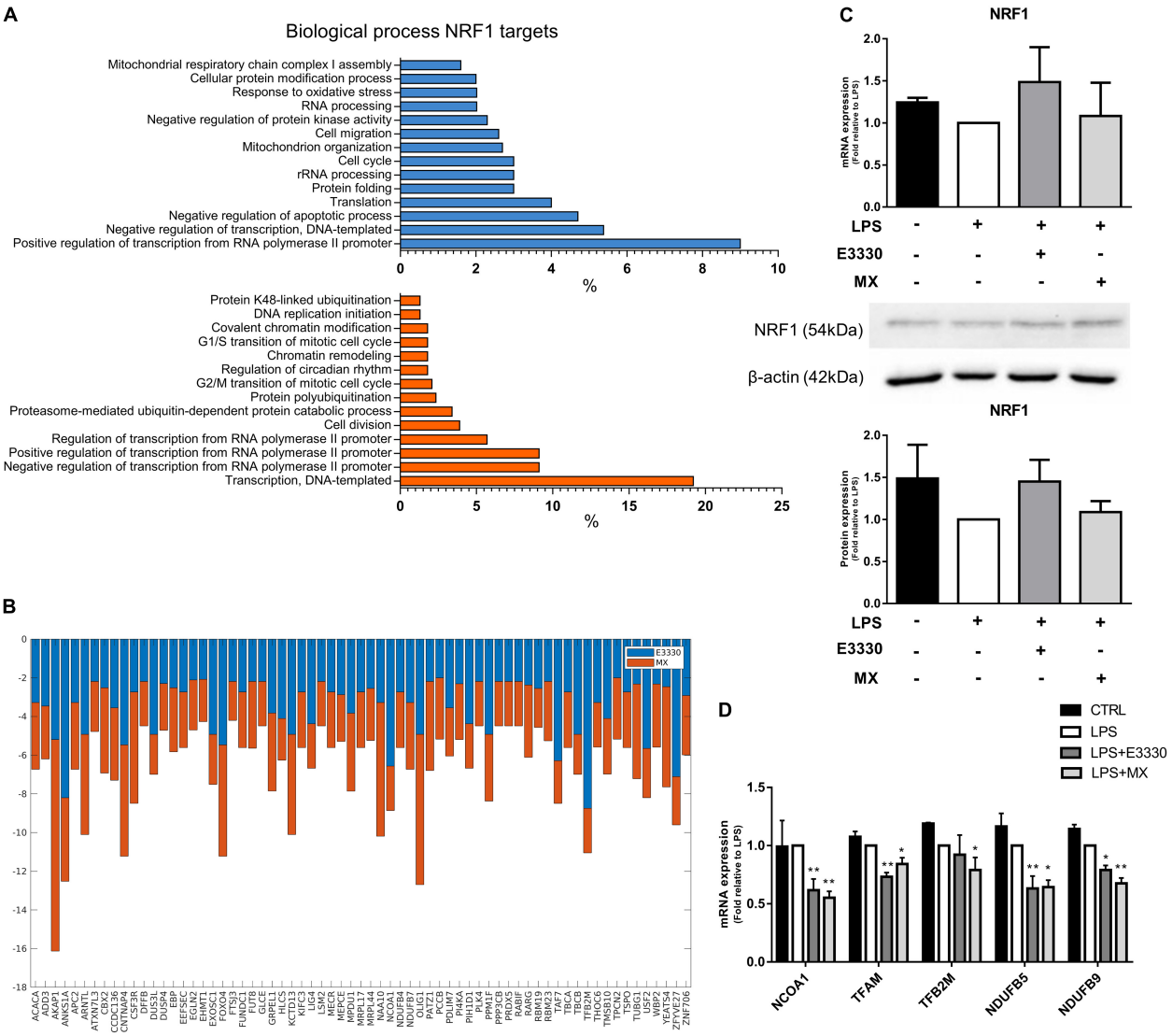


FIGURE 5 | E3330 and MX treatment decreased the expression of nuclear respiratory factor 1 (NRF1) targets in U937 cells during LPS stimulation. **(A)** Biological process enriched between NRF1 targets downregulated after E3330 treatment (blue) and MX treatment (orange). All processes described were significant ($p < 0.05$). **(B)** Expression of NRF1 targets common to both treatments in RNAseq. **(C)** Protein and mRNA NRF1 expression analyzed by western blot and q-PCR in U937 cells. **(D)** mRNA expression of NRF1 target genes upon LPS, MX, and E3330 treatment. Unpaired Student's *t*-test was performed and $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$.

a reduction of viable cells at the time point of 48 h of treatment with E3330 was observed, and flow cytometry analysis revealed a slight increase in the ratio of subG1 cells (**Figure 7A**), indicating a role for APE1/REF-1 redox regulation in the control of cell growth. Furthermore, we could observe that p65 (RELA) reduced expression at the protein level after E3330 treatment (**Figures 7B,C**), corroborating the transcriptome data. A statistically significant increase in the expression of MDM2 (60 kDa cleaved portion) was also observed after E3330 treatment. However, no significant difference in the protein expression of EGR1 and Casp3 was observed (**Figures 7B,C**). These data corroborate our findings for the upregulated networks.

DISCUSSION

Our data revealed the downregulation of several transcriptional regulators and immune response-activating signal transduction genes for both E3330 and MX treatments. These data indicate that APE1/Ref-1 acts on the transcriptional regulation of cytokines and inflammatory modulators during LPS signaling. NF-κB is a crucial transcriptional factor involved in the inflammatory process and is a redox APE1/Ref-1 target; its DNA-binding activity is impaired when APE1/Ref-1 redox function is inhibited (Mitomo et al., 1994; Hiramoto et al., 1998). In this study, we observed the downregulation of p65 (RELA gene), the catalytic subunit of NF-kB, after APE1/Ref-1 redox inhibition

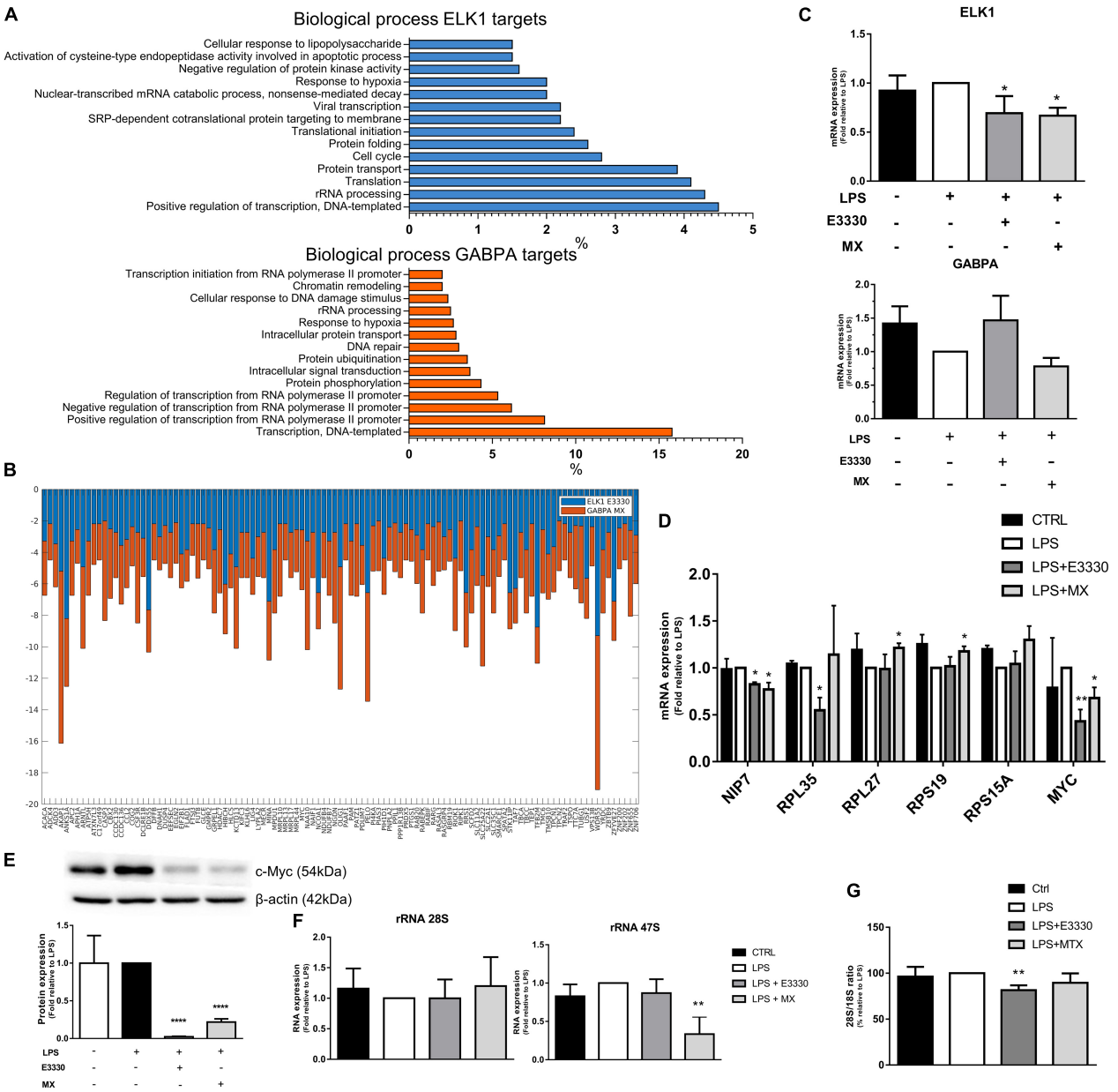


FIGURE 6 | The ELK1 and GABPA targets related to ribosomal biogenesis were regulated differently after E3330 and MX treatment. **(A)** Biological process enriched between downregulated ELK1 targets genes after E3330 treatment (blue) and downregulated GABPA targets genes after MX treatment (orange). All processes described were significant ($p < 0.05$). **(B)** Expression of ELK1 and GABPA targets common to both treatments. **(C)** mRNA expression of ELK1 and GABPA analyzed by qPCR in U937 cells. **(D)** mRNA expression of genes related to ribosomal biogenesis upon LPS, MX, and E3330 treatment. **(E)** MYC protein expression was analyzed by western blot. **(F)** Ratio 28S/18S and expression of rRNA after MX and E3330 treatment. Unpaired Student's *t*-test was performed and $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

by E3330. Therefore, inhibiting APE1/Ref-1 redox activity might decrease inflammatory modulators' expression by inhibiting NF-κB binding in gene promoters and reducing the expression of its p65 catalytic subunit in U937 cells.

We also showed a new close relationship between APE1/Ref-1 and a region of the TNF promoter, called TNF4. Interestingly, APE1/Ref-1 was found attached to TNF4, and reduced levels of TNF-α were observed after E3330 treatment in LPS-stimulated cells, suggesting that APE1/Ref-1 redox activity is associated with repression of TNF4 in U937 cells. Furthermore, this promoter region contains an ELK1 binding site, which can act as a corepressor linked to complexes with HDAC-1 and LSD1 (Yang et al., 2001; Gerosa et al., 2020), both of which are APE1/Ref-1 partners (Bhakat et al., 2003; Amente et al., 2010). Conversely, MX treatment did not promote APE1/Ref-1 attachment on TNF4, but reduced expression of TNF-α were observed.

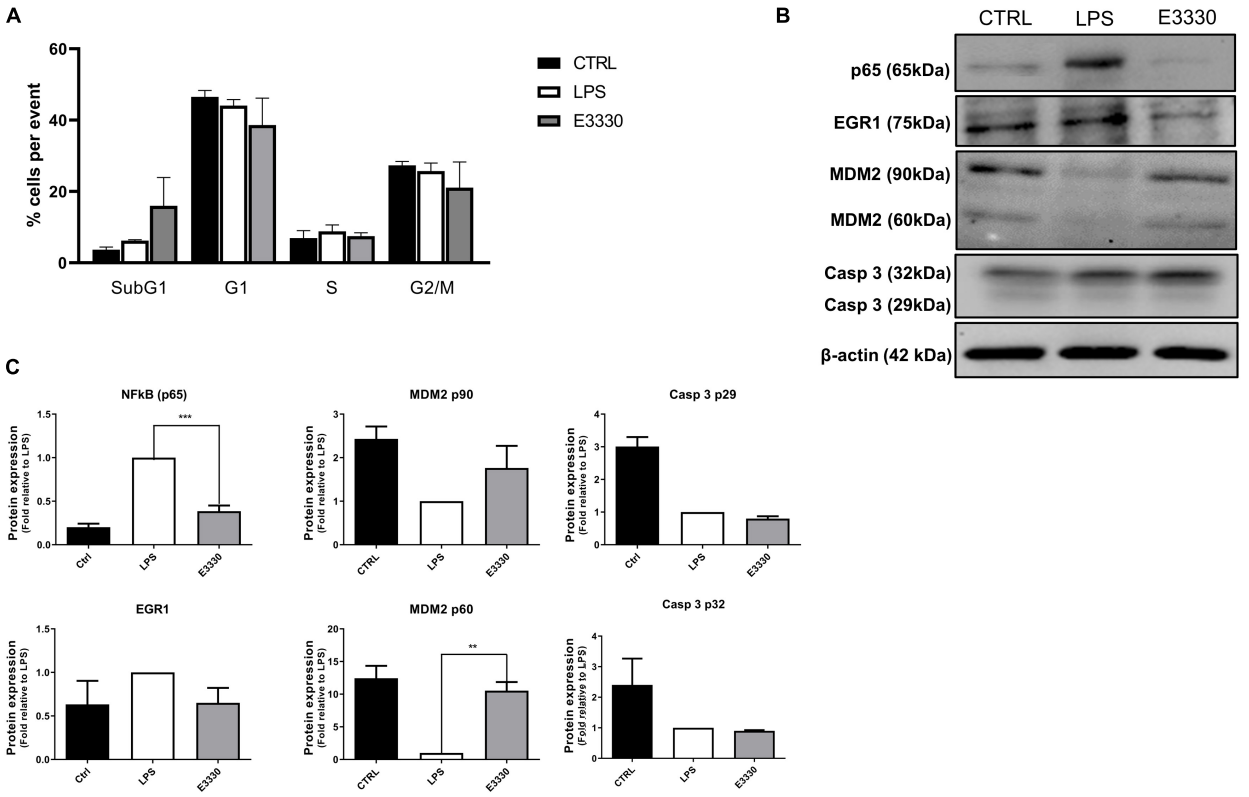


FIGURE 7 | Inhibition of APE1/Ref-1 redox function affects the cell growth and expression of different proteins related to stress response. **(A)** Cell cycle assays after stimulation with LPS for 24 h followed by incubation with E3330 for 4 h show a discrete increase ratio (%) of subG1cells. **(B)** Representative western blot analysis of whole-cell extracts and **(C)** histogram reporting densitometric quantification of western blotting signals from at least three independent experiments. β-actin was used as the loading control. Unpaired Student's *t*-test was performed and $p < 0.05$ was considered statistically significant. $^{**}p < 0.01$, $^{***}p < 0.001$.

In cells stimulated with TNF-α, the binding of OGG1 to 8-oxoG in regions close to the TNF-α promoter increased the DNA occupancy of NF-κB and gene expression via epigenetic regulation (Pan et al., 2016). Furthermore, we also observed a decrease in TNF-α and MCP1 mRNA expression during impairment of endonuclease activity by MX. These results indicate that the resolution of 8-oxoG, more specifically AP sites, is crucial for cytokine expression.

In this study, we have demonstrated that both inhibitors decreased the expression of genes related to mitochondrial gene expression and rRNA metabolic process in LPS-stimulated monocytes (**Figures 3C,D**). In addition, these genes showed enrichment of binding motifs to the transcriptional factor NRF1 (**Figure 4**). NRF1 is one of the main regulatory factors of mitochondrial biogenesis, often referred to as a transcription activator (Gleyzer et al., 2005; Piantadosi and Suliman, 2012). In addition, chip-on-chip and chip-seq studies have revealed that NRF1 binds to genes associated with RNA metabolism, DNA damage repair, chromosome organization, and cell cycle (Cam et al., 2004; Satoh et al., 2013; Bhawe and Roy, 2018). It has already been observed that the lack of aprataxin leads to reduced levels of APE1/Ref-1, which in turn is related to the reduction of NRF1 levels and consequent mitochondrial dysfunction (Garcia-Diaz et al., 2015). Furthermore, APE1/Ref-1 redox function is involved in controlling the DNA-binding activity of NRF1. In the absence of APE1/Ref-1 redox function, the expression of NRF1 target genes was significantly reduced (Li et al., 2012).

Amente et al. (2010) observed that LSD1 produces H_2O_2, increasing the oxidation of guanines in MYC target gene promoters. The presence of 8-oxoG in DNA recruited OGG1 and APE1/Ref-1 and improved gene expression (Amente et al., 2010). LSD1 is also a member of the transcriptional corepressor complex CoREST, a unique complex containing both a histone demethylase (LSD1) and a deacetylase enzyme (HDAC1) (Song et al., 2020). The association between the NRF1 motif and LSD1 occupancy has been reported in different cell lines (Benner et al., 2013). Hence, blockage of AP sites by MX during gene regulation may be the reason for the decrease in NRF1 targets after MX treatment. This hypothesis should be tested in future studies.

Here, we also identified a consensus signature for the ETS family of TFs, which have 28 members in the human genome and significant redundancy among their binding motifs; consequently, diverse targets were also identified among several regulators (Sizemore et al., 2017). Among ETS factors, GABPA and ELK1 were shown to be master regulators of downregulated genes. Both GABPA and ELK1 exhibit target redundancy and control the same biological processes, including ribosome biogenesis, mitochondrial processes, cytoskeleton, and

cell migration. However, despite the redundancy of targets and functions, these regulators also present a cohort of specific target genes (Boros et al., 2009; Odrowaz and Sharrocks, 2012).

Although a set of ETS target genes was found to be mainly downregulated upon MX inhibition, ELK1 mRNA expression was decreased after both treatments (**Figures 6C,D**). Furthermore, we noted that the biological processes involved in ribosomal biogenesis were more representative of the E3330 transcriptome. Interestingly, ELK1 targets were related to rRNA processing and ribosomal biogenesis, mainly in the E3330 network (**Figure 6A**). In addition, the 28S/18S ratio was significantly lower in cells treated with E3330, suggesting inefficient rRNA processing. These data indicate that the redox inhibition of APE1/Ref-1 is more effective in regulating rRNA processing. In contrast, MX treatment decreased the expression of rRNA 47S without affecting rRNA processing.

ETS transcription factors are generally activated by phosphorylation and binding in specific sequences such as RAS-responsive elements (RREs) and, in TCF subfamily cases, serum response elements (SRE). ETS-binding sequences act as RREs when flanked by AP-1 binding sites, and enhancer activation requires ETS1 and AP-1 activation (Wasylyk et al., 1998; Yordy and Muise-Helmericks, 2000; Hollenhorst et al., 2011). It is known that the redox activity of APE1/Ref-1 facilitates AP-1 DNA binding and activity (Xanthoudakis and Curran, 1992; Ando et al., 2008). Therefore, E3330 treatment can decrease AP-1 activation and disturb the expression of genes that have RREs. In contrast, we observed that MX treatment decreased the expression of the TCF subfamily (ELK1, ELK4, and ELK3), including SRF. Thus, these TFs can act as transcription activators and repressors that bind to the SRE (For review, Yordy and Muise-Helmericks, 2000; Shaw and Saxton, 2003; Buchwalter et al., 2004). We observed an enrichment of ETS motifs among MX-upregulated genes, indicating activation of transcription activators or the absence of a repressor.

Several pathways play an essential role in response to LPS stimulation. The ERK pathway is responsible for the phosphorylation of TFs such as ELK-1 and FLI1, leading to their activation and consequent induction of genes related to inflammatory response, differentiation, and cell growth (Guha and Mackman, 2001). Furthermore, changes in the redox state of ERK proteins are associated with their activation and inhibition (Keyes et al., 2017). For example, it was demonstrated that APE1/Ref-1 forms a complex with ERK2 and rescues ERK oxidative inactivation through its redox function, favoring cyclin D1 expression and cell cycle progression G1-to-S passage (Wang et al., 2013). Thus, in our model, E3330 treatment can compromise cellular responses dependent on the ERK pathway, which was not observed in the MX treatment.

We should also consider TFs that were not enriched by iRegulon analysis but are classic APE1/Ref-1 redox activity targets; examples include EGR1 and Jun/Fos (AP-1) (Xanthoudakis and Curran, 1992; Huang and Adamson, 1993; Pines et al., 2005; Ando et al., 2008; Fantini et al., 2008). Binding sites to the SP family transcription factor were found to be enriched exclusively in E3330 downregulated genes (represented by SP8; **Supplementary Figure 5**), which has an overlap of targets with EGR1. Similarly, genes such as *RPL35*, *ESRRA*, and *RelA*, downregulated by E3330 treatment in monocytes, also have binding sites to EGR1 and Jun. In addition, EGR1 is a known activator of the ELK1 gene promoter in monocytes (Lehmann et al., 1999). In addition, we also observed decreased expression of ELK1 after MX treatment, which can be associated to the presence of CpG-rich regions in the ELK1 gene promoter that targets active demethylation by TED enzymes (Qu et al., 2017), suggesting that APE1/Ref-1 may also be related to DNA repair-dependent ELK1 expression control. These results indicate that the redox and repair activities of APE1/Ref-1 can regulate gene expression through independent but overlapping mechanisms.

In addition, in the iRegulon analysis, we observed enrichment of binding sites for RARgamma (RARG) into downregulated genes exclusively after MX treatment. RARG is a nuclear retinoic acid receptor (RAR) that forms heterodimers with RXRs. The redox function of APE1/Ref-1 mediates the binding of RARs to retinoic acid-responsive elements (RARE) (Robertson et al., 2006; Fishel et al., 2010). RAR and estrogen receptor (ER) have overlapping DNA-binding sites and may act cooperatively or antagonistically (Liu et al., 2014). The RAR and ER pathways control cell differentiation, stress response, and immune homeostasis (Straub, 2007; Oliveira et al., 2018). In our study, we observed downregulation of RARG after both treatments. However, ESRRA (an estrogen receptor member) is upregulated in MX and downregulated after E3330 treatment. Estrogen and retinoic acid-responsive gene promoters are DNA oxidation targets mediated by LSD1, which recruits BER enzymes, favoring chromatin remodeling (Perillo et al., 2008; Zuchegna et al., 2014).

Binding sites for YY1/YY2 were also enriched between MX-downregulated and E3330 upregulated genes. YY1 and YY2 are homologous proteins that show overlapping DNA binding sites and can act as synergistic or antagonistic activators or repressors, and are involved in regulating cellular processes such as inflammation, stress response, and cell cycle control (Klar and Bode, 2005; Chen et al., 2010; Li et al., 2020). Li et al. (2014) showed the direct repression of CBF/NF-Y/YY1 DNA-binding activities by E3330, suggesting that YY1 is an APE1 target.

Several studies have revealed a connection between DNA damage response, DNA repair, and rRNA metabolism pathways (Larsen and Stucki, 2016; Vohhodina et al., 2016). In the E3330 treatment, we observed the upregulation of several genes involved in cell cycle control, DNA damage response, and DNA repair, such as PIK3CA, CDK1, and ATR (**Supplementary Figure 4**), which are classified as hub-bottlenecks, as well as an increase in the expression of MDM2 when compared to LPS (**Figure 7**). MDM2 is a stress sensor, and MDM2-mediated ubiquitination can signal APE1/REF-1 degradation following treatment with genotoxicants (Busso et al., 2009). APE1/REF-1 redox inhibition seems to induce cell stress higher than the inhibition of DNA repair activity in our experimental model.

In summary, the selective inhibition of APE1/Ref-1 can alter several cellular processes and understanding the mechanism underlying protein regulation would be a valuable target for both preventative and curative treatment paradigms. Furthermore, the molecular mechanisms responsible for the various functions

of APE1/Ref-1 need to be elucidated to develop more targeted therapies for a wide range of human diseases. Our data showed that the AP site repair and redox functions of APE1/Ref-1 are essential for modulating genes related to the global inflammatory response through direct and indirect pathways. In addition, redox and repair activities are also necessary for the transcription of genes related to basal transcription, cell cycle, ribosomal biogenesis, and mitochondrial biogenesis, suggesting that both functions affect transcriptional regulation by different but overlapping mechanisms, thus, indicating that these functions are not entirely independent, as initially proposed. Finally, this work indicates several new TFs that may be APE1/Ref-1 function targets.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GEO, accession no: GSE182813.

AUTHOR CONTRIBUTIONS

TO, FF-D, RM, DP, and LC developed the experimental assays. TO, FF-D, VS, and SS performed RNAseq data analysis. LA-L, TO, and FF-D conceived the study and its design, performed data analysis, and drafted the manuscript. The final manuscript was reviewed and authorized by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.731588/full#supplementary-material

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REVIEW
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DNA Damage-Induced Inflammatory Microenvironment and Adult Stem Cell Response

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Adult stem cells ensure tissue homeostasis and regeneration after injury. Due to their longevity and functional requirements, throughout their life stem cells are subject to a significant amount of DNA damage. Genotoxic stress has recently been shown to trigger a cascade of cell- and non-cell autonomous inflammatory signaling pathways, leading to the release of pro-inflammatory factors and an increase in the amount of infiltrating immune cells. In this review, we discuss recent evidence of how DNA damage by affecting the microenvironment of stem cells present in adult tissues and neoplasms can affect their maintenance and long-term function. We first focus on the importance of self-DNA sensing in immunity activation, inflammation and secretion of pro-inflammatory factors mediated by activation of the cGAS-STING pathway, the ZBP1 pathogen sensor, the AIM2 and NLRP3 inflammasomes. Alongside cytosolic DNA, the emerging roles of cytosolic double-stranded RNA and mitochondrial DNA are discussed. The DNA damage response can also initiate mechanisms to limit division of damaged stem/progenitor cells by inducing a permanent state of cell cycle arrest, known as senescence. Persistent DNA damage triggers senescent cells to secrete senescence-associated secretory phenotype (SASP) factors, which can act as strong immune modulators. Altogether these DNA damage-mediated immunomodulatory responses have been shown to affect the homeostasis of tissue-specific stem cells leading to degenerative conditions. Conversely, the release of specific cytokines can also positively impact tissue-specific stem cell plasticity and regeneration in addition to enhancing the activity of cancer stem cells thereby driving tumor progression. Further mechanistic understanding of the DNA damage-induced immunomodulatory response on the stem cell microenvironment might shed light on age-related diseases and cancer, and potentially inform novel treatment strategies.

Keywords: DNA damage, inflammation, microenvironment, immune response, stem cells, cancer

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INTRODUCTION

Stem cells are undifferentiated cells essential for tissue growth and maintenance (Blanpain and Simons, 2013). They can be classified according to their origin in embryonic stem cells, induced pluripotent stem cells (iPSCs) and tissue-specific stem cells (also known as adult or somatic stem cells) (Shevde, 2012). Embryonic and iPSCs are pluripotent stem cells, derived from an early stage

embryo and reprogramming of somatic cells, respectively, able to differentiate into any specialized tissue cell type (Kingham and Oreffo, 2013). On the other hand, tissue-specific stem cells are multi- or unipotent cells able to give rise to specialized cell type(s) present in a specific tissue and belonging to a particular lineage. Tissue-specific stem cells are present in small numbers in several adult tissues or organs ensuring tissue homeostasis and regeneration upon damage (Wagers and Weissman, 2004). Similarly to normal tissues, most tumors also possess a population of cells characterized by stem cell-like features that are defined as cancer stem cells (CSCs) (Coppes and Dubrovska, 2017). Like normal stem cells, CSCs have the ability to self-renew and generate differentiated cell types, which foster the growth and maintenance of many types of neoplasms alongside being often attributed to treatment resistance and cancer recurrence (Vitale et al., 2017).

Stem cells constantly receive signals from the surrounding microenvironment, also known as the stem cell niche, a 'home' that supports the maintenance and proper function of stem cells to ensure tissue homeostasis and respond to damage (Jones and Wagers, 2008). These niche signals can be either extrinsic, mediated by secreted factors, cell surface molecules/receptors, cell-cell interactions and gap junctions, or intrinsic resulting in persistent intracellular changes in the stem cell epigenetic profile and metabolism (Pennings et al., 2018). Additionally, the stem cell microenvironment is composed by specific cells, often distributed in a defined spatial order, such as differentiated cells, stromal cells, immune cells, vasculature- and nervous system-related cells (**Figure 1**, left panel). These cells work together to ensure structure and appropriate reception of local as well as systemic signals (Lane et al., 2014).

The non-cellular physical properties of the microenvironment itself, such as the extra-cellular matrix (ECM) molecule composition and oxygen levels, can also affect stem cell behavior by affecting stem cell related pathways. A classic example of this is the role of the YAP/TAZ signaling pathway in mechanotransduction (Dupont et al., 2011). While hypoxia inducible factors (HIFs) have been shown to modulate other stem cell-related pathways, such as Notch signaling (Keith and Simon, 2007) and autophagy (Li et al., 2015).

The structure and function of the stem cell microenvironment have been extensively reviewed in Jones and Wagers (2008). Additionally, notions described for the hematopoietic stem cell (HSC) niche (Mendelson and Frenette, 2014; Crane et al., 2017; Pinho and Frenette, 2019), such as the maintenance of HSCs via specific factors secreted by endothelial and stromal cells as well as immune cells and sympathetic nerve fibers, can be applied to solid tissues. Importantly, the stem cell microenvironment has been shown to be a dynamic compartment rapidly adapting in response to insults, diseases (including oncogenesis) and aging (Scadden, 2006). Aging is a pleiotropic process characterized by multiple factors, including increased levels of DNA damage (Schumacher et al., 2021) due to various sources (as described in the following section) coupled with a reduced cellular DNA repair capacity.

Throughout life stem cells are significantly exposed to DNA damage due to their longevity and functional requirements, such as ensuring tissue homeostasis and replenishment of damaged or lost cells via prolonged proliferation (self-renewal) and differentiation (Mandal et al., 2011; Schumacher et al., 2021). In particular, cell proliferation is intrinsically related to replication stress, a phenomenon characterized by DNA synthesis slow down and stalled replication forks that in turn can result in DNA damage as previously shown in aged HSCs (Flach et al., 2014).

In this review, we describe how the recently discovered immunomodulatory responses initiated by DNA damage can

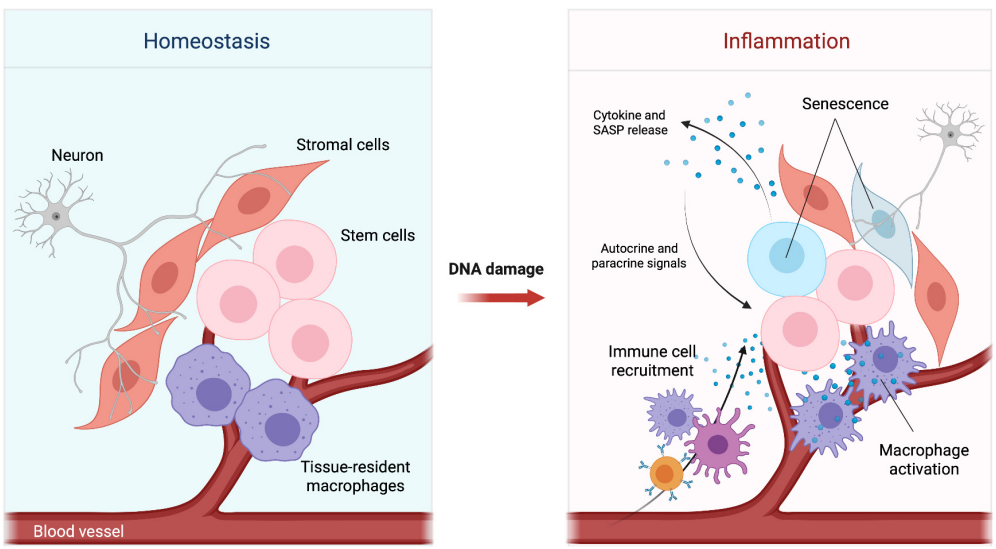

FIGURE 1 | Changes in the stem cell microenvironment upon DNA damage. Simplified schematic representation of the tissue microenvironment during homeostasis **(Left)** and inflammation **(Right)**. DNA damage can impair tissue homeostasis by promoting senescence, cytokine and SASP release. Cytokines and chemokines present in the microenvironment can lead to recruitment of immune cells and activation of tissue-resident macrophages.

alter different aspects of the stem cell microenvironment thereby affecting the function of both tissue-specific and cancer stem cells. The main cytosolic nucleic acid sensing pathways activated upon cytosolic DNA and RNA recognition and involved mechanisms, which can induce microenvironmental changes that affect stem cell function, are discussed. Additionally, an overview of immune cell infiltration and the importance of DNA damage-induced cellular senescence in tissue homeostasis, stem cell regeneration potential and CSC recognition is provided.

DNA DAMAGE IN ADULT STEM CELLS

Different endogenous sources of DNA damage can affect adult stem cells and their microenvironment, such as reactive oxygen species (ROS) produced by metabolic intermediates and dysfunctional mitochondria, alcohol and endogenous aldehydes, glycolytic by-products and advanced glycation end products, replication stress depending on the proliferation status of the cells, transcriptional disruption and telomere shortening. Importantly, although it is difficult to reliably assess such endogenous sources of DNA damage, they are thought to increase with age (Chaudhuri et al., 2018; Schumacher et al., 2021). As a result of such physiological cellular processes, in a day each cell may be exposed to nearly 100,000 DNA lesions, including different types of base modifications, single-strand breaks (SSBs) and double-strand breaks (DSBs) (Madabhushi et al., 2014). Additionally, external sources can lead to DNA damage, such as ionizing radiation (most commonly UV and X-rays) and certain chemicals. This is especially relevant for CSCs and normal tissue stem cells co-exposed to DNA damaging cancer therapies, such as radiotherapy and many chemotherapeutic agents.

In response to DNA damage cells initiate a coordinated series of events known as the DNA damage response (DDR), which encompasses various DNA repair pathways, cell cycle checkpoints and cell death pathways, and extensively described in Jackson and Bartek (2009). The DDR initiates with the sensing of DNA damage by protein complexes and kinases, and the subsequent signaling mediated by post-translational modifications, such as protein phosphorylation. Although these complex molecular mechanisms can vary between different types of somatic cells and stem cells (Vitale et al., 2017), the main DNA repair pathways are usually conserved and depending on the type of DNA lesion comprise: base excision repair (BER) and single strand break repair (SSBR), which promote the repair of small DNA lesions, such as base modifications and SSBs, through the excision of damaged bases; nucleotide excision repair (NER), which promotes the repair of DNA lesions such as adducts and structures that distort the DNA double helix; DNA mismatch repair (MMR), essential for the correction of base mismatches and small insertions or deletions; non-homologous end joining (NHEJ) and homologous recombination (HR), which are the classical pathways involved in DSB repair (Jackson and Bartek, 2009; Scully et al., 2019). Collectively with DNA repair, DNA damage signaling can lead to the activation of cell cycle checkpoints, which are points throughout the cell cycle in which movement is paused or slowed down to allow time for the cell to repair the damage, or to the induction of cell death, mainly by apoptosis and necrosis, or to an irreversible state of growth arrest so-called cellular senescence (Jackson and Bartek, 2009).

As stem cells age, DNA damage coupled with a reduced DNA repair capacity has been shown to contribute to the development of age-related disorders from cancer to tissue degeneration (Behrens et al., 2014). Oncogenesis is often a result of aging due to DNA damage misrepair and the consequent accumulation of mutations (Jeggo et al., 2016). A decline in stem cell function has also been related to defects in different DDR components, which in the HSC system cause reduced self-renewal and long-term exhaustion leading to bone marrow failure and anemia (Vitale et al., 2017) or pre-mature differentiation in non-HSCs such as neural stem cells (NSCs) (Barazzuol et al., 2017) and melanocyte stem cells (McSCs) (Inomata et al., 2009). Additionally, as a consequence of DNA damage, the microenvironment, the resulting signaling pathways and infiltrating cells adapt to this new state (**Figure 1**, right panel).

Although long-term self-renewal and differentiation capabilities are the defining features of stem cells, their regeneration potential is restricted to a definite number of times during the lifespan of an organism (Pazhanisamy, 2009). This can represent an important limitation; indeed, stem cells can be induced to self-renew more often upon damage or genotoxic stress, such as irradiation (Sémont et al., 2006). A prolonged activation of this process can inevitably lead to stem cell exhaustion and loss of tissue homeostasis maintenance (Pazhanisamy, 2009). Furthermore, many types of stem cells, including HSCs and NSCs (Barazzuol et al., 2017; Schumacher et al., 2021), reside in a quiescent state, which, although limits the amount of endogenous DNA damage (such as that caused by replication stress or metabolic by-products), may lead to the accumulation of DNA lesions due to the restricted availability of error prone NHEJ in non-cycling cells with subsequent stem cell functional impairment and premature aging (Schumacher et al., 2021). Moreover, DNA damage upon genotoxic stress has been shown to promote premature differentiation of McSCs, hair follicle stem cells and NSCs, thus preventing their expansion (Inomata et al., 2009; Matsumura et al., 2016; Barazzuol et al., 2017). However, it remains unclear whether DNA damage-induced premature differentiation can be linked to the DNA damage-mediated inflammatory process. Defects in DNA repair as in rare genetic disorders can also promote the decline of tissue stem cell functions leading to age-related diseases, such as bone marrow failure, and tumor formation via the generation of CSCs (Biechonski et al., 2017; Vitale et al., 2017; Tiwari and Wilson, 2019). For example, similarly to other stem cells, NSCs have the ability to migrate from their niche in order to differentiate and promote the repair of damaged brain tissue; however, abnormalities in this process may lead to stem cell transformation and glioblastoma formation (Vescovi et al., 2006). These events can be extended also to other tissues and organs, such as skin, liver, muscle and gut,

whose repair depends on the activity of specific adult stem cells (Kenyon and Gerson, 2007).

INTERPLAY BETWEEN DNA DAMAGE AND INFLAMMATION IN THE STEM CELL MICROENVIRONMENT

One of the main roles of the immune system is to mediate the recognition of dangerous and invasive elements through the expression of specific pattern recognition receptors (PRRs). These elements can be distinguished in pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Gong et al., 2019). PAMPs are exogenous components that are unique to invading microorganisms, such as specific membrane-associated lipids, lipopolysaccharides (LPS) and lipoglycans (Silva-Gomes et al., 2014). In contrast, DAMPs are endogenous molecules released by damaged or dying cells and are not related to a pathogen infection (Gong et al., 2019). Although PAMPs and DAMPs have different origins, the recognition of such molecules is mediated by similar PRRs, such as Toll-like receptors (TLRs), NOD-like receptors, intracellular nucleic acid-sensing receptors and C-type lectin receptors. Activation of these proteins is essential for the secretion of cytokines and attracting innate immune cells into the infected or damaged tissue (Gasteiger et al., 2017). The innate immune system can then intervene with cell-dependent mechanisms, such as phagocytosis, cytotoxicity and secreted factors, in order to eliminate the pathogens or damaged cells (Gasteiger et al., 2017). Several *in vitro* and *in vivo* studies have demonstrated that an aberrant activation of these mechanisms can trigger the host immune response leading to inflammatory events and autoimmune diseases (Nakad and Schumacher, 2016). Interestingly, receptors and adaptor proteins related to DAMP and PAMP recognition processes strongly overlap and interconnect often in a positive feedback loop. This tight relation might explain why infection as well as stress factors like DNA damage can trigger the activation of similar inflammatory pathways leading to pro-inflammatory cytokine release and immunity activation (Jounai et al., 2013; Foell et al., 2007; Paludan and Bowie, 2013).

It is well established that in normal physiological conditions DNA is largely located within the nucleus and mitochondria. However, DNA leakage within the cytosol can occur as result of adverse events, such as DNA damage, triggering the induction of specific cytosolic DNA sensors and activation of DAMP-related immune responses (Ishikawa et al., 2009; MacKenzie et al., 2017; Maekawa et al., 2019). Genotoxic events are usually accompanying by the formation of micronuclei into the cytoplasm, small extra-nuclear bodies formed by lagging chromosomes and chromosome fragments upon mitotic errors or DNA damage (Kwon et al., 2020). Importantly, due to defects in nuclear lamina organization (Hatch et al., 2013), the envelope of these isolated nuclear structures is fragile (Kwon et al., 2020). It has been shown that its rupture can lead to release of the micronuclear content into the cytoplasm with consequent chromothripsis (a mutational process characterized by the shattering and reassembly of a chromosome from a micronucleus) (Crasta et al., 2012; Koltsova et al., 2019). Alongside these processes, ruptured micronuclei have also been linked with the activation of various cytoplasmic nucleic acid sensors that boost the production of pro-inflammatory cytokines and activate the immune response (Crasta et al., 2012; MacKenzie et al., 2017). Interestingly, the activation of these pathways reflects a sign of microbial infections, which also alert the host innate immune system to mount a defense response.

These pro-inflammatory events can have important consequences on a variety of cell types, including stem cells, which function is modulated by their microenvironment (Voog and Jones, 2010). In fact, it has been shown that stem cell activity and their ability to self-renew can be strongly affected by either stress events, such as oxidative stress (Vono et al., 2018), or by chronic inflammatory diseases, such as bone marrow failure (Pronk et al., 2011; Vono et al., 2018). These events lead to the release of specific cytokines and chemokines into the microenvironment with consequent abnormal stem cell proliferation, mobilization and differentiation as well as premature quiescence and self-renewal decline (Jahandideh et al., 2020). Thus, excessive production and release of pro-inflammatory cytokines upon DNA damage can highly affect the stem cell regeneration capacity contributing to long-term dysfunction in aging tissues, including skin, bone marrow and adipose tissue (Crop et al., 2010; Shin et al., 2017; Hormaechea-Agulla et al., 2020).

Like normal tissue-specific stem cells, also CSCs can be influenced by changes in their surrounding microenvironment; indeed, it has been shown that pro-inflammatory stimuli are an essential component of the CSC niche (Zhang S. et al., 2018) able to potentially alter their function. The presence of inflammatory cytokines can have conflicting effects on CSCs. Growing evidence suggest that inflammation may be an important source of tumor progression and CSC expansion (Jeong et al., 2018), while other studies showed that specific type of cytokines, in particular interferons, can exert anti-tumor activity and obstruct angiogenesis (Martin-Hijano and Sainz, 2020).

Cytosolic DNA Sensors and STING Activation

Cytokines are in fact powerful mediators of stem cell function and their wide range of effects highlights the importance of DNA-sensing pathways, and activation and release of inflammatory molecules. In particular, one of the main adaptor proteins activated upon cytosolic DNA recognition and essential for the initiation of these inflammatory responses is stimulator of interferon genes (STING) (Ishikawa and Barber, 2008).

STING has been identified as an essential component for the initiation of innate immune signaling processes following activation of pathways related to cytosolic nucleic acid recognition (Ishikawa and Barber, 2008). The activation of this endoplasmic reticulum adaptor protein is essential for the triggering of transcription pathways and efficient production of type 1 interferon (IFN1) in several mammalian cell types (Ishikawa and Barber, 2008; Ishikawa et al., 2009). Specifically,

STING activation promotes the nuclear translocation of the transcription factors NF-κB and IRF3 leading to cytokine production and innate immune gene transcription with a strong impact on cell fate and tissue homeostasis (Yum et al., 2021) (**Figure 2**). The specific mechanisms and pathways involved in NF-κB and IRF3 activation, cytokine release, IFN1 expression and immunity regulation have been extensively reviewed in Liu et al. (2017) and Jefferies (2019).

Upon conformational changes, STING translocates to the Golgi apparatus thereby inducing the activation of IRF3 and NF-κB, which function together in order to promote the transcription of cytokines like IFN1 (Ablasser and Chen, 2019; Li and Chen, 2018).

Interferons are a group of cytokines able to modulate the immune response and related inflammatory events. IFN1 shows autocrine, paracrine, and systemic functions and upon interaction with its receptor is able to induce the expression of more than 200 interferon stimulated genes (ISGs), which reinforce the expression of IFN1 leading to inflammation and innate immune signaling activation (Dunphy et al., 2018; Lee and Ashkar, 2018; Martin-Hijano and Sainz, 2020). Although the secretion of IFN1 can be beneficial for the resolution of viral infection events, the chronic exposure to this cytokine can influence stem cell proliferation and thereby induce functional defects. For example, chronic expression of IFN1 as a consequence of DNA damage has been shown to be a critical mechanism that connects DNA damage accumulation with premature aging and inhibition of intestinal stem cell function both *in vitro* and *in vivo* (Yu et al., 2015). Furthermore, IFN1 was shown to be implicated in proliferation and exhaustion of HSCs, and suppression of IFN signaling safeguards stem cell self-renewal and differentiation capacity providing the basis for potential improvements of bone marrow transplantation (Sato et al., 2009). Due to the large spectrum of ISGs produced upon IFN1 activation, the presence of this cytokine might be critical for the treatment of some cancer types

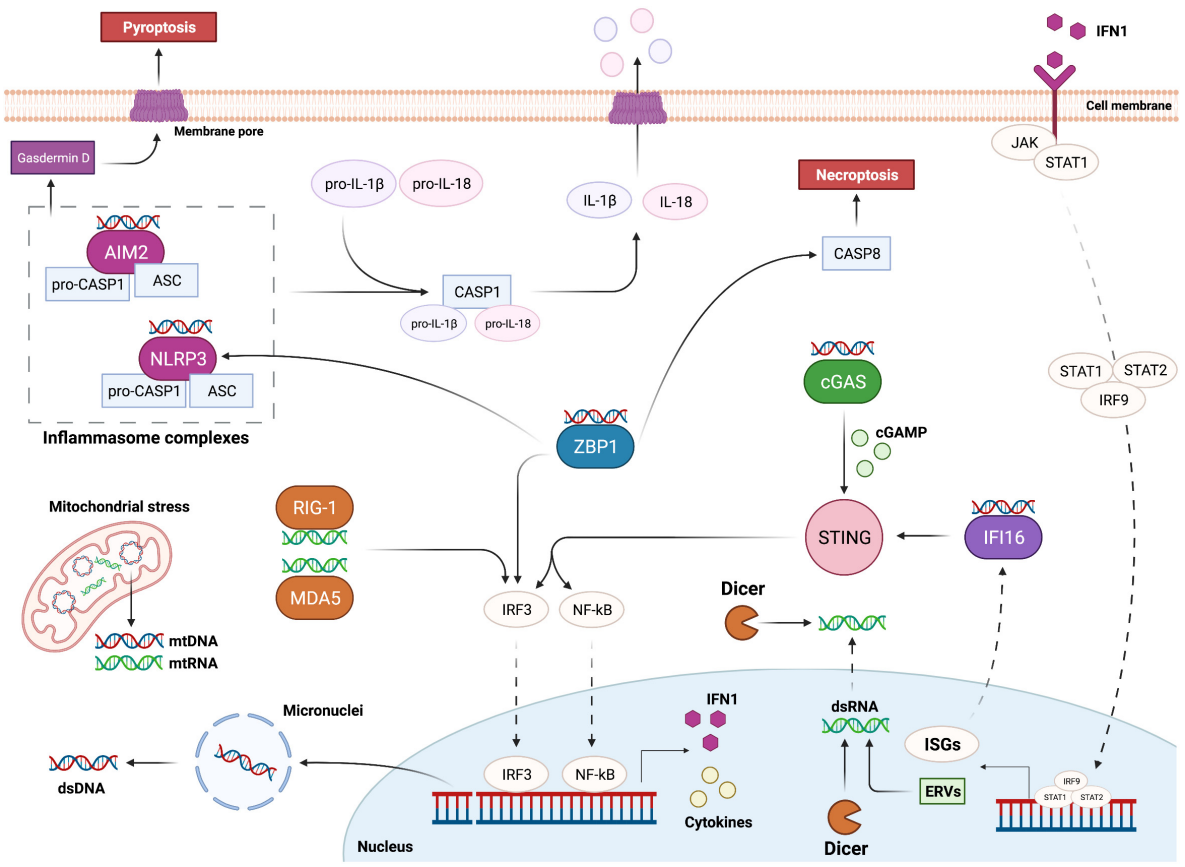

FIGURE 2 | Overview of the main pathways responsible for cytosolic nucleic acid recognition. DNA damage can trigger the formation of micronuclei and the release of double stranded DNA (dsDNA) into the cytoplasm. Mitochondria can also be a source of cytoplasmic DNA and RNA upon genomic stress. AIM2 and NLRP3 are part of two distinct inflammasome complexes, responsible for the bioactivation of Caspase-1 (CASP1); activated CASP1 in turn cleaves and promotes the activation of IL-1β and IL-18, which leave the cell upon inflammasome-mediated pyroptosis. cGAS is the main protein responsible for cytoplasmic dsDNA recognition; upon dsDNA binding cGAS promotes the formation of cGAMP, which binds and activates STING; STING in turn promotes the activation of IRF3 and NF-kB transcription factors responsible for the expression of IFN1 and various cytokines. IFN1 is then able to leave the cell and interact with its receptor; this interaction leads to STAT1, STAT2 and IRF9 complex (ISGF3) translocation into the nucleus and transcription of interferon stimulated genes (ISGs). ISGF3 activation can also lead to expression of ancestor endogenous retroviruses (ERVs) in form of double stranded RNA (dsRNA). Cytoplasmic dsRNA is recognized by RIG-1 and MDA5, which trigger the activation of IRF3. On the other hand, Dicer can sense and cleave both cytoplasmic and nucleic dsRNA. IFI16 is an ISG able to recognize dsDNA and activate STING. ZBP1 promotes IRF3 and NLRP3 activation, and Caspase-8 (CASP8)-mediated necroptosis upon recognition of Z forms of dsDNA.

leading to the regression of CSCs and an overall decrease in tumor viability (Doherty et al., 2017; Martin-Hijano and Sainz, 2020). In fact, IFNs have been shown to impede tumor expansion by inducing prolonged cell cycle arrest and angiogenesis downregulation (Shang et al., 2011). Furthermore, IFNs are fundamental regulators of the immune response against tumors as exemplified by the IFN1-mediated immunogenicity of tumor cells increasing the immune system recognition (Martin-Hijano and Sainz, 2020).

One of the main proteins related to genotoxicity and mediation of immune responses upon cytosolic double-stranded DNA (dsDNA) recognition is the DNA-sensing enzyme cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) (**Table 1**). Activated cGAS promotes the conversion of ATP and GTP into cyclin GMP-AMP (cGAMP), which binds and activates STING (Xia et al., 2016).

In addition to cGAS, other key DNA sensors are known to be important modulators of STING-dependent IFN1 production (**Table 1**). An example is the IFNγ-inducible protein 16 (IFI16), which can recognize both cytosolic and nuclear dsDNA (Almine et al., 2017). Importantly, this protein is not only a DNA sensor but it is itself an ISG, and the activation of this positive feedback loop can further enhance the inflammatory response and immune activation triggered by DNA damage and cytosolic DNA recognition (Dunphy et al., 2018) (**Figure 2**). Hence, excessive accumulation of IFI16 may have important consequences on IFN1-related autoimmune diseases, such as systemic lupus erythematosus and Sjogren syndrome (Li et al., 2019). Interestingly, a recent study showed that upon etoposide-induced DNA damage, DNA damage response factors, such ATM and PARP1, are able to activate p53 and TRAF6, which assemble into a protein complex together with IFI16 outside the nucleus. This complex can then activate STING in a non-canonical way leading to a more pronounced activation of NF-κB compared to IRF3 (Dunphy et al., 2018). Importantly, a higher induction of NF-κB can result in the expression of various pro-inflammatory related-genes, adhesion molecules and cell cycle regulators, such as IL-6, TNFα, RANTES, CXCL10, MMPs and BCL-2 family proteins (Liu et al., 2017; Dunphy et al., 2018).

Both canonical and non-canonical activations of NF-κB are known to be involved in immune and inflammatory responses. Promotion of this family of transcription factors leads to expression of a broad range of molecules leading to inflammation as well as cell survival, proliferation, angiogenesis, cell adhesion and metastasis (Liu et al., 2017). It has been observed that genotoxic stress and NF-κB autocrine and paracrine signaling are able to influence mesenchymal and hematopoietic stem cell characteristics affecting their proliferation capacity and regeneration potential (Ping et al., 2019). Furthermore, chronic exposure to inflammatory molecules induced through activation of NF-κB has been associated with uncontrolled NSC proliferation with consequent risk of mutagenesis and hence cancer development (Widera et al., 2008). NF-κB is also known to be a powerful activator of immune cells through the secretion of several chemokines and cytokines (Hayden et al., 2006). Immune cell infiltration in normal tissue can compromise tissue homeostasis and physiological function. Indeed, a recent study showed that loss of sensory neurons and decreased olfactory function in chronic rhinosinusitis can be linked to a prolonged inflammatory state alongside immune cell infiltration (Chen M. et al., 2019). NF-κB deregulation can indeed lead to overexpression of specific cytokines and chemokines, such as CCL19 and CCL20, with consequent recruitment and proliferation of macrophages and T cells causing a loss in olfactory mucosa horizontal basal stem cell regeneration potential and tissue homeostasis *in vivo* (Chen M. et al., 2019).

TABLE 1 | List of DNA and RNA sensors mentioned in this review, the relative downstream effect and examples of stem cell types shown to express these sensors.

Sensor	Ligand	Source	Downstream effect	Stem cell type	References
cGAS	dsDNA	Virus infection; cytosolic self-DNA; micronuclei	STING activation; NF-kB activation; IFN1 expression	MSCs; HSCs; stem cell-like CD8+ T cells; embryonic stem cells	Yang et al., 2015; Li et al., 2020; Sharma et al., 2020; Zheng et al., 2020
IFI16	dsDNA or ssDNA	Virus infection; cytosolic self-DNA; micronuclei	Modulation of STING activity; NF-kB activation; IFN1 expression	Hair follicle stem cells; HSCs	Piccaluga et al., 2015; Orvain et al., 2020
ZBP1 (DAI)	Z-dsDNA or RNA	Virus infection; cytosolic self-DNA; endogenous RNA	IFN1 expression; cell death; NLRP3 activation	MSCs; intestinal stem cells	Wang et al., 2020; Zhao et al., 2020
AIM2	dsDNA	Virus infection; cytosolic self-DNA	AIM2 inflammasome complex assembly; IL-18 and IL-1β activation; cell death	Intestinal stem cells; epithelial stem cells; MSCs	Man et al., 2015; Yang et al., 2015; Naik et al., 2017
NLRP3	dsDNA	Virus infection; cytosolic self-DNA	NLRP3 inflammasome complex assembly; IL-18 and IL-1β activation; cell death	HSCs; MSCs; CSCs	Huang et al., 2017; Adamiak et al., 2020; Ahn et al., 2020
RLRs	dsRNA	Virus infection; endogenous dsRNA; ERV expression	IFN1 and ISGs expression	NSCs; MSCs; HSCs	Yang et al., 2013; Lin et al., 2019; Clapes et al., 2021
Dicer	dsRNA or ncRNA	Virus infection; endogenous dsRNA; ERV expression; miRNA	dsRNA cleavage and IFN1 repression	Embryonic stem cells; NSCs; CSCs; hair follicle stem cells; intestinal stem cells	Kawase-Koga et al., 2010; Iliou et al., 2014; Park et al., 2017; Vishlaghi and Lisse, 2020; Gurung et al., 2021

Recent studies have also shown a strong relation between specific types of stem cells and cytosolic DNA sensors (Liao et al., 2020). For example, proteins of the cGAS-STING pathway might be highly expressed in hematopoietic stem and progenitor cells (HSPCs) allowing a quick response to stress events and thus becoming critical components of HSPC-driven hematopoiesis (Qian et al., 2016; Liao et al., 2020). Hence, dysregulation of this pathway may lead to myeloid malignancies and inflammation-related diseases such as cardiovascular and metabolic diseases (Liao et al., 2020). Furthermore, recent works showed that activation of STING enhances the formation of a stem cell-like memory phenotype in T cells with a potential beneficial effect for immunotherapy (Li et al., 2020). STING may also play a pivotal role in the differentiation of neuronal progenitor cells (NPCs) into neurons by sensing DNA damage during brain development (Zhang et al., 2020).

Z-DNA binding protein 1 (ZBP1), also known as DLM-1 and DAI (DNA-dependent activator of IFN-regulatory factors), is another potential cytoplasmic recognition receptor able to sense nucleic acids from endogenous and exogenous sources (**Table 1**). Its role as Z-DNA/Z-RNA sensor has been long questioned and further studies are required to elucidate its exact function in inflammation and cell death. However, it has been observed that its activation upon cytoplasmic DNA recognition is sufficient to induce the expression of IFN1 through the activation of IRF3 and IRF7, independently from STING, as well as to induce necroptosis and the NLRP3 inflammasome complex (Takaoka et al., 2007; Kuriakose and Kanneganti, 2018) (**Figure 2**). Importantly, an uncontrolled execution of necroptosis and the release of immunogenic molecules by dying cells may result in detrimental inflammatory responses further driving autoimmune and chronic diseases such as skin inflammation, pulmonary diseases, kidney fibrosis, cardiovascular diseases, and neurodegenerative disorders (Choi et al., 2019; Devos et al., 2020).

Inflammasome Activation and Cytokine Release

Activation of the inflammasomes, formed by innate immune system receptors and sensors (Guo et al., 2015), is another key event for the regulation and induction of inflammation. Upon sensing of PAMP and DAMP molecules inflammasomes are assembled by self-oligomerization into a caspase-1-activating scaffold leading to proinflammatory IL-1 family cleavage and bioactivation (Guo et al., 2015). Inflammasomes are mostly expressed by immune cells and the activation of caspase-1 is not only linked to pro-inflammatory cytokine promotion, but it is also a defining feature of a peculiar type of cell death called pyroptosis. This type of immunogenic cell death is caused by the formation of pores into the cell membrane that are generated upon cleavage of gasdermin D (Liu et al., 2016). This leads to a rapid plasma-membrane rupture and consequent release of proinflammatory molecules into the extracellular environment (Bergsbaken et al., 2009) (**Figure 2**). IL-1β together with IL-18 are the main pro-inflammatory cytokines produced upon inflammasome activation and after being released they can activate a broad spectrum of immunological and inflammatory responses (Strowig et al., 2012; Dinarello, 2018).

Different types of inflammasomes have been identified and described in literature (Schroder and Tschopp, 2010) and among them NLRP3 and AIM2 inflammasomes are the ones that are mostly related to DNA damage and cytokine release (Inoue and Shinohara, 2013; Wei et al., 2019). NLRP3 is part of the NLR protein family (He et al., 2016) and can be activated upon recognition of viral components as well as cytosolic danger signals (Zhao and Zhao, 2020). In the activated form, NLRP3 inflammasome is a multi-protein complex, constituted by NLRP3, ASC and procaspase-1, that is able to bioactivate IL-1β and IL-18 upon caspase-1 activation (Sharma and de Alba, 2021) (**Figure 2**). In particular, it has been observed that DNA damage in skin cells does not cause apoptosis but activation of a fibroblast-specific NLRP3 inflammasome and IL-1β secretion, which lead to defects in stem cell specification and consequent epithelial and dermal hyperplasia (Seldin and Macara, 2020). Similar results have been observed in human keratinocytes upon exposure to UV light. UV-induced DNA damage mediates an increase in NLRP3 gene expression and inflammatory cytokine production, such as IL-1β, IL-6 and TNFα, indicating that DNA damage induces the activation of NLRP3 inflammasome potentially leading to cutaneous tissue disorders (Hasegawa et al., 2016). Moreover, IL-1β and TNFα have been shown to affect adipogenic and osteogenic potential of murine mesenchymal stem cells (MSCs) *in vitro*, which might correlate with collagen-induced arthritis *in vivo* (Sullivan et al., 2014).

Like NLRP3, AIM2 inflammasome activation is also important for the recognition of cytosolic dsDNA (**Table 1**). Indeed, AIM2 is an HIN-200 protein family member able to activate caspase-1 upon sensing of cytoplasmic DNA (Fernandes-Alnemri et al., 2009). Similarly to the NLRP3 inflammasome, interaction of AIM2 with ASC allows the activation of procaspase-1 leading to IL-1β and IL-18 activation and pyroptosis induction (Hornung et al., 2009; Sagulenko et al., 2013) (**Figure 2**). Interestingly, a recent study showed that AIM2 can also sense radiation-induced DNA damage into the nucleus of epithelial and bone marrow cells leading to AIM2 inflammasome assembly (Hu et al., 2016). As a consequence, AIM2 inflammasome can promote caspase-1 activation, immunogenic cell death and release of mature cytokines into the surrounding environment (Hu et al., 2016). As mentioned previously, CSCs also strongly depend on their microenvironment and events, like pyroptosis, with the consequent release of IL-1β and IL-18 into the extracellular environment might potentially lead to stimulation of dormant CSCs, leading to increased tumor treatment resistance and metastases (Tulotta et al., 2019; Van Gorp and Lamkanfi, 2019). This further supports pharmacologic inhibition of IL-1β as a potential cancer treatment strategy (Tulotta et al., 2019).

Role of RIG-1-Like Receptors in dsRNA Sensing

The activation of nucleic acid sensors is not only limited to the recognition of cytosolic self-DNA but can also be induced by the presence of endogenous double-stranded RNA

(dsRNA). Activation of retinoic acid-inducible gene 1 (RIG-1) like receptors (RLRs) can be triggered by both viral and host-derived RNAs leading to strong immune activation and inflammatory responses (**Table 1**) (Rehwinkel and Gack, 2020; Onomoto et al., 2021).

Several studies have shown a connection between the promotion of the IRF3-IFN1 axis and activation of RLRs in response to DNA damage. In particular, deregulation of both RIG-1 and MDA5 has been associated to autoimmune and inflammatory diseases induced by a STING-mediated IFN1 production (Ghosh et al., 2018; Onomoto et al., 2021).

Although why and how dsRNA is being released after DNA damage largely remains to be elucidated, recent studies showed an activation of latent endogenous retroviruses (ERVs) upon irradiation and DNA DSB formation. ERVs are originated from retroviruses that are thought to have infected early ancestor's germ cells millions of years ago and most of these ERVs are normally silent or suppressed (Gao et al., 2021). However, it has been observed that after stress events, such as ionizing radiation-induced DNA damage, there is an activation of these dormant genes with consequent formation and release of dsRNA and IFN1 expression through the activation of STING (Lee et al., 2020). The promotion of these ERVs upon DNA damage may enhance the activation of transcription factors leading to innate immunity activation and secretion of molecules with similar consequences as the ones observed upon dsDNA recognition and cytosolic DNA sensor activation (**Figure 2**).

Another protein involved in dsRNA sensing is the endoribonuclease Dicer (**Table 1**), this enzyme is usually required for the processing and maturation of miRNAs (a class of small ncRNAs important for the regulation of gene expression at the post-transcriptional level) (Kuehbacher et al., 2007). However, recent studies showed its essential role also in the recognition and clearance of dsRNA localized in both the cytoplasm and the nucleus (Much et al., 2016; Burger et al., 2017). In fact, its downregulation has been shown to correlate with dsRNA accumulation and consequent IFN1 production *in vitro* (White et al., 2014). Interestingly, Dicer and its isoform (aviD) (Poirier et al., 2021) have been shown to be upregulated in NSCs, embryonic stem cells and adult intestinal stem cells (Kawase-Koga et al., 2010; Park et al., 2017; Gurung et al., 2021), and knock down of aviD has been shown to be related to higher levels of stem cell apoptosis upon viral infection (Poirier et al., 2021). Moreover, a DNA damage-inducible phospho-switch of Dicer has been linked with accumulation of this protein into the nucleus and consequent dsRNA clearance and prevention of RLR activation (Burger et al., 2017). Although central in dsRNA clearance upon viral infection, it remains unknown whether Dicer and aviD might play a role in the modulation of DNA damage-induced IFN1 expression in both normal and cancer stem cells.

Role of Mitochondrial DNA in Inflammation and Immunity

Mitochondria are dynamic and essential organelles important for cellular bioenergetic maintenance, calcium metabolism and apoptotic processes (Detmer and Chan, 2007), and a proper function of this organelle is also important for stem cell self-renewal and differentiation (Zhang H. et al., 2018). Next to the DNA in the nucleus, also mitochondria contain several copies of their own circular DNA and changes in mitochondrial DNA (mtDNA) are often implicated in gene expression alterations, loss of tissue function, cancer and diseases (Singh et al., 2015; Castellani et al., 2020). DNA damage has been associated to mitochondrial dysfunction either via direct damage to the mtDNA or via depletion of nicotinamide adenine dinucleotide (NAD+) through activation of PARP1, a DDR protein that consumes NAD+, causing an imbalance in energy levels (Schumacher et al., 2021). Other processes such as DNA damage-associated defects in mitophagy and mtDNA replication might also contribute to mitochondrial dysfunction (Fang et al., 2014).

Mitochondrial disfunction and the consequent mtDNA leakage into the cytoplasm have been related to inflammatory responses upon activation of DNA sensor molecules. Similar to cytosolic self-DNA, recognition of cytosolic mtDNA can trigger the induction of inflammatory pathways, such as cGAS and NOD-like receptors leading to STING activation, promotion of IFNβ expression, release of NF-κB-related cytokines release as well as increased transcription and activation of IL-1β and IL-18 (Dib et al., 2015; Mussil et al., 2019; Guo et al., 2020). As mentioned previously, the release of these pro-inflammatory cytokines into the stem cell microenvironment can strongly affect stem cell function and self-renewal. Additionally, mitochondria are a major source of ROS and mitochondrial dysfunction can lead to the release of ROS, which in turn further damages the mitochondria and their mtDNA reinforcing the associated inflammatory response. Interestingly, specific inhibition of mitochondrial ROS was shown to prevent the activation of the NLRP3 inflammasome (Chen et al., 2018).

Furthermore, DSBs of mtDNA upon genotoxic events have been associated with mitochondrial disfunction and consequent mitochondrial RNA (mtRNA) release thereby triggering RIG-1-dependent pathways. Recognition of cytoplasmic mtRNA leads to IFN1 activation and ISG promotion in order to cope with the damaged mtDNA emphasizing the importance of mitochondria-nucleus communication and immunity activation (Tigano et al., 2021). In conclusion, release and recognition of mtDNA upon DSBs can trigger similar immunostimulatory events as the ones observed upon recognition of cytoplasmic self-DNA suggesting that also mtDNA can potentially be implicated in stem cell functional defects and immunity activation. The specific role of mtDNA recognition in inflammation has been recently reviewed by Riley and Tait (2020) (**Figure 2**).

IMMUNE CELL INFILTRATION IN THE STEM CELL MICROENVIRONMENT

The immune system works as the main body's line of defense against pathogens, toxins and tumors while simultaneously being involved in other cellular processes such as tissue development, homeostasis, and repair. The immune response is traditionally classified into innate and adaptive (Vivier and Malissen, 2005).

The innate immune cells, such as natural killer cells, mast cells and phagocytic cells, are activated by PAMPs and DAMPs and their non-specific response includes phagocytosis, cell locomotion, killing of pathogens or cells and cytokine production. On the other hand, the adaptive immune response is antigen specific and able to create a long-term immunological memory mediated by cells, like dendritic cells, specialized T cells and B cells (Netea et al., 2020). Interestingly, the innate immune response can be also mediated by stem cells such as HSCs. Indeed, it has been shown that HSCs can create an epigenetic memory in response to genotoxic stress or pathogens and that IFNγ can specifically lead to proliferation and myeloid-biased progenitor differentiation during an innate immune response to infection (Aurora and Olson, 2014; Matatall et al., 2014).

As previously described pathways related to immune cell activation and DNA damage recognition are strongly interconnected and the release of immune modulators upon DNA damage responses can recruit and activate immune cells emulating pathogen infections or tissue injury. An uncontrolled infiltration of immune cells may severely compromise stem cell self-renewal capacity by affecting the local stem cell microenvironment leading to a loss in cellular homeostasis in healthy organs and tissues (Naik et al., 2018). Indeed, it has been observed that infiltration of macrophages can influence skeletal muscle regeneration by affecting the activity and recruitment of fibroadipogenic progenitor cells into the injury site (Low et al., 2017; Dort et al., 2019).

Macrophages are highly dynamic and essential innate immune cells implicated in tissue homeostasis and regeneration. Their activity is highly controlled by specific signals and their polarization toward a proinflammatory (M1) or anti-inflammatory (M2) phenotype is strongly dependent on the presence of specific cytokines within the environment (Barcellos-Hoff et al., 2005). Pro-inflammatory macrophages are mostly induced my microbial elements as well as TLR ligands and cytokines such as IFNs and TNFα, while anti-inflammatory macrophages can be induced by cytokines like IL-4 and IL-13 (Barcellos-Hoff et al., 2005; Viola et al., 2019). IRFs and NF-κB are the main transcription factors involved in cytokine and chemokine release and chronic activation of these pathways upon DNA damage can thereby influence the recruitment and functionality of unpolarized macrophages (Platanitis and Decker, 2018) (**Figure 3**, points 1–3).

Other immune regulatory cells, such as MSCs, are able to differentiate into a variety of cell types promoting repair and remodeling of tissues such as bone, cartilage, muscle, adipose and connective tissues (DiMarino et al., 2013; MacDonald and Barrett, 2020). MSCs foster the regeneration of these tissues by controlling immune cell activation, angiogenesis, and extracellular matrix deposition (DiMarino et al., 2013).

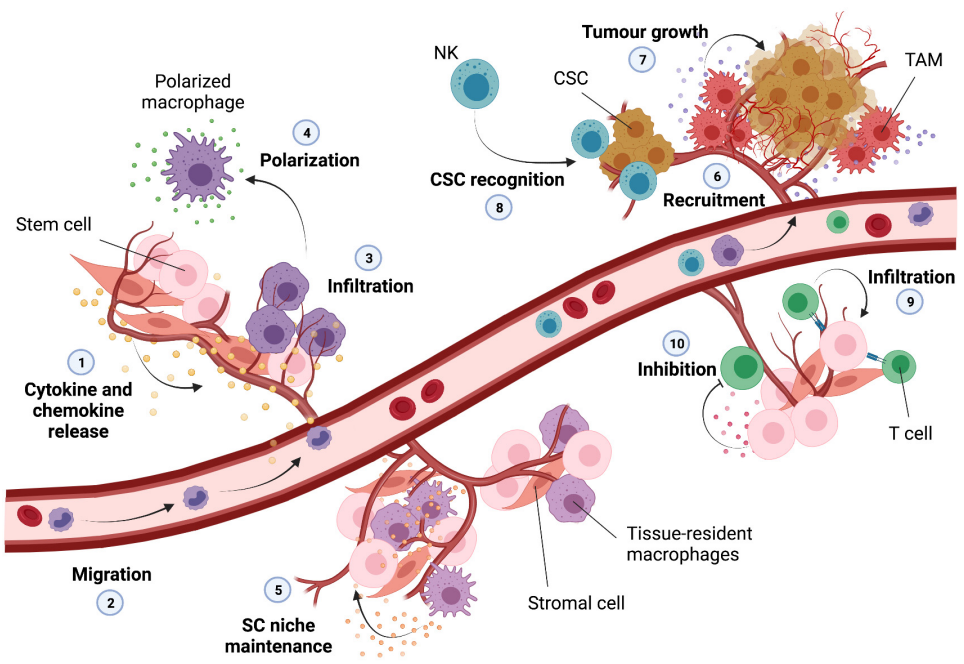

FIGURE 3 | Infiltration of immune cells into the normal stem cell and cancer stem cell niche. DNA damage can trigger the release of various cytokines and chemokines into the microenvironment (1); these can lead to migration and recruitment of macrophages (2) that exit the circulation and infiltrate into the tissue (3). The inflammatory microenvironment is then able to trigger the polarization of these macrophages (4) into M1 or M2 subtypes. Tissue-resident macrophages are normally present into the stem cell microenvironment, and they are important for the maintenance of the stem cell niche (5). The tumor microenvironment can lead to recruitment of various immune cells (6); tumor associated macrophages (TAM) can secrete inflammatory cytokines important for tumor growth and expansion (7); natural killer (NK) cells can potentially recognize and eliminate cancer stem cells (CSC) upon infiltration (8). T-cells can infiltrate into the normal stem cell niche (9) influencing stem cell proliferation and homeostasis; some types of stem cells are able to secrete molecules able to inhibit activation and differentiation of infiltrated T-cells (10).

Recent studies showed that inflammatory cytokines produced by polarized macrophages (**Figure 3**, point 4) can influence migration and differentiation of human MSCs toward an osteoblastic lineage essential for effective bone tissue regeneration and spinal cord repair (Maldonado-Lasunción et al., 2018; Vallés et al., 2020). Furthermore, it has been shown that MSCs can maintain tissue homeostasis upon injury by inhibiting macrophage activity and T-cell mediated immune responses and thus preserving the activity of corneal epithelial precursor cells (Ko et al., 2020).

Macrophage's polarization has been suggested to influence not only MSCs but also HSC self-renewal capacity and quiescence status. In fact, macrophages are essential regulators of HSC pool size and mobilization, and the cytokines produced by pro-inflammatory macrophages can directly affect haematopoiesis by acting on the HSC niche and function (McCabe and MacNamara, 2016; Seyfried et al., 2020). It has been described that in physiological conditions tissue resident-macrophages positively contribute to bone marrow homeostasis by promoting HSC niche maintenance and activity. Indeed, these tissue-resident immune cells can produce matrix metalloproteinases in order to degrade the matrix that surrounds HSCs leading to HSC escape into the circulation (Winkler et al., 2010). However, another study showed that resident macrophages are also important for the retention of HSCs in the spleen through the expression of adhesion molecules (**Figure 3**, point 5). Thus, this demonstrates that elimination of tissue resident macrophages can cause HSC escape into the circulation influencing extramedullary hematopoiesis (Dutta et al., 2015).

The dual proinflammatory and anti-inflammatory feature that macrophages can acquire upon infiltration plays a pivotal role in tissue homeostasis and stem cell function. This seems to be particularly important also for the CSC niche and tumor progression. Macrophage infiltration has been recently associated with many types of tumors and their presence in the tumor environment together with their paracrine signaling have been linked to glioblastoma growth and spread of the CSC phenotype (Shi et al., 2017). Recent works have observed a correlation between the presence of tumor associated macrophages (TAMs) and CSC niche modification and expansion. TAMs are crucial components of the tumor microenvironment, able to exert pro-tumor features through the activation of specific signaling pathways and secretion of a broad range of inflammatory cytokines. For instance, it has been observed that TAMs are able to physically interact with CSCs leading to induction of NF-κB and release of cytokines for the sustainment of the CSC phenotype (Lu et al., 2014). Furthermore, TAMs are also able to promote CSC-like properties through the secretion of higher levels of TGFβ1 compared to other type of macrophages (Fan et al., 2014). Lastly, the presence of TAMs has been positively associated with CSC density in human tumors and the consequent release of pro-inflammatory cytokines has been correlated with an increase of CSC-like cells and invasiveness (Fan et al., 2014) (**Figure 3**, point 6–7).

Unlike macrophages, natural killer (NK) cells possess abilities to infiltrate and selectively kill CSCs. They are major effectors of innate immunity and therefore able to display a strong cytolytic activity against many tumors or virus-infected cells. Although the role of NK cells in cancer surveillance remains still under debate, recent *in vivo* and *in vitro* studies suggested that NK cells may be able to specifically detect CSCs through the recognition of surface markers leading to a possible decrease in tumor malignity (Tallerico et al., 2017). Furthermore, reduced function of NK cells has been associated with increased risk of developing tumors together with an increased risk in tumor-related mortality (Luna et al., 2017) (**Figure 3**, point 8).

T cells coordinate multiple adaptive immune responses and are responsible for the recognition of pathogens, antigens and tumors. They originate from bone marrow progenitor cells and upon maturation in the thymus migrate to the periphery of the body in order to exert their patrolling functions. Upon antigen encounter, T cells are able to differentiate into effector cells, a key event for the elimination of pathogens through the production of several cytokines and cytotoxic mediators (Kumar et al., 2018; Goswami and Awasthi, 2020). It has been shown that MSCs can influence T cell proliferation and differentiation *in vitro* through the secretion of a number of soluble factors (Duffy et al., 2011). Galectin-1 in particular is highly expressed by MSCs and able to directly inhibit T cell activation. Indeed, knockdown of this protein *in vitro* has been shown to partially rescue proliferation of both killer and helper T lymphocytes (Gieseke et al., 2010). T cells can differentiate into different subsets of cells; however, dysregulation of this process can lead to immunological deficiencies and autoimmune diseases. For example, it has been observed that NPCs are able to selectively inhibit differentiation of pathogenic T cells through the expression of leukaemia inhibitory factor (LIF) receptors providing potential new insights into multiple sclerosis (Cao et al., 2011). On a separate study, using scRNA sequencing T cells have been shown to infiltrate the adult sub-ventricular zone NSC niche during aging. T cells in old brains secrete IFNγ which promotes a decrease in proliferation of NSCs in both co-culture experiments and *in vivo* (Dulken et al., 2019) (**Figure 3**, point 9).

Furthermore, MSCs are also able to indirectly modulate T cell activity by affecting maturation and differentiation of antigen presenting cells, such as dendritic cells, important for antigen processing and presentation (Jiang et al., 2005). While neoantigen presentation has been mostly studied in cancer (stem) cells with a focus on T cells and the expression of major histocompatibility complex I (MHC-I) after radiation- or chemotherapy-induced DNA damage in connection with an increased mutational load and subclonal neoantigen generation (McLaughlin et al., 2020), little is known about DNA damage and neoantigen presentation in normal tissue-specific stem cells and their microenvironment (**Figure 3**, point 10).

THE DUAL ROLE OF CELLULAR SENESCENCE IN STEM CELL FUNCTION

Cellular senescence is described as an irreversible state of growth arrest triggered by a number of oncogenic events, such as telomere shortening, chromatin perturbation, replication stress, DNA damage and chronic exposure to anti-proliferative

cytokines like IFNβ. Unlike quiescence, the senescence state is permanent and cannot be reversed by known physiological stimuli (Campisi and D'Adda Di Fagagna, 2007; Collado et al., 2007). Senescent cells undergo morphological changes accompanying by apoptosis resistance and an altered gene expression pattern that leads to deep metabolic reprogramming and secretion of a wide range of soluble and insoluble factors collectively named senescence-associated secretory phenotype (SASP) (Herranz and Gil, 2018). Furthermore, senescence is associated with persistent DDR activation often correlated with high expression of cell-cycle inhibitors, such as p21 (also termed CDKN1a) and p16 (also termed CDKN2a) (Campisi and D'Adda Di Fagagna, 2007; Faget et al., 2019).

The SASP is a common feature of senescent cells, able to induce paracrine signaling through the secretion of factors including inflammatory cytokines and chemokines. Importantly, it has been observed that there is not a singular SASP phenotype and its composition can change depending on the senescence-inducing triggering factor and cell type (Gonzalez-Meljem et al., 2018). Recent studies have demonstrated that a prolonged DDR activation (Fumagalli et al., 2014) as well as activation of previously mentioned DNA damage-related mechanisms, such as cGAS/STING (Yang et al., 2017), inflammasomes (Yin et al., 2017) and mitochondrial stress pathways (Passos et al., 2007), are able to induce autocrine and paracrine senescence of neighboring cells through the secretion of SASP factors. The promotion of these pathways seems to converge on C/EBPB and NF-κB activation, responsible for the direct regulation of inflammatory cytokine expression and release, in particular IL-8, IL-6 and IL-1α (Herranz and Gil, 2018). IL-1 signaling has been linked to *in vivo* paracrine senescence upon activation of the inflammasome complex (Acosta et al., 2013), while IL-6 and IL-8 are specific interleukins able to act in a paracrine manner to promote senescence development, and their depletion has been shown to prevent senescence entry *in vitro* (Kuilman et al., 2008). Furthermore, STING activation and a sustained IFNβ signaling also cause senescence. Indeed, it has been shown that acute IFNβ stimulation can reversibly arrest cell growth while its chronic stimulation leads to p53-dependent cell cycle arrest and subsequent senescence (Moiseeva et al., 2006).

Although stem cells are able to divide and renew over a long period of time, they are also susceptible to cell cycle arrest and senescence upon exposure to genotoxic stress thereby affecting tissue regeneration and homeostasis (Vitale et al., 2017). A clear example is given by radiation induced-DNA damage, which can promote senescence in different types of stem cells (Chen Z. et al., 2019). In a recent study it has been observed that irradiation induces senescence of bone marrow-derived mesenchymal stem cells (BMSCs), which can be associated with decline in bone formation, a typical side effect of anticancer therapies. Importantly, the secretion of SASP components by senescent BMSCs worsens bone marrow remodeling by inducing osteogenic differentiation dysfunction via paracrine signaling. SASP can therefore be a potential target to ameliorate radiation-induced bone loss (Bai et al., 2020). Although quiescent HSCs are relatively radioresistant, they can be affected by radiation-induced ROS production (Shao et al., 2014; McBride and Schaue, 2020). Indeed, HSCs appear to be quite sensitive to oxidative stress generated upon irradiation or through the activation of proinflammatory pathways (McBride and Schaue, 2020). These events can cause HSCs to undergo premature senescence leading to long-term bone marrow suppression and decrease repopulation capacity (Shao et al., 2014). Cellular senescence has also been identified in NPCs of primary progressive multiple sclerosis (MS)-derived tissue linking DNA damage to remyelination failure and thus offering potential new treatments against MS (Nicaise et al., 2019). In salivary glands irradiation is able to induce accumulation of senescent cells in or near the salivary gland stem and progenitor cell (SGSC) niche both *in vitro* and *in vivo* leading to tissue-specific functional impairment. Selective elimination of senescent cells was shown to improve the self-renewal of SGSCs and to partially rescue salivary secretion activity (Peng et al., 2020). Furthermore, SASP release has been shown to play a pivotal role in salivary gland homeostasis and function upon radiation-induced senescence (Marmary et al., 2016). Sustained expression of IL-6 in particular is known to be essential for both induction of senescence and tissue hypofunction. However, exposure to this cytokine prior irradiation has been shown to enhance DNA repair preventing senescence and salivary gland dysfunction (Marmary et al., 2016). Senescence and SASP promotion can also cause perturbation of the intestinal stem cell niche contributing to potential gastrointestinal disorders, inflammation and carcinogenesis upon heavy ion irradiation and DNA damage (Kumar et al., 2019).

In contrast to normal tissue-specific stem cells, due to its pro-inflammatory features, cellular senescence plays a pivotal role in cancer promotion and stemness. SASP can create an immunosuppressive environment driving tumorigenesis, tumor progression and metastasis (Faget et al., 2019). In multiple myeloma, the release of chemokines, like IP-10 and RANTES, by senescent cells was shown to favor the emergence and maintenance of cancer stem-like cells (Cahu et al., 2012). In CSCs, a gain of stem cell-like features can severely impact tumor progression and aggressiveness. A recent study showed that chemotherapy-induced senescence can lead to a significant upregulation of stem-cell like markers, such as Kit and Sca1, in senescent cells compared to non-senescent cells thereby leading to a much more aggressive tumour phenotype (Milanovic et al., 2018).

Although senescence and SASP are generally associated with aging-related diseases and tumorigenesis, recent studies have also brought to light a positive impact of senescence and SASP in the regeneration and cell reprogramming of some tissues. In acute and chronic muscle injury, cellular plasticity and skeletal muscle reprogramming were shown to be promoted by muscle-damage-induced senescence and SASP release (Chiche et al., 2017). Furthermore, it has been observed that transient exposure to SASP factors promotes cell plasticity, tissue regeneration and stemness while chronic SASP exposure counteracts the regeneration stimuli by inducing cell cycle arrest. Specifically, Ritschka et al. (2017) showed that incubation of primary mouse

keratinocytes with conditioned medium from oncogene-induced senescent cells during a short-term period of 2 days led to increased stem cell features and that transplantation of these cells generated more hair follicles compared to untreated cells. However, upon a prolonged exposure to the same SASP both cell-intrinsic and paracrine senescence was observed (Ritschka et al., 2017). Similar findings have been reported by Mosteiro et al. (2018), whose work showed that senescence is important for *in vivo* tissue reprogramming mediated by OCT4, SOX2, KLF4, and MYC (OSKM), four transcription factors used to reprogram somatic cells into iPSCs (Cai et al., 2015; Cevallos et al., 2020). Following this approach, the authors found that paracrine secretion of IL-6 and other soluble factors is essential for the reinforcement of cellular senescence and regeneration upon damage *in vivo* (Mosteiro et al., 2018). This has been recently observed also in fibro-adipogenic progenitor cells highlighting the importance of SASP and senescence in tissue remodeling and plasticity (Saito et al., 2020). Senescence is clearly a powerful mechanism important not only for cell cycle arrest but also for the induction of a state of regenerative inflammation that enhances tissue repair and function upon DNA damage.

CONCLUDING REMARKS

DNA damage profoundly affects the inflammatory microenvironment where stem cells reside, which can have detrimental consequences for their maintenance and long-term function. Indeed, it has been shown that DNA damage-induced immunostimulatory events can lead to tissue-specific stem cell exhaustion leading to degenerative conditions. Conversely, the release of specific cytokines can also positively impact tissue-specific stem cell plasticity and regeneration of damaged tissues in addition to enhance CSC activity leading to tumor progression.

This review provides an overview of the main biological mechanisms linked to changes in the stem cell microenvironment and activation of immune processes upon DNA damage induction. Although recent findings have brought to light new insights into these DNA damage-related inflammatory events, some questions remain unanswered. For instance, it is still not clear how to exploit the production of inflammatory cytokines in order to promote on one side immunostimulatory responses against the tumor and on the other side immunosuppressive responses against aging-related degenerative conditions. Especially since the activation of DNA and RNA sensors might change depending on the specific stimulus and cell type. For example, it has been shown that cytosolic DNA in keratinocytes of psoriatic lesions or exposure of hematopoietic cells to ionizing radiation can both trigger the activation of the AIM2 inflammasome (Dombrowski et al., 2011). However, exposure of keratinocytes to UVB has been shown to induce NLRP3 and cGAS activation with consequent production of a broad spectrum of cytokines including IL-1, IL-6, TNFα, and IFN1 (Hasegawa et al., 2016; Li et al., 2021). These studies exemplified how different stimuli can trigger the activation of different sensors within the same cell type. Whether such observations might also apply to adult stem cells and their self-renewal capacity leading to organ-/tissue-specific outcomes remains to be elucidated.

DNA damage-induced senescence plays a pivotal role in cell cycle arrest and can be used as a barrier against tumor expansion; however, due to the accompanying SASP, it is also responsible for loss of tissue function, aging-related diseases and tumor progression. Therefore, further studies are required to understand how to properly modulate the exposure to SASP factors toward the promotion of a regenerative state and against detrimental effects, such as paracrine senescence of neighboring cells and chronic inflammation. Furthermore, it would be interesting to explore how different types of DNA damage can influence senescence and its SAPS phenotype in different adult stem cells. It has been shown that the SASP associated with radiation-induced DNA damage can differ from SASP induced by other stress factors, such as mitochondrial dysfunction (Wiley et al., 2016; Aratani et al., 2018). Moreover, p53 plays a central role in the DDR and its activity is essential for the prevention of cancer development by promoting cell death and senescence (Mijit et al., 2020). However, it has been shown that in some types of adult stem cells, such as hair follicle bulge stem cells and HSCs, reduced p53 activation upon irradiation mediates resistance to apoptosis or senescence (Sotiropoulou et al., 2010; Insinga et al., 2013) indicating that DDR proteins can actually trigger distinct responses in different types of stem and progenitor cells (Lee et al., 2013).

Further understanding of DNA damage immunomodulatory mechanisms, cell- and stimulus-specific variability might unravel novel strategies to regulate the stem cell microenvironment. Recently developed 3D *in vitro* models, such as organoids and organs-on-a-chip (OOC), represent innovative strategies to address these questions by closely resembling the normal and cancer stem cell microenvironment. Furthermore, organoid co-culture systems and assembloids, organoids generated by incorporating multiple cell types or by fusing organoids of different identities (Vogt, 2021), can be used to study the interaction between immune cells and normal or cancer stem cells upon genotoxic stress. For instance, OOC allow to mimic the combined response to several stimuli and environmental changes by using multiple cell types/stimuli in different chambers (Wu et al., 2020; Almela et al., 2021). These advanced 3D *in vitro* models together with 3D bioprinting techniques, which can reproduce complex tissue structures by using a combination of multicomponent bioinks and cell types (Almela et al., 2021), allow to generate a similar organizational complexity to *in vivo* tissues/organs with the ability to regulate key parameters, such as tissue interactions, concentration gradients and cell patterning (Wu et al., 2020; Almela et al., 2021).

As mentioned in this review, genotoxic stress can affect the stem cell microenvironment leading to stem cell exhaustion, likely through a combination of a decline in cell number and functional capacity, with the emergence of aging-related pathologies (Schumacher et al., 2021). On the other hand, due to their self-renewal properties, CSCs are also affected by DNA damage and the associated inflammatory microenvironment, which can worsen tumor control and treatment efficacy. Understanding the mechanistic links between stem cell

properties and microenvironmental changes initiated upon DNA damage will be critical to counteract the functional decline of adult stem cells in aging-related diseases and effectively diminish CSC activity and expansion.

AUTHOR CONTRIBUTIONS

DC, RC, and LB wrote the manuscript. DC and LB designed the figures. All authors contributed to the article and approved the submitted version.

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Functions of the CSB Protein at Topoisomerase 2 Inhibitors-Induced DNA Lesions

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Topoisomerase 2 (TOP2) inhibitors are drugs widely used in the treatment of different types of cancer. Processing of their induced-lesions create double-strand breaks (DSBs) in the DNA, which is the main toxic mechanism of topoisomerase inhibitors to kill cancer cells. It was established that the Nucleotide Excision Repair pathway respond to TOP2-induced lesions, mainly through the Cockayne Syndrome B (CSB) protein. In this paper, we further define the mechanism and type of lesions induced by TOP2 inhibitors when CSB is abrogated. In the absence of TOP2, but not during pharmacological inhibition, an increase in R-Loops was detected. We also observed that CSB knockdown provokes the accumulation of DSBs induced by TOP2 inhibitors. Consistent with a functional interplay, interaction between CSB and TOP2 occurred after TOP2 inhibition. This was corroborated with *in vitro* DNA cleavage assays where CSB stimulated the activity of TOP2. Altogether, our results show that TOP2 is stimulated by the CSB protein and prevents the accumulation of R-loops/DSBs linked to genomic instability.

Keywords: Topoisomerase 2 inhibitors, Nucleotide Excision Repair (NER), R-loops, DNA repair, CSB, Topoisomerase 2

INTRODUCTION

Topoisomerase are essential enzymes required for transcription, replication, and chromatin remodeling. Topoisomerases TOP1 and TOP2 mediate the cleavage, respectively, of single or double stranded DNA for relaxing generated DNA supercoiling, untangle catenanes, and condense chromosomes, avoiding DNA over winding. Topoisomerases are particularly vulnerable to topoisomerase poison (topoisomerase inhibitors) during their cleavage reaction. These drugs block the re-ligation step of the enzyme-induced DNA break through the formation of the drug-DNA-topoisomerase complexes, which is referred to as the cleavage complex (TOPcc). The cytotoxic activity of TOP1 inhibitors such as camptothecin is related to the interference of trapped TOP1cc with DNA replication and transcription. Processing of these complexes creates double-strand breaks (DSBs) in the DNA, which is their main toxic mechanism to kill cancer cells (Pommier, 2013; Pommier et al., 2016; Marinello et al., 2018). Similarly, Topoisomerase 2 (TOP2) inhibitors such as Doxorubicin (DOX) and Mitoxantrone (MXT) are drugs widely used in the treatment

of different types of cancer, such as breast, prostate, lung, bladder, testis, leukemia, lymphomas, and osteosarcomas.

We have previously reported that NER pathway deficiency reenforces TOP2 inhibitors suggesting a role of the (NER) pathway in processing the TOP2cc intermediate (Saffi et al., 2010; Rocha et al., 2016a,b). Thus, deficiency in Cockayne syndrome B (CSB), a protein from TC-NER (Transcription Coupled – NER), accumulates more Top2ccs in response to MXT than cells deficient in XPC, a protein from GG-NER (Global Genome – NER) (Rocha et al., 2016a). These results strongly indicate an involvement of the NER pathway, or at least of CSB, in processing of these complexes and, maybe, mediating the generation of DSBs.

The CSB protein, also known as ERCC6, is a multifunctional protein belonging to the SWI/SNF2 superfamily that completes other non-canonical functions besides the classical functions NER pathway, including DSBs repair (Batenburg et al., 2015). Batenburg et al. (2017) have shown that CSB is involved in the pathway choice to repair DSBs, once it removes histones from the damaged area in the DNA, allowing HR proteins to access it. It was also shown that CSB could imply in DSBs signaling when they occur in active-transcribed genes, once these are important regions in the genome (Wei et al., 2015, 2016; Teng et al., 2018). CSB also seems to be involved in resolving R-loops (Sollier et al., 2014), which are DNA:RNA hybrids that can occur physiologically during different processes, including transcription and replication (Chédin, 2016; Bhatia et al., 2017). Persistent R-loops forming in the head-on direction can block DNA replication and, if unresolved, can cause DNA breaks and genomic instability (Aguilera and Gómez-González, 2017; Hamperl et al., 2017). CSB is also involved in recognizing such hybrids at active-transcribed regions, promoting mRNAs release for their use as a template by HR factors (Teng et al., 2018).

It has also been shown that R-loops are powerful and reversible topology sink that cells may use to relieve superhelical stress during transcription (Stolz et al., 2019). Coordinated action of Top1 and Top2 counteract the accumulation of torsional stress at replication forks, thus preventing the diffusion of topological changes along large chromosomal regions (Bermejo et al., 2007). Hence cells treated with Camptothecin increases topological stress which accumulate R-loops and result into more genome instability (Sollier et al., 2014; Manzo et al., 2018). We hypothesized that CSB recognizes Top2cc mediated R-loops in response to TOP2 inhibitors. Such R-loops accumulation might be a consequence of RNA Polymerase (RNA Pol) arrest causing by the complexes TOP2ccs. Therefore, this study aimed to understand the role of CSB in response to TOP2 inhibitors and the relation with DSB repair pathways.

MATERIALS AND METHODS

Cell Culture, siRNAs, Plasmids and Transfection

All mammalian cells were cultured at 37°C with 5% CO_2. U2OS human osteosarcoma cells (ATCC) was cultured in McCoy's medium and HEK-293T cells (ATCC) and U2OS-TRE reporter cells were cultured in Dulbecco's modified Eagle's medium, all of them containing 10% fetal bovine serum (FBS). All cells were transfected with plasmid DNAs using Lipofectamine 2000 and siRNA oligonucleotides using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. All siRNAs were purchased from Dharmacon as a SMARTpool and 50 nM siRNA was used for transfection. pTREX-FLAG-TOP2A and pTREX-FLAG-TOP2B were provided from Dr. YP lab (NIH) and 1 μg DNA was used to overexpress TOP2A and TOP2B in HEK-293T cells. pBroad3 TA-KR and pBroad3 tetR-KR were provided by Dr. Li Lan lab (MGH) and 1 μg DNA was used for damage at RNA transcription (DART) assay.

Reagents and Antibodies

Doxorubicin (DOX), Mitoxantrone (MXT) and Etoposide (ETO) were purchased from Sigma Aldrich. Antibodies anti-CSB (ab96089), anti-topoisomerase 2 alpha (ab12318) and anti-topoisomerase 2 beta (ab72334) were purchased from Abcam. Monoclonal anti-FLAG M2 antibody and monoclonal anti-vinculin antibody (V9131), used as loading control in western blot analysis, were purchased from Sigma Aldrich. Anti-phospho-histone H2A.X (Ser139) (clone JBW301) was purchased from Merck Millipore and 53BP1 antibody was purchased from Novus Biologicals (NB100-304). For DRIP and DART experiments, S9.6 antibody was purified from the hybridoma purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States), by Dr. Masson. ANTI-FLAG® M2 Affinity Gel (Sigma Aldrich) and protein-A/G agarose beads (Pierce™ Protein A/G UltraLink™ Resin – Thermo Fisher Scientific) were used for CSB and TOP2 Immunoprecipitation (IP) experiments and R-Loops IP at DRIP-qPCR assay. To confirm XPC and XPA knockdown through qPCR 2 pair of primers were used for each gene as follows: 5′ TTGTCGTGGAGAAGCGGTCTAC/3′ CTTCTCCAAGCCTCACCACTCT and 5′ GACAAGCAGGA GAAGGCAAC/3′ GGTTCGGAATCCTCATCAGA for XPC; 5′ GAAGTCCGACAGGAAAACCGAG/3′ GATGAACAATCG TCTCCCTTTTCC and 5′ GCAGCCCCAAAGATAATTGA/3′ TGGCAAATCAAAGTGGTTCA for XPA. Primers used for DRIP-qPCR analysis are presented in **Supplementary Table 1**.

Damage at RNA Transcription Assay

U2OS-TRE cells were first transfected with siRNAs (siERCC6, siTOP2A, and siTOP2B) and 24 h after siRNA transfection the same cells were transfected with plasmids expressing KillerRed (KR) (pBroad3 TA-KR and pBroad3 tetR-KR). 36–48 h after plasmids transfection, cells were exposed for 25 min to a 15W Sylvania cool white fluorescent lamp for ROS-induced damage through light-induced KR activation and let for 1 h to recover before fixation to start the S9.6 Immunofluorescence (IF) protocol. Cells were rinsed with PBS and fixed in 4% PFA for 15 min at room temperature. They were washed three times with PBS, permeabilized by 0.2% Triton X-100 in PBS for 10 min, and then washed three times with PBS. After that, cells were incubated in buffer (10 mM Tris-HCl, 2 mM EDTA, pH = 9) and steamed on a 95°C heating block for 20 min to expose the antigen. The dish was cooled down, washed three times with PBS and cells were blocked using 5% BSA in 0.1% PBST for 30 min at room

temperature. The first and secondary antibodies were diluted in the same blocking buffer (anti-S9.6 1:500 and anti-mouse Alexa-Fluor 488 1:1,000). Primary antibody was incubated for 2 h at room temperature, then cells were washed three times with PBS and incubated for 1 h with the secondary antibody, following three more washes with PBS and incubation with DAPI 1 mg/mL. Images were taken using Volocity (Quorum Technologies) and S9.6 intensity in the KillerRed foci area was quantified using the same software.

DRIP-qPCR

DRIP assays were performed as described by Mersaoui et al. (2018). Briefly, nucleic acids were extracted from U2OS cells by SDS/proteinase K treatment at 37°C overnight followed by phenol-chloroform extraction using MaXtract™ High Density (100 × 15mL from QIAGEN) and ethanol precipitation at room temperature. The harvested nucleic acids were digested for 24 h at 37°C using a restriction enzyme cocktail (50 units/100 μg nucleic acids, each of *Bsr*GI, *Eco*RI, *Hin*dIII, *Ssp*I, and *Xba*I) in the New England Biolabs CutSmart buffer with 2 mM Spermidine and 1X BSA. Digested DNAs were cleaned up by phenol-chloroform extraction using MaXtract™ High Density (200 × 2mL from QIAGEN) followed by treatment or not with RNase H (20 units/100 μg nucleic acids) overnight at 37°C in the New England Biolabs RNase H buffer. DNA:RNA hybrids from 4 μg digested nucleic acids, treated or not with RNase H, were immunoprecipitated using 10 μg of S9.6 antibody and 50 μl of protein-A/G agarose beads (Pierce™ Protein A/G UltraLink™ Resin – Thermo Fisher Scientific) at 4°C for overnight and 2 h, respectively, in IP buffer (10 mM $NaPO_4$, 140 mM NaCl, 0.05% Triton X-100). Beads were then washed three times with IP buffer for 10 min at room temperature and nucleic acids were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS and 70 μg of protease K) at 55°C for 1 h. Immunoprecipitated DNA were then cleaned up by a phenol-chloroform extraction followed by ethanol precipitation at −20°C for 1 h. Quantitative PCR was performed at the indicated regions using the primers listed in **Supplementary Table 1**. Enrichment of DNA:RNA hybrids was calculated as percentage of input.

Immunofluorescence

For γH2AX and 53BP1 immunostaining U2OS cells were at first transfected with siERCC6, siXPC, and siXPA. 24 h after siRNA transfections cells were treated with 0.025 μg/ml DOX and 0.0125 μg/ml MXT for additional 24 h, and right after that the IF protocol was performed. Briefly cells were rinsed three times with PBS 1X, fixed using PFA 2% for 30 min, rinsed three times with PBS 1X and permeabilized with PBS-Triton X-100 0.3% for 30 min, followed by three washes with PBS 1X. Before incubation with antibodies, cells were blocked for 30 min using a blocking buffer (10% goat serum; 0.5% NP40, 0.5% saponin in PBS 1X). Primary and secondary antibodies were diluted in blocking buffer (anti-γH2AX 1:10,000, anti-53BP1 1:1,000, Alexa-fluor 488 goat anti-rabbit 1:1000 and Alexa-fluor 568 goat anti-mouse 1:1,000). Incubation with primary antibodies was done for 1 h 30 min and with secondary antibodies for 1 h. The slides were prepared using ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) to stain the nucleus. Three independent experiments were performed, and the images were taken using Volocity software (Quorum Technologies). A hundread cells per condition were analyzed for foci number using Cell Profiler software (Broad Institute). For S9.6 immunostaining the details are described in the DART assay method.

Cell Lysis and Immunoprecipitation

For co-immunoprecipitation experiments of endogenous proteins, U2OS cells were treated with DOX or MXT for 24 h and right after lysed with a lysis buffer containing 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.5% NP40 and a cocktail of protease inhibitors and phosphatase inhibitors. After a brief sonication (5 cycles 30 s on/off) followed by high speed centrifugation, the supernatant was precleared and protein quantification was done using Bradford method (Bradford, 1976). 1–2 mg protein lysate was separated for IP, and 0.6 μL of benzonase was added. The IP was performed incubating at first the lysate with anti-CSB and anti-IgG for the IgG control, according to the antibody manufacturer instructions, for 1 to 2 h at 4°C in rotation. After that, this lysate that was previously incubated with the antibody was then incubated for 1 h at 4°C in rotation with approximately 40 μL of beads. After IP, beads were washed with the lysis buffer (without protease and phosphatase inhibitors), and eluted. Samples were applied in a SDS-PAGE gel and blotted against anti-TOP2 or anti-CSB to check the interaction between the endogenous proteins.

For overexpression TOP2 immunoprecipitations, HEK-293T cells overexpressing FLAG tagged TOP2A and TOP2B were treated with DOX, MXT, and ETO for 2 h. After lysis and quantification following the same procedure described for endogenous proteins IP, the FLAG-tagged proteins IP was performed using 50 μL of beads ANTI-FLAG® M2 Affinity Gel (Sigma Aldrich), and the lysate containing the beads was incubated for 3 h at 4°C in rotation. After IP, beads were washed with washing buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5% NP40, and eluted. Samples were applied in a SDS-PAGE gel and blotted against anti-FLAG or anti-CSB to check the interaction between proteins.

In vitro Topoisomerase 2 Cleavage Assay

Cockayne Syndrome B protein purification was performed by the GST-His protein purification method as described by Maity et al. (2013). Human TOP2α and TOP2β were purified from yeast strains JEL1 top1Δ transformed with 12-URA-B 6 × His-hTOP2α and 12-URA-C 6 × His-hTOP2β, respectively. Induction of TOP2 by galactose as described previously (Dong et al., 2000). Yeast cells were lysed in equilibration buffer [300 mM KCl, 10 mM imidazole, 20 mM Tris HCl pH 7.7, 10% glycerol, and protease inhibitor cocktail (Sigma-Aldrich, catalog no. P8215)] by glass bead homogenization. Lysates were incubated with Ni-NTA resin and washed using wash buffer #1 (300 mM KCL, 30 mM imidazole, 20 mM Tris HCl pH 7.7, 10% glycerol, and protease inhibitors) then wash buffer #2 (150 mM KCl, 30 mM imidazole, 20 mM Tris HCl pH 7.7,

10% glycerol, and protease inhibitor cocktail). TOP2α and β were eluted on a Poly-Prep chromatography column (Bio-Rad, catalog no. 7311550) with elution buffer (150 mM KCl, 300 mM imidazole, 20 mM Tris HCl pH 7.7, 10% glycerol, and protease inhibitors). The peak protein fractions were dialyzed in dialysis buffer (750 mM KCl, 50 mM Tris HCl pH 7.7, 20% glycerol, 0.1 mM EDTA, and 0.5 mM DTT) and His tag was removed using Tobacco Etch Virus (TEV) protease.

Topoisomerase plasmid cleavage assay was carried out as described previously (Nitiss et al., 2012). In brief, 5 nM pBR322 supercoiled plasmid DNA and 100 nM recombinant TOP2α or TOP2β were incubated in 20 μL TOP2 reaction buffer containing

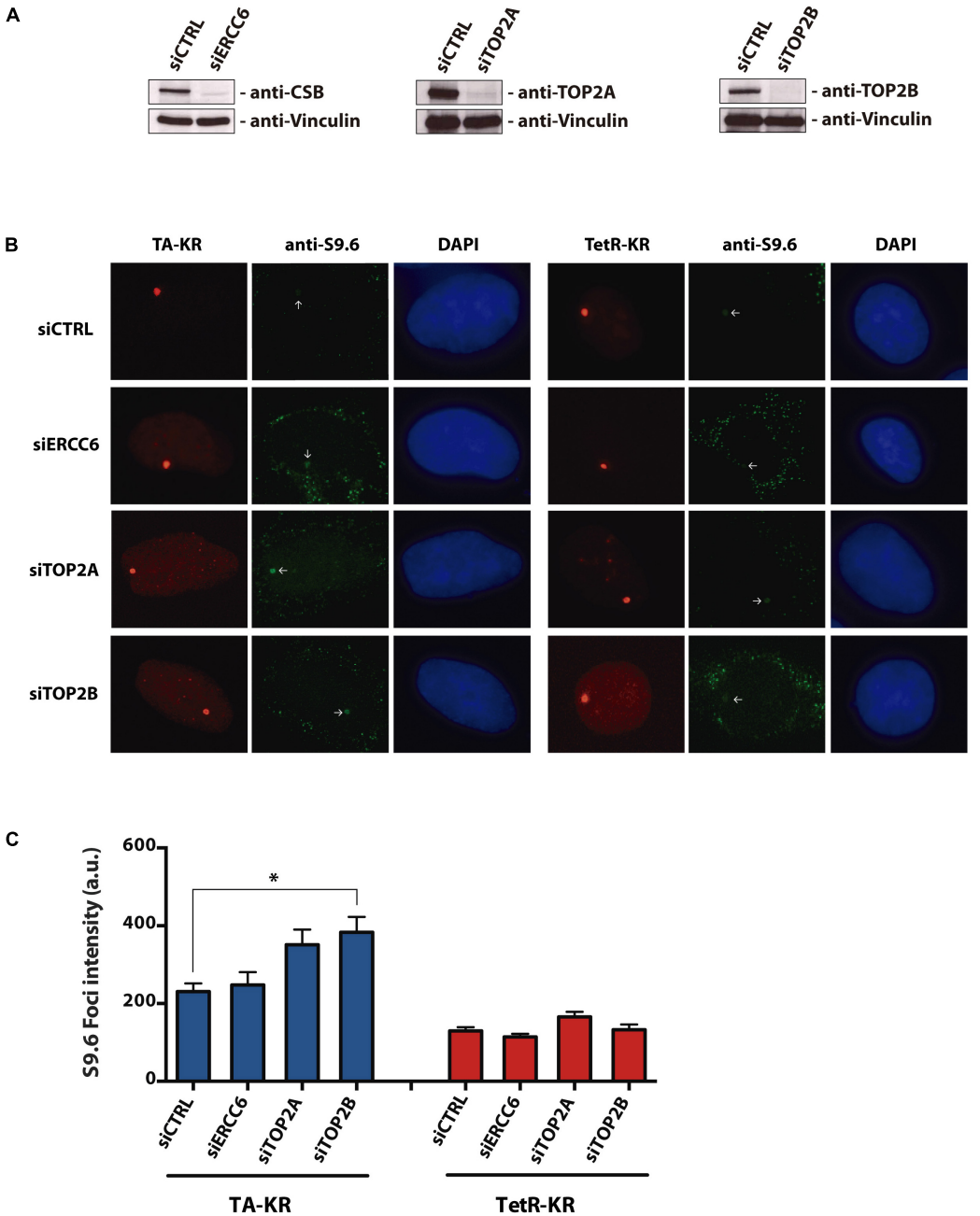

FIGURE 1 | Depletion of TOP2A and TOP2B increases R-loops at transcribed regions with local ROS-induced DNA damage. **(A)** Western blotting of U2OS-TRE cells with ERCC6, TOP2A, and TOP2B knockdowns. **(B)** Representative images of S9.6 staining in siCTRL, siERCC6, siTOP2A and siTOP2B knockdown at transcription on (TA-KR) or off (tetR-KR) genomic loci in U2OS TRE cells. **(C)** Quantification of the S9.6 foci intensity in the indicated conditions. Bars represent mean of S9.6 foci intensity quantification ± SEM from three independent experiments. The statistical analysis was performed by two-tailed Student's *t*-test (Mann-Whitney test *U*-test). 100 cells per condition were analyzed at each independent experiment.

20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM KCL, 1 mM ATP, 1 mM EDTA, 1 mM DTT, and 30 μg/mL acetylated BSA (TOP2) in the presence of 50 μM etoposide and indicated concentrations of CSB 37°C for 30 min. The reactions were terminated by adding 2 μL 10% SDS, 0.75 μL of 500 mM EDTA, pH 8.0, and 2 μL 0.8 mg/mL proteinase K and further incubated for 2 h at 30°C. DNA samples were electrophoresed in 0.8% agarose gels containing 0.5 μg/mL ethidium bromide.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software, version 6.0 (Cherwell Scientific, Oxford, United Kingdom). Results are the mean of at least three independent experiments with error bars showing S.E.M. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test and two-tailed Student's *t*-test (Mann-Whitney test *U*-test). An alpha level of 0.05 was used to determine significance in all statistical analysis.

RESULTS

Topoisomerase 2 Knockdown, but Not Inhibition, Increases R-Loops

Previous reports show an increase in R-loops formation after TOP1 inhibition by Camptothecin (Sollier et al., 2014; Marinello et al., 2016; Manzo et al., 2018) and also concerning CSB role in resolving these DNA:RNA hybrids (Sollier et al., 2014), we wanted to evaluate the R-loops accumulation in the context of CSB and TOP2 knockdowns. For that we performed the DNA DART assay in siCTRL, siERCC6 (CSB), siTOP2A, and siTOP2B cells. This system can measure a DNA:RNA hybrid accumulation at a particular locus (Lan et al., 2014; Teng et al., 2018). The light-inducible chromophore-modified KillerRed (KR) is fused with either transcription activator (TA) or repressor (tetR). KR generates reactive oxygen species (ROS) through the excited chromophore and induces DNA damage and transcriptional activation at the genome-integrated tet response element (TRE) locus in U2OS TRE cells. Elevated R-loop at the TRE locus over background is visualized by immunofluorescence using the S9.6 antibody (Lan et al., 2014; Teng et al., 2018). TOP2B knockdown led to a significant increase in R-loops specifically at the TA-KR marked locus, while the level of R-loops was similar to the control at the tetR-KR locus (**Figure 1**). Although it was not statistically different, we also saw an increase in TOP2A knockdown in relation to the control in TA-KR. These findings further confirm the accumulation of R-loops in the absence of TOP2A and TOP2B using an independent assay i.e., DART, in the presence or not of damage.

We also chose to investigate the occurrence of the same pattern after TOP2 pharmacological inhibition, which would induce different lesions from the ROS induced lesions. To assess R-loops levels in U2OS cells treated with a TOP2 inhibitor, we immunoprecipitated the DNA:RNA hybrids performing a DRIP-qPCR assay, which is a specific method to detect R-loops at different loci known to accumulate these structures. Surprisingly, in 4 out of 5 loci analyzed we did not find a significant increase in R-loops after TOP2 inhibition with ETO (**Figures 2A,B**) and MXT (data not shown) at any siRNA condition. Furthermore, even though we saw a significant R-loops increase in siCTRL cells treated with ETO for 24 h at HIST1H1E loci, this was not observed for other genes that were analyzed. In fact, in general, for all siRNAs tested R-loops levels were either the same or lower

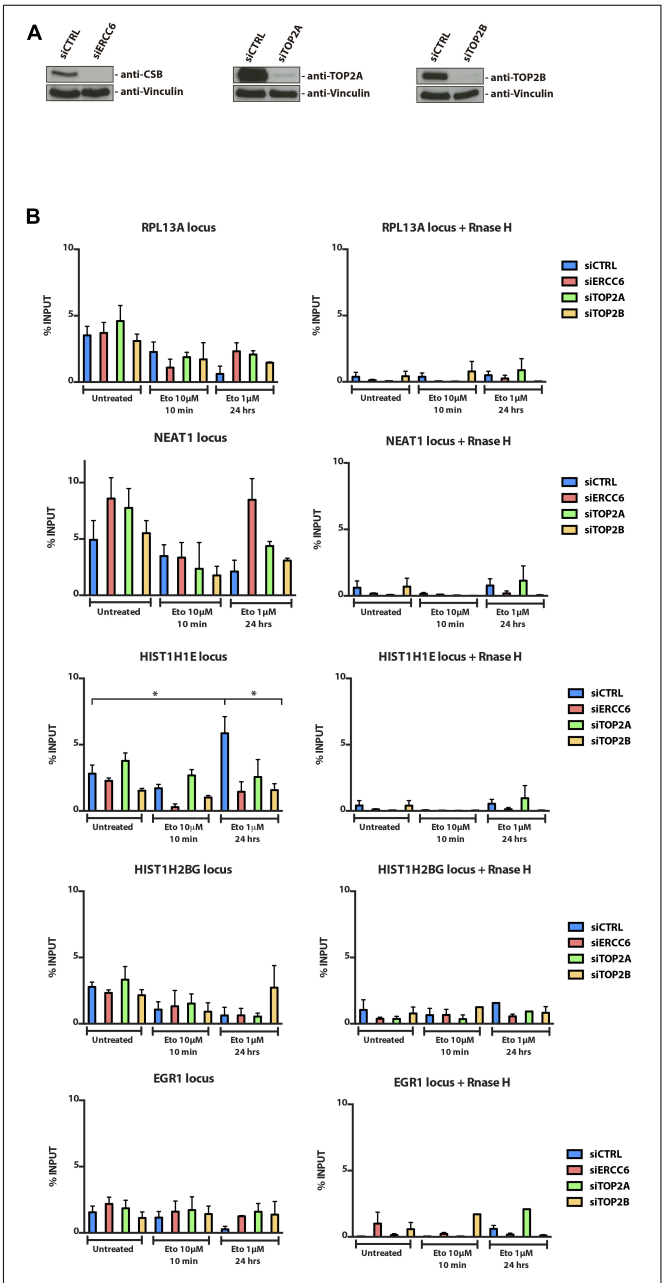

FIGURE 2 | R-loops immunoprecipitation through DRIP-qPCR. **(A)** U2OS cells were transfected with siCTRL, siERCC6, siTOP2A, and siTOP2B and **(B)** treated with ETO for 10 min and 24 h. After treatments, cells were subjected to DRIP-qPCR analysis. The average ± SEM. from three independent experiments is shown. Statistical analysis were performed using one-way ANOVA with Tukey's multiple comparison test; $p < 0.05$ was considered as significative.

after ETO treatments. However, in 4 out of 5 locus there is a slight increase in siTOP2A condition in untreated cells, but when treated with ETO for short (10 min) or long exposure (24 h) we could not find a pattern.

siERCC6 and siXPC Present Different 53BP1 and γH2AX Foci Pattern

In order to determine if TOP2 inhibitor induces DSBs we assessed γH2AX and 53BP1 foci formation in U2OS cells. Then we investigated the influence NER genes in this response, after siRNA knockdown for ERCC6, XPC and XPA (**Figure 3A**). After 24 h treatments with DOX and MXT, we could see differences in the foci formation among the knockdowns (**Figures 3B,C**). After DOX treatments siXPC cells present less 53BP1 and γH2AX foci compared to siCTRL, siXPA, and specially siERCC6. The same pattern is observed in γH2AX foci at untreated and MXT conditions, when siXPC cells present less foci than siERCC6 and siCTRL. This can indicate that the absence of XPC does not affect the signaling to repair DSBs, once the evaluation was done 24 h

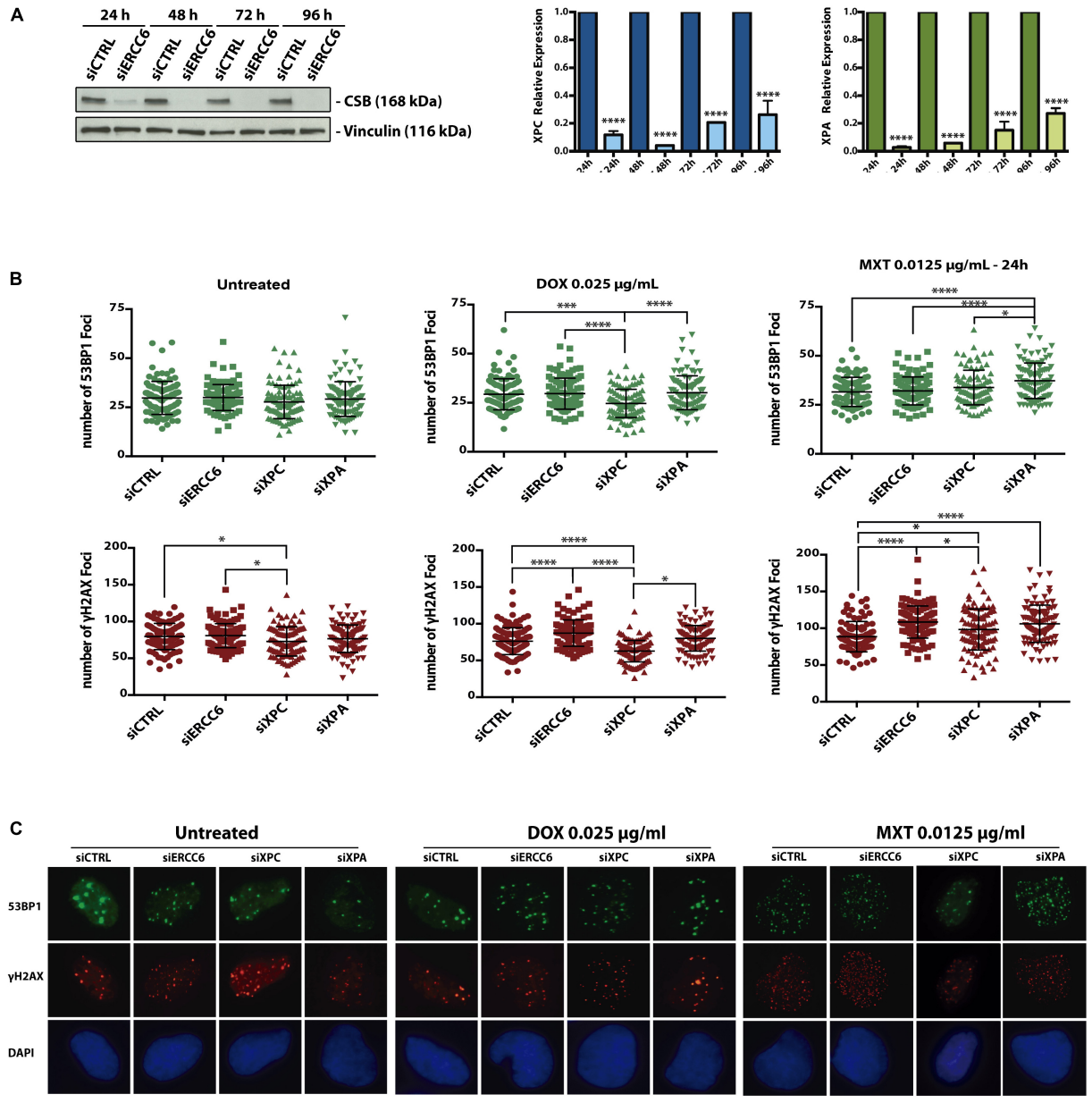

FIGURE 3 | Cockayne Syndrome B depletion induces more 53BP1 foci formation upon TOP2 inhibition. U2OS cells were transfected with siCTRL, siERCC6, siXPC, and siXPA **(A)** and treated with DOX and MXT for 24 h. **(B)** represents 53BP1 and γH2AX foci quantification and **(C)** shows representative images. Graphs represents the average ± SEM from three independent experiments. Statistical analysis were performed using one-way ANOVA with Tukey's multiple comparison test; $p < 0.05$ was considered as significative. 100 cells per condition were analyzed at each independent experiment.

after the treatments and by this time we could think the DSBs generated by these drugs are already resolved. On the other hand, the absence of CSB, evaluated by siERCC6, seems to increase γH2AX foci, but not 53BP1, in relation to siCTRL with both TOP2 inhibitors. This can indicate that there are more DSBs at these conditions or the absence of CSB delays the resolution of this DSBs, maintaining the phosphorylation of H2AX even after 24 h later.

Cockayne Syndrome B Interacts With TOP2A and TOP2B

Considering that ERCC6 knockdown increases DSBs levels upon TOP2 inhibition (**Figure 3B**), and CSB depleted cells accumulate more Top2ccs than XPC-deficient cells in response to MXT we hypothesized a direct interaction between CSB and TOP2 before or after TOP2 inhibition. This interaction could be necessary to process the Top2ccs-complexes. To test this, we performed a Co-Immunoprecipitation (Co-IP) of the endogenous CSB using U2OS cells, before or after 24 h treatment with TOP2 inhibitors (DOX or MXT). Western blotting with Anti-TOP2A, revealed a slight interaction between TOP2A and CSB in normal condition (**Figure 4**). Interestingly, the TOP2A-CSB interaction is increased after MXT treatments.

We also evaluated this protein interaction in HEK 293T cells overexpressing both isoforms, TOP2A or TOP2B, FLAG-tagged and treated with three different TOP2 inhibitors, DOX, MXT, and ETO for 2 h. When endogenous CSB was analyzed in FLAG IP samples through western blot, we found a slight interaction between TOP2A and CSB in untreated condition that was more pronounced in cells treated with DOX and MXT, but less intense in ETO treatment (**Supplementary Figure 1A**). However, for TOP2B we observed a very slight interaction in untreated and more pronounced in MXT treated cells (**Supplementary Figure 1B**). Different from TOP2A, the same interaction was not observed in DOX and ETO treatments when TOP2B was immunoprecipitated.

In order to verify if CSB stimulates TOP2 function, we performed an *in vitro* cleavage assay with both proteins in the presence or not of the TOP2 inhibitor ETO. Our results show that CSB stimulates DNA cleavage by both TOP2 isoforms (alpha and beta) *in vitro* (**Figure 5**). This effect seems to happen since the addition of lower CSB concentrations (5 nM), but mainly at 10 nM for both isoforms, remaining still active in stimulating DNA cleavage at CSB 20 nM for TOP2A, as it is better observed by the quantifications of the image from **Figure 5B**, presented in **Figure 5A**. Although there is a trend that CSB presence stimulates more TOP2A than TOP2B, it was not statistically different. We can also see that the presence of CSB without the damage generated by ETO does not affects DNA cleavage. This indicates again that this interaction tends to occur in the face of any TOP2 interaction that might end in DNA damage, analyzed here by the trapping of TOP2 in the DNA caused by the ETO treatment.

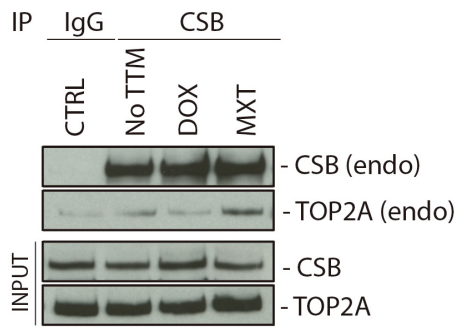

FIGURE 4 | Cockayne Syndrome B physically interacts with TOP2A. U2OS cells were treated for 24 h with DOX or MXT and a CSB IP was performed after the treatments. CTRL cells did not perform CSB IP, but IgG instead. NO TTM: cells were CSB-IP, but no treatment was applied with the TOP2 inhibitors.

DISCUSSION

R-Loops and Topoisomerase 2 Inhibition/Knockdown

Nucleotide Excision Repair (NER) is known to repair adducts and bulky lesions in the DNA, that can occur at different parts of the genome. When these lesions are at transcribed active genes it causes RNA Pol arrest, and in response to this event CSB is recruited to start the signaling to other NER proteins that will remove the DNA containing the lesion. However, recent evidences have shown different roles for CSB (Sollier et al., 2014; Batenburg et al., 2015, 2017; Wei et al., 2015, 2016; Teng et al., 2018).

Sollier et al. (2014) have shown an involvement of CSB and other NER factors to remove R-loops that can be generated by different mechanisms, including inhibition of TOP1. Based on that and our previous interest in TOP2 inhibitors mechanisms and NER involvement to solve the induced lesions, we wondered if we could identify R-loops without an active TOP2 enzyme. Therefore, we investigated R-loops levels through two different methodologies, one using TOP2 inhibition with ETO, besides their knockdowns (through DRIP-qPCR), and the other with TOP2 knockdowns but inducing a different type of damage (through DART assay).

In our analysis, we could conclude that TOP2 presence is important to avoid R-loops formation/accumulation. However, its inhibition does not seem to change much as its knockdown. This makes sense considering that TOP2 is an important enzyme to keep DNA topology. Hence, its complete absence makes more difference than its inhibition that creates other lesions besides the complexes in the DNA. Although it was not evaluated in our study, it is still worth mentioning the role of Tdp2 protein, an important endonuclease that can remove Top2ccs (Nitiss and Nitiss, 2013; Pommier et al., 2014). Therefore, an interesting approach would be analyzing R-loops formation in response to TOP2 inhibition in the absence of Tdp2. This approach could show if the Top2ccs generated are removed by the endonuclease Tdp2 and how it influences the generation of R-loops in the presence or not of CSB.

Marinello et al. (2016), have seen increased R-loops formation in response to Campthotecin, a TOP1 inhibitor, after 2 and

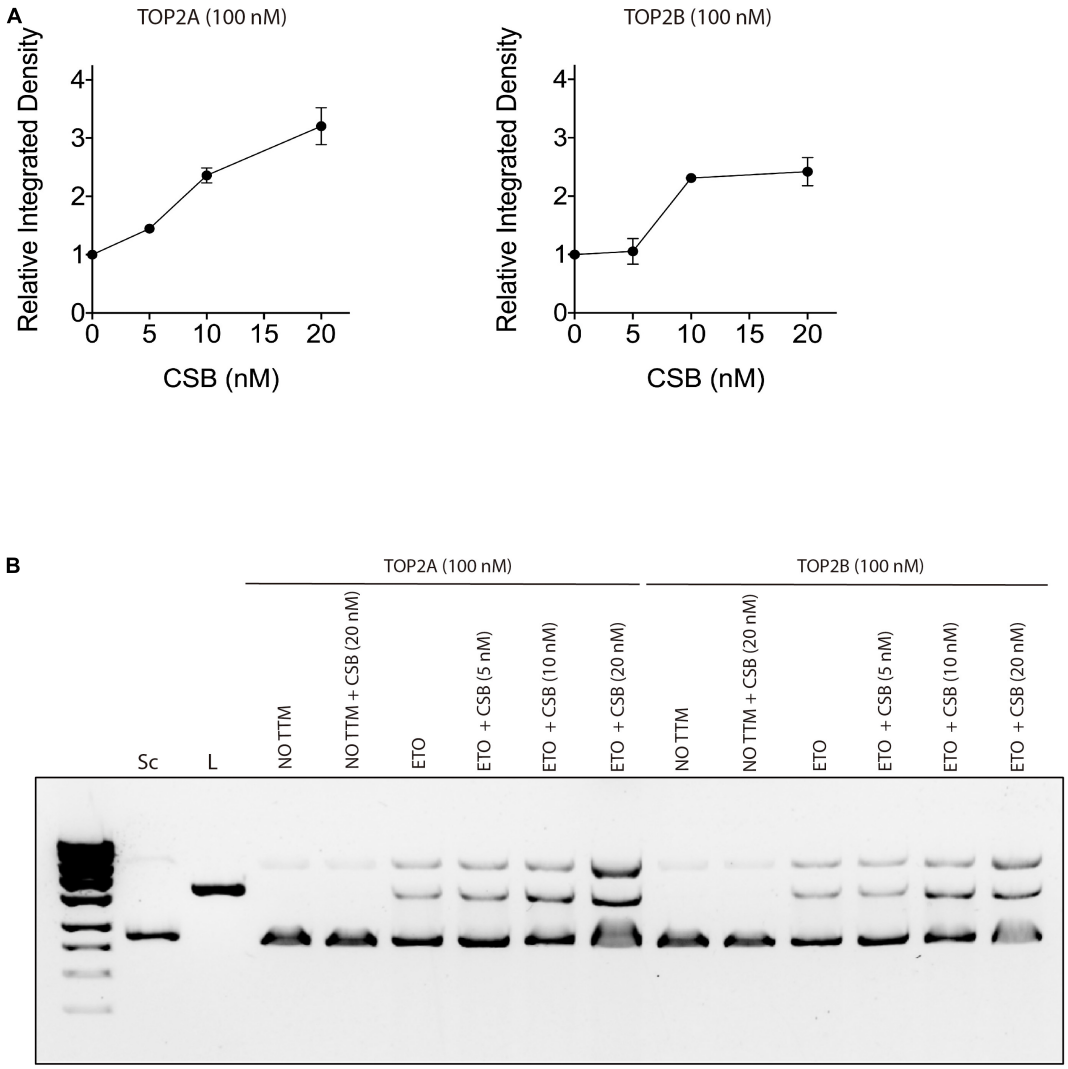

FIGURE 5 | Cockayne Syndrome B stimulates TOP2A and TOP2B DNA cleavage in the presence of a TOP2 inhibitor treatment. **(A)** *in vitro* TOP2 cleavage assay quantification in the presence of CSB. **(B)** *in vitro* TOP2 cleavage assay representative gel. Sc, supercoiled DNA; L, linear DNA; NO TTM, no treatment; ETO, Etoposide.

10 min, but this was completely lost after 4 h of treatment, at different loci, including RPL13A, which is one locus also analyzed in our study. Based on that they affirm that TOP1 inhibition by Campthotecin can stabilize antisense and sense R-loops at active divergent promoters, but only for a short time (Marinello et al., 2016). We also analyzed R-loops levels at short and even longer-term ETO treatments through DRIP-qPCR. However, the difference in time does not seem to change the results in our case. It would be interesting to evaluate it in an even shorter time, such as 1 or 2 min after drug exposure. On the other side, it is known that R-loops are dynamic structures that are continuously formed and resolved and that the retention of nascent transcripts at their site of transcription is also a dynamic feature of the mammalian chromatin (Chédin, 2016).

It is known that negative supercoiling in the DNA (DNA under-winding) stabilizes R-loops, while positive supercoiling (DNA over-winding) tends to resolve them (Belotserkovskii et al., 2018). This could explain why different studies have observed R-loops induction after inhibiting TOP1 and we have not seen the same after TOP2 inhibition. However, this does not explain our findings in TOP2 knockdowns accumulating more R-loops in 4 of the 5 analyzed locus and at the specific TA-KR locus of the DART assay, which is transcriptionally activated.

We observed in DART assay results that siTOP2B cells presented more R-loops measured by S9.6 intensity in the KR foci area at the transcription activated locus. Although, in DRIP-qPCR, it was siTOP2A that showed higher levels of R-loops. This can be explained by the fact that TOP2B is more related to transcription, while TOP2A is usually related to replication. When we look at the data in tetR locus at DART assay, where transcription is not activated, we do see

siTOP2B cells presenting a similar level of S9.6 intensity to the control. However, siTOP2A cells still present a slightly higher signal, which suggests that the absence of TOP2A really makes a difference in terms of R-loops formation/resolution, independently of transcription-activated or not. We did not evaluate a condition with transcription inhibition.

Cockayne Syndrome B Stimulates Topoisomerase 2 DNA Cleavage Preventing Genome Instability

We have previously shown that CSB deficient cells accumulate more Top2ccs in response to MXT than XPC deficient or NER proficient cells (Rocha et al., 2016a). We investigated in this work if there is a physical interaction of CSB and TOP2 that could explain the difficulty in resolving these complexes generated by TOP2 inhibition with DOX, MXT, and ETO. ETO is known to be more specific in creating Top2ccs as this is its primary toxic mechanism, while DOX and MXT can create other lesions besides these complexes in the DNA (Parker et al., 1999; Bromberg et al., 2002; Minotti et al., 2004; Baldwin and Osheroff, 2005; Swift et al., 2006).

We did find the interaction through co-IP of CSB and TOP2 after TOP2 inhibition and we also showed that CSB stimulates TOP2 DNA cleavage *in vitro*. The TOP2 inhibition creates Top2ccs in the DNA which is known that can block transcription, promoting then RNA PolII arrest. Taking these facts in consideration, we speculate at first that this arrest could

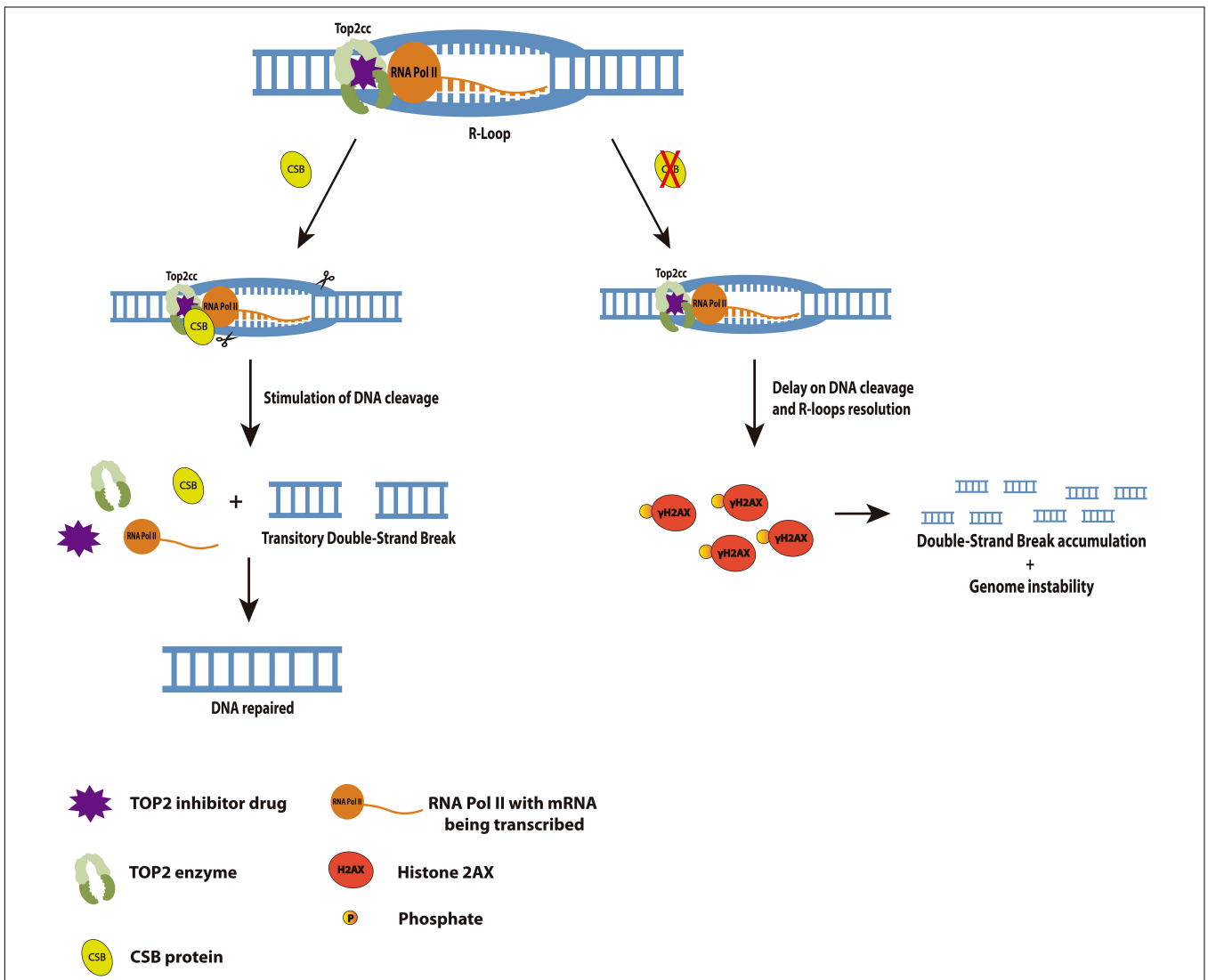

FIGURE 6 | Cockayne Syndrome B interaction with TOP2 at a transcription arrest provoked by a TOP2 inhibition favors DNA cleavage, R-loops resolution and avoids genome instability. We propose here in this model that in the presence of CSB, the TOP2 inhibition generated lesions would be easily removed by TOP2 cleavage stimulated by CSB. These lesions could be R-loops formed by the hybridization of mRNA at the transcription bubble, since it is known RNA Pol II arrests when it encounters a lesion at the transcription active region. It is also known that this arrest recruits CSB, so in its absence, cells would accumulate R-loops for longer and consequently generates long-term DSBs (measured in our study by γH2AX), leading to genome instability.

favor R-loops formation and recruit CSB to this system. CSB presence could be essential to help in the removal of these R-loops by recruiting then other factors such as endonucleases that could relieve the super torsions, that result from the TOP2 trapped in the DNA, and consequently remove the R-loops. Considering that and our findings at the *in vitro* TOP2 cleavage assay, we could raise the hypothesis presented at our final model in **Figure 6**. In the presence of CSB, Top2ccs can be more easily removed after a Top2 inhibition once CSB stimulates the DNA cleavage by TOP2. This release of the Top2cc from the DNA would not favor for the R-loops accumulation, and cells might have some transitory DSBs during the process. However, when CSB is absent, the lack of stimulation for TOP2 to cleave the DNA might impact the accumulation of Top2ccs and R-loops and as a consequence DSBs generation that last longer and could end up in genome instability.

More studies are needed to elucidate better CSB or NER involvement in lesions mediated by TOP2 inhibition. Although we could not prove the formation of R-loops in TOP2 inhibition treatments and the participation of CSB in this process, we did show a functional interaction of CSB and TOP2. This interaction might be important to release TOP2 from the DNA when trapped due to an inhibition, for example. We also showed in this work the importance of TOP2 presence in preventing R-loops accumulation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FB was responsible for main idea conceptualization, performing all methodologies except for the TOP2 cleavage assay, data analysis, making figures and writing-original draft preparation. SM was responsible for conceptualization and support at the DRIP-qPCR methodology and for reviewing the manuscript. YS and YP were responsible for performing TOP2 cleavage assay. JYM and JS were responsible for main idea conceptualization, supervision, writing, reviewing, and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.727836/full#supplementary-material

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An Eye in the Replication Stress Response: Lessons From Tissue-Specific Studies *in vivo*

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Several inherited human syndromes that severely affect organogenesis and other developmental processes are caused by mutations in replication stress response (RSR) genes. Although the molecular machinery of RSR is conserved, disease-causing mutations in RSR-genes may have distinct tissue-specific outcomes, indicating that progenitor cells may differ in their responses to RSR inactivation. Therefore, understanding how different cell types respond to replication stress is crucial to uncover the mechanisms of RSR-related human syndromes. Here, we review the ocular manifestations in RSR-related human syndromes and summarize recent findings investigating the mechanisms of RSR during eye development *in vivo*. We highlight a remarkable heterogeneity of progenitor cells responses to RSR inactivation and discuss its implications for RSR-related human syndromes.

Keywords: genome stability, cell cycle, DNA damage, chekcpoint, ATR, organogenesis, retina, lens

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INTRODUCTION

Maintenance of genome stability is essential for development and homeostasis, and failures in processes required for genomic stability are associated with various human syndromes (Ciccia and Elledge, 2010; Negrini et al., 2010; O'Driscoll, 2012). DNA replication, transcriptional regulation and chromatin modifications must be precisely coordinated to ensure faithful transmission of genetic information to stem/progenitor cell pools that expand during development (Prioleau and MacAlpine, 2016). During DNA synthesis, many sources of genotoxic stress may slow or stall the progression of replication forks, a condition defined as replication stress. As a consequence, cells trigger the replication stress response (RSR). Activation of the RSR signaling pathways may slow DNA replication and allow extra time for DNA repair, preventing DNA mutations, chromosomal rearrangements and, therefore, genomic instability (Zeman and Cimprich, 2014; Techer et al., 2017; Tubbs and Nussenzweig, 2017). Due to its essential role during replication and development, mutations in genes that code proteins required for RSR are associated with several developmental syndromes (Zeman and Cimprich, 2014; Munoz and Mendez, 2017). Here, we review the ocular

manifestations in RSR-related human syndromes and discuss recent findings investigating tissue-specific RSR in the developing eye that may contribute to understanding how defective-RSR drives developmental malformations.

REPLICATION STRESS RESPONSE

Single-stranded DNA (ssDNA) breaks are proposed to be the most frequent DNA lesion (∼75%) and those are normally generated during DNA replication (Lindahl and Barnes, 2000; Tubbs and Nussenzweig, 2017). The formation ssDNA stretches and aberrant replication fork structures lead to the activation of the ATR kinase, the master regulator of the RSR (**Figure 1A**). When exposed, long ssDNA stretches are coated by the replication protein A (RPA) complex. ATR-interacting protein (ATRIP), a mutually dependent partner of ATR, directly binds to RPA and recruits ATR to the RPA-ssDNA sites (Hekmat-Nejad et al., 2000; Cortez et al., 2001; Zou and Elledge, 2003; Dart et al., 2004; Ball et al., 2005) (**Figure 1A**). ATR recruitment is not sufficient for its full activation and many regulatory partners are necessary (Saldivar et al., 2017). In double-stranded DNA-ssDNA (dsDNA-ssDNA) junctions, such as the ones found in stalled replication forks, ATR activation requires DNA topoisomerase II-binding protein 1 (TOPBP1) (Kumagai et al., 2006). TOPBP1 recruitment to dsDNA-ssDNA junctions depends on its interaction with RAD9, member of the 9-1-1 clamp complex (RAD9-RAD1-HUS1) that is recruited by the clamp load factor RAD17 (Bermudez et al., 2003; Kumagai et al., 2006; Delacroix et al., 2007) (**Figure 1A**). TOPBP1 recruitment depends on other proteins, including the MRE11-RAD50-NBS1 (MRN) complex and RHINO (Cotta-Ramusino et al., 2011; Duursma et al., 2013). Importantly, NBS1 and the MRN complex are directly involved in ATR activation and cells from patients with inactivating mutations in *NBS1* exhibit defective RSR (Stiff et al., 2005; Duursma et al., 2013; Shiotani et al., 2013). In ssDNA regions without ssDNA-dsDNA junctions, RSR activation can be mediated by ETAA1, that directly interacts with RPA and activates ATR through its ATR-activating domain (AAD) domain (**Figure 1A**; Bass et al., 2016; Haahr et al., 2016; Lee et al., 2016; Thada and Cortez, 2019). Studies in human cell lines suggested that ATR activation by TOPBP1 and ETAA1 may occur in different contexts. TOPBP1 would activate ATR upon induced replication stress and ETAA1 would trigger ATR activation in unchallenged replication to avoid under-replicated DNA during the S-M transition (Saldivar et al., 2018). In addition, ATR can be directly activated by NBS1, although the mechanisms are not clear since NBS1 does not have an AAD domain (Kobayashi et al., 2013).

RSR depends not only on ATR-mediated signal transduction but also on its downstream effectors, specially the checkpoint protein 1 (CHK1) (Saldivar et al., 2017). ATR phosphorylates CHK1 in multiple sites and CHK1 activation depends on its partner CLASPIN (Kumagai and Dunphy, 2000; Liu et al., 2000, 2006; Zhao and Piwnica-Worms, 2001; **Figure 1A**). Once activated, the ATR-CHK1 signaling triggers local (e.g., dormant replication fork firing) and global (e.g., cell cycle arrest) responses to ensure the faithful duplication of the genome (Saldivar et al., 2017).

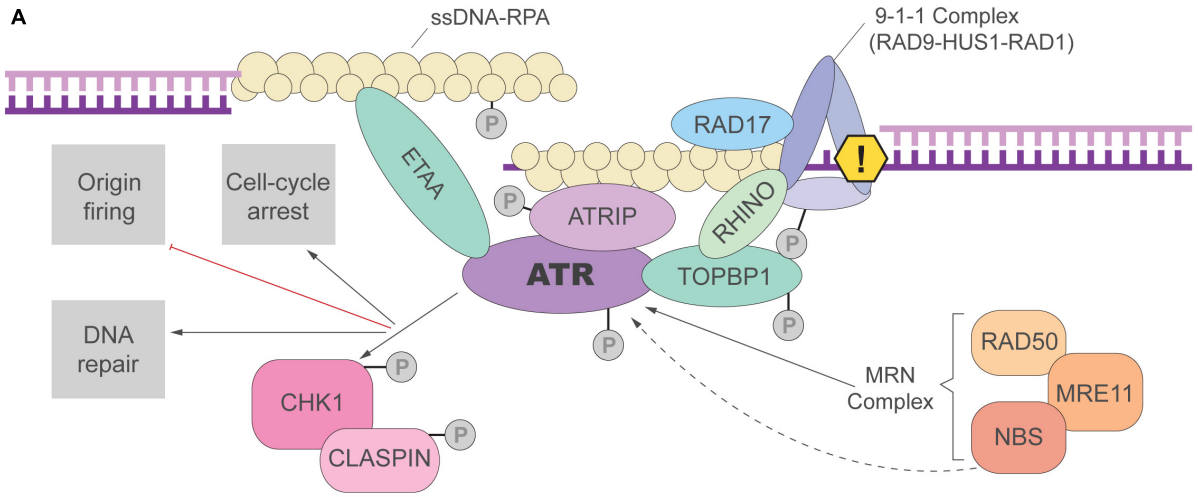
INACTIVATION OF THE REPLICATION STRESS RESPONSE *IN VIVO*

Highlighting the importance of ATR activation for unchallenged cell proliferation during development *in vivo*, inactivation of various "RSR genes" (here defined as genes necessary for full activation of ATR-CHK1 signaling following replication stress) is embryonic lethal in mice (Luo et al., 1999; Brown and Baltimore, 2000; de Klein et al., 2000; Liu et al., 2000; Weiss et al., 2000; Zhu et al., 2001; Dumon-Jones et al., 2003; Budzowska et al., 2004; Hopkins et al., 2004; Wang et al., 2005; Han et al., 2010; Jeon et al., 2011; Yang et al., 2016; Miosge et al., 2017). Although RSR has been extensively studied in various models, the mechanisms of the ATR activation and, therefore, the exact roles of ATR regulators in unchallenged replication *in vivo* are still not completely understood. For example, it was clear that ATR protein stability and function depend on its interaction with ATRIP in human cells (Cortez et al., 2001), however, prior to our recent work (Matos-Rodrigues et al., 2020a,b) ATRIP function had not been investigated *in vivo*. Moreover, while ETAA1 plays an essential role in an ATR-regulated S-G2 checkpoint in immortalized cells (Saldivar et al., 2018), ETAA1 null mice show a mild phenotype of partial embryonic lethality (Miosge et al., 2017). In contrast, ATR activation by TOPBP1 has an essential role in unchallenged replication *in vivo*, since disruption of ATR activation by TOPBP1 leads to embryonic lethality in mice (Zhou et al., 2013). These data indicate that ATR activation by TOPBP1, but not ETAA1, is essential for unchallenged replication in mice. The reason behind these distinct requirements in cultured human cells and in mouse development remains unclear.

REPLICATION STRESS RESPONSE *IN VIVO*: FOCUS ON THE EYE

The eye is the sensory organ responsible for vision and is composed of three main tissues: cornea, lens and retina (**Figure 2A**). The anterior segment of the eye comprises the cornea, the iris and the lens, a transparent structure that focus the light to the back of the eye. The main tissue of its posterior segment is the retina, the neural part of the eye responsible for detection and preprocessing of the visual stimuli before transmission to the visual centers of the brain through the optic nerve (Dowling, 1987). The development of these ocular tissues is extremely interdependent. In mice, on the ninth day of embryonic development (E9), a projection of the diencephalon, the optic vesicle, encounters the surface ectoderm of the head and starts eye organogenesis by triggering the invagination of both structures. While the invagination of the surface ectoderm gives rise to the lens, the retina originates from the invaginating optic vesicle (Miesfeld and Brown, 2019).

Importantly, the eye represents a unique model to study the impact of defective RSR to organogenesis because: (1) of the



B

Human disease (OMIM)	Affected pathway	Reported ophtalmological issues
Seckel Syndrome (#210600)	ATR signaling	Microphtalmia, pigmentary retinopathy, myopia, astigmatism, lack of cone and rod responses, hypermetropia, macular atrophy, retinal hyperpigmentation, bilateral pigmentary retinopathy, glaucoma, retinal detachment, lens dislocation.
Nijmegen Breakage Syndrome (#251260)	MRN complex; ATR/ATM activation	Reduced visual acuity, small eyes, cataract, refractive errors.

FIGURE 1 | ATR activation and the replication stress response (RSR). **(A)** The ATRIP-ATR complex is recruited to RPA coated ssDNA. ATR can be directly activated by TOPBP1 or ETAA1 *via* their AAD domain. In regions of dsDNA-ssDNA junctions, the 9-1-1 complex is responsible for TOPBP1 recruitment and ATR activation. ETAA1-mediated ATR activation is not dependent on ssDNA-dsDNA junctions as ETAA1 directly binds to RPA-coated ssDNA. RHINO and the MRN complex are also important for ATR activation that phosphorylates different targets, including the CHK1 kinase. Once the RSR is activated, ATR and its downstream targets can modify different aspects of cell metabolism to prevent genome instability. **(B)** Ocular manifestations reported in patients of the RSR-related syndromes: Seckel (Lim and Wong, 1973; Guirgis et al., 2001; Reddy and Starr, 2007; Aktas et al., 2013; Krzyzanowska-Berkowska et al., 2014) and Nijmegen breakage syndromes (Varon et al., 1998; Gralek et al., 2011).

vast knowledge about its development in mammals; (2) it is a non-essential organ, therefore a powerful model to analyze genetic interactions, and evaluate the long term consequences of essential genes inactivation; (3) there is a substantial amount of genetic tools available; (4) it is composed of tissues derived from distinct developmental lineages, making it ideal to study progenitor cells of different lineages. In addition, although clinical studies have shown ophthalmological manifestations in RSR-related syndromes (**Figure 1B**), the origins of these manifestations in these syndromes have been underexplored and raising awareness to this topic may bring important contributions to patients.

Loss-of-function mutations in ATR/ATRIP or in NBS1 are among the known causes of Seckel or Nijmegen breakage syndrome, respectively. These syndromes are characterized by moderate to severe tissue-growth impairments, neurodevelopmental defects and a series of ocular manifestations that have been reported in patients (Lim and Wong, 1973; Varon et al., 1998; Guirgis et al., 2001; Reddy and Starr, 2007; Gralek et al., 2011; Aktas et al., 2013; Krzyzanowska-Berkowska et al., 2014). Due to the recent advances on the understanding of these genes in eye development, we focus on their functions and its related human syndromes.

OCULAR MANIFESTATIONS IN REPLICATION STRESS-RELATED HUMAN SYNDROMES

Microphthalmia

Microphthalmia is a disorder characterized by abnormally small eyes that display high genetic heterogeneity and may occur as part of a syndrome. Disproportional ocular growth may contribute to

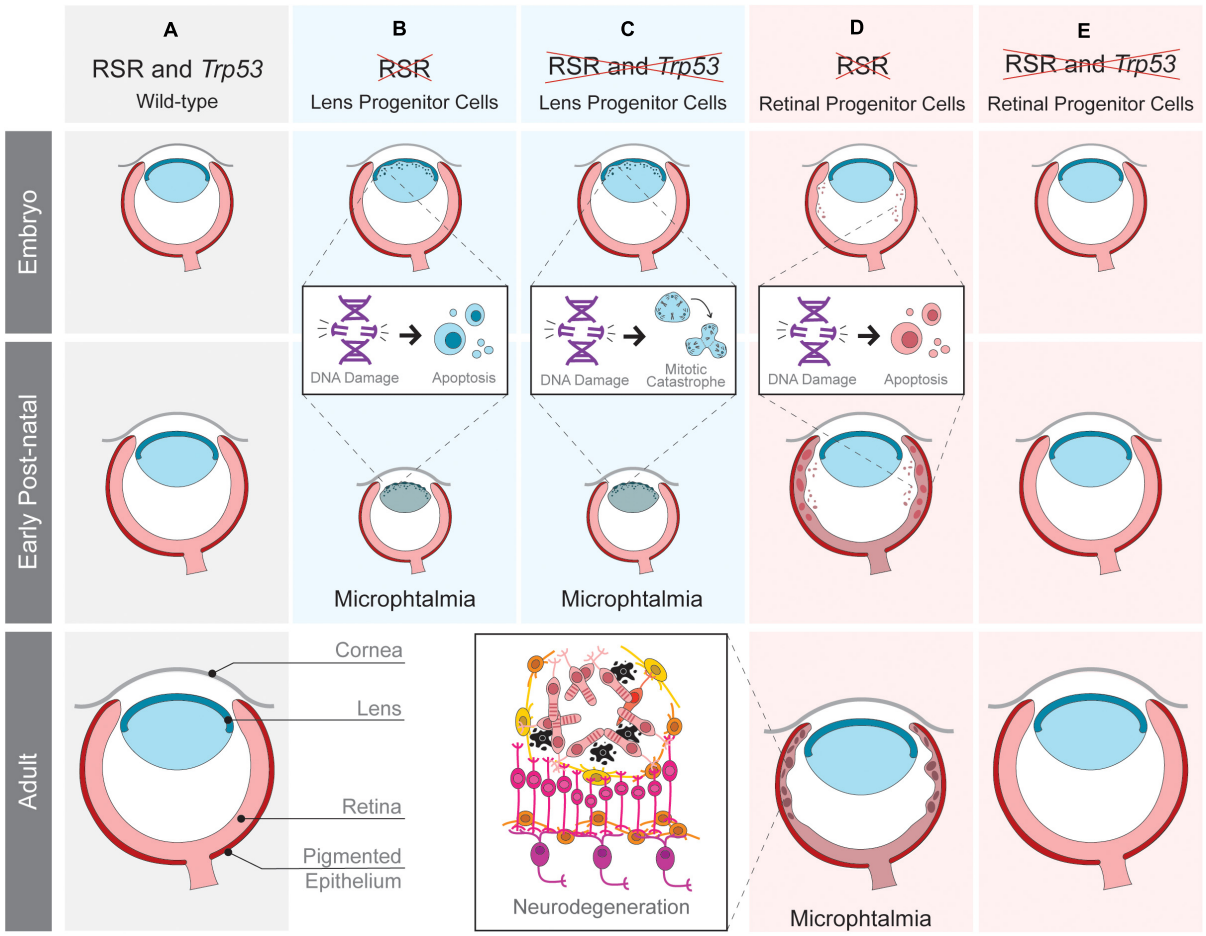


FIGURE 2 | Tissue-specific effects of replication stress response (RSR) inactivation in developing mouse eye. Schematic representation of ocular development in wild-type mice **(A)** and the consequences of RSR inactivation in lens **(B,C)** or retinal progenitor cells **(D,E)** in *Trp53*-proficient **(B,D)** and *Trp53*-deficient scenarios **(C,E)**. RSR inactivation by the loss of ATRIP leads to progenitor cell apoptosis in both the lens and retina. Only in the retina, *Trp53*-deficiency rescued embryonic apoptosis and the consequent secondary phenotypes.

microphthalmia, since microphthalmic eyes are more affected in the posterior segment than the anterior (Verma and Fitzpatrick, 2007). Microphthalmia has been reported in both Seckel and Nijmegen breakage syndromes (**Figure 1B**). Studies in animal models (discussed in the next sections) suggested that defective cell proliferation and increased cell death may be the cause of microphthalmia following the inactivation of RSR genes (Yang et al., 2006; Rodrigues et al., 2013; Matos-Rodrigues et al., 2020a,b). However, the mechanisms driving eye growth defects in syndromes caused by mutations in RSR-genes are far from being completely understood.

Cataract

Although treatable, cataracts are the most common cause of blindness. Congenital cataracts, the ones in which the opacification of the lens is detected at birth, are a clinical feature of almost 200 syndromic genetic diseases (Liu et al., 2017; Berry et al., 2020). Many evidences directly associates cataractogenesis and DNA damage. Increased DNA oxidation has been found in cataract patients and is thought to trigger cataractogenesis (Osnes-Ringen et al., 2013; Zhang et al., 2014; Erol Tinaztepe et al., 2017; Uwineza et al., 2019). DNA repair genes are known risk factors for cataract (Su et al., 2013; Cui et al., 2017; Yang et al., 2018) and cataracts have been reported in Seckel syndrome patients (Rao et al., 2011) (**Figure 1B**). Human lens progenitor cells from cataract patients display increased levels of DNA single strand breaks, a hallmark of replication stress (Kleiman and Spector, 1993). Finally, sources of replication stress, such as oxidative damage, UV-light and ionizing radiation cause cataract (Liu et al., 2017). As expected, induced DNA damage disturbs the proliferation and differentiation of lens progenitor cells, which is proposed to be an underlying cause of ionizing radiation induced cataract (Uwineza et al., 2019). The molecular mechanisms driving these processes are still to be determined.

Retinal Neurodegeneration

Glaucoma is characterized by structural damage to the optic nerve and retinal ganglion cell degeneration, leading to loss of vision due to the interruption of the transmission of

information from the eye to the brain (Quigley, 2011; Calkins, 2012; Gemenetzi et al., 2012). Other retinopathies leading to neurodegeneration and vision loss include macular degeneration, retinopathy diabetic and retinitis pigmentosa (Massengill et al., 2018). Glaucoma, photoreceptors degeneration and lack of photoreceptor electrical responses were reported in patients with Seckel syndrome (**Figure 1B**). Importantly, replication stress has also been associated with the activation of pro-inflammatory pathways, which might fuel retinal neurodegeneration (Charlier and Martins, 2020; Ragu et al., 2020).

LESSONS FROM MOUSE MODELS

Genetic inactivation of NBS1 in mice was key to understanding the etiology of Nijmegen breakage syndrome (Frappart and McKinnon, 2008). While NBS1 knockout in mice led to early embryonic lethality (Zhu et al., 2001), neural tissue-specific inactivation of NBS1 resulted in abnormalities similar to patients including microcephaly, growth retardation, cerebellar defects and ataxia (Frappart et al., 2005). Importantly, NBS1 loss in the developing brain led to distinct outcomes depending on the progenitor cell affected. For example, NBS1 deficiency in progenitor cells of the neocortex induced cell cycle arrest. In the cerebellum, growth defects are driven by progenitor cell death (Frappart et al., 2005; Li et al., 2012; Rodrigues et al., 2013).

In the developing eye, NBS1-deficiency in the lens leads to cell death, proliferation defects and microphthalmia (Yang et al., 2006; Baranes et al., 2009; Rodrigues et al., 2013). During retinogenesis, NBS1 is also required for retinal progenitor cell survival, but its inactivation does not affect eye growth (Rodrigues et al., 2013), most likely due to a minor contribution of retinal growth to eye size. Finding that NBS1 loss led to microphthalmia only when inactivated in lens progenitor cells provided a first hint of how RSR inactivation could affect eye development in a tissue-specific manner (Yang et al., 2006; Rodrigues et al., 2013). Interestingly, NBS1-deficient mature retinas undergo degeneration of the optic nerve and loss of retinal function (Baranes et al., 2009), but the molecular and cellular mechanisms underlying this neurodegeneration remain unclear.

Interestingly, a specific synergy between NBS1 loss and TRP53 was also revealed in lens progenitor cells. In the developing brain, TRP53 inactivation rescues cell death and proliferation defects and brain growth defects caused by NBS1 loss (Frappart et al., 2005; Li et al., 2012). In the lens, however, *Trp53* inactivation rescued progenitor cell death caused by NBS1 loss, but it did not rescue the defects in eye growth or cataract (Yang et al., 2006). Therefore, in NBS1-deficient lens progenitors, cell proliferation is blocked even when TRP53 is not functional, but the underlying mechanisms are still unknown. Importantly, in addition to its roles in RSR, NBS1 also participates in double-strand break signaling (Lee and Paull, 2005; Syed and Tainer, 2018), which could also factor in the diversity of outcomes observed.

Recently, we explored the function of another RSR gene by analyzing the function of ATRIP following tissue-specific inactivation in mice (**Figure 2**). As shown in transformed human cells (Cortez et al., 2001), ATR protein stability also depends on ATRIP in embryonic neural progenitor cells (Matos-Rodrigues et al., 2020a). Nestin-Cre-mediated inactivation of ATRIP in the developing central nervous system and in the eye leads to tissue growth defects (microphthalmia and microcephaly) that mirror the ones observed upon *Atr* inactivation (Lee et al., 2012). To understand the mechanisms underlying microphthalmia caused by ATRIP loss, we evaluated its contribution to cell cycle progression in *Trp53*-proficient and *Trp53*-deficient lens progenitor cells. In the presence of *Trp53,* ATRIP loss increases DNA damage and cell death, while in *Trp53*-deficient progenitors, ATRIP loss does not increase cell death, but leads to mitotic DNA damage and mitotic defects (Matos-Rodrigues et al., 2020a). These data suggest that inactivation of both genes might confer the ability to bypass the TRP53-mediated checkpoint and avoid cell death in S-phase, but ultimately culminating in mitotic catastrophe. Finally, as observed for NBS1, TRP53 deficiency does not rescue the microphthalmia caused by *Atrip* inactivation in lens progenitor cells.

We have also evaluated the effects of RSR inactivation in the mouse retina. ATRIP loss in embryonic retinal progenitor cells induces DNA damage accumulation and cell death, leading to lamination defects, photoreceptor degeneration and loss of vision (Matos-Rodrigues et al., 2020b). A previous study revealed photoreceptor degeneration in mice carrying an *Atr* hypomorphic mutation (Valdes-Sanchez et al., 2013). A role of ATR in the photoreceptor cilia was suggested to explain the observed neurodegeneration. Importantly, we found no evidence for a role of ATRIP in photoreceptors, since inactivation of *Atrip* specifically in these post-mitotic neurons did not affect retinal morphology or function. Because ATRIP is essential for ATR stability and all of its known functions are interdependent, further research is required to define the possible roles of the ATR-ATRIP complex in post-mitotic photoreceptor neurons.

In contrast to the lens, inactivation of *Trp53* rescues the cell death of retinal progenitor cells, neurodegeneration and visual impairment caused by ATRIP loss, indicating that TRP53-dependent apoptosis is the driver of retinal malformations caused by *Atrip* inactivation (Matos-Rodrigues et al., 2020b). These findings reinforced the existence of tissue-specific effects of RSR inactivation in the developing eye. An intact RSR is essential for lens progenitor cell proliferation since *Atrip* inactivation in the lens either abolishes lens formation (aphakia) or causes microphthalmia (Matos-Rodrigues et al., 2020a). In retinal progenitor cells, *Atrip* inactivation also leads to DNA damage accumulation and cell death. However, retinal development is not completely impaired by the slight modifications in proliferation and differentiation caused by defective RSR (Matos-Rodrigues et al., 2020b). These results suggest that lens progenitor cells are more sensitive to RSR inactivation than retinal ones and point to a different synergy between *Atrip* and *Trp53* when comparing retinal and lens progenitors. *Trp53* inactivation rescues lens progenitor cells apoptosis, but does not rescue eye growth defects, which were likely caused by enhanced mitotic DNA damage and mitotic defects (Matos-Rodrigues et al., 2020a). In opposition, *Trp53* inactivation completely rescues the developmental defects and the consequent neurodegeneration of the *Atrip*-deficient retinas (**Figure 2**). These observations are in agreement with

previous data on the effects of NBS1 inactivation during mouse eye development.

DISCUSSION

Based on the above-described studies we propose that the eye growth defects observed in replication-stress related syndrome patients are caused by the essential function of the affected genes in RSR in progenitor cells during embryogenesis. For example, tissue dysplasia and photoreceptor degeneration observed in *Atrip*-deficient retinas are a secondary consequence of progenitor apoptosis caused by the defective RSR in progenitor cells during embryonic development (Matos-Rodrigues et al., 2020b). Reports of retinal malformations and degeneration have been found in Seckel and Nijmegen breakage syndrome (**Figure 1B**). However, possible non-canonical functions of RSR genes in post-mitotic cells should not be overlooked, as it has been recently shown that ATR-CHK1 pathway can have a direct function on post-mitotic neurons activity and regeneration in model organisms (Kirtay et al., 2021; Li et al., 2021). Clinical investigations performing follow up in RSR-related syndromes patients associated with molecular diagnosis can bring important insights on the eye manifestations of these disorders.

The DDR is an evolutionarily conserved process that is often believed to operate by universal uniform principles. However, given that different progenitor cells have distinct transcriptional programs, metabolism, microenvironment and face different DNA-damaging insults, the DDR presents cell type- and developmental stage-specific adaptations (Blanpain et al., 2011; Rodrigues et al., 2013; Kafer and Cesare, 2020). The heterogeneous cellular outcomes of RSR inactivation in retinal and lens progenitor cells leads to the question of why progenitor cells show different sensitivity to RSR inactivation. Future studies in this field might bring exciting new contributions to the understanding of the RSR and its implications for developmental syndromes.

AUTHOR CONTRIBUTIONS

Both authors wrote the manuscript and read and agreed to the published version of the manuscript.

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REVIEW
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Mechanistic Insights From Single-Molecule Studies of Repair of Double Strand Breaks

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DNA double strand breaks (DSBs) are among some of the most deleterious forms of DNA damage. Left unrepaired, they are detrimental to genome stability, leading to high risk of cancer. Two major mechanisms are responsible for the repair of DSBs, homologous recombination (HR) and nonhomologous end joining (NHEJ). The complex nature of both pathways, involving a myriad of protein factors functioning in a highly coordinated manner at distinct stages of repair, lend themselves to detailed mechanistic studies using the latest single-molecule techniques. In avoiding ensemble averaging effects inherent to traditional biochemical or genetic methods, single-molecule studies have painted an increasingly detailed picture for every step of the DSB repair processes.

Keywords: non homologous end joining (NHEJ), homologous recombination (HR), single-molecule, DNA repair, optical tweezers (OT), magnetic tweezers, DNA curtain, FRET—fluorescence resonance energy transfer

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INTRODUCTION

Maintenance of genome stability is paramount to the survival of all living organisms. Both extrinsic environmental factors, as well as intrinsic, routine cellular processes such as transcription and replication can lead to DNA damage and contribute to genome instability. Understanding DNA damage and genome maintenance is a crucial aspect of cancer research, as they are involved in carcinogenesis and cancer therapies (Hoeijmakers, 2009).

Though accounting for only 0.01% of the ~10^5 spontaneous DNA lesions that a cell experiences per day, double strand breaks (DSBs) pose a unique challenge to repair in that the physical continuity of the DNA molecule is disrupted (Lindahl, 1993; Vilenchik and Knudson, 2003). DSBs can arise from a variety of exogenous factors such as ionizing radiation and chemotherapeutic drugs, as well as endogenous sources such as replication stress, V(D)J recombination, and meiosis. In addition, DSBs can also be generated when single strand breaks (SSBs), which are much more common, are encountered by DNA replication forks (Ohnishi et al., 2009). Mis-repair of DSBs can lead to deleterious consequences, causing large-scale chromosome rearrangements or local genetic mutations (Aparicio et al., 2014). Therefore, the repair process of DSBs is tightly controlled, employing complementary pathways consisted of intricately linked and carefully orchestrated steps.

Two major, well conserved, pathways in DSB repair are homologous recombination (HR) and canonical nonhomologous end joining (NHEJ). Together with pathways of alternative end joining (alt-NHEJ) and single-strand annealing (SSA), these four mechanisms are tasked to minimize undesired loss of genetic information in the process of restoring the physical continuity of DNA. Canonical and alternative end joining repair pathways directly join and ligate the two broken ends of a DSB after minimal end processing (Lieber, 2010). As these pathways require little to minimal sequence context, NHEJ and alt-NHEJ have typically been viewed as error-prone in repair. In contrast, homologous recombination is based on the search and pairing of the broken DNA end(s) to

existing homologous sequence elsewhere in the genome, thus maximizing repair fidelity (San Filippo et al., 2008). HR is cell cycle dependent and takes place in S and G2 phases, when homologous sequences in sister chromatids are available as repair templates. Whereas the end-joining pathways remain functional throughout the cell cycle (Symington and Gautier, 2011).

Over the past two and half decades, single-molecule microscopy and spectroscopy have made significant contributions to characterizations of systems previously considered intractable, thanks in no small part to technological innovations in fields from physics to nanotechnology and protein engineering. In this review, we begin with a brief description of single-molecule techniques frequently used in the studies of protein-DNA interactions. The sections that follow will be dedicated to homologous recombination and nonhomologous end joining, where we first provide an overview of each of these pathways in mammalian cells. We highlight and discuss in detail the findings from single-molecule studies that contributed to mechanistic understanding of steps involved in each repair mechanism. While the focus is on eukaryotic DSB repair, insights from pioneering studies of bacterial repair proteins will also be presented when appropriate.

OVERVIEW OF SINGLE-MOLECULE TECHNIQUES

A crucial hurdle that all *in vitro* single-molecule imaging studies of protein-DNA interactions must overcome is that as flexible polymers, DNA molecules, especially those significantly longer than their persistence length, collapse into random coils in the absence of external forces on the ends. Under most circumstances, unambiguous characterization of protein-DNA transactions is only possible when imaging is unencumbered by the presence of multiple DNA segments in close vicinity. To that end, several experimental approaches have been developed to maintain extended conformation of DNA molecules by exerting forces on their ends. In the sections below, we briefly describe these implementations.

Broadly speaking, there are two strategies for extending single DNA molecules to a desired end-to-end distance: mechanical force extension, typically through the use of optical or magnetic tweezers, and hydrodynamic force extension.

Optical Tweezers (OT) is an implementation of optical manipulation that controls and measures motion of trapped microscopic dielectric particle(s) using optical/electromagnetic forces (Ashkin, 1970; Ashkin et al., 1986). Beyond its applications that led to two separate awards of the Nobel Prize in Physics (Steve Chu in 1997 and Arthur Ashkin in 2018), optical trapping has been widely adopted today as a tool to study biophysical and biochemical properties of biological macromolecules and processes (Moffitt et al., 2008; Bustamante et al., 2020). The basic principles of optical trapping involve creating a tightly focused laser beam where the spatial gradient of its intensity exerts a restoring force on an object within the beam, balancing out the scattering force that pushes the object along the direction of light propagation (Ashkin et al., 1986). While the object is near (~150 nm) the center of the beam, the restoring force is linearly related to the displacement of the object from the center, acting as a Hookean spring (Neuman and Block, 2004). Single DNA molecules are typically extended using optical tweezers by fixing one end of the DNA to an optically trapped bead, while the other end is attached to either a physically fixed part of the flow cell assembly such as the surface or a micropipette, or another optically trapped bead (i.e., DNA dumbbells) (**Figure 1A**).

Similar to optical tweezers, magnetic tweezers utilize paramagnetic microspheres that are held in magnetic fields generated by external magnets (De Vlaminck and Dekker, 2012). Typically, the two ends of a DNA molecule are attached to the magnetic bead and the flow cell surface, with the molecule being extended by vertical positioning of the magnetic field relative to the flow cell (**Figure 1B**). While implementation of torque measurements has been developed for optical tweezers by using nanofabricated quartz cylinders held in angular optical traps (La Porta and Wang, 2004), the ability and ease to apply torque to a tethered molecule in magnetic tweezers by simply rotating the external magnetic field remain unparalleled. In addition to indirectly measuring torque through monitoring the DNA end-to-end distance, direct torque and twist measurements are also possible on magnetic tweezers with circularly symmetric or near-zero torque fields.

In comparison to mechanical force extension, hydrodynamic flow represents a more straightforward, albeit less precise, method to unravel DNA molecules. Flow stretching readily complements optical tweezers where only one end of the DNA is attached to a bead held in an optical trap, as such combination was initially used to study DNA conformational dynamics and polymer physics models (Perkins et al., 1995). When combined with surface-tethered DNA, flow stretching allows parallelization of measurements on multiple DNA molecules (**Figure 1C**). Briefly, one end of the DNA molecules is first immobilized on the surface of the flow cell, where they are randomly distributed spatially. These DNA molecules are then extended in the presence of applied buffer flow. Depending on the application, the down-flow ends may be left free, thus requiring continuous flow for the duration of these single-tethered experiments for real time observations. Alternatively, the second ends may also be anchored to the surface, forming double-tethered DNA and enabling steady-state observations in the absence of any buffer flow (**Figure 1C**). One common area of concern in these experiments is the potential of interference from the flow cell surface in protein-DNA interactions that are being studied.

A variation of surface-tethered single-molecule imaging technique named DNA curtains has been developed to minimize potential of surface interference and maximize parallelization (Graneli et al., 2006; Visnapuu et al., 2008). The platform uses nanofabricated chromium structures on flow cell surfaces to precisely align hundreds of DNA molecules at pre-determined positions (**Figure 1D**). Such alignment is achieved by first forming a biotinylated lipid bilayer, to which one end of the DNA molecules are tethered, on the surface of the microfluidic

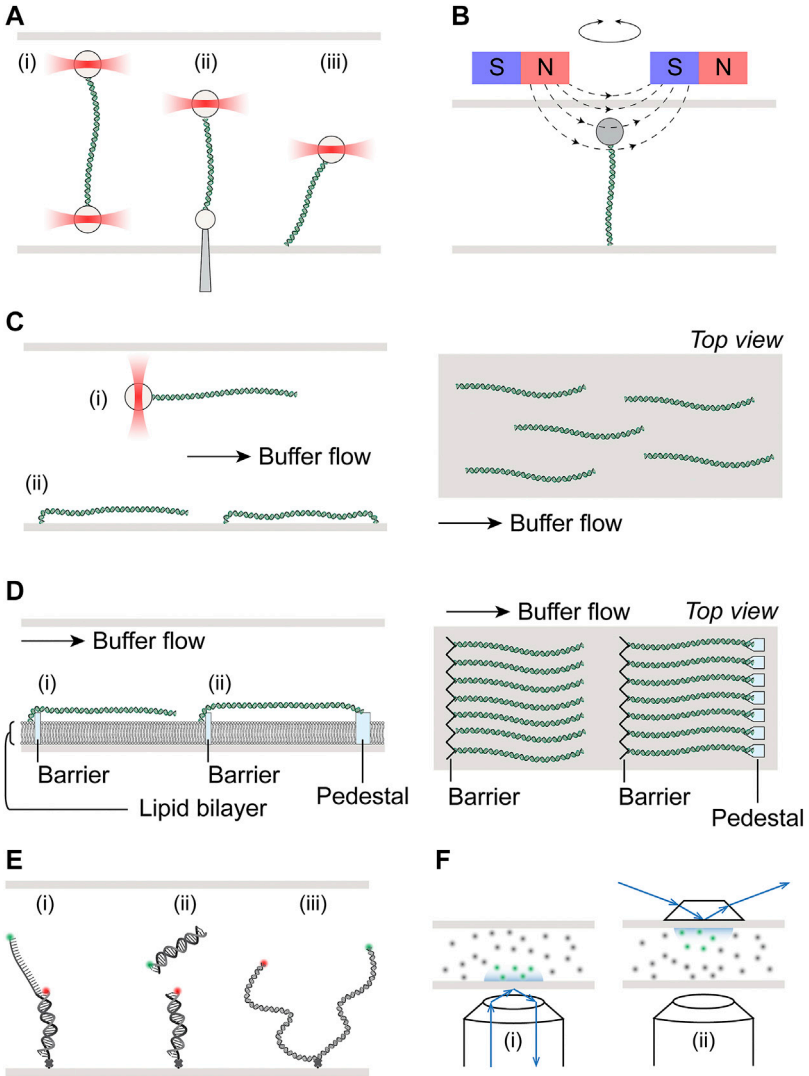

FIGURE 1 | Schematics of single-molecule setups. **(A)** Schematic illustration of optical tweezers, where a single DNA molecule is tethered at one end to an optically trapped bead, and the other end to **(i)** another bead held in a second optical trap, **(ii)** a micro-pipette fixed to the flow cell, or **(iii)** the surface of the flow cell. **(B)** Schematic illustration of magnetic tweezers, where a single DNA molecule is held between a magnetic bead and the flow cell surface. Torsional stress can be applied by rotation of the external magnets. **(C) Left:** Hydrodynamic flow extension of DNA molecules attached to either **(i)** an optically trapped bead or **(ii)** the flow cell surface. DNA molecules may be tethered to the surface at both ends if desired. **Right:** Top view of flow extended and surface tethered DNA in a flow cell, illustrating the random spatial distribution of these molecules. **(D) Left:** Schematic of DNA curtains where molecules tethered at one end to the lipid bilayer are aligned at the diffusion barrier. Single-tethered DNA may be extended by buffer flow **(i)**. Alternatively, DNA maybe double-tethered at the pedestal **(ii)**. **Right:** Top view of single- or double-tethered DNA curtains, where molecules are aligned in uniformity. **(E)** Schematic illustration of smFRET, where the energy transfer may be either intramolecular **(i)** and **(iii)** or intermolecular **(ii)**. DNA molecules are immobilized on the flow cell surface via biotin-streptavidin linkage with biotin placed at the ends of short substrates **(i)** and **(ii)**, or internally for ~kbp length substrates **(iii)**. **(F)** Schematic illustrate of total internal reflection fluorescence microscopy (TIRFM), achieved either through objective **(i)** or through prism **(ii)**. Green and gray dots represent excited inside and dark fluorophores outside the evanescence field, respectively.

device. The fluidity of lipid molecule allows these single-tethered DNA molecules to diffuse freely without flow. In the presence of buffer flow, DNA molecules are pushed against the chromium diffusion barriers and uniformly extended in parallel. Furthermore, the lipid bilayer also serves as a close mimic to biological membranes in cellular environments, minimizing non-specific surface adsorption of protein or DNA. Directional double-tethering is achieved by using orthogonal attachment chemistry at both the lipid bilayer and the chromium anchors, the latter deposited a specified distance away from the alignment barriers (**Figure 1D**). Further development of the initial dsDNA curtain technique allowed tethering of ssDNA and greatly expanded repertoire of biological processes that could be investigated with this technology (Ma et al., 2017b; De Tullio et al., 2018).

Visualization of protein-DNA interactions on extended DNA molecules is commonly based on fluorescence microscopy. dsDNA can be visualized by staining with fluorescent

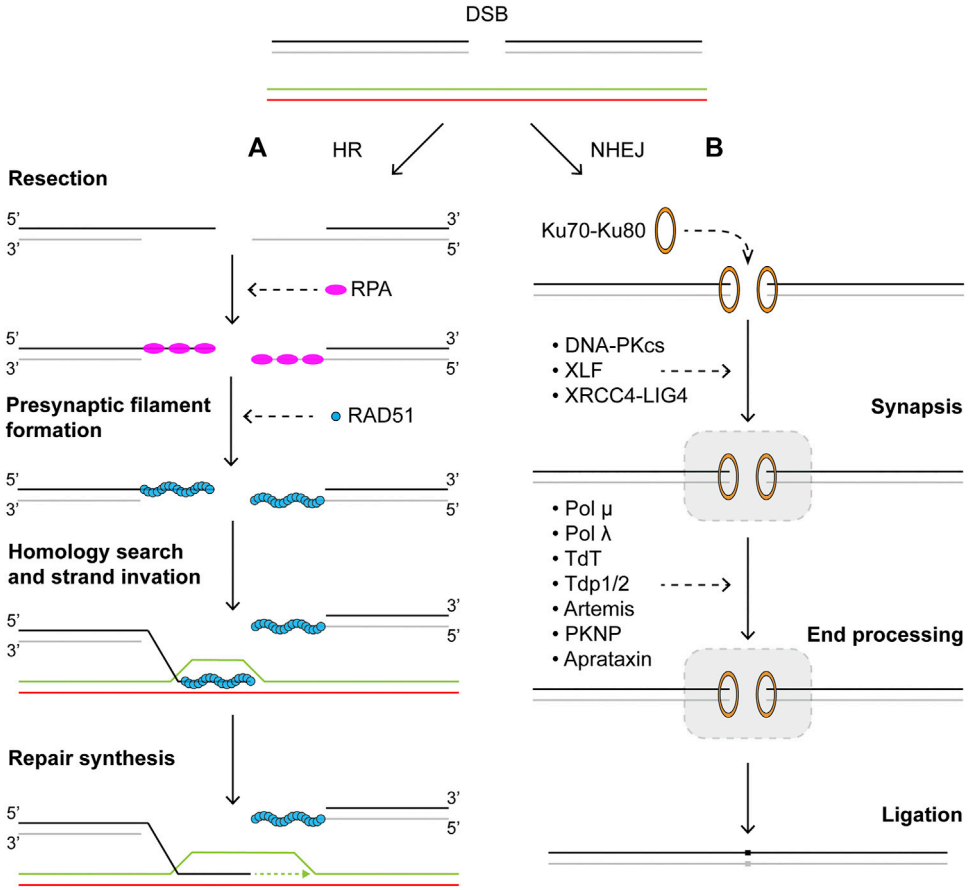

FIGURE 2 | Repair of DNA double strand breaks *via* **(A)** homologous recombination or **(B)** nonhomologous end-joining pathways. Schematics of homologous recombination and nonhomologous end-joining pathways. See main text for details.

intercalating dyes such as YOYO-1 or SYTOX Orange. Multiple options exist for protein labeling, including fluorescent fusion proteins, fluorescent nanocrystals (quantum dots), and a myriad of increasingly bright and photostable small-molecule fluorescent probes such as Alexa Fluor, ATTO, Janelia Fluor dyes (Ha and Tinnefeld, 2012). Fluorescent excitation may be readily accomplished *via* epi-fluorescence, total internal reflection, or confocal illumination. Each illumination scheme has its own advantages and disadvantages. For example, while total internal reflection reduces background noise significantly compared to epi-fluorescence, it is also restricted to imaging within ~200 nm of the surface, due to the depth reachable by evanescence waves produced by total internal reflection at that surface (Selvin and Ha, 2008) (**Figure 1E**).

In contrast to the direct imaging approaches described above, single-molecule Forster Resonance Energy Transfer (smFRET) experiments shed light on interactions that occur at much smaller distance scale (Roy et al., 2008). smFRET monitors the distance, usually between 1 and 10 nm, between single pairs of donor and acceptor fluorophores, by measuring their intensities and the extent of non-radiative energy transfer (Ha et al., 1996). Unencumbered by diffraction limited resolution (~250 nm) in typical fluorescence based single-molecule imaging experiments, smFRET has been widely employed in biophysical studies on topics ranging from replication, transcription and repair to RNA and protein conformational dynamics (Feng et al., 2021). *In vitro* smFRET experiments usually requires immobilization of fluorescently labeled macromolecules, either DNA or protein, on the passivated flow cell surface, where excitation of fluorophores is achieved through total internal reflection (**Figure 1F**). Although the length of surface-immobilized DNA used in smFRET experiments is typically short (~100 bp), longer DNA substrates on the order of kbp have also been successful in experiments under conditions such that DNA could become chromatinized (Graham et al., 2017).

OVERVIEW OF HOMOLOGOUS RECOMBINATION

Usually considered the error-free repair pathway for DSBs, HR can be divided into four distinct stages: end resection, formation of presynaptic filament, homology search, and repair synthesis (**Figure 2A**). In mammalian cells, resection is initiated first by the MRN complex consisting of Mre11, Rad50, and Nbs1, in complex with CtIP (Sartori et al., 2007; Shibata et al., 2014; Anand et al.,

2016). This short-range resection begins with Mre11 nicking the strand with a 5′ terminal at the break. The nick is then extended towards the break in the 3′–5′ manner by the exonuclease activity of Mre11. The single-stranded DNA gap created by short-range resection acts as a platform for long-range resection machineries to land. Proteins involved in long-range resection include EXO1, DNA2, BLM, and WRN (Eid et al., 2010; Nimonkar et al., 2011; Sturzenegger et al., 2014). EXO1 is a versatile and active 5′–3′ exonuclease. BLM and WRN are RecQ family helicases that can processively translocate on ssDNA in a 3′–5′ direction. Strand separation by BLM and WRN generates 5′ DNA flaps which are substrates for DNA2 activity. Together, their actions generate long 3′ ssDNA tails that are rapidly bound by the heterotrimeric ssDNA binding protein complex RPA to protect the integrity of DNA. Given that resection commits repair to homologous recombination, the process is subject to many forms of regulation. Phosphorylation of CtIP by CDK and ATM is essential for resection, through stimulating endonuclease activity of Mre11 as well as mediating interactions with BRCA1-BARD1 (Peterson et al., 2013; Wang et al., 2013). Furthermore, BRCA1 also plays an important role in removal of 53BP1, which is recruited to DSB sites and blocks 5′ end resection in G1 phase (Bunting et al., 2010; Mirman and de Lange, 2020). The assembly of presynaptic filament begins with binding of recombinase RAD51, homolog of bacterial RecA, to ssDNA, replacing RPA (Sung et al., 2003; Bonilla et al., 2020). Formation of the RAD51-ssDNA nucleofilament must overcome the inhibitive effects of RPA and is facilitated by recombination mediator proteins such as yeast Rad52 and human BRCA2 (Sung, 1997a; New et al., 1998; Jensen et al., 2010). BRCA2 interacts with RAD51 and together they are targeted to RPA-bound ssDNA by DSS1, a stable interaction partner of BRCA2 that also helps displacement of RPA from ssDNA. Stability of the Rad51-ssDNA filament is regulated by a number of pro- and anti-recombination proteins. Paralogues of human RAD51 form two distinct complexes, RAD51B-RAD51C-RAD51D-XRCC2 (BCDX2) and RAD51C-XRCC3 (CX3) (Masson et al., 2001a; Masson et al., 2001b). Together with the yeast complex Rad55-Rad57, as well as the Shu complexes, these paralog complexes are known to promote RAD51 filament formation and stability (Sung, 1997b; Bernstein et al., 2011; Liu T. et al., 2011; Bonilla et al., 2020). The RAD51-ssDNA nucleofilament must then undergo a homology search in an effort to locate and pair with homologous sequence elsewhere in the genome to be used as template for potentially error-free repair (Renkawitz et al., 2014; Haber, 2018). The search process is mediated by many proteins, including RAD54 (Petukhova et al., 1998; Petukhova et al., 2000; Zhang et al., 2007; Renkawitz et al., 2013). During the search, the presynaptic filament interrogates the dsDNA template and samples base pairing for homology. After recognition of homologous sequence is established, a stable heteroduplex called the displacement loop (D-loop) is formed, where the invading 3′ ssDNA tail is base paired with the complementary strand in the template DNA, displacing the homologous strand. D-loops formation, similar to that of the presynaptic filament, offers another opportunity for regulation. BRCA1-BARD1, RAD51AP1-UAF1, and PALB2 have all been shown to stimulate D-loop formation (Dray et al., 2010; Liang et al., 2016; Zhao et al., 2017). In order to initiate nascent DNA synthesis using the now paired strand as template, RAD51 is removed from the heteroduplex to expose the 3′ of the invading ssDNA, where DNA replication machineries including PCNA, RFC, and polymerase δ are assembled and can commence repair synthesis (Li et al., 2009; Sebesta et al., 2011; McVey et al., 2016). Finally, for DSBs with two free DNA ends, the repair may be completed through synthesis dependent strand annealing (SDSA), where the invading strand now extended through DNA synthesis dissociates from the D-loop structure and reanneals with the other broken end (San Filippo et al., 2008; Mehta and Haber, 2014). No crossover events occur as a result of SDSA. Alternatively, the second broken end may be captured by and annealed to the displaced strand of the D-loop, leading to the formation of a double Holliday junction (dHJ). Dissolution of dHJs by BLM and Topo IIIα will result in non-crossover events, while resolution can lead to either crossover or non-crossover events, depending on the resolvases involved (Xue et al., 2013; Bizard and Hickson, 2014; Chen et al., 2014; Matos and West, 2014).

SM Studies of DNA End Resection

The *Escherichia coli* RecBCD is a helicase and nuclease complex that plays a critical role in repair of DSBs in bacteria by homologous recombination, whose function in promoting recombination is regulated by the Chi (χ, crossover hotspot instigator) sequence in DNA (Dillingham and Kowalczykowski, 2008; Smith, 2012). Biophysical properties of RecBCD have been extensively characterized over the past two decades using a plethora of different single-molecule techniques. Though known to be a highly processive helicase, the actions of individual RecBCD complexes had not been directly observed (Roman et al., 1992). Using YOYO-stained λ-DNA conjugated to an optically trapped bead and extended by flow (**Figure 3A**, left), velocity and processivity of single RecBCD enzymes were measured by quantifying the loss of YOYO signal as the dsDNA was converted to ssDNA through actions of the enzyme (**Figure 3A**, right) (Bianco et al., 2001). Using the same imaging technique, the mechanism of regulation for the recombination hotspot χ sequence was elegantly elucidated. Single RecBCD complexes were observed to pause precisely upon encountering the χ site and slow down afterwards, which was revealed to be due to a change in the lead helicase from RecD to RecB, rather than the loss of RecD as previously believed (Spies et al., 2003; Handa et al., 2005; Spies et al., 2007). Moving from naked DNA towards a more physiological environment, further studies on RecBCD using the high throughput DNA curtains showed that the powerful complex is capable of ejecting stably bound proteins from DNA (Finkelstein et al., 2010; Terakawa et al., 2017).

An early responder in mammalian DNA damage response, 53BP1 is an enigmatic factor that is known to prevent the formation of long 3′ overhangs by limiting 5′ end resection at DSB sites in G1 cells (Mirman and de Lange, 2020). The 53BP1-mediated block to end resection mediated is accomplished through effector proteins RIF1 and PTIP, whose recruitment

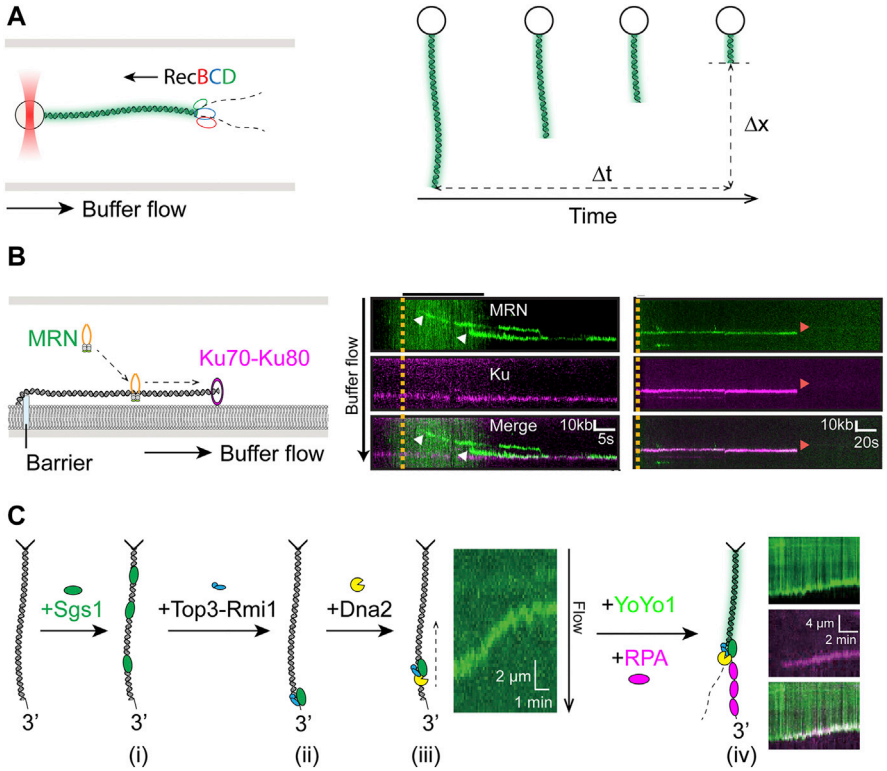

FIGURE 3 | Single-molecule studies of resection in HR. **(A) Left:** Schematics of unlabeled RecBCD resecting YOYO1-stained DNA attached to an optically trapped bead and extended by hydrodynamic flow. **Right:** Velocity ($\Delta x/\Delta t$) and processivity of RecBCD resection could be measured by quantifying shortening of YOYO1 tract (Δx) over time (Δt). **(B) Left:** Schematics of fluorescently labeled MRN binding to and sliding on single-tethered dsDNA, with end-bound Ku, in a single-tethered DNA curtain assay. **Right:** Kymographs showing the Mre11-dependent nucleolytic reaction leading to MRN and Ku release from DNA. White arrows indicate MRN binding. Red arrows indicate dissociation of both MRN and Ku. Adapted from (Myler et al., 2017). **(C)** Sgs1 bound randomly to single-tethered dsDNA in DNA curtain **(i)**, but was targeted to the free ends in the presence of Top3-Rmi1 **(ii)**. Translocation (green tract in kymograph) by Sgs1-Top3-Rmi complex was only activated by addition of Dna2 **(iii)**. End resection required further addition of RPA as evident by the shortening of YOYO1-staining of dsDNA accompanied by increase of fluorescence RPA signal at the DNA end **(iv)**. Adapted from Xue et al. (2019b).

depends on 53BP1 N-terminal phosphorylation by ATM (Callen et al., 2013; Zimmermann et al., 2013). For HR to proceed, restoration of end resection in S/G2 cells relies on the antagonistic functions of BRCA1 towards 53BP1 (Chapman et al., 2012; Densham et al., 2016; Hustedt and Durocher, 2016). Super-resolution light microscopy techniques such as the single-molecule localization microscopy (SMLM) has proven invaluable in elucidating the behavior of 53BP1 in response to DNA damage by ionizing radiation (Depes et al., 2018). SMLM measurements have shown cell type specific recruitment patterns of 53BP1, as well as dynamic changes in chromatin architecture, after high and low linear energy transfer irradiations (Bobkova et al., 2018; Hausmann et al., 2020).

End resection in human cells begins with the short-range resection initiated by the MRN complex with its phosphorylated cofactor CtIP, which produces a nick ~20 nt away from the end (Anand et al., 2016; Cannavo and Cejka, 2014). Single-molecule imaging of fluorescently labeled MRN showed that the protein utilizes facilitated diffusion to reach the DNA ends (**Figure 3B**, left) (Myler et al., 2017). Because the NHEJ initiating factor Ku also binds tightly to DNA ends, it raises the question of how MRN behaves when encountering DNA-bound Ku, as it also relates to the problem of pathway choice in DSB repair (Scully et al., 2019). Myler et al. showed in the same study that MRN is able to release DNA-bound Ku *via* an Mre11-dependent nucleolytic reaction (**Figure 3B**, right), thus providing a mechanism for initiation of HR even when Ku is the first to arrive at DSB sites (Myler et al., 2017). Recent follow-up on the topic from the same groups corroborated and extended the initial finding by including CtIP and DNA-PKcs in the DNA curtain assay, showing nucleolytic release of DNA-PK by MRN/CtIP (Deshpande et al., 2020).

The short overhang generated by MRN allows long-range resection factors BLM/DNA2 or EXO1 to assemble and carry out extensive resection. Significant insights into the biophysical characteristics of these enzymes as well as their regulation have been gained from single-molecule studies. Fluorescence imaging on DNA curtains demonstrated that human and yeast Exo1 are both processive nucleases that are susceptible to displacement by multivalent ssDNA binding proteins such as RPA, though extensive resection by human EXO1 was supported by the SOSS1, another ssDNA binding complex essential for HR in human cells (Myler et al., 2016). The coordination and regulation of long-range end resection among its participants

was well illustrated in a recent DNA curtain study focused on Sgs1, the yeast ortholog of BLM (**Figure 3C**) (Xue et al., 2019b). The authors showed that Sgs1 unwound dsDNA from internal positions in the presence of RPA (**Figure 3Ci**) and can be targeted to dsDNA ends in either Top3-Rmi1-dependent or independent manner (**Figure 3Cii**). However, Sgs1 remained inactive at DSBs until the addition of Dna2, which activated long-range translocation by Sgs1 from DNA ends (**Figure 3Ciii**). Furthermore, this complex lacked nucleolytic activity, which was only triggered through addition of RPA (**Figure 3Civ**), thus underscoring the importance of RPA in end resection as previously reported (Cejka et al., 2010a; Niu et al., 2010). Simultaneously, Sgs1 functions were also being studied using magnetic tweezers, where dsDNA unwinding initiated from a ssDNA gap with a 5′ flap produced comparable velocities to those from DNA curtain measurements, though rewinding of dsDNA, suggested to involve strand switching by Sgs1, was also observed (Kasaciunaite et al., 2019). In addition to the role in end recruitment of Sgs1 observed on DNA curtains, Top3-Rmi1 was shown to increase Sgs1 velocity when initiating translocation from flapped gap substrate, consistent with previously observed stimulatory effects (Cejka et al., 2010b; Kasaciunaite et al., 2019). The role of RPA in human RECQ helicase BLM-mediated resection was examined by two recent DNA curtain studies. BLM exhibited high speed and robustness in DNA unwinding regardless of the presence of RPA, while its end resection activity was dependent on the phosphorylation status of RPA (Xue et al., 2019a; Soniat et al., 2019). In the latter paper, resection by BLM/EXO2 or BLM/DNA2 on single-tethered DNA curtain was quantified in the presence of RPA or its phosphomimetic or phosphoblocking mutants. Phosphorylation on residues in RPA32 was found to reduce velocity and processivity of end resection by both BLM/EXO2 and BLM/DNA2, as well as inhibit their resection past individual nucleosomes, therefore acting as a negative regulator of resection (Soniat et al., 2019).

SM Studies of Presynaptic Filament Formation and Dynamics

As the 3′-ssDNA tail is being generated by long-range resection, it is rapidly bound and protected from nucleases by single strand binding proteins, which must then be replaced by recombinases. The bacterial SSB and recombinase RecA were among the first to be studied using single-molecule methods. Efforts to characterize fundamental behavior of SSB typically favored smFRET experiments with surface-immobilized single-stranded DNA that is labeled at the ends with a donor/acceptor fluorophore pair. Wrapping of ssDNA around the SSB tetramer during binding would bring the donor and acceptor closer and allow FRET efficiency to be used as a main observable. It was elegantly shown that tetrameric SSB could spontaneously diffuse on ssDNA, capable of removing secondary structure such as a small stem-loop hairpin and promoting formation of RecA filaments (Roy et al., 2009). Coupling an optically trapped bead to the smFRET substrate to apply pN-level of force on the complex, Zhou et al. was able to discern the molecular mechanism for SSB sliding as reptation, where the motion is facilitated by the formation of a DNA bulge and its propagation around the protein opposite its direction of sliding (Zhou et al., 2011).

Following binding of SSB to the 3′-ssDNA overhang, the *E. coli* recombinase RecA must be loaded to form a nucleoprotein filament capable of homology search and strand invasion. Observation of this process at the single-molecule level was first reported using smFRET and DNA substrates with short ssDNA overhang, where the donor/acceptor pair was placed at the junction and end of ssDNA (**Figure 4A**) (Joo et al., 2006). Five monomers of RecA were determined necessary for nucleation and dynamic binding and dissociation of single monomers from both ends of the filament contributed to filament growth. Notably, it was shown that RecA could displace SSB from ssDNA when a preformed nucleation cluster was present (Joo et al., 2006). Having first shown RecA binding of flow-extended double-stranded λ-DNA tethered to an optically trapped bead, Kowalczykowski et al. then reported formation of fluorescently labeled RecA filament on SSB-coated surface-tethered ssDNA (Galletto et al., 2006; Bell et al., 2012). In this work, Bell et al. observed that a RecA dimer was required for filament nucleation through titration of RecA concentration and its relationship with nucleation frequency. Using two-color labelling of RecA, it was demonstrated that RecA filament growth was bidirectional but faster in the 5′-3′ direction, consistent with previous findings (Galletto et al., 2006; Joo et al., 2006). Furthermore, *E. coli* recombination mediator proteins RecOR were shown to stimulate both RecA nucleation and filament growth (Bell et al., 2012).

Many of the same characteristics exhibited by SSB and RecA are conserved in their eukaryotic counterparts. Human RPA has been shown by smFRET to diffuse on ssDNA and melt secondary structures (Nguyen et al., 2014). Dynamics of RPA filament were thoroughly investigated using single-stranded DNA curtains (Gibb et al., 2012; Ma et al., 2017b). RPA filament formed on ssDNA was shown to be stable for over 2 h when unbound proteins were flushed out (Gibb et al., 2014a; Deng et al., 2014; Ma et al., 2016). When challenged with free protein in solution, it was observed that ssDNA-bound RPA could be exchanged with those in solution in a manner dependent on concentrations of the free RPA, consistent with facilitated dissociation previously reported for DNA binding proteins with multiple contacts (Graham et al., 2011; Gibb et al., 2014a; Deng et al., 2014; Ma et al., 2016). Direct visualization of fluorescently labeled human RAD51 showed conserved end-biased bidirectional filament growth on dsDNA, initiated by nucleation of ~2–3 monomers (Hilario et al., 2009). Assembly of RAD51 filament on the physiologically relevant RPA-coated DNA as well as its disassembly characteristics were also recapitulated on ssDNA curtains (Ma et al., 2016).

To overcome RPA-mediated inhibition of RAD51 filament formation, mediator proteins such as BRCA2 and RAD51 paralogs are needed (Bonilla et al., 2020). Effects of yeast Rad52, considered a possible functional ortholog of human BRCA2, on the dynamics of presynaptic filaments were revealed using ssDNA curtains (**Figure 4B**, left) (Gibb et al.,

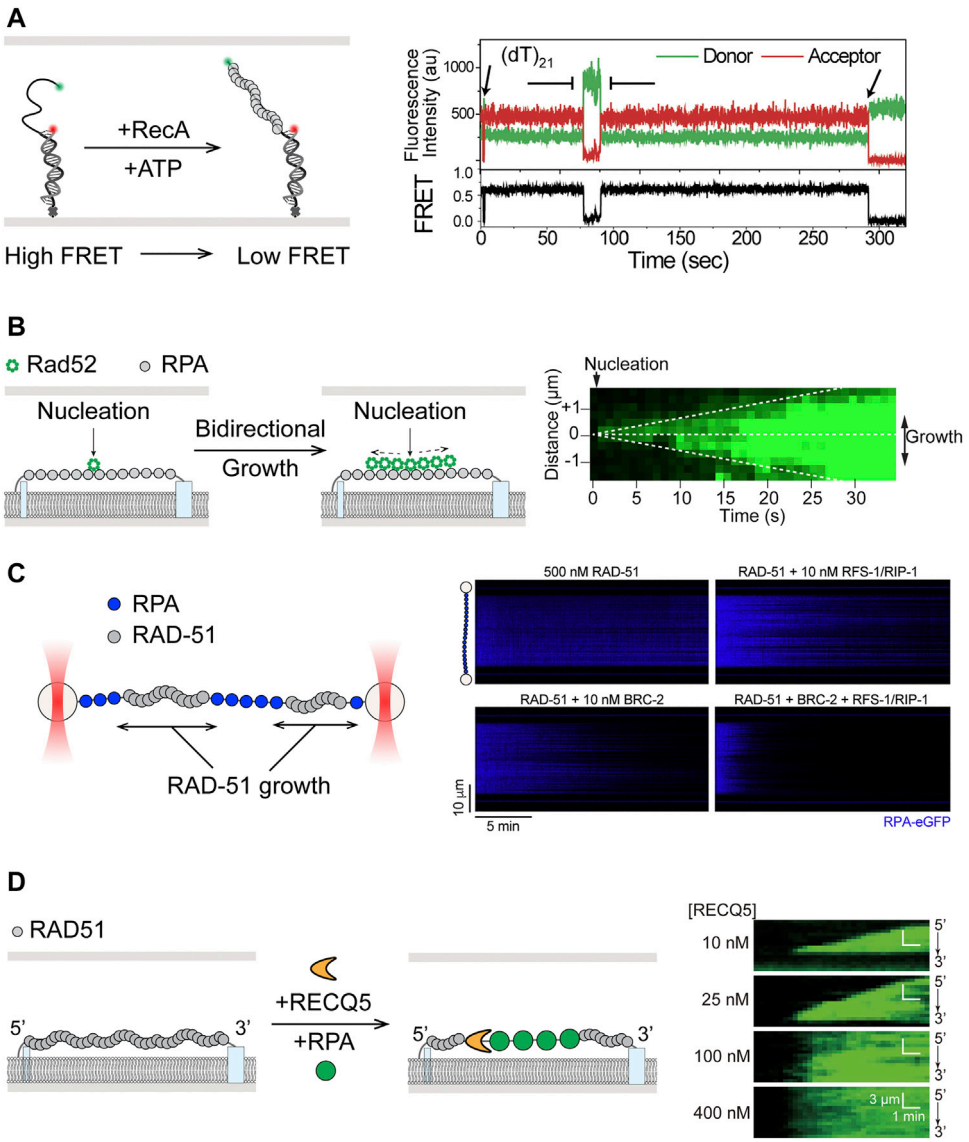

FIGURE 4 | Single-molecule studies of filament assembly and dynamics in HR. **(A)** smFRET study of assembly of RecA filaments on 3′-ssDNA overhang of duplex DNA substrates, where formation of RecA filaments leads to further separation of the donor and acceptor fluorophores and lower FRET efficiency. Adapted from Joo et al. (2006). **(B) Left:** Single-stranded DNA curtain showing bidirectional growth of Rad52 after nucleation on unlabeled RPA-coated single stranded DNA. **Right:** Kymograph of ssDNA shows double-sided wedge shape in fluorescence signal over time. Adapted from Gibb et al. (2014b). **(C) Left:** Scanning confocal fluorescence imaging of ssDNA held between two optically trapped beads. **Right:** RAD-51 paralogues RFS-1/RIP-1 was shown to work synergistically with BRC-2 in stimulation of unlabeled RAD-51 filament assembly, as reflected by the loss of fluorescent RPA signal in the kymographs. Adapted from Belan et al. (2021). **(D) Left:** Translocation by RECQ5 on RAD51 filaments in ssDNA curtain assay, where RAD51 was displaced in the process, as shown by the increase in fluorescent RPA signal. **Right:** Kymographs showing increase of fluorescent RPA signal as RAD51 was removed by RECQ5 from ssDNA. Wedge shape growth, from 3′ to 5′, of the fluorescence signal at low RECQ5 concentrations shows the direction of translocation. Adapted from Xue et al. (2021).

2014b). Fluorescently labeled Rad52 was shown to nucleate on RPA-coated ssDNA and promote bidirectional growth for additional Rad52 binding (**Figure 4B**, right). Rad52-RPA clusters were also observed to remain after formation of extended Rad51 filaments and served as nucleation sites for additional binding of RPA and Rad52 (Gibb et al., 2014b). Recent smFRET work suggests that the Rad52 destabilizes the DBD-D DNA binding domain of RPA, thereby increasing access to ssDNA previously occluded by RPA (Pokhrel et al., 2019). Many of these same characteristics of Rad52 were also recapitulated in a DNA curtain study of human RAD52, whose deletion in vertebrates does not produce a strong phenotype, with the exception that human RAD52 and RPA could not rebind to remaining clusters after assembly of human RAD51 filaments (Ma et al., 2017a). In addition, effects of RAD51 paralogs on presynaptic filaments have been the subject of several recent single-molecule studies. In a smFRET study of *C. elegans* RAD-51 paralogs RFS-1/RIP-1, surfaced-immobilized substrates

were labeled with donor and acceptor dyes seven nucleotides apart in the ssDNA region (Taylor et al., 2015). Addition of RAD-51 to naked ssDNA led to transition from high FRET to low FRET, reflecting the stretching of ssDNA upon RAD-51 binding. RFS-1/RIP-1 bound RAD-51 filament exhibited intermediate FRET value along with broadening of the FRET signal distribution, suggesting that these paralogs remodeled RAD-51 filaments to a more flexible conformation (Taylor et al., 2015). Most recently, optical tweezers with confocal fluorescence imaging (Lumicks C-trap) and DNA curtains were separately applied to better understand the actions of RFS-1/RIP-1 and the yeast Rad51 paralogs Rad55-Rad77, respectively (Belan et al., 2021; Roy et al., 2021). Both studies showed that the paralogs promote RAD51 filament assembly through transient interactions, dissociating rapidly by hydrolyzing ATP. In addition, Belan et al. found that RFS-1/RIP-1 synergize with BRC-2 (human BRCA2 homolog) in promoting presynaptic filament assembly, specifically by engaging with the 5′ end of the RAD-51 filament to stimulate growth in a 3′→5′ direction (**Figure 4C**) (Belan et al., 2021). Roy et al. also showed that Rad55-Rad57 antagonism of anti-recombinase Srs2 might be through promoting faster re-assembly of Rad51 rather than inhibiting the anti-recombinase itself, as previously suggested (Liu J. et al., 2011; Roy et al., 2021).

Excessive recombination, also referred to as hyper-recombination, however, can be genotoxic and must be prevented. Counteracting the effects of RAD51 mediators that promote filament assembly are the negative regulators, or anti-recombinases. Several SF1 helicases are known to display anti-recombinase activity. Bacterial UvrD and PcrA have been implicated in dismantling of RecA filaments in genetic and biochemical experiments (Veaute et al., 2005; Bidnenko et al., 2006; Lestini and Michel, 2007; Petrova et al., 2015). In particular, smFRET studies showed that PcrA strips RecA filaments off DNA through a reeling motion (Park et al., 2010). The yeast SF1 helicase Srs2 has also been shown to prevent recombination by dismantling the Rad51-ssDNA nucleofilament through stimulation of ATP hydrolysis by Rad51 and its dissociation (Krejci et al., 2003; Veaute et al., 2003; Antony et al., 2009). Actions of Srs2 on different HR intermediates have been visualized at the single-molecule level. In one smFRET study, Srs2 cleared Rad51 bound to short ssDNA overhangs and exhibited repetitive motion at the ssDNA/dsDNA junction, proposed to prevent reformation of Rad51 filament (Qiu et al., 2013). Single-stranded DNA curtain experiments showed that Srs2 was capable of processively translocating on naked ssDNA, as well as RPA-coated ssDNA, Rad51-ssDNA, and ssDNA bound by both RPA and Rad52 (De Tullio et al., 2017; Kaniecki et al., 2017). While translocating on protein-bound ssDNA, Srs2 also efficiently removed RPA, Rad51, Rad52, and short heteroduplexes formed with Rad51. Remarkably, this robust anti-recombination function of Srs2 was strongly inhibited by the presence of meiosis-specific recombinase Dmc1 within the presynaptic filament (Crickard et al., 2018).

In addition to SF1 helicases, members of the RecQ subfamily of SF2 helicases have also been implicated in anti-recombination functions (Branzei and Szakal, 2017; Larsen and Hickson, 2013). A DNA curtain study showed that Sgs1, apart from its role in end resection, also acted on presynaptic filaments (Crickard et al., 2019). Sgs1 was observed translocating on RPA-coated ssDNA and, in accordance with its expected anti-recombinase activity, displacing Rad51 while translocating on the Rad51-ssDNA filament. Sgs1-mediated Rad51 removal was found to be independent of Rad51 ATP hydrolysis, in stark contrast to the mechanism employed by Srs2 (Antony et al., 2009; Kaniecki et al., 2017). Though similar to the case of Srs2, Sgs1 action was also inhibited by Dmc1 (Crickard et al., 2019). Functional conservation of RECQ helicases in anti-recombination was recently demonstrated for the human RECQ5 on DNA curtain (**Figure 4D**, left) (Xue et al., 2021). RECQ5 not only translocated on ssDNA bound by RPA, RAD51, or DMC1, but also removed these proteins in the process (**Figure 4D**, right). Real-time observation of RAD51 removal by RECQ5 is consistent with previous results from biochemical assays (Hu et al., 2007). Similar to Sgs1, RECQ5 was able to strip ATPase-deficient RAD51 from ssDNA, suggesting a mechanism not coupled to RAD51 ATP hydrolysis. The ability of RECQ5 to translocate and disrupt DMC1 filaments contrasts with the inhibitory effects of Dmc1 on Sgs1 and Srs2, suggesting that it may play a role in meiosis (Xue et al., 2021). Finally, as mentioned above in the context of DNA end resection, BLM showed robust dsDNA unwinding but little interaction with RPA- or active ATP-bound RAD51-coated ssDNA in DNA curtain assays, even though it was considered an anti-recombinase capable disrupting inactive ADP-bound RAD51 filaments (Bugreev et al., 2007; Xue et al., 2019a). The apparent differences in the abilities of RECQ5 and BLM to interact with different HR intermediates may arise from differences in protein domain architecture and reflect division of labor among RECQ helicases in HR.

SM Studies of Homology Search

Once the stable presynaptic filament forms, it must locate sequence homology elsewhere in the genome, against a vast background of heterologous sequence. The recombinase then catalyzes a strand exchange reaction to form a heteroduplex containing the ssDNA base paired with the complementary strand and displacing the strand containing the homologous sequence (D-loop). At its core, the homology search process is highly similar to target search by other ubiquitous sequence specific DNA-binding proteins. A theoretical solution to the target search problem has been known for four decades as facilitated diffusion (Berg et al., 1981). Understanding of this process, involving a combination of 1D and 3D diffusion as well as microscopic hopping and intersegmental transfer, has seen significant contributions from single-molecule experiments. Following their earlier work on RecA filament assembly, the Kowalczykowski and Ha groups were the first to shed light on the mechanism behind homology search by RecA presynaptic filaments (Forget and Kowalczykowski, 2012; Ragunathan et al., 2012). In the first study, dsDNA serving as the homologous sequence donor was held between two optically trapped beads in a "dumbbell" configuration (**Figure 5A**). By systematically varying the distance between the beads, hence the contour length and 3D conformation of

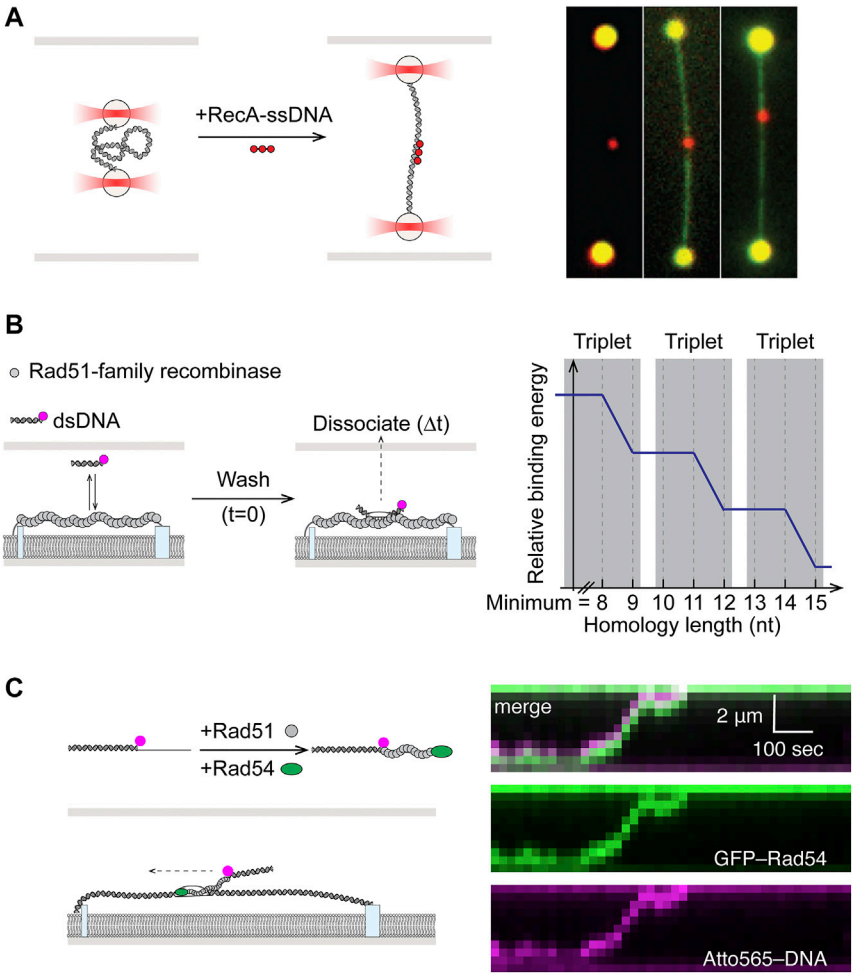

FIGURE 5 | Single-molecule studies of homology search in HR. **(A) Left:** Dual optical trap setup allowed precise control of dsDNA end-to-end distance in study of homology search by RecA. **Right:** Fluorescent RecA-ssDNA bound to expected homology positions in dsDNA after incubation. dsDNA could be visualized by staining with YOYO1. Adapted from Forget and Kowalczykowski (2012). **(B)** Fluorescently-labeled and microhomology-containing dsDNA fragments were incubated with nucleofilaments formed by Rad51-family recombinases on ssDNA curtain. Dissociation times of bound-particles post-wash (Δ*t*) showed energy stabilization occurring in steps of Watson-Crick base triplets. **(C) Left:** Presynaptic complexes were assembled by mixing Rad51 and Rad54 with dye-labeled partial duplex DNA containing homology in the ssDNA overhang region. **Right:** Dual color imaging of labeled DNA and Rad54 showed that Rad54 drove active homology search along DNA in an ATP hydrolysis-dependent manner. Adapted from Crickard et al. (2020).

the DNA, it was shown that RecA filaments conducted homology search via multiple weak contacts for sampling DNA sequence within a 3D volume, a mechanism the authors termed "intersegmental contact sampling" (**Figure 5A**) (Forget and Kowalczykowski, 2012). In the second smFRET study, free dsDNA homology donor and surface-immobilized RecA-ssDNA were labeled with donor and acceptor dyes, respectively. By observing the dynamic FRET values while controlling for sequence homology, a sliding model was proposed, in which RecA filaments can diffuse along the dsDNA track while efficiently sampling for homology as short as six nucleotides (Ragunathan et al., 2012). Together these studies demonstrate that homology search by RecA filaments occurs through facilitated diffusion using a combination of 1D sliding and 3D diffusion, expedited *via* intersegmental contact sampling.

More detailed understanding of minimum sequence homology requirements and kinetics of sampling were uncovered in a pair of papers using single-stranded DNA curtains (Lee et al., 2015; Qi et al., 2015). In these experiments, presynaptic filaments were assembled on long ssDNA tethered to the lipid bilayer surface, while fluorescently labeled duplexes containing varying degrees of homology were free in solution (**Figure 5B**). The first study revealed that eight nucleotides of homology was the minimum requirement for recognition by Rad51 and stable capture, while subsequent strand exchange occurred in precise three nucleotide steps (Qi et al., 2015). The follow-up work illustrated that the base triplet stepping for homology recognition (**Figure 5B**) was a conserved feature in the RecA family of recombinases from RecA to Rad51, including the meiosis-specific Dmc1. Dmc1, however, was also unique in its ability to stabilize internal mismatches. Whereas

mismatches in RecA or Rad51 filaments could be tolerated but would not contribute to stabilizing recognition complex (Lee et al., 2015).

Adding to the complexity of homology search is the fact that the process in eukaryotic cells also involves the multi-functional SWI2/SNF2 motor proteins Rad54 and Rdh54 (Ceballos and Heyer, 2011). Early single-molecule work had shown that both Rad54 and Rdh54 are highly processive translocases on dsDNA (Amitani et al., 2006; Nimonkar et al., 2007; Prasad et al., 2007). While Rad54 is also known to facilitate Rad51-mediated homologous DNA pairing *in vitro* and homology search *in vivo*, its exact mechanism of action remained unknown (Petukhova et al., 1998; Renkawitz et al., 2013). Recently, using double-tethered dsDNA curtains as sequence donor and labeled partial duplex DNA with 3′ Rad51-ssDNA filament, Rad54 was shown to promote targeting to homologous DNA by translocating with the presynaptic filament on dsDNA (**Figure 5C**) (Crickard et al., 2020). This ATP-dependent behavior adds to the 3D diffusion mechanism of the homology search and serves in reducing dimensionality and increasing search efficiency. Moreover, while driving active translocation, Rad54 induced transient strand opening coupled to RPA binding, potentially allowing the Rad51 presynaptic complex to sample both strands of dsDNA donor for homology (Crickard et al., 2020).

OVERVIEW OF NHEJ

Upon formation of a DSB, the "canonical" NHEJ pathway proceeds through three distinct steps: synapsis, end processing, and ligation (**Figure 2B**). The DNA ends are first recognized by Ku70-Ku80, a ring-shaped heterodimer with high affinity to DNA ends (Walker et al., 2001). After binding, Ku70-Ku80 (Ku) serves as a 'tool belt' that interacts and stabilizes many subsequence NHEJ proteins (Lieber, 2008). One of the first factors recruited to DNA-bound Ku is DNA-PKcs (DNA-dependent protein kinase, catalytic subunit), a member of the phophoinositide 3-kinase family (Gottlieb and Jackson, 1993; Smith and Jackson, 1999; Falck et al., 2005). Together they form the DNA-PK holoenzyme, whose kinase activity is required for NHEJ, as it phosphorylates many other NHEJ accessory factors as well as itself (Uematsu et al., 2007; Jette and Lees-Miller, 2015; Jiang et al., 2015). In the next step, the two broken DNA ends must be brought to close proximity to enable synapsis in a dynamic process. The mechanism of synapsis depends on binding of LIG4 (DNA ligase IV), XRCC4, and XLF (XRCC4-like factor) (Stinson and Loparo, 2021). LIG4 and XRCC4 form an active complex, through interactions between XRCC4 and the region between the BRCT motifs in the C-terminal of LIG4 (Critchlow et al., 1997; Grawunder et al., 1997; Grawunder et al., 1998; Sibanda et al., 2001; Wu et al., 2009). XLF was identified to interact with LIG4-XRCC4 to promote NHEJ (Ahnesorg et al., 2006; Buck et al., 2006). Evidence also suggests that XRCC4 and XLF may form filaments that help bridge DNA ends (Hammel et al., 2011; Ropars et al., 2011; Andres et al., 2012; Mahaney et al., 2013). Post synapsis, blunt ends that do not require further processing may be ligated directly by XRCC4-LIG4. However, naturally occurring DSBs typically have incompatible ends that cannot be readily ligated. Therefore, end processing in the forms of resection by nucleases and/or addition and filling-in by the X family of DNA polymerases are often needed before generating compatible ends for ligation (Waters et al., 2014; Chang et al., 2016). Artemis is a nuclease associated with NHEJ and essential for V(D)J recombination (Ma et al., 2002; Riballo et al., 2004). Its C-terminal region has been found to interact with LIG4 and DNA-PKcs (Niewolik et al., 2006; De Ioannes et al., 2012; Malu et al., 2012). While members of the X family DNA polymerase, pol λ, pol μ, and TdT (terminal deoxynucleotidyl transferase) are all implicated in NHEJ with different levels of template dependence (Nick McElhinny et al., 2005). Recruitment of these polymerases to sites of NHEJ is known to occur through interactions with Ku and XRCC4-LIG4 via their N-terminal BRCT domain (Mahajan et al., 2002; Fan and Wu, 2004; Ma et al., 2004). Notably, recent structural evidence has indicated that synapsis of DNA ends with single nucleotide homology could be mediated solely by TdT or pol μ, in the absence of other NHEJ core factors (Kaminski et al., 2020; Loc'h et al., 2016). In cases of unligatable chemical blocks at DNA ends, PNKP (polynucleotide kinase 3′-phosphatase), aprataxin and PNKP-like factor (APLF), or tyrosyl-DNA phosphodiesterase 1/2 (TDP1/2) may be recruited to DSB sites for processing (Zhao et al., 2020a). Finally, PAXX (paralogue of XRCC4 and XLF) is a recently discovered factor that promotes ligation and assembly of core NHEJ proteins (Ochi et al., 2015; Xing et al., 2015). Although its functions in NHEJ appear to overlap with those of XLF (Tadi et al., 2016).

SINGLE-MOLECULE STUDIES OF NHEJ

Fundamental Mechanism of Synapsis

Synapsis is the step in which the two broken DNA ends are brought together to close proximity such that NHEJ machinery may assemble in a stable complex and assess the actions needed to restore the structural integrity of DNA. Detailed mechanistic insights on this critical early step are therefore prerequisite to understanding of the pathway. The dynamic nature of the process involving two DNA ends has made smFRET the single-molecule platform of choice in studying the system. By measuring the fluorescence energy transfer between the donor-labeled surface-immobilized fragment and the acceptor-labeled freely-diffusing fragment, smFRET allows real-time monitoring of intermolecular synapsis. High FRET indicates close proximity of the two DNA ends, while fluctuating FRET values would suggest dynamics in the process of DNA end alignment.

Early models derived from work using purified proteins in bulk biochemical assays, electron microscopy, and x-ray scattering as well as laser microirradiation of cells followed by immunofluorescence imaging suggested that DNA-PKcs is recruited by Ku to DNA breaks and together they are able to bridge the broken DNA ends (DeFazio et al., 2002; Hammel et al., 2010b; Kim et al., 2005; Weterings et al., 2003). Nonetheless, the lack of spatiotemporal resolution precluded these studies from

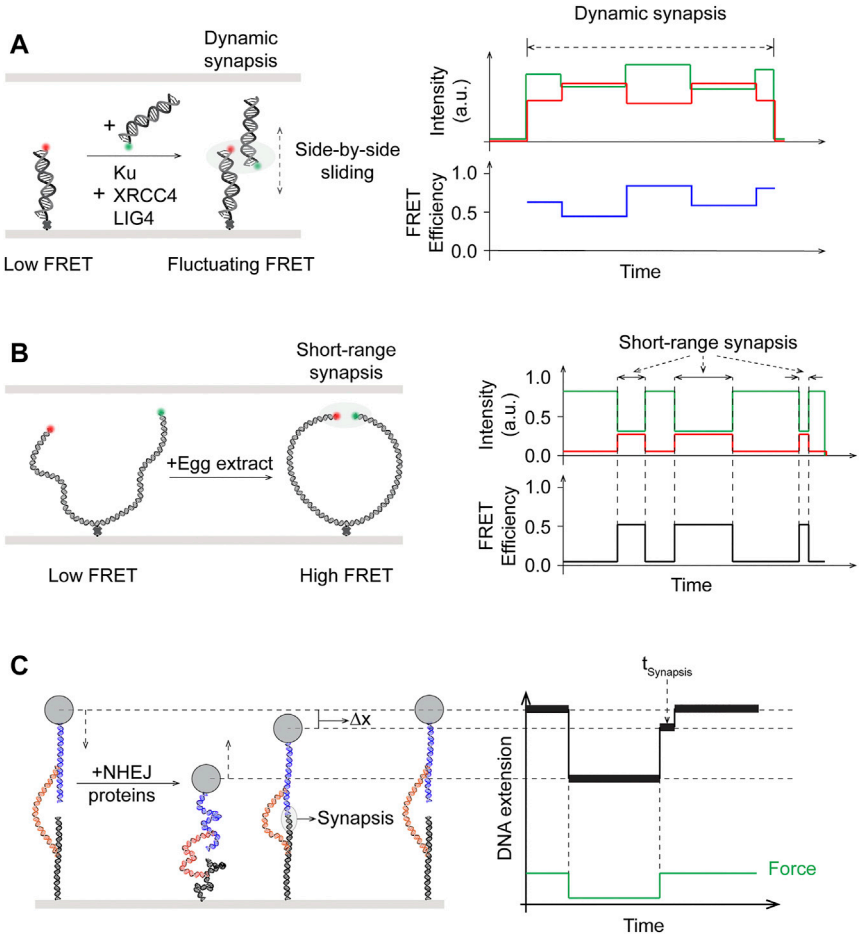

FIGURE 6 | Single-molecule studies of end synapsis in NHEJ. **(A) Left:** Schematics of intermolecular smFRET showing synapsis upon addition of purified human Ku, XRCC4, LIG4, but in the absence of DNA-PKcs, to be dynamic with fluctuating and widely distributed FRET efficiency, suggesting that the two broken ends may slide relative to and past each other during synapsis. **Right:** Side-by-side sliding of the donor with respect to the acceptor could give rise to the fluctuating FRET efficiency values. **(B) Left:** Schematics of intramolecular smFRET used to monitor end synapsis mediated by NHEJ factors in egg extract. **Right:** Distinct high FRET state reverted back to low FRET state, showing short-range synapsis was dynamic. **(C)** Magnetic tweezers with novel DNA substrate design tracks DNA extension and enables measuring dwell times (Δt) of transient synapsis, by cycling between low force/extension that allows formation of synaptic complex and high force/extension to disrupt synapsed but unligated ends. A change in DNA extension (Δx) at the same high force is observed when the synapsed ends are disrupted.

revealing any transient intermediate steps or subcomplexes in the process. Using smFRET with two DNA fragments containing four nucleotide homology at the ends and differentially labeled with donor and acceptor fluorophores, Rothenberg and coworkers observed co-localization of the donor/acceptor pairs after addition of purified NHEJ components except DNA-PKcs (**Figure 6A**, left) (Reid et al., 2015). Although aggregated joining was observed in the presence of DNA-PKcs, this result cast doubt over the requirement of DNA-PKcs in synapsis. This end joining process mediated by Ku70-Ku80, XRCC4-LIG4, and XLF was revealed to be dynamic, as shown by FRET efficiency distributions (**Figure 6A**, right). These distributions exhibited widths indicative of the possibility that the DNA ends may be positioned in a side-by-side manner, in addition to end-to-end. Examination of fluctuating FRET trajectories in conjunction with using substrates that varied in end chemistry also supported the notion of DNA ends in "adjacent configuration" during the synaptic process that is highly dynamic (**Figure 6A**). More mechanistic details were uncovered in a later follow-up smFRET study by the Rothenberg and Lieber laboratories (Zhao et al., 2019). It was shown that the first "flexible" stage of blunt end synapsis (FS), mediated by Ku and XRCC4-LIG4, involves the dsDNA ends being brought into a parallel side-by-side configuration where they can still slide along each other, as evidenced by fluctuating FRET efficiency values. Flexible synapsis, shown to be independent of DNA-PKcs, can then be converted to a close synaptic state (CS) by XLF or PAXX, where the two DNA ends are aligned in close proximity in an end-to-end manner.

The lack of DNA-PKcs requirement in synapsis as monitored by smFRET contradicts existing evidence for its role in NHEJ *in vivo* (Baumann and West, 1998; Cottarel et al., 2013; Jiang et al., 2015; Zhao et al., 2006). This apparent discrepancy was further investigated by Loparo and coworkers using smFRET and cell-

free extract of *X. laevis* eggs (**Figure 6B**) (Graham et al., 2016). Xenopus egg extract represents a more physiological system compared to biochemical reconstitution of purified proteins, and has been established for single-molecule imaging studies as well as being capable of Ku- and DNA-PK-dependent DNA end joining (Di Virgilio and Gautier, 2005; Labhart, 1999; Yardimci et al., 2012). In addition to intermolecular synapsis of two separate DNA fragments, a longer 2 kbp DNA fragment with donor/acceptor-labeled blunt ends and an internal biotin for surface immobilization was used in this study to facilitate intramolecular end joining (**Figure 6B**, left) (Graham et al., 2017). Based on the distance between the donor/acceptor dyes, synapsis was observed to occur through two distinct stages: long-range (LR) where both dyes were present but no FRET, and short-range (SR) where FRET was seen between the dye pair (**Figure 6B**, right). In contrast to previous single-molecule work, LR synapsis in Xenopus egg extracts required both Ku70-Ku80 and DNA-PKcs, though the kinase activity of the latter is not needed. Transition from LR to SR synapsis would occur after several seconds and require the catalytic activity of DNA-PK, as well as the presence of XRCC4-LIG4 and XLF, though not the catalytic activity of LIG4.

Unlike order of assembly studies, quantifying biophysical observables such as step-wise reaction energetics has mostly been intractable for bulk biochemistry. In particular, a novel DNA substrate featuring two free DNA ends tethered *via* a leash held by magnetic tweezers has been developed as a unique single-molecule force spectroscopy approach to probe the energetics of NHEJ synapsis with reconstitution of purified proteins (Kostrz et al., 2019; Wang et al., 2018). By cycling between low and high forces on a single tether and monitoring changes in tether length, Strick and coworkers demonstrated that Ku and DNA-PKcs are required to first establish a brief (~100 ms) stage of synapsis of DNA ends (Wang et al., 2018) (**Figure 6C**). This initial step is further stabilized by either XRCC4-LIG4 and XLS and/or PAXX, each contributing k_BT-scale energy, leading to long-lived (~seconds) intermediate stages and stable (~minute) synaptic complexes. Notably, these results support the two distinct stages of synapsis observed by Graham et al. using smFRET. The subcomplex containing Ku and DNA-PKcs and stabilized by PAXX (~2 s) appears consistent with the long range synapsis, while the full complex further stabilized by XRCC4-LIG4 and XLF (~66 s) would correspond to the short range synapsis (Stinson and Loparo, 2021). Most recently, the same technique was applied to demonstrate the dynamic properties of prokayrotic NHEJ synapsis involving just the Ku heterodimer and Ligase D (Oz et al., 2021). Although debates remain regarding whether DNA-PKcs is required for synapsis, as the results appear to be dependent on the system employed, recent single-molecule work have unambiguously shown the process to be a dynamic process with distinct stages.

Roles of XLF in Synaptic Complexes

XRCC4-like factor (XLF, or Cernunnos) is identified as an interactor of XRCC4 and regulator of ligation (Ahnesorg et al., 2006; Buck et al., 2006). X-ray crystallography and electron microscopy studies have shown that XLF and XRCC4 can form filaments in crystals (Andres et al., 2012; Hammel et al., 2010a; Mahaney et al., 2013; Ropars et al., 2011). Filamentous structures of XRCC4, XLF, and LIG4 have also been observed at DSB sites using super-resolution fluorescence microscopy (Reid et al., 2015). The mode of interaction between XLF-XRCC4 complexes and DNA remained elusive until the collaborative work from the Modesti, Peterman, and Wuite groups. In a single-molecule tour de force, dual- and quadruple optical traps were combined with wide-field fluorescence imaging to demonstrate that XRCC4-XLF complexes robustly bridged two independent DNA fragments (Brouwer et al., 2016). These complexes acted like sleeves that were able to withstand high applied forces and capable of sliding along DNA molecules (**Figure 7A**). Specific contributions from XLF in synapsis in the presence of other NHEJ core proteins were elucidated using smFRET (**Figure 7B**, left). Mutagenesis in XLF and XRCC4 showed that close alignment of donor/acceptor dye labeled DNA ends in the xenopus egg extracts system required interactions between these two proteins (Graham et al., 2018). Moreover, binding of a single dye-labeled XLF dimer was sufficient to mediate this short-range synapsis, which is shown to also be dependent on interactions of both XLF head domains with XRCC4 (**Figure 7B**, right). These findings call into question the requirement and relevance of XLF-XRCC4 filaments, as observed in bulk, in NHEJ. More corroborating evidence incompatible with the XLF filament hypothesis emerged in a subsequent smFRET study by Rothenberg and Lieber laboratories using reconstituted human NHEJ proteins. XLF was found to drive DNA ends into close proximity in a manner that is not strongly dependent on XLF concentrations, suggesting that only one to a few XLF dimers are needed at the DNA ends (Zhao et al., 2019).

End Processing and Ligation

Many DNA ends at DSB sites, regardless of their origins, require end processing before repair. The arsenal of NHEJ end-processing enzymes include nucleases, polymerases, kinases, phosphatases, and phosphodiesterases (Chang et al., 2017). The effects of chemically diverse DNA ends have on the dynamics of how they come together during synapsis is a question uniquely suited for single-molecule studies. Pairing efficiency as monitored by smFRET was shown to be strongly affected by phosphorylation status of the 5′ end of compatible DNA ends with four nucleotide overhangs in a minimal reconstituted system (Reid et al., 2017). Two distinct kinetic regimes, transient (<5 s) and persistent (>30 s), were found to exist for end pairing during the process, and that their energetics are modulated by the 5′ phosphate, through recognition by LIG4. In the absence of other end processing factors in this single-molecule work, a model involving an iterative process was proposed, where incompatible ends within a synaptic complex would fall apart to provide access by the processing enzymes and thus generating new compatible ends for synapsis and ligation (Reid et al., 2017). Subsequent smFRET work further examined the ability of LIG4 to sense complex ends in the minimal reconstituted system. At DNA ends with overhangs containing varying degrees of complementarity, LIG4 was shown to promote alignment of complementary ends in pre-catalytic positions, but

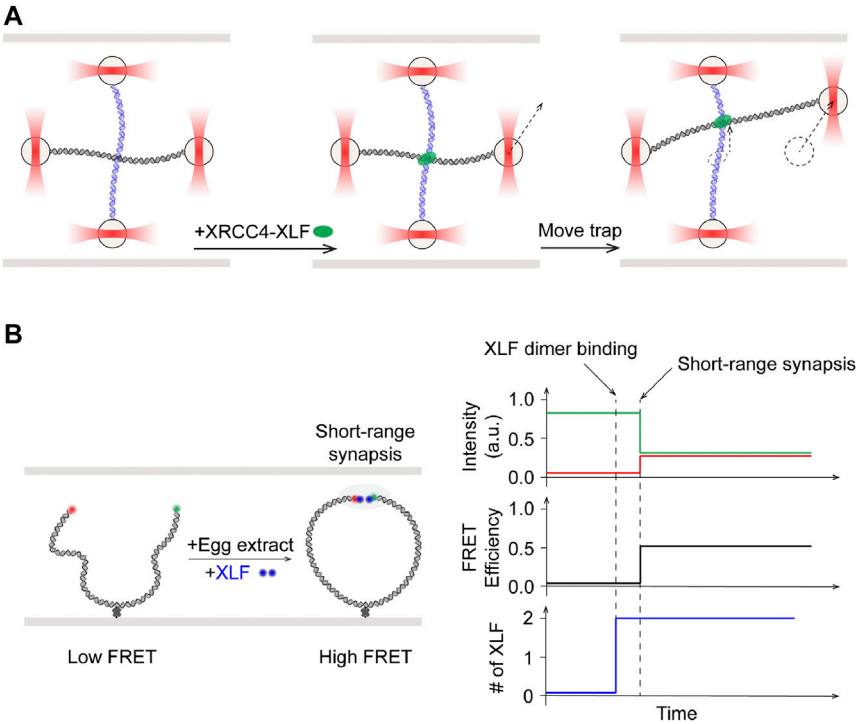

FIGURE 7 | Single-molecule studies of roles of XLF in NHEJ synapsis. **(A) Left:** Quad optical trap was used to show bridging of two separate DNA molecules by XRCC4-XLF. **Middle:** One trap was moved to shift one of the two DNA molecules. **Right:** XRCC4-XLF was able to slide while maintaining the bridge. **(B) Left:** Schematics of smFRET study using fluorescently labeled XLF dimer with egg extract. **Right:** Onset of high FRET, indicative of short-range synapsis between the DNA ends, was preceded by increase in fluorescence signal corresponding to binding of one XLF dimer.

allow dynamic sampling of alignments for terminal mismatches or ends with embedded complementarity that requires nucleolytic end process before ligation (Conlin et al., 2017). While the above-mentioned work demonstrated the participation of LIG4 in the alignment of DNA ends, in order to understand how end processing is coordinated with alignment of DNA, simultaneous observation of end processing enzymes and synapsis would be required. Taking advantage of the xenopus egg extracts system, Stinson et al. recently expanded on the requirement of LIG4-mediated close alignment of the DNA ends and showed that end processing is coordinated to take place within this synaptic complex (Stinson et al., 2020). Synaptic complex formation was monitored through smFRET with donor/acceptor dye-labeled DNA ends as before. To observe pol λ activity, the first incoming nucleotide was labeled with a fluorescent quencher, which once incorporated leads to quenching of the donor fluorophore. To observe Tdp1 activity, one of the 3′ adducts is conjugated to the donor fluorophore, which once processed by Tdp1 will be lost. It was observed that donor signal loss was preceded by high FRET, indicating close alignment of DNA end (Stinson et al., 2020). These data clearly demonstrated that end processing by pol λ and Tdp1 occurs within the short-range synaptic complex. This level of coordination between end processing and ligation during synapsis has thus been proposed as a regulatory mechanism to minimize errors and maximize fidelity of NHEJ (Stinson et al., 2020; Stinson and Loparo, 2021). Finally, attesting to the flexibility of NHEJ, it was recently reported in a smFRET study that pol μ, another X family polymerase participating in NHEJ alongside pol λ, alone can mediate synapsis of 3′ overhangs with at least 1 nt homology, in the absence of Ku (Zhao et al., 2020b).

CONCLUSION AND PERSPECTIVES

Single-molecule techniques have advanced and matured by leaps and bounds, thanks to technological improvements in equipment and reagents such as cameras and fluorescent dyes. The field has also expanded and benefited from commercialization of single-molecule instruments. An underlying technical challenge in single-molecule work has always been to achieve higher spatial and temporal resolutions. And this drive has steadfastly pushed technical innovations in the field. As single-molecule studies are typically built with a bottom-up approach, the field is constantly striving for increased levels of complexity in biological systems under examination. For mechanistic studies of homologous recombination, challenges remain, including but not limited to, in addressing functions of RAD51 mediator proteins and incorporating other accessory proteins in reconstituting the process from filament assembly to strand invasion, among others. Studying repair in general within the physiologically

relevant context of chromatin has also proven challenging. Since multiple repair pathways exist and are available to cells for DSB repair, pathway choice is an overarching subject that bridges studies of individual repair mechanisms. Though initial work exists, the molecular mechanism for how competing repair mechanisms cooperate at the single-molecule level has largely been elusive. Biochemical reconstitutions of repair using purified recombinant proteins provide a clear, pre-defined set of parameters and have been the preferred system for single-molecule studies. However, functional cell extracts that already contain the proteins of interest may be the key to the pursuit of higher degrees of reaction complexity.

The unprecedented level of detail in mechanistic insights from single-molecule experiments may at times be seemingly at odds with existing biochemical or *in vivo* evidence and require careful reconciliation. It is worth repeating that gaps exist among these approaches, such that a comprehensive picture is best constructed when all evidence is considered together. Indeed, differences exist even between comparable single-molecule studies using the same techniques, resulting in apparently incompatible interpretation of the mechanism. Building on existing imaging platforms that focus on studies of particular stages of the process, further developments of single-molecule imaging *in vivo*, complemented by biochemical and *in vitro* studies, will undoubtedly help uncover deeper understanding of DSB repair.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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The Making and Breaking of Serine-ADP-Ribosylation in the DNA Damage Response

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ADP-ribosylation is a widespread posttranslational modification that is of particular therapeutic relevance due to its involvement in DNA repair. In response to DNA damage, PARP1 and 2 are the main enzymes that catalyze ADP-ribosylation at damage sites. Recently, serine was identified as the primary amino acid acceptor of the ADP-ribosyl moiety following DNA damage and appears to act as seed for chain elongation in this context. Serine-ADP-ribosylation strictly depends on HPF1, an auxiliary factor of PARP1/2, which facilitates this modification by completing the PARP1/2 active site. The signal is terminated by initial poly(ADP-ribose) chain degradation, primarily carried out by PARG, while another enzyme, (ADP-ribosyl)hydrolase 3 (ARH3), specifically cleaves the terminal seryl-ADP-ribosyl bond, thus completing the chain degradation initiated by PARG. This review summarizes recent findings in the field of serine-ADP-ribosylation, its mechanisms, possible functions and potential for therapeutic targeting through HPF1 and ARH3 inhibition.

Keywords: DNA damage, PARP, ADP-ribosylation, cancer, PARG, neurodegeneration, posttranslational modification (PTM), ARH3

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INTRODUCTION

ADP-ribosylation refers to the transfer of ADP-ribose (ADPr) moiety from NAD^+ onto substrate proteins or nucleic acids by enzymes termed (ADP-ribosyl)transferases (ARTs; **Figure 1**; Liu and Yu, 2015; Wei and Yu, 2016; Munnur and Ahel, 2017; Zarkovic et al., 2018; Munnur et al., 2019; Groslambert et al., 2021). ADP-ribosylation can occur as mono- or poly(ADP-ribosyl)ation (MARylation or PARylation, respectively) and is a highly conserved and widespread posttranslational modification (PTM) that controls many cellular processes, including cell proliferation and differentiation, the cellular stress response, maintenance of genome stability, behavior, viral infection, and microbial metabolism (Perina et al., 2014; Wei and Yu, 2016; Cohen and Chang, 2018; Palazzo et al., 2019; Crawford et al., 2021; Mikolčević et al., 2021). Proteins participating in ADPr signaling are often described in terms of "writers," i.e., ARTs, "readers" that contain ADPr-binding domains, and "erasers" which modify or remove the ADP-ribosylation signal (Gupte et al., 2017).

One of the ART families, the diphtheria toxin-like ARTs (ARTDs), consists of seventeen members in humans, of which PARP1-3 are directly involved in the DNA damage response (DDR)(Schreiber et al., 2002; Boehler et al., 2011; Liu et al., 2017; Lüscher et al., 2021). The

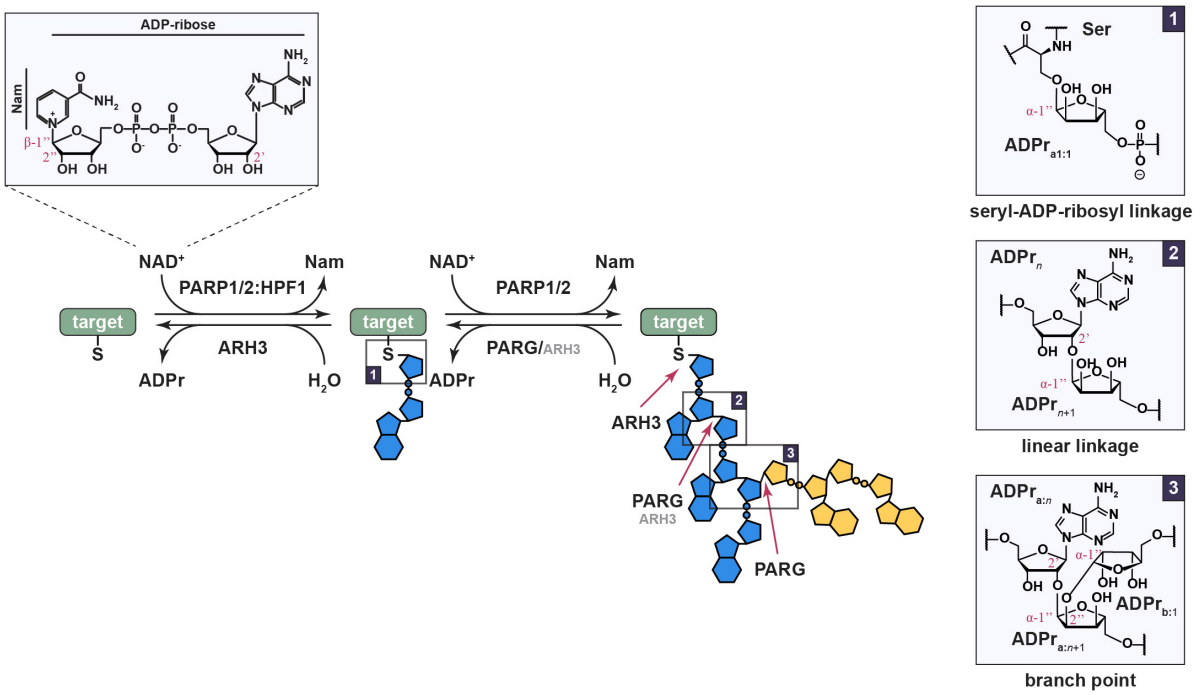

FIGURE 1 | Ser-ADPr is a reversible and complex modification. The reaction involves the transfer of the ADPr moiety from β-NAD$^+$ under inversion at the anomeric carbon, thus resulting in a modification in the α-conformation. The initial modification of a serine residue is catalyzed by the PARP1/2:HPF1 complex (box 1), while further chain extension is catalyzed by PARP1/2 alone. The latter occurs as linear, ribose(1″→2′)ribose (box 2), or infrequently branched, ribose(1″→2″)ribose(1″→2′)ribose (box 3), continuations leading to a large and diverse polymer structure. Linear polymers are primarily degraded by PARG, and to a lesser extend ARH3, while branch pruning, hydrolysis of the 1″→2″ bond, is carried out solely by PARG and precedes the cleavage of the 1″→2′ bond at branch points. In contrast, the proximal seryl-ADP-ribosyl bond can only be cleaved by ARH3. Identified target proteins in the context of the DDR include PARP1 and 2 themselves (automodification), histones (primarily H2B, H3, H4, and H1), FEN1, LIG3, and NUCKS1.

latter, also termed DNA repair PARPs, are specifically activated by binding to DNA lesions and subsequently ADP-ribosylate a variety of different targets within the vicinity of the damage site (Langelier et al., 2012; Eustermann et al., 2015; Pascal, 2018). Unlike most other PARPs, PARP1 and 2 can PARylate proteins by elongating pre-existing MARylation sites (**Figure 1**). (ADP-ribose)polymers come in varying lengths and morphologies, linear or branched, which was shown to have physiological effects including the alteration of gene expression, affecting PAR reader recruitment, and signal persistence (Hatakeyama et al., 1986; Aberle et al., 2020; Rack et al., 2021; Reber and Mangerich, 2021). Amongst the DNA repair PARPs, PARP1 is the earliest and most prolific DNA damage sensor with sub-second recruitment onset in laser micro-irradiations experiments (Haince et al., 2008) and is responsible for up to 90% of DNA damage-induced PAR in cells (D'Amours et al., 1999). Targets of the modification include PARP1 automodification as well as other chromatin and repair associated proteins, such as histones (Chapman et al., 2013; Zhang et al., 2013; Daniels et al., 2014; Pic et al., 2014; Teloni and Altmeyer, 2016; Bonfiglio et al., 2017; Palazzo et al., 2018). The locally generated ADP-ribosylation signal serves as a recruitment scaffold for a variety of PAR-binding factors and supports the assembly of the DNA repair machinery (Teloni and Altmeyer, 2016). Moreover, ADP-ribosylation has regulatory roles in the DDR, including facilitating chromatin reorganization and altering transcription (Wei and Yu, 2016; Polo et al., 2019). In comparison, PARP2, a close homolog of PARP1, is recruited to DNA lesions at a slower rate, potentially due to the absence of the N-terminal zinc finger motifs that facilitate PARP1 damage recognition, but persists longer than PARP1 (Perina et al., 2014; Liu et al., 2017; Chen et al., 2018). While both PARP1 and 2 can establish initial modification and elongate these into polymers, the differences in recruitment dynamics and signal production have been suggested to indicate that PARP1 and 2 play only partly overlapping roles in the establishment of the complex and context-specific "PAR code" (Mortusewicz et al., 2007; Liu et al., 2017; Chen et al., 2018). Indeed, PARP1-derived linear PAR, in addition to DNA damage, can activate PARP2 and stimulate the PARP2-dependent production of branched polymers, which are subsequently recognized by histone chaperone APLF and facilitate effective DNA repair (Chen et al., 2018). How this induction of branching is achieved, how it mechanistically differs from the normal, stochastic PARP1 and 2 branching background, whether the branch frequency of PARP1 can be altered, and whether establishment of specific branching patterns is possible remains, as yet, elusive.

Initially, PARP1-3 have been shown to modify glutamate/aspartate residues (Sharifi et al., 2013; Zhang et al., 2013; Daniels et al., 2015; Gibson et al., 2016). Lysine

residues have been also suggested, but many of the suggested sites turned out to be mis-assignments (Crawford et al., 2017). Recently, serine residues have been identified as the most abundant acceptor of ADP-ribosylation, especially in the context of DDR (Leidecker et al., 2016; Bonfiglio et al., 2017; Larsen et al., 2018; Palazzo et al., 2018). It was shown that PARP1 and 2 are required, but not sufficient, for serine-ADP-ribosylation (Ser-ADPr). Histone PARylation Factor 1 (HPF1) (Gibbs-Seymour et al., 2016) forms a non-obligate, transient complex with either PARP1 or 2 (PARP1/2), thus enabling modification of serine residues by extending the catalytic center. Moreover, formation of the complex increases the efficiency of the ADP-ribosylation reaction (**Figure 1**; Bonfiglio et al., 2017; Prokhorova et al., 2021b). Importantly, Ser-ADPr is specifically removed by a single enzyme, ARH3 (Fontana et al., 2017), in conjunction with PARG that acts on PAR chains (Lin et al., 1997; Slade et al., 2011).

This review focuses on Ser-ADPr as the most prominent protein ADP-ribosylation type of the DDR and explains the details of its synthesis and removal, influence on cellular outcomes of DNA damage and the therapeutic potential of targeting Ser-ADPr signaling.

HISTONE PARylation FACTOR 1 AS AN AUXILIARY FACTOR OF PARP1/2

HPF1 was initially linked to DNA repair PARPs due to the presence of a poly(ADPr)-binding zinc finger (PBZ) domain in the orthologs from insects and molluscs (Ahel et al., 2008). Later, it was shown that human HPF1 interacts specifically with PARP1 and 2, and promotes their efficient modification of histones (Gibbs-Seymour et al., 2016). The recruitment of HPF1 to DNA damage sites depends on direct physical interaction with PARP1 and does not require the prior presence of an ADP-ribosylation signal (Gibbs-Seymour et al., 2016; Suskiewicz et al., 2020; Prokhorova et al., 2021b). Loss of HPF1 greatly increases cellular sensitivity to treatment with DNA alkylating agents, such as methyl methanesulfonate (MMS) and sensitizes cells to PARP inhibition (Gibbs-Seymour et al., 2016). HPF1 was further shown to limit PARP1 hyper-automodification *in vivo* and *in vitro*, instead redirecting its catalytic activity toward histones and other substrates (Gibbs-Seymour et al., 2016). HPF1 not only boosts the ADP-ribosylation activity on histones and other targets (see below), but also is the determining factor in shifting PARP1-specificity from Glu/Asp residues to the generation of Ser-ADPr (Bonfiglio et al., 2017). Proteomic and cell-based analyses further confirmed that HPF1 is essential for the widespread Ser-ADPr following DNA damage with targets including histones, PARP1 and hundreds of other proteins (Bonfiglio et al., 2017; Hendriks et al., 2021).

The interaction of HPF1 with PARP1 is strengthened by DNA and NAD^+, providing a potential mechanism how HPF1, which is estimated to be twenty-times less abundant than PARP1 (Hein et al., 2015; Gibbs-Seymour et al., 2016), could be preferentially recruited to PARP1 molecules that become activated upon detecting DNA damage (Suskiewicz et al., 2020). PARP enzymes directly involved in DNA repair, PARP1-3, are defined by their helical subdomain (HD), an autoinhibitory domain that rapidly unfolds upon recognition of DNA damage, thereby exposing the NAD^+ binding site (Dawicki-McKenna et al., 2015). Deleting the HD enhances the HPF1:PARP1/2 interaction both *in vitro* and in cells (Suskiewicz et al., 2020), suggesting that this subdomain inhibits HPF1 binding and its DNA-induced unfolding could explain the enhancement of the interaction by DNA breaks.

Recently, the crystal and cryo-EM structures of HPF1 bound to the PARP2 catalytic domain were solved, providing first insights into the structural basis for the HPF1-mediated serine switch (Bilokapic et al., 2020; Suskiewicz et al., 2020). These data were confirmed by NMR and crystallographic analyses of the HPF1:PARP1 interaction (Suskiewicz et al., 2020; Sun et al., 2021). The HPF1:PARP1/2 interaction was found to critically depend upon a conserved aspartate residue (Asp283) in the C-terminal region of HPF1 that contacts His826 in PARP1 (His381 in PARP2) as well as the highly conserved leucine-tryptophan C-terminal residues of PARP1/2 that lock into a groove on HPF1 (Suskiewicz et al., 2020; Rudolph et al., 2021; Sun et al., 2021; Suskiewicz et al., 2021).

Structural and mutational analysis of the HPF1:PARP2 complex also revealed that the HPF1-mediated amino acid preference switch of PARP1/2 can be explained by the provision of a catalytic glutamate residue by HPF1 (Suskiewicz et al., 2020). PARP1 and PARP2 by themselves contain a single catalytic glutamate residue (Glu988 and Glu545, respectively), which was shown to be critical for PAR chain elongation (Marsischky et al., 1995), but this is not sufficient for Ser-ADPr (Bonfiglio et al., 2017). Interaction of HPF1 and PARP1/2 places Glu284 of HPF1 near the catalytic glutamate of PARP1/2 and the NAD^+ molecule, allowing the formation of a composite active site that is capable of catalyzing efficient Ser-ADPr (Suskiewicz et al., 2020). Glu284 of HPF1 could act as a general base in this reaction, abstracting a proton from the acceptor serine residue in a substrate (Suskiewicz et al., 2020) analogously to a conserved catalytic aspartate found in protein-serine/threonine/tyrosine kinases (Endicott et al., 2012). The deprotonation step is dispensable when the acceptor is a glutamate or aspartate residue, possibly explaining why ADP-ribosylation of acidic residues does not require HPF1. The HPF1:PARP1/2 complex contains a putative peptide-binding cleft with a strong negative charge provided by HPF1 (Suskiewicz et al., 2020), which was suggested to explain the abundance of Ser-ADPr within lysine-serine (KS) consensus motifs (Leidecker et al., 2016; Bonfiglio et al., 2017).

Interestingly, HPF1 also limits auto-PARylation of PARP1/2, leading to the formation of shorter polymers (Gibbs-Seymour et al., 2016; Suskiewicz et al., 2020). Asp283 of HPF1 was shown to occupy the negative-charge binding pocket, which during the PAR chain elongation reaction recognizes the pyrophosphate group of the acceptor ADPr unit (Suskiewicz et al., 2020). As a result, HPF1 binding to PARPs is mutually exclusive with PAR chain formation. This leads to the idea of distinct PAR chain initiation and elongation steps, catalyzed by HPF1:PARP1/2 or PARP1/2 alone, respectively. Indeed, MARylation of histones primed by the HPF1:PARP1/2

complex can be efficiently extended by PARP1 alone (**Figure 1**; Prokhorova et al., 2021a).

REVERSAL OF SERINE-ADP-RIBOSYLATION BY (ADP-RIBOSYL)HYDROLASE 3

The consumption of the metabolic cofactor NAD^+, associated with the formation of extensive linear and branched (ADPr)polymers following DNA damage, exerts a high energetic cost, and hence has to be tightly regulated. This cost is partly offset by the degradation of the polymer into free ADP-ribose by macrodomain- or ARH-type hydrolases and subsequent conversion into ATP by ADPr pyrophosphorylase, thus directly supporting ATP-dependent repair processes (Tanuma, 1989; Oei and Ziegler, 2000; Wright et al., 2016; Rack et al., 2020). In addition, ADPr can feed into nucleotide salvage pathways through the conversion into AMP by Nudix hydrolases (Dölle et al., 2013; Rack et al., 2016). Poly(ADP-ribosyl)glycohydrolase (PARG) is the dominant degrader of linear and branched chains, which hydrolzes the ribose-ribose bonds within PAR chains with high efficiency (**Figure 1**; Hatakeyama et al., 1986; Alvarez-Gonzalez and Jacobson, 1987; Braun et al., 1994; Rack et al., 2021). ARH3 can also degrade linear chains, albeit with a one-to-two orders of magnitude lower activity than PARG and is incapable of cleaving branched PAR (**Figure 1**; Oka et al., 2006; Drown et al., 2018; Rack et al., 2021). Consequently, PARG is the dominant force controlling PAR chain degradation in cells (Fontana et al., 2017); however, PARG activity is lowered on PAR chains shorter than four ADPr units (Hatakeyama et al., 1986; Barkauskaite et al., 2013). Moreover, PARG cannot hydrolyze the seryl-ADP-ribosyl bond (Slade et al., 2011; Fontana et al., 2017) and ARH3 is the only known human enzyme that can catalyze this reaction (**Figure 1**). This suggests that PAR signaling is a multi-step process not only on the level of synthesis (incl. initiation, elongation, and branching), but also on that of reversal (incl. cleavage, branch pruning, and termination). This complexity suggests that ADP-ribosylation signaling acts not only as a generic repair factor recruitment scaffold, but is utilized to fine-tune the DDR in a context specific manner. This is further highlighted for example by the diversity of PAR-substructure readers (Teloni and Altmeyer, 2016) or the influence of polymer composition on its stability (Rack et al., 2021). Furthermore, inactivation of both hydrolases is required to induce uncontrolled PAR accumulation with severely increased chain length and abundance (Prokhorova et al., 2021a).

Phylogenetically and mechanistically, PARG and ARH3 belong to distinct families of hydrolases, the macrodomains and (ADP-ribosyl)hydrolases, respectively (Rack et al., 2020). ARH3 is a compact, mainly α-helical orthogonal bundle with a catalytic binuclear Mg^{2+} center situated within the ligand-binding cleft (Mueller-Dieckmann et al., 2006; Pourfarjam et al., 2018; Rack et al., 2018; Wang et al., 2018). Substrate binding was proposed to be gated by a conformationally flexible region, termed Glu41-flap due to the presence of the catalytic Glu41 residue (Pourfarjam et al., 2018). In the auto-inhibitory closed state, Glu41 interacts with Mg_{II}, thus locking the active site and sequestering the catalytic residue (Pourfarjam et al., 2018; Rack et al., 2018; Wang et al., 2018). It was recently shown that substrate binding not only displaces Glu41 from Mg_{II} leading to the opening of the Glu41-flap, but actually positions Glu41 in close proximity to Mg_I, where it contributes to activation of a water molecule for the nucleophilic attack on the scissile bond, which initiates the catalytic cycle (Rack et al., 2021). Moreover, substrate binding induces changes in the coordination of Mg_{II}, which adopts a higher-energy square-pyramidal geometry, thus contributing to substrate activation (Rack et al., 2021). In contrast, the PARG structure is composed of a three-layer α/β/α sandwich with a substrate binding groove along the crest of the domain (Slade et al., 2011; Dunstan et al., 2012; Tucker et al., 2012). The catalytic mechanism involves the induction of a strained substrate binding conformation as well as substrate activation by a catalytic glutamate dyad (Patel et al., 2005; Slade et al., 2011; Lambrecht et al., 2015).

Deficiency of PARG and ARH3 leads to sensitivity to DNA damage (Cortes et al., 2004; Mashimo et al., 2013; Shirai et al., 2013). PARG was found to be an essential gene, with deletion leading to embryonic lethality in both mice and flies (Hanai et al., 2004; Koh et al., 2004). Continued culture at 29°C upon pupation allowed a minority (<25%) of flies to survive into adulthood, although these flies showed a progressive neurodegenerative phenotype linked to PAR accumulation in neurons (Hanai et al., 2004). In mice, knock-out of $PARG_{110}$, the longest and primary nuclear isoform, induces a hypersensitivity to exogenous DNA damage (Cortes et al., 2004).

Loss of cellular ARH3 activity, recently described in patients with the autosomal recessive disorder stress-induced childhood-onset neurodegeneration with variable ataxia and seizures (CONDSIAS), was linked with episodic infection-/stress-associated neurological deterioration resulting in impaired or declining cognitive development, physical impairments including muscle weakness, seizures and gait ataxia, and in several cases childhood lethality (Danhauser et al., 2018; Ghosh et al., 2018). ARH3 localizes to the cytoplasm, nucleus, and mitochondria (Oka et al., 2006; Niere et al., 2008), but it has been suggested that its nuclear function is critical to prevent neurodegeneration (Beijer et al., 2021). While the precise molecular causes are not fully understood, accumulation of both chromatin-linked and free PAR was observed (Danhauser et al., 2018; Ghosh et al., 2018; Mashimo et al., 2019) and both processes are linked to aberrant cellular functions. First, cytoplasmic ARH3 protects cells from oxidative-stress induced cell death (parthanatos) by preventing PAR-induced AIF release from the mitochondria (Mashimo et al., 2013). ARH3 thus counteracts PARG by degrading PARG-generated free PAR chains induced by severe oxidative DNA damage (Mashimo et al., 2013), providing a potential therapeutic target not only for CONDSIAS patients, but also other forms of parthanatos-induced cell death, for instance in ischemic brain injury and other neurodegenerative illnesses (Mashimo et al., 2013, 2019). Second, histone ADP-ribosylation was shown to affect other modifications, including acetylation and phosphorylation, and to influence the local histone code (Bartlett et al., 2018;

Palazzo et al., 2018; Hanzlikova et al., 2020). Recent cell biological data further suggest that persistent chromatin serine ADP-ribosylation can lead to dysregulated transcription and abnormal telomere structure (Prokhorova et al., 2021a).

DISCUSSION

While the discovery of Ser-ADPr has greatly expanded the research in the DNA-damage dependent ADP-ribosylation signaling field, our understanding of the exact role of this PTM is still in its infancy. One emerging role of Ser-ADPr is the control of the chromatin state, which is supported by initial findings of cross-talk between histone Ser-ADPr and other canonical histone marks (Bartlett et al., 2018; Prokhorova et al., 2021a). One example stems from histone H3, where neighboring Ser-ADPr and acetylation marks were found to be mutually exclusive (Bartlett et al., 2018; Liszczak et al., 2018). In addition, HPF1 was recently also implicated in regulation of replication. HPF1-directed PARP1 activity was shown to be required for recruitment of XRCC1/DNA ligase 3 complexes, which provide a back-up mechanism for Okazaki fragment ligation, and thus promoting repair of replication-associated DNA damage (Kumamoto et al., 2021). HPF1 also cooperates with the methyltransferase CARM1 to stimulate PARP1 activity and thereby promotes slowing down of replication fork progression (Genois et al., 2021).

So far, the only consequence of site-specific Ser-ADPr that is understood is the effect of the PARP inhibitor response through PARP1 automodification (Prokhorova et al., 2021b). Mutation of PARP1 Ser499, Ser507 and Ser519, or loss of HPF1, leads to greater sensitivity to PARP inhibitors by resulting in increased PARP trapping on chromatin (Prokhorova et al., 2021b). As such, HPF1 loss could be considered a potential biomarker for cancer therapy.

Similarly, ARH3 also emerges as a potential cancer biomarker and drug target, partially due to being the "opposing force" to HPF1. Specifically, either HPF1 deficiency or ARH3 overexpression led to PARP inhibitor sensitivity (Prokhorova et al., 2021b). In line with this, ARH3-deficient cells are sensitive to PARG inhibitors and resistant to PARP inhibitors (Prokhorova et al., 2021b). ARH3 deficiency is therefore a potential novel PARP1 inhibitor resistance mechanism, similar to what has been described for loss of PARG, which causes PARP inhibitor resistance in cancer cells due to stabilization of the PARylation signal (Gogola et al., 2018). Moreover, pharmacological inhibition of ARH3 appears to negatively impact DNA damage repair (Liu et al., 2020). With several lines of evidence pointing at a protective role of ARH3 against neurodegeneration there exists a further pathway to therapeutic application of ARH3 antagonists that can be explored in the future (Danhauser et al., 2018; Ghosh et al., 2018; Mashimo et al., 2019). Deepening our understanding of the opposing forces of HPF1 and ARH3 in the making and breaking of Ser-ADPr will certainly aid our progress in many therapeutically relevant avenues in the future.

AUTHOR CONTRIBUTIONS

KS, JGMR, and IA wrote the review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Roles for the 8-Oxoguanine DNA Repair System in Protecting Telomeres From Oxidative Stress

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Telomeres are protective nucleoprotein structures that cap linear chromosome ends and safeguard genome stability. Progressive telomere shortening at each somatic cell division eventually leads to critically short and dysfunctional telomeres, which can contribute to either cellular senescence and aging, or tumorigenesis. Human reproductive cells, some stem cells, and most cancer cells, express the enzyme telomerase to restore telomeric DNA. Numerous studies have shown that oxidative stress caused by excess reactive oxygen species is associated with accelerated telomere shortening and dysfunction. Telomeric repeat sequences are remarkably susceptible to oxidative damage and are preferred sites for the production of the mutagenic base lesion 8-oxoguanine, which can alter telomere length homeostasis and integrity. Therefore, knowledge of the repair pathways involved in the processing of 8-oxoguanine at telomeres is important for advancing understanding of the pathogenesis of degenerative diseases and cancer associated with telomere instability. The highly conserved guanine oxidation (GO) system involves three specialized enzymes that initiate distinct pathways to specifically mitigate the adverse effects of 8-oxoguanine. Here we introduce the GO system and review the studies focused on investigating how telomeric 8-oxoguanine processing affects telomere integrity and overall genome stability. We also discuss newly developed technologies that target oxidative damage selectively to telomeres to investigate roles for the GO system in telomere stability.

Keywords: Telomeres, oxidative stress, 8-oxoguanine, Base excision repair, Telomerase

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INTRODUCTION: TELOMERES ON THE GO

Telomere caps at the ends of linear chromosomes are nucleoprotein-DNA structures essential for genome stability, sustained cellular proliferation, and the overall health of an organism. Telomeres lie at the interface between aging and cancer because dysfunctional telomeres contribute to degenerative diseases that occur with aging, but also cause genetic alterations that drive carcinogenesis [reviewed in (Chakravarti et al., 2021)]. To prevent aging-related diseases and cancer, telomeres solve two problems that chromosome ends present 1) the end replication and 2) end protection. First, telomeres shorten progressively with each round of DNA replication and cell division due to the inability of replicative DNA polymerases to completely copy chromosome ends. Telomeres solve this end replication problem by recruiting a specialized reverse transcriptase called telomerase, which synthesizes telomeric DNA to restore the DNA that is lost each time the cell divides (Greider and Blackburn, 1985). However, while telomerase activity is sufficient in germ cells, some stem cells, and

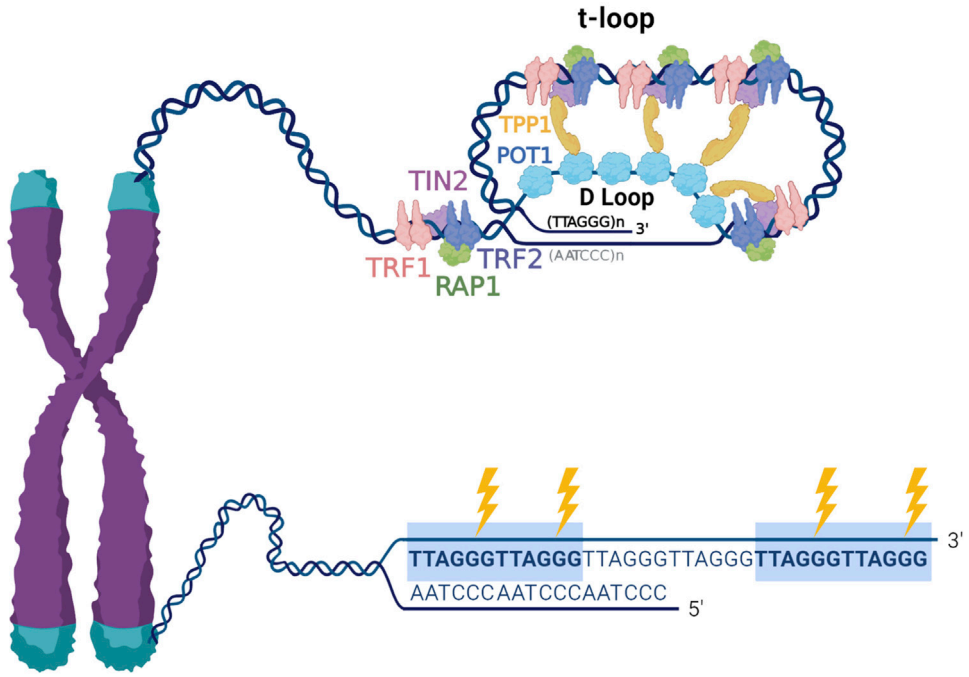

FIGURE 1 | Telomere structure and sensitivity to oxidative damage. Telomeres are nucleoprotein structures composed of repetitive TTAGGG sequence and associated telomere-specific proteins, named shelterin. Telomeric DNA terminates in a 3′ single stranded overhang, which invades the double stranded telomeric DNA to form a lariat-like t-loop. The formation of the t-loop is mediated by the protective shelterin complex, which consists of TRF1, TRF2, RAP1, TIN2, TPP1 and POT1. The highly repetitive G-rich telomeric repeats are preferred sites for production 8-oxoG (indicated by the lightning bolts), therefore, telomeric DNA is remarkably susceptible to oxidative stress.

most cancer cells, it is insufficient or lacking in most human somatic cells, which experience telomere shortening with age (Harley et al., 1990; Bodnar et al., 1998; Opresko and Shay, 2017). When telomeres become critically short they cannot perform their end protection role. Functional telomeres prevent chromosome ends from being inappropriately recognized and processed by the DNA damage response (DDR) and double strand break (DSB) machineries, through the engagement of a 6-member protein complex termed shelterin (D'adda Di Fagagna et al., 2003; De Lange, 2018). DDR activation at dysfunctional, unprotected telomeres can trigger irreversible growth arrest (senescence) or cell death. Cells that bypass senescence experience chromosome end-to-end fusions and genomic instability, and enter crisis which kills most of the cells. However, the survivors that emerge either upregulate telomerase or activate a recombination-based method of telomere maintenance termed alternative lengthening of telomeres (ALT) [reviewed in (Opresko and Shay, 2017; Hoang and O'Sullivan, 2020; Chakravarti et al., 2021)].

Mammalian telomeres consist of long (tens of kilobases) arrays of tandem 5′-TTAGGG-3′ repeats on one strand and 5′-CCCTAA-3 on the complementary strand. Telomeres terminate in a 3′ single stranded overhang comprising about 50–200 nucleotides of TTAGGG repeats, that can invade the telomere duplex DNA to form a large lasso-like t-loop (Griffith et al., 1999). When the overhang pairs with the duplex it displaces a portion of the G-rich strand and forms a D-loop, and thus, single stranded TTAGGG repeats are present at the telomeres regardless of conformation. This is significant because the G-rich sequences can form stable four stranded structures termed G-quadruplexes (G4s) (Hwang et al., 2014). Shelterin mediates t-loop formation, and while this structure functions in telomere protection, evidence suggests t-loop structures are dynamic (Doksani et al., 2013; Markiewicz-Potoczny et al., 2021; Ruis et al., 2021). The shelterin complex engages telomeric DNA through proteins TRF1 and TRF2 binding to duplex TTAGGG repeats, and POT1 binding to single stranded 5′-TTAGGGTTAG-3′ sequences. These proteins modulate telomere function by recruiting the other members TPP1, RAP1 and TIN2 (De Lange, 2018) (**Figure 1**). The presence of repetitive G-rich sequence, single stranded DNA, and shelterin proteins, makes the telomeres a unique context for the processing of DNA damage. However, these features combined with the fact that telomeres represent less than 0.02% of the genome, also make them challenging to study, requiring and fueling innovative approaches for examining DNA damage and repair.

Nearly 2 decades of work have revealed that telomeres are particularly sensitive to DNA damage caused by oxidative stress [reviewed in (Barnes et al., 2019)]. Cells in tissues and organs are continuously exposed to endogenous and exogenous factors that lead to the generation of reactive oxygen species (ROS). Primary sources of endogenous ROS include mitochondrial respiration, inflammatory responses and by-products of cellular signaling, while environmental pollution, ionizing radiation, ultraviolet

light, cigarette smoking, certain foods and drugs are the major exogenous sources of ROS (reviewed in (Nakamura and Takada, 2021)). Low physiological levels of ROS play critical roles in cellular signaling (Sies and Jones, 2020). However, oxidative stress is caused by an imbalance between excess ROS production and deficiencies in the antioxidant defenses that regulate and detoxify ROS. Oxidative DNA damage caused by ROS can promote mutagenesis and carcinogenesis, as well as senescence and degenerative diseases associated with aging (Kregel and Zhang, 2007; Kryston et al., 2011). One of the most common oxidative DNA base modifications is 8-Oxo-7,8-dihydroguanine (8-oxoG), which arises in the genome at an estimated 2,800 lesions per cell per day in unstressed cells (Tubbs and Nussenzweig, 2017). This relatively high prevalence is partly due to the low redox potential of guanine, making it highly susceptible to oxidation (Kino et al., 2017). Telomeric TTAGGG repeats are preferred sites for 8-oxoG formation (Oikawa et al., 2001) (**Figure 1**), and numerous studies have shown that telomeres are highly sensitive to oxidative stress arising from both endogenous and environmental sources [reviewed in (Barnes et al., 2019)]. Data ranging from human population studies, to model organisms and cultured cells reveal a general association of oxidative stress and accelerated telomere shortening and dysfunction (Zhang et al., 2016; Graham and Meeker, 2017; Reichert and Stier, 2017; Ahmed and Lingner, 2018a). A previous model suggested this is due to unrepaired oxidative base damage, or repair intermediates, interfering with replication fork progression at telomeres (Von Zglinicki, 2000; Von Zglinicki, 2002; Wang et al., 2010). Previous work showed that 8-oxoG lesions and abasic repair intermediates within telomeric DNA disrupt TRF1 and TRF2 binding *in vitro* (Opresko et al., 2005). Collectively, these studies suggest that telomeric oxidative damage greatly impacts telomere length homeostasis and integrity, and underscores the need to better understand the role of 8-oxoG processing and repair in telomere maintenance. In this review we will focus on the known mechanisms for managing 8-oxoG damage arising within the genome, collectively termed the "guanine oxidation" (GO) system. We will discuss recent advances in elucidating the function of the GO system at telomeres, along with the development of new tools for investigating the consequences of telomeric 8-oxoG damage on telomere integrity, overall genome stability, and cellular health.

FROM BACTERIA TO HUMANS: THE GO SYSTEM AND BER ARE EVOLUTIONARILY CONSERVED

Oxidative stress resulting from excess cellular ROS represents one of the most common and significant threats to DNA integrity and genome stability, therefore, multiple systems have evolved to counteract the harmful consequences of oxidative base damage. Generally, the repair of small and often non-helix-distorting DNA base lesions, such as 8-oxoG, is carried out by the base excision repair (BER) pathway, which utilizes several highly conserved proteins involved in the essential steps of damage recognition and DNA restoration. First, a specific DNA glycosylase recognizes and excises the damaged DNA base through the cleavage of the N-glycosydic bond. DNA glycosylases are classified as mono- or bifunctional according to the enzymes' ability to both excise the modified base by hydrolysis and then cleave the DNA backbone at the resulting apurinic/apyrimidinic (abasic/AP) product. For monofunctional DNA glycosylases, the AP site is further processed by an AP endonuclease, which incises the sugar phosphate backbone 5′ of the lesion leaving behind a nucleotide gap with 3′-hydroxyl and 5′-terminal abasic deoxyribose phosphate (5′-dRP) residues. Lyase activity removes the 5′-dRP, DNA polymerase fills the gap, and then DNA ligase seals the nick to restore the DNA backbone [for extensive review see (Wallace, 2014; Beard et al., 2019; Caldecott, 2020)]. In contrast, bifunctional DNA glycosylases remove the damaged base and then cleave the sugar-phosphate backbone 3′ of the AP site. Endonuclease activity removes the 3′ unsaturated hydroxyaldehyde (3′dRP), enabling gap filling and repair completion. The processing after DNA glycosylase activity is considered "short-patch" (SP) BER if a single nucleotide gap is canonically generated, filled and ligated, or "long-patch" (LP) BER if the generated gap is 2–10 nucleotides and further processed by additional enzymes [reviewed in (Fortini and Dogliotti, 2007; Wallace et al., 2012)].

The proteins specifically involved in the removal of 8-oxoG constitute the GO system, a term first used to describe the DNA repair enzymes that prevent mutagenesis caused by 8-oxoG in bacteria (mutT, mutM and mutY) (Michaels et al., 1992; Michaels and Miller, 1992). In brief, mutT sanitizes the nucleotide pool by hydrolyzing 8-oxo-dGTP to 8-oxo-dGMP (Ito et al., 2005). If 8-oxodGTP escapes removal it can be inserted into nascent DNA by a polymerase during DNA replication or repair. 8-oxoG can also arise in the genome when guanine is directly oxidized. Formamidopyrimidine DNA Glycosylase (Fpg or mutM) recognizes and excises 8-oxoG base paired with cytosine, initiating BER (Jiricny, 2010). If 8-oxoG remains unrepaired in the template DNA strand, a round of replication can lead to adenine insertion opposite 8-oxoG. This happens because 8-oxoG preferentially adopts a *syn* conformation in the DNA due to steric repulsion between the deoxyribose and the O8 of the modified G, allowing 8-oxoG to stably pair with adenine [reviewed in (Beard et al., 2010)]. Hence, as the ultimate protection from mutagenesis, mutY removes the adenine mispaired opposite 8-oxoG to initiate BER (Whitaker et al., 2017). Several studies have identified proteins involved in a functional equivalent of the GO system in human cells, which includes Nudix hydrolase (NUDT1, also known as MutT human homolog 1, or MTH1), 8-oxoG glycosylase (OGG1) and the adenine glycosylase MutY homolog (MUTYH) (**Figure 2**) (reviewed in (Banda et al., 2017)). In this section we explore the repair mechanisms and activities of these enzymes, with special focus on their known roles at telomeres.

OGG1 Function at 8-oxoG:C Base Pairs in Telomeres

Since the discovery of yeast OGG1 and the subsequent identification of the mammalian orthologue, a plethora of

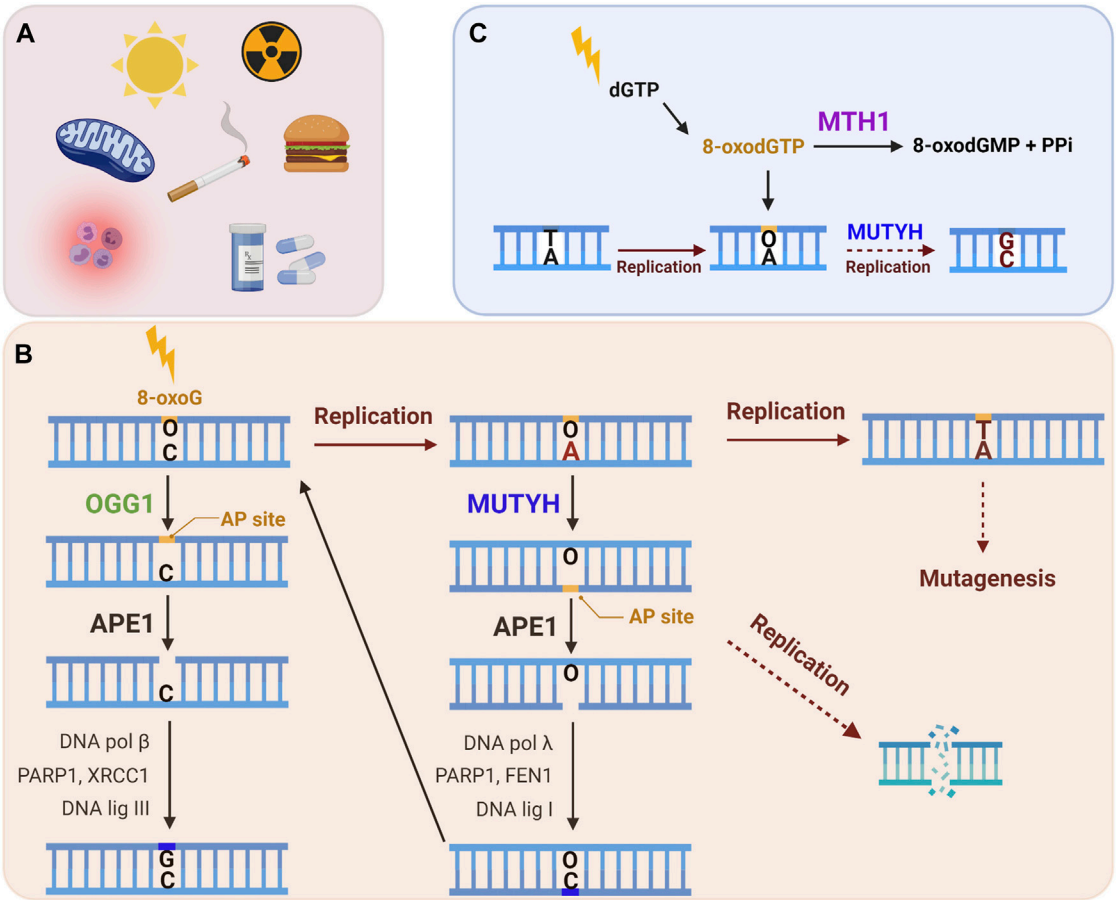

FIGURE 2 | The human GO repair system. **(A)** 8-oxoG lesion is among the most common forms of oxidative DNA damage, which can arise following exposure to endogenous and/or exogenous ROS. **(B)** An 8-oxoG:C base pair is recognized and excised by the OGG1 glycosylase, producing an apurinic (AP) site, which is cleaved by APE1, and then processed by downstream BER to restore the correct G:C base pair. If 8-oxoG escapes repair it can miscode for adenine upon DNA replication. MUTYH glycosylase recognizes a 8-oxoG:A mispair and excises the undamaged adenine, thereby initiating long-patch BER to restore the 8-oxoG:C base pair. This allows OGG1 another chance to excise 8-oxoG and to initiate BER to restore the G:C base pair. If the 8-oxoG:A mispair is not repaired, a further round of replication converts the damage to a G:C to T:A transversion mutation. Lesion processing by BER generates repair intermediates, including AP sites and SSBs, which can cause replication fork collapse and subsequent DSBs. **(C)** MTH1 sanitase provides further protection against 8-oxoG mutagenesis through removing 8-oxodGTP from the nucleotide pool by hydrolyzing it to 8-oxodGMP and pyrophosphate. This prevents misincorporation of 8-oxodGTP opposite a template adenine during DNA replication or repair. The mismatch repair (MMR) enzymes (not shown) can also eliminate 8-oxoG from newly synthesized DNA that has been misinserted opposite adenine. (Black arrows: canonical repair steps. Brown arrows: Rounds of replication. Dashed arrows: mutagenesis or DNA damage generating steps).

studies have elucidated this enzyme's structural features, mechanism of action and repair activity. OGG1 is a bifunctional glycosylase, able to hydrolyze the N-glycosydic bond of 8-oxoG (DNA glycosylase activity) and cleave the DNA backbone through a β-elimination step (β-lyase activity) *in vitro* (Svilar et al., 2011). The glycosylase first searches for, and finds, the target lesion among a myriad of undamaged bases, through a combination of rotational diffusion along the DNA via consistent contact (sliding), and rapid dissociations and rebinding to the DNA (hopping) (Blainey et al., 2006). Once the enzyme selectively recognizes 8-oxoG opposite cytosine, the damaged base is flipped out from the DNA double helix into the OGG1 active site and excised. However, OGG1 lyase activity is very weak and OGG1 remains bound to the abasic site upon 8-oxoG excision, resulting in product inhibition. AP endonuclease-1 (APE1) enhances OGG1 turnover, preventing its reassociation with the AP site (Hill et al., 2001). APE1 cleaves the phosphodiester backbone 3′ of the abasic site, and then DNA polymerase (pol) β removes the 5′dRP with its lyase activity and fills the gap with its DNA synthesis activity. DNA ligase III (LIG3) seals the nick, facilitated by scaffold protein X-ray repair cross complementing 1 (XRCC1) (for more comprehensive review see (Boiteux et al., 2017; Ba and Boldogh, 2018; D'Augustin et al., 2020)). While not essential for BER *in vitro*, Poly(ADP-ribose) polymerase-1 (PARP1) binds the single strand break (SSB) repair intermediates generated by APE1 and activates poly(ADP-ribose) (PAR) synthesis to recruit downstream proteins (Schreiber et al., 2006). A recent study showed XRCC1, which interacts with and stabilizes the Pol β and

LIG3, prevents excessive PARP1 engagement and activity at the SSB intermediate, enhancing access and repair by the downstream BER enzymes (Demin et al., 2021).

The predominance of guanines in the telomeric sequence and their high susceptibility to oxidative modification, have stimulated a longstanding interest in uncovering the importance and activity of OGG1 at telomeres. *In vitro* studies demonstrated the ability of OGG1 to remove 8-oxoG in the context of telomeric sequences. OGG1-excision assays performed on 8-oxoG containing double-stranded oligonucleotides with telomeric or non-telomeric repeats, revealed that OGG1 excision activity is not impacted by the number of 8-oxodG within GGG runs. However, OGG1 excision is affected by the position of 8-oxoG in different telomere configurations (e.g., fork, 3′-overhang, and D-loop). For example, OGG1 excises less efficiently an 8-oxoG placed at the 3′ terminal end of the invading strand of a telomeric D-loop (Rhee et al., 2011). Studies in *S. Cerevisiae* provided the first direct evidence for OGG1 processing of telomeric 8oxo-G damage in telomere length regulation *in vivo*, by showing that OGG1 deficiency leads to telomere lengthening in yeast under non-stressed conditions (Askree et al., 2004; Lu and Liu, 2010). Subsequent work in transgenic mice confirmed that OGG1 depletion caused telomere lengthening *in vivo*, and in primary mouse embryonic fibroblasts (MEFs) cultured under low oxygen tension. However, this study also reported the novel discovery that loss of OGG1 increased telomeric 8oxo-G in primary MEFs under high oxidative stress conditions, and increased telomere attrition and aberrations (Wang et al., 2010). These findings provide evidence that OGG1 is involved in the repair of oxidative guanine lesions in telomeres *in vivo*, and that low basal telomeric 8-oxoG levels are associated with telomere lengthening in unstressed mice. This may be due to the ability of 8-oxoG to disrupt blocking G4 structures (see *8-oxoG Formation and Repair in the Context of Telomeric G-Quadruplex Structures: Beneficial or Detrimental for Telomere Stability?*). However, too much 8-oxoG arising from oxidative stress is clearly detrimental, and causes telomere shortening and aberrations in repair-deficient cultured cells. Whether unrepaired telomeric 8-oxoG cause similar defects *in vivo* in Ogg1 deficient mice experiencing oxidative stress remains unknown.

Previous studies examining the role of 8-oxoG repair at the telomeres in genome stability and cellular or organism health, suffered from the limitation that oxidants used to produce oxidative stress and 8-oxoG, also damage numerous cellular components and produce a myriad of oxidative DNA lesions. The KillerRed-TRF1 system (KR-TRF1) was one approach developed to investigate whether oxidative stress-induced damage at telomeres could directly and singularly induce telomere shortening and dysfunction. KR is a fluorescent protein which generates superoxide upon excitation with visible light illumination (550–580 nm). Expression of a KR fusion protein with shelterin TRF1 enables localized superoxide production at telomeres upon cellular light exposure. This system provided evidence that oxidative telomeric damage induces telomere shortening and related chromosomal aberrations, such as chromatid telomere loss and telomere associations (Sun et al., 2015). However, superoxide production is not selective for 8-oxoG, as evidenced by KR-TRF1 induction of SSBs and double strand breaks (DSBs) at telomeres, making it difficult to determine the specific consequences of 8-oxoG formation and repair. We overcame this technical hurdle by developing a novel targeting tool that specifically generates 8-oxoG at telomeres. In brief, this system expresses a fusion protein of fluorogen-activating peptide (FAP) and TRF1. The FAP binds with high affinity to the photosensitizer dye di-iodinated malachite green (MG2I) that, when bound and excited by 660 nm light, produces singlet oxygen ($^1O_2$), which reacts specifically with guanine to generate 8-oxoG (**Figure 3**) (Sies and Menck, 1992; Fouquerel et al., 2019). We estimated a production of at least one 8-oxoG per 28-kb telomere in HeLa LT cells after treatment with dye and light for 5 min (acute exposure). Exploiting this spatially and temporally controlled tool, we showed that $^1O_2$ production at the telomeres stimulates OGG1 recruitment, but not the NEIL1 glycosylase which instead processes oxidized pyrimidines and hydantoin lesions. OGG1 was followed by downstream BER factors, as shown by PARP1 activation and XRCC1 recruitment. While acute telomeric 8-oxoG formation did not cause telomere dysfunction in cancer cells, repeated lesion production over a month decreased cell growth, and caused telomere shortening and losses, chromosome fusions and genomic instability, all of which were greatly exacerbated by OGG1 deficiency (Fouquerel et al., 2019). A recent study impaired BER with OGG1 inhibitor TH5487 in cancer cells under oxidative stress, and showed reduced XRCC1 recruitment and increased 8-oxoG levels in telomeric DNA. This study found pharmacological OGG1 inhibition recapitulated the increased telomere loss observed in OGG1 deficient cells challenged with targeted telomeric 8-oxoG formation using FAP-TRF1 (Fouquerel et al., 2019; Baquero et al., 2021). Both direct 8-oxoG production at telomeres in OGG1 deficient cells, and pharmacological OGG1 inhibition, provide evidence that unrepaired 8-oxoG causes telomere dysfunction by inducing replication stress. On the other hand, another recent study reported evidence that OGG1 processing of lesions induced by H_2O_2 leads to SSBs at telomeres. Depleting OGG1 in cells deficient for the antioxidant enzyme Peroxiredoxin 1 (PRDX1) attenuated the formation of SSBs, suggesting OGG1 may generate repair intermediates at telomeres that could be detrimental (Ahmed and Lingner, 2020).

Further study about OGG1 roles at telomeres in the context of chromatin revealed a surprising role for the UV-damaged DNA-binding (UV-DDB) protein complex in 8-oxoG repair. UV-DDB is well known for recognizing UV photoproducts and initiating the global genome nucleotide excision repair pathway. The discovery that UV-DDB binds to 8-oxoG lesions and abasic sites, led to the novel finding that UV-DDB enhances OGG1-mediated excision of 8-oxoG, facilitating OGG1 enzymatic turnover by displacing it from the abasic site *in vitro*. Use of the FAP-TRF1 tool showed that UV-DDB colocalizes with OGG1 at telomeric 8-oxoG lesions, but precedes OGG1 (Jang et al., 2019). These data suggest that UV-DDB serves as a BER sensor and makes the damage site available to OGG1, most likely by

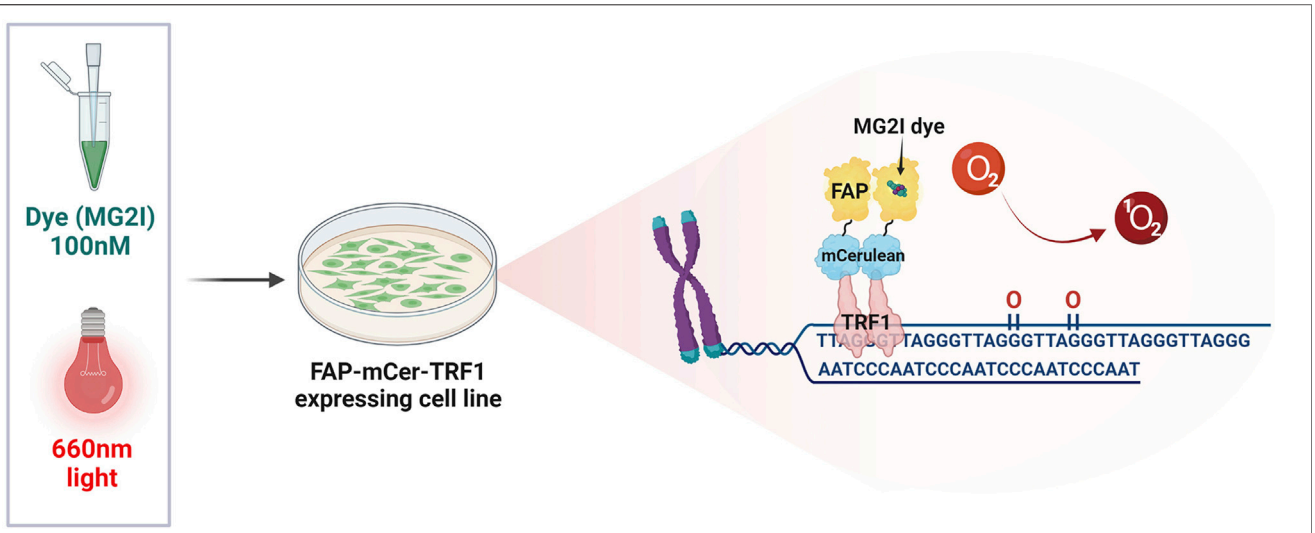

FIGURE 3 | The FAP-mCer-TRF1 system. The targeting tool that specifically generates 8-oxoG at telomeres, consists of an overexpressed shelterin protein TRF1 fused with the fluorogen-activating peptide (FAP), along with the fluorescent protein m-Cerulean (mCer) to visualize expression. FAP-mCer-TRF1 expressing cells are preincubated with 100 nM of the photosensitizer dye di-iodinated malachite green (MG2I), which when bound to the FAP, produces singlet oxygen ($^1O_2$) upon excitation with 660 nm light. The singlet oxygen then reacts specifically with the telomeric guanines to generate 8-oxoG in a temporally and spatially controlled manner.

opening chromatin, and enhances OGG1 turnover allowing further downstream BER reactions.

MUTYH Function at 8-oxoG:A Mispairs in Telomeres

The human monofunctional DNA glycosylase homologue of *E. Coli* mutY is encoded by the MUTYH gene (Slupska et al., 1996), and has the unique ability to recognize and excise an undamaged adenine positioned opposite 8-oxoG, rather than removing the damaged base. 8-oxoG may occur in the template strand during DNA replication if it escapes removal by OGG1 or arises during S-phase. 8-oxoG has dual coding properties and can form the correct 8-oxoG(anti):C(anti) by canonical Watson-Crick-Rosalind base pairing, or the incorrect 8-oxoG(syn): adenine(anti) by Hoogsteen base pairing. Most DNA polymerases can insert C and/or A opposite 8-oxoG, but preferentially extend from the misinserted base pair (Maga et al., 2007; Beard et al., 2010; Katafuchi and Nohmi, 2010). The potential for 8-oxoG to cause a mutation varies among polymerases and depends on the ability of the polymerase active site to accommodate the altered correct or incorrect base pair with 8-oxoG for extension (Rechkoblit et al., 2021). With its adenine glycosylase activity, MUTYH counteracts the mutagenic properties of 8-oxoG and prevents C:G to T:A transversion mutations. Following adenine excision and AP site formation, MUTYH interaction with key factors in replication-associated LP-BER recreates an 8-oxoG:C base pair, offering OGG1 another chance to restore the undamaged DNA. APE1 stimulates MUTYH glycosylase activity and turnover, and then cleaves the DNA backbone at the AP site (Yang et al., 2001). MUTYH is recruited to oxidative damage with downstream proteins involved in LP-BER including replication protein A (RPA), PCNA, and DNA polymerase λ (pol λ), which promotes gap filling with a cytosine (Parker et al., 2001; Yang et al., 2001; Maga et al., 2007; Van Loon and Hubscher, 2009). Biochemical reconstitution studies show that pol λ incorporates 2 nt at the gap, causing strand displacement that is processed by flap endonuclease 1 (FEN1), followed by DNA ligase I sealing the nick (Van Loon and Hubscher, 2009). However, MUTYH can also initiate SP-BER, independently of the cell cycle status, for example under high oxidative stress. This is due to the reinsertion of an adenine opposite an 8-oxoG during futile BER cycles by DNA polymerases including pol β and pol κ (Hashimoto et al., 2004; Vasquez-Del Carpio et al., 2009; Beard et al., 2010). Such futile BER cycles can lead to SSB accumulation due to repeated incision of the AP sites generated by MUTYH, causing PARP1 activation, prolonged accumulation of poly(ADP-ribose) polymers, depletion of nicotinamide adenine dinucleotide (NAD) and ATP, finally triggering apoptotic cell death (Oka et al., 2008). These studies have led to the hypothesis that loss of MUTYH function may contribute to malignant transformation by sustained cell death evasion under oxidative stress (Sakamoto et al., 2007). The discovery that biallelic germline mutations in the MUTYH gene cause the colorectal predisposition disorder named MUTYH-associated polyposis (Al-Tassan et al., 2002), confirmed its roles in cancer prevention, and revealed domains indispensable for its repair activity. MUTYH's primary function in suppressing tumorigenesis is likely by preventing somatic mutations in proto oncogenes or tumor suppressor genes, which would otherwise develop as a consequence of oxidative DNA damage. Noteworthy, two of the most common MUTYH mutations in humans, Y165C and G382D, are located in the adenine glycosylase active site and in the 8-oxoG recognition domain, respectively, underscoring the importance of MUTYH recognition of adenine in the 8-oxoG

mispair [for extensive review see (Banda et al., 2017) and (Markkanen et al., 2013)]. This first step in lesion discrimination was confirmed with recent single molecule fluorescence microscopy studies, which showed that while MUTYH binds to both 8-oxoG:A and 8-oxoG:C, its interaction with the correct base pair, which it cannot cleave, is shorter-lived (Nelson et al., 2019). However, this raises the possibility that in a context of high oxidative stress, MUTYH may interact at multiple sites of oxidative lesions, without necessarily initiating the repair. This may have harmful consequences if non-productive binding hinders replication fork progression or transcription. Currently, information regarding a direct role for MUTYH activity in modulating telomere homeostasis and integrity remains very limited. Studies in fission yeast *Schizosaccharomyces pombe* provided the first evidence for enrichment of Myh1 at telomeres following oxidative stress (Chang et al., 2011). Later, the histone deacetylase SIRT6 was found to interact with and stimulate the activities of human MUTYH and APE1, and to interact with the DNA-damage-checkpoint complex Rad9/Rad1/Hus1 (9-1-1) *in vitro*. Consistent with the known association of human SIRT6, APE1, and 9-1-1 with telomeres and their roles in preserving telomere stability (Francia et al., 2006; Michishita et al., 2008; Madlener et al., 2013), a subsequent study also found mMUTYH enrichment at telomeres in mouse cells following oxidative damage by H_2O_2 treatment (Hwang et al., 2015). Very recently, this same group employing the KR-TRF1 system to produce superoxide at mouse telomeres, showed evidence that SIRT6 and 9-1-1 together recruit MUTYH to oxidatively damaged telomeres. SIRT6 recruitment prior to MUTYH may enhance repair through nucleosome remodeling (Tan et al., 2020). However, H_2O_2 and superoxide lead to multiple DNA lesion types, DSBs and SSBs, making it difficult to determine which damage recruits SIRT6. A similar damage sensor and nucleosome remodeling role has also been proposed for UV-DDB, which is recruited to telomeres upon targeted production of 8-oxoG with the FAP-TRF1 system, and stimulates MUTYH activity and turnover (Jang et al., 2019; Jang et al., 2021). It is not clear whether MUTYH recruitment is dependent on replication, particularly since MUTYH can bind 8-oxoG:C base pairs, although in an unproductive manner.

Interestingly, WRN protein, a helicase of the RecQ family has also been implicated in BER and in telomere preservation. Mutations in the gene encoding WRN protein cause Werner Syndrome, a rare human genetic disorder characterized by features of premature aging, predisposition to sarcoma and thyroid cancers, oxidative stress, genomic instability, and increased telomere loss (Crabbe et al., 2007; Muftuoglu et al., 2008; Croteau et al., 2014). WRN facilitates telomere replication by resolving complex DNA structures found at telomeres such as T-loops, D-loops and G4s (Opresko et al., 2004; Nora et al., 2010; Damerla et al., 2012). However, WRN can also promote long-patch BER DNA synthesis by Polλ during MUTYH initiated repair at 8-oxo-G:A mispairs (Kanagaraj et al., 2012). Together with findings that WRN deficiency is associated with 8-oxoG accumulation (Von Kobbe et al., 2004; Das et al., 2007), it is tempting to speculate that WRN may also contribute to telomere preservation by stimulating MUTYH processing of 8-oxoG:A mispairs in the telomeric sequences. Whether MUTYH, and associated proteins, play a critical role preserving telomere sequence integrity, counteracting the harmful promutagenic effects of oxidative stress, remains to be determined. However, whole genome sequencing has revealed the presence of telomere repeat variants, including TTATGG, which could have derived from unrepaired TTA(8-oxoG) GG sequences (Lee et al., 2014; Barnes et al., 2019), and suggests a role for MUTYH at telomeres in preventing mutagenesis. More study is required in MUTYH deficient cells using specific oxidative targeting systems to establish MUTYH's contribution in telomere integrity preservation.

MTH1 Function in Removal of Oxidatively Damaged dNTPs at Telomeres

The nucleotide pool is highly vulnerable to cellular oxidants and free 2′-deoxyguanosine 5′-triphosphate (dGTP) is more susceptible to oxidation than guanine in chromatin-protected DNA (Haghdoost et al., 2006). 8-oxo-dGTP generated upon reaction of dGTP with ROS, can be inserted into DNA opposite either cytosine or adenine by DNA polymerases with different efficiencies depending on the polymerase [as reviewed in (Katafuchi and Nohmi, 2010)]. Thus, transversion mutations can be induced during replication not only by misinsertion of A opposite a template 8-oxoG in DNA, but also by misinsertion of 8-oxo-dGTP opposite template A. As an additional defense against the harmful effects of oxidative stress-induced 8-oxoG accumulation in the genome, mammalian cells rely on the activity of MTH1, also known as nudix hydrolase 1 (Sakumi et al., 1993; Furuichi et al., 1994). Similar to MutT in bacteria, MTH1 hydrolyzes 8-oxo-dGTP into 8-oxoGMP, which cannot be incorporated into DNA (Hayakawa et al., 1999). MTH1 also hydrolyzes oxidatively damaged dATPs, including 2-OH-dATP and 8-oxo-dATP, which are also mutagenic but arise less frequently than 8-oxodGTP (Rai and Sobol, 2019). MTH1 not only counteracts mutagenesis, but also prevents DNA double strand breaks that can arise following insertion of oxidized dNTPs, which can trigger senescence or apoptosis [for extensive review see (Rai, 2010)]. Furthermore, Pol β insertion of 8-oxo-dGTP during BER can impair downstream ligation, preventing the completion of repair (Freudenthal et al., 2015; Caglayan et al., 2017). Several studies have shown a correlation between MTH1 overexpression in cancer and poor prognosis (Rai and Sobol, 2019). Some studies suggest cancer cells may be more sensitive to MTH1 inhibitors, due to higher levels of ROS compared to non-diseased cells (Gad et al., 2014). However, despite the demonstrated effectiveness of some newly developed MTH1 inhibitor drugs, the potential efficacy in targeting MTH1 to treat cancer remains controversial and may depend on the tumor properties (Warpman Berglund et al., 2016; Yin and Chen, 2020).

Considering how sensitive telomeres are to oxidative damage, a deeper understanding of MTH1 in telomere stability is necessary to shed more light on its cellular

importance and potential effectiveness as a target in cancer therapy. Recent studies showed that MTH1 functions in telomere length regulation because oxidized dNTPs impair the ability of telomerase to lengthen telomeres. Telomerase is a reverse transcriptase that uses an inherent RNA template to add GGTTAG repeats to the 3' telomeric ssDNA overhang, and then translocates and ratchets back to add additional repeats to restore the telomere (Wu et al., 2017). The number of repeats telomerase adds prior to complete dissociation from the substrate is termed repeat addition processivity (RAP). Moreover, like all DNA polymerases, telomerase contains in its catalytic cycle a nucleotide addition processivity (NAP), which represents the number of nucleotides added prior to enzyme dissociation from the 6-nt CCAAUC template (Sanford et al., 2021). We and others showed that telomerase can insert 8-oxodGTP during telomeric DNA synthesis, but the damaged nucleotide acts as a telomerase chain terminator, halting further telomere elongation after addition (Aeby et al., 2016; Fouquerel et al., 2016). Telomerase can also insert 2-OH-dATP, but this addition impairs telomere lengthening by interfering with telomerase translocation. Even the telomerase repeat addition processivity factor POT1–TPP1 is unable to rescue the 8-oxo-dGTP or 2-OH-dATP inhibition of telomerase extension (Sanford et al., 2020). Consistent with oxidized dNTPs inhibiting telomerase, MTH1 depletion in telomerase expressing cancer cells with short telomeres causes telomere loss and dysfunction, and apoptosis (Fouquerel et al., 2016). However, cancer cells with long telomeres were less affected by MTH1 depletion in the short term. A separate study showed antioxidant enzyme PRDX1 is enriched at telomeres, and PRDX1 loss increases oxidative stress induced damage at telomeres, as detected by SSBs (Aeby et al., 2016). PRDX1 reduces ROS partly by scavenging hydrogen peroxide, and therefore, may decrease oxidative damage of free nucleotides within the vicinity of the telomeres. A follow up study further demonstrated that MTH1 and PRDX1 cooperate in preventing ROS-mediated telomere shortening. Telomerase expressing colon cancer cells lacking both MTH1 and PRDX1 showed greater telomere shortening compared to the single knockout and wild type cells, when cultured under oxidative stress at 20% O_2 (Ahmed and Lingner, 2018b). As evidence this telomere shortening was caused by telomerase inhibition, they elegantly showed a reduction in telomerase-mediated new telomeric DNA synthesis in cultured cells. This study overexpressed a mutant telomerase (TSQ1-hTR) that adds variant telomeric repeats to monitor new telomeric DNA synthesis, and found addition of the variant repeats was greatly reduced in MTH1 knockout and PRDX1/MTH1 double knockout cells cultured at 20% O_2, compared to wild-type cells (Ahmed and Lingner, 2018b). Collectively, these studies show MTH1 provides an antioxidant protection by counteracting the inhibitory effects of oxidized dNTPs on telomerase activity, to ensure telomere maintenance. However, the bulk of the telomere is duplicated by the canonical DNA replication machinery, and more work is required to determine whether insertion of oxidized dNTPs during telomere replication or repair can impair telomere stability or cause telomere mutagenesis.

DOES MMR FUNCTION AT TELOMERES AS AN ADDITIONAL 8-OXOG REPAIR PATHWAY?

DNA mismatch repair (MMR) is an evolutionary conserved repair system which canonically removes errors associated with DNA replication (for extensive review see (Jiricny, 2013; Ijsselsteijn et al., 2020)). In humans, the heterodimer MutSα (hMSH2-MSH6) recognizes single base mismatches and small insertion/deletion loops, while the heterodimer MutSβ (hMSH2-hMSH3) searches for larger insertion/deletion loops. The heterodimer MutLα (hMLH1-hPMS2) is then recruited and repair is completed by EXO1 exonuclease-mediated degradation of the error-containing strand, DNA pol δ gap filling DNA synthesis, and DNA ligase I sealing of the nick (Jiricny, 2006). MMR deficiency, mainly due to inactivation of MSH2 and MLH1, leads to increased spontaneous mutagenesis, microsatellite instability and the development of Lynch syndrome, a genetic disorder marked by increased risk for colorectal cancers (Peltomaki, 2001; Peltomaki, 2005). The association of MMR with the repair of 8-oxoG lesions has been shown in yeast and mouse (Deweese et al., 1998; Ni et al., 1999). Later *in vitro* studies established that the hMSH2-hMSH6 heterodimer can bind specifically to mismatched 8-oxoG containing DNA substrates (Mazurek et al., 2002). Further studies in MEFs showed that MSH2 and OGG1 act independently, and have an additive effect on maintaining low levels of both spontaneous and exogenously induced 8-oxoG in genomic DNA, and that overexpression of MTH1 mitigates the mutator effect of MMR deficiency (Colussi et al., 2002; Russo et al., 2004). Based on these results, the authors proposed that MMR acts at 8-oxoG:A mispairs formed by 8-oxodGTP incorporation into the daughter DNA strand opposite a template A on the parental strand, thus contributing to the elimination from newly synthesized DNA of the misincorporated 8-oxoG (Colussi et al., 2002). When 8-oxodGTP is misincorporated from the nucleotide pool opposite A, MMR activity is preferred because it allows restoration of the original T:A base pair. Conversely, MUTYH removal of A in the parental strand would be mutagenic if C is then inserted in the parental strand opposite 8-oxoG on the daughter strand. This converts the original T:A base pair to 8oxoG:C.MUTYH physically interacts with MSH6 (MutSα), and this interaction stimulates MUTYH DNA binding and glycosylase activity (Gu et al., 2002). These studies suggest that MMR can repair 8-oxoG in newly synthesized DNA, and raise the possibility that the GO repair enzymes crosstalk with MMR proteins at telomeres to process oxidative damage.

There is very limited information on potential MMR roles in telomere maintenance and protection from oxidative damage. MMR deficiency is associated with telomere shortening in leukocytes of cancer patients with Lynch Syndrome, in tumors with microsatellite instability and in normal primary human lung

fibroblasts depleted of hMSH2 (Rampazzo et al., 2010; Mendez-Bermudez and Royle, 2011; Segui et al., 2013; Garrido-Navas et al., 2020). Two studies showed knock out of PMS2 or MSH2 in telomerase (*Terc*) deficient mice partly rescued the reduced lifespan and degenerative pathologies caused by shortened, dysfunctional telomeres (Siegl-Cachedenier et al., 2007) (Martinez et al., 2009). The improvement of these phenotypes was due to an attenuated p21 induction in response to telomere attrition. Despite evidence for MMR proteins in modulating cellular responses to dysfunctional telomeres *in vivo*, many questions remain regarding the potential roles for processing mismatches in both cancerous and non-diseased cells. Some studies suggest MMR prevents aberrant recombination at telomeres [reviewed in (Jia et al., 2015)]. Whether MMR proteins may function as a backup repair system for 8-oxoG:C or 8-oxoG:A base pairs that escaped the GO repair activity, or that arise in excess under high oxidative stress, remains to be determined. Therefore, it will be interesting to determine whether MMR proteins are recruited at telomeres after oxidative damage, and how MMR may coordinate with the GO system at telomeres.

ALTERATIONS CAUSED BY 8-OXOG PROCESSING AT REPETITIVE SEQUENCES

Studies of the GO system in other repetitive regions of the genome beyond the telomeres demonstrate how DNA structure can cause aberrant BER, leading to changes in repeat lengths. Trinucleotide repeat (TNR) inherited disorders are caused by unstable repetitive DNA sequences, which can occur in different genomic contexts, including the coding sequence of a gene which leads to an aberrant protein product [reviewed in (Jones et al., 2017)]. The TNR disorders are characterized by repeat expansion, that can occur in dividing and non-dividing cells, and exhibit genetic anticipation causing an earlier onset of disease with successive generations (Orr and Zoghbi, 2007). Huntington's disease (HD) is a well-studied example of a TNR progressive neurodegenerative disorder, caused by expansion of CAG repeats in the huntingtin (HTT) gene, in which the expansion length determines the age of onset (Duyao et al., 1993). Several studies showed that both in HD patients and in transgenic mouse models, mutant HTT expression is associated with mitochondrial alterations, increased ROS and accumulation of oxidative DNA damage (Polidori et al., 1999; Askeland et al., 2018). Similar to telomeric repeats, CAG repeats are considered hotspots for oxidative DNA damage and can form secondary structures which are processed during replication and/or repair, thereby generating deletions or expansions (Kovtun and McMurray, 2008; Jarem et al., 2009; Volle et al., 2012). A proposed mechanism for the repeat expansion in HD is BER processing of 8-oxoG lesions within or near CAG repeats. Acute H_2O_2 treatment of human HD fibroblasts caused expansion of medium- and disease-length alleles, that correlated with increased SSBs. The age-dependent expansion *in vivo* was significantly suppressed or delayed when knocking out OGG1 in HD mouse models. As confirmation, *in vitro* experiments showed that OGG1-mediated BER initiates repeat expansion by subsequent APE1 production of a nick that leads to stable CAG hairpin formation, which causes an expansion following ligation and repair completion (Kovtun et al., 2007; Kovtun and McMurray, 2008). This explains how oxidative stress can cause sequence expansion in quiescent and non-replicating cells such as neurons. Furthermore, the nucleotide pool sanitizing activity of MTH1 protects both nuclear and mitochondrial DNA from the increased oxidative damage, and MTH1 over expression attenuates the HD symptoms in mice (De Luca et al., 2008; Ventura et al., 2013). Moreover, DNA pol β can incorporate 8-oxodGTP in CAG repeat sequences *in vitro*, leading to the formation of 8-oxodG:C and 8-oxodG:A mispairs, which can be processed by the OGG1 and MUTYH DNA glycosylases, further generating closely spaced SSBs on opposite DNA strands that cause TNR expansion. Interestingly, the authors of this study also found high levels of oxidized bases in the genome together with increased oxidized dNTPs in the nucleotide pool in the areas affected by neurodegeneration of an HD mouse model (Cilli et al., 2016). Collectively, these studies demonstrate how the processing of 8-oxoG lesions by the GO enzymes affects the stability of repetitive DNA sequences capable of forming secondary structures. Whether 8-oxoG processing can similarly impact telomere repeat length dynamics in replicating and quiescent cells remains to be established.

8-OXOG FORMATION AND REPAIR IN THE CONTEXT OF TELOMERIC G-QUADRUPLEX STRUCTURES: BENEFICIAL OR DETRIMENTAL FOR TELOMERE STABILITY?

The ability of telomeric sequences to spontaneously fold into G-quadruplex (G4) structures greatly influences the efficiency of damage recognition and processing by the GO system. G4s are non-canonical secondary structures that can form in single-stranded DNA and RNA containing four or more runs of guanine bases [reviewed in (Bryan and Baumann, 2011)]. The guanine bases of G4 structures interact by Hoogsteen base-pairing forming planar G-quartets, whereby two or more G-quartets stack on top of each other, stabilized by centrally-located monovalent cations, particularly potassium or sodium ions. The conformations of G4 structures vary depending on the sequence.

The repetitive TTAGGG sequence and single-stranded regions makes telomeres ideally suited for quadruplex formation, and intra-molecular G4s readily fold in oligonucleotides containing at least four telomeric repeats (Lee et al., 2005; Hwang et al., 2014). The biological roles of G-quadruplexes throughout the genome include modulation of DNA replication, DNA repair, gene expression, and telomere maintenance (Rhodes and Lipps, 2015; Johnson, 2020). Folded G4s prevent the binding of proteins that normally interact with double-stranded B-DNA,

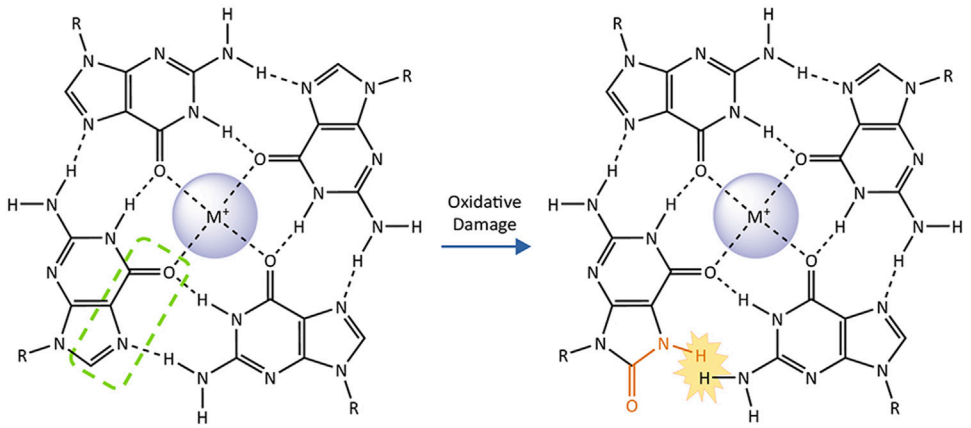

FIGURE4 | The impact of 8-oxoguanine on a G-quartet. G-quadruplexes consist of two or more stacked G-quartets and central monovalent cations. These planar arrangements of four guanine bases are stabilized by hydrogen bonding on their Hoogsteen face (highlighted by green dashed line on one representative base). Conversion of the guanine to 8-oxoguanine (affected atoms and bonds in orange) introduces a steric clash with the adjacent guanine that prevents them from forming a Hoogsteen binding interaction, destabilizing the G-quartet and the G-quadruplex as a whole.

effectively masking tracts of DNA from binding and recognition factors. For example, the folding of guanine-rich regions in gene promoters in G4 structures can inhibit gene expression (Cogoi and Xodo, 2006). G4 folding also influences processing of DNA lesions by BER enzymes, and can thereby influence gene expression when the lesion resides in a G-rich promoter (for review see (Fleming and Burrows, 2020). While NEIL1 and NEIL3 glycosylases can remove hydantoin lesions from a G4, OGG1 is unable to recognize and excise 8-oxoG residing in a telomeric or promoter G4 (Zhou et al., 2013; Zhou et al., 2015; Ferino and Xodo, 2021). Whether MUTYH can excise A in the context of a G4 is unknown but is unlikely given that the A:8-oxoG base pair is disrupted in a G4. While APE1 can bind an abasic residue within a telomeric or promoter G4, its cleavage activity is attenuated depending on the G4 conformation (Broxson et al., 2014; Zhou et al., 2015). Thus, G4 structures impact the GO system, and may decrease 8-oxoG repair within telomeric G4s by inhibiting OGG1 and APE1. Whether 8-oxoG repair is less efficient at telomeres *in vivo* is not clear. Telomeres can also take advantage of adjacent repeats to remodel a G4, which may enable 8-oxoG repair. In this "spare tire" model (Fleming et al., 2015), when an 8-oxoG arises in four G-tracks (i.e., telomeric repeats) folded into a G4, a nearby G-track (i.e., spare tire or fifth telomeric repeat) can participate in the G4 and thereby extrude the 8-oxoG, to a loop, making it accessible to repair enzymes. We previously demonstrated that increasing the number of telomeric repeats beyond four in oligonucleotides, increases the structural dynamics and conformations (Hwang et al., 2014), suggesting G4 remodeling within a telomere may promote lesion accessibility and repair.

The relationship between G4 and the GO system is further complicated by the alterations and dynamics that guanine oxidation imparts on G4 structures. Conversion of guanine to an 8-oxoG, disrupts the hydrogen-bonding pattern on the Hoogsteen face for the base within a G-quartet (**Figure 4**). Solution NMR studies of single-stranded oligonucleotides with G-rich telomeric sequences show that the formation of G4s is substantially disrupted by the substitution of guanine for 8-oxoG. The tendency for 8-oxoG to adopt a syn-orientation instead of the anti-orientation typically assumed by guanine bases, changes the preferred loop conformations assumed by the oligonucleotide. While telomeric G4s containing 8-oxoG can still fold, they melt at significantly lower temperatures compared to undamaged G4s (Cheong et al., 2015; Bielskute et al., 2019). 8-oxoG substitution at the 2nd G in TTAGGG within the middle G-quartet, is significantly more disruptive than substitution at the 1st or 3rd Gs which participate in an outer quartets (Bielskute et al., 2019). These structural studies are complemented by single-molecule Forester Resonance Energy Transfer (smFRET) experiments to monitor G4 folding in real time. In this approach G4 folding brings two strategically placed dyes within a telomeric oligonucleotide close enough to FRET, whereby one dye donates its energy to a proximal acceptor dye, which then fluoresces. SmFRET shows that 8-oxoG substitution for a single guanine in a telomeric sequence does not completely unfold the G4, but instead induces dynamic fluctuations between partially-unfolded and short-lived folded G4 conformations. Consistent with the position-dependent effects of 8-oxoG seen in NMR, conversion of the central 2nd guanine to 8-oxoG also has the strongest destabilizing effect (Lee et al., 2020). Furthermore, the 8-carbon of most guanines within a G4 is solvent-exposed, which allows for guanine oxidation in the context of a folded G4. Single-electron oxidation experiments demonstrate that, while guanine oxidation is slower than in duplex DNA, 8-oxoG can form in folded telomeric G4s (Merta et al., 2019).

The ability of 8-oxoG to alter G4 conformation and stability suggests that guanine oxidation at telomeres may lead to a reduction of telomere G4s, even when quadruplexes folded prior to oxidative damage. Given that G4s have been implicated in inhibiting telomere replication and telomerase mediated telomere lengthening, 8-oxoG modulation of G4

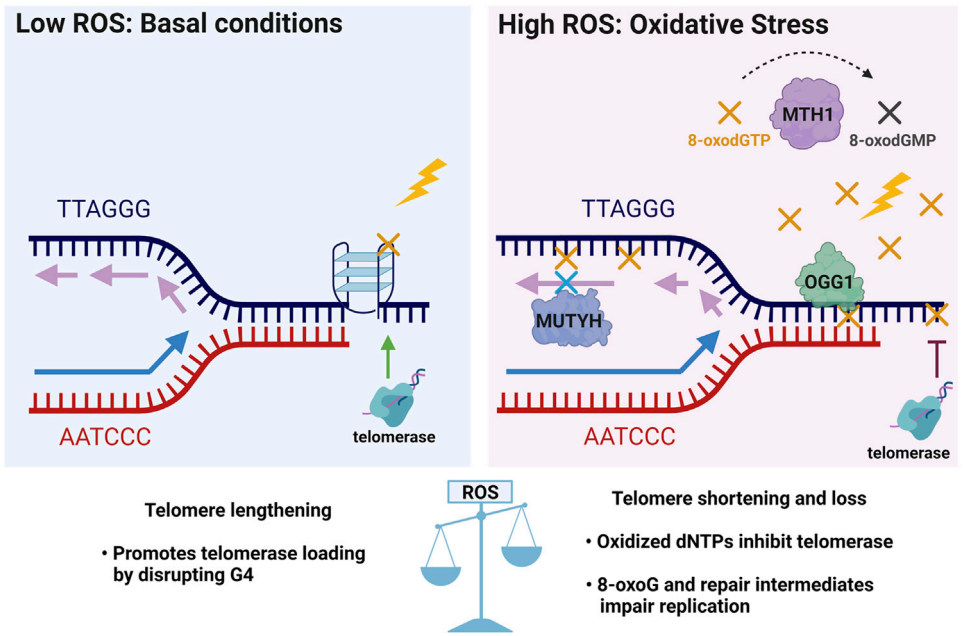

FIGURE 5 | Telomere 8-oxoG hormesis model and the crucial roles of the GO system enzymes in telomere stability. In unstressed conditions of basal ROS, low levels of 8-oxoG (yellow X) may promote telomere maintenance by destabilizing G4s structures (as shown in **Figure 4**) which block telomerase loading and impair replication, and may thereby facilitate telomere lengthening. Conversely, elevated ROS under oxidative stress inhibits telomere maintenance by producing excess 8-oxoG lesions (yellow X) and repair intermediates that impair telomere replication, and by producing 8-oxodGTP (yellow X) which inhibits telomerase. Thus, under oxidative stress the GO system may play a critical role in telomere preservation through MTH1 hydrolysis and removal of 8-oxodGTP, OGG1 initiated BER of 8-oxoG opposite C, and MUTYH initiated BER removal of A misinserted opposite 8-oxoG in the template strand. Thus, a little telomeric 8-oxoG may be beneficial for telomere maintenance, but too much telomeric 8-oxoG is detrimental for telomere stability.

structure likely influence telomere maintenance. SmFRET studies revealed that substitution of G with 8-oxoG in telomeric oligonucleotides enhances accessibility and binding of a complementary DNA strand, telomerase, and telomeric ssDNA binding protein POT1 (Lee et al., 2017). As a result, 8-oxoG substitution also improved telomerase extension of telomeric oligonucleotides that were pre-folded into G4s (Fouquerel et al., 2016; Lee et al., 2020). POT1 can partially unravel G4s as well (Zaug et al., 2005; Wang et al., 2011), and may cooperate with 8-oxoG to modulate telomeric G4s. The ability of 8-oxoG to destabilize G4 may partly explain why OGG1 loss leads to telomere lengthening *in vivo* under non-stress conditions (Lu and Liu, 2010; Wang et al., 2010). The role for G4 structures in telomere regulation and protection, and for 8-oxoG modulation of G4 at telomeres remain unclear. More research into the interplay between G-quadruplexes, oxidative damage, shelterin, and telomerase is needed to fully understand how 8-oxoG and the GO system influence telomere stability and cellular health.

PERSPECTIVE

During the last 2 decades, a large number of studies have revealed that telomeres are highly susceptible to oxidative stress, and that oxidative damage to telomeric DNA is associated with accelerated telomere shortening and/or dysfunction. As we discussed in this review, one of the most common oxidative lesions within the genome is 8-oxoG. The biological importance of this lesion is underscored by the evolution of the highly conserved GO system that involves three distinct enzymes that recognize and process 8-oxoG in various contexts to preserve the genome. The recently developed cutting-edge FAP-TRF1 technology has made it possible to specifically produce 8-oxoG selectively at telomeres, in the absence of damage elsewhere in the genome. Since oxidative stress damages numerous cellular components, targeted lesion production allows researchers to determine what damage is collateral and what damage drives the cellular response and genomic alterations. Both OGG1 genetic depletion and pharmacological inhibition have provided evidence for a crucial OGG1 role in protecting telomeres from the harmful effects of high oxidative stress in cancer cells. More investigation is needed to uncover the role of OGG1 in preserving telomere integrity and modulating cellular responses to telomeric oxidative damage in non-diseased and primary cells. Furthermore, despite the lack of studies assessing the roles of the GO system enzymes at telomeres in quiescent cells, findings in HD cellular and animal models show how TNR expansion in quiescent and non-replicating cells can result from the repair of oxidative damage (Kovtun et al., 2007; Kovtun and McMurray, 2008). This raises the possibility that 8-oxoG processing in non-replicating cells at other repetitive sequences such as telomeres, may affect their integrity and length dynamics. Finally, potential activation of ATM and ATR kinases by 8-oxoG processing in normal cells with intact DNA damage response pathways may alter telomere length

based on evidence that these kinases regulate telomerase recruitment (Lee et al., 2015; Tong et al., 2015). The consequences of OGG1 processing at telomeres will likely differ depending on the cell and tissues type, underscoring the need for future studies.

Notwithstanding evidence for MUTYH association with telomeres undergoing oxidative stress, more investigation is required to understand the impact of 8-oxoG:A mispairs at telomeric repeats for telomere function and stability. In regards of studying potential mutagenesis at telomeres, sequencing of telomeric DNA has been challenging because of its repetitive nature. However, the advent of long-read or third-generation sequencing, including PacBio single-molecule real-time (SMRT) sequencing and Oxford Nanopore Technologies (ONT) sequencing, enables detection of mutations in repetitive regions of the genome, where short reads cannot be mapped uniquely (Amarasinghe et al., 2020). Therefore, these recent advances in third-generation sequencing, or new developments in bioinformatic tools able to accurately map telomeric sequences even from short-reads, may help to elucidate whether telomeres undergo mutagenesis due to 8-oxoG formation in contexts of functional or disrupted repair. The possibility of targeting 8-oxoG at telomeres in human cellular models, singly, doubly, or triply deficient for MUTYH, OGG1, and MTH1 will further uncover the role various GO system components play in safeguarding telomeric repeats.

Based on evidence that OGG1 promotes telomere lengthening under non-stressed conditions *in vivo*, but accelerates telomere shortening and dysfunction under oxidative stress (Wang et al., 2010; Fouquerel et al., 2019), we propose a hormesis model for 8-oxoG roles in telomere stability (**Figure 5**). According to this model, low 8-oxoG levels may facilitate telomere maintenance by disrupting G4s thus promoting replication fork progression and telomerase loading. Alternatively, studies in yeast suggest low 8-oxoG can promote telomere elongation by RAD52-mediated homologous recombination [not shown, (Lu and Liu, 2010)]. In contrast, elevated 8-oxoG levels under oxidative stress inhibit telomere maintenance because persistent 8-oxoG lesions and repair intermediates impair telomere replication and 8-oxodGTP inhibits telomerase, thereby accelerating telomere shortening and loss. In the proposed model, the GO system enzymes play crucial roles in telomere stability particularly under oxidative stress conditions. For example, MTH1 depletion only causes telomere shortening when cells are cultured at 20% oxygen, not when cultured at low 5% oxygen (Ahmed and Lingner, 2018b). Establishing how the GO system enzymes OGG1, MUTHY and MTH1 cooperate and cross-talk with additional repair pathways to safeguard telomere integrity from oxidative stress, will be valuable for developing new therapeutic strategies that preserve telomeres and delay aging-related diseases, or that conversely target telomeres in cancer cells to halt proliferation.

AUTHOR CONTRIBUTIONS

MDR wrote the majority of the review and prepared the figures. SJ contributed section 5 on telomeric G-quadruplex. PO supervised the review preparation, wrote the introduction and edited the review for content and clarity.

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REVIEW
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Cyclin E/CDK2: DNA Replication, Replication Stress and Genomic Instability

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DNA replication must be precisely controlled in order to maintain genome stability. Transition through cell cycle phases is regulated by a family of Cyclin-Dependent Kinases (CDKs) in association with respective cyclin regulatory subunits. In normal cell cycles, E-type cyclins (Cyclin E1 and Cyclin E2, *CCNE1* and *CCNE2* genes) associate with CDK2 to promote G1/S transition. Cyclin E/CDK2 complex mostly controls cell cycle progression and DNA replication through phosphorylation of specific substrates. Oncogenic activation of Cyclin E/CDK2 complex impairs normal DNA replication, causing replication stress and DNA damage. As a consequence, Cyclin E/CDK2-induced replication stress leads to genomic instability and contributes to human carcinogenesis. In this review, we focus on the main functions of Cyclin E/CDK2 complex in normal DNA replication and the molecular mechanisms by which oncogenic activation of Cyclin E/CDK2 causes replication stress and genomic instability in human cancer.

Keywords: cyclin E, CCNE, CDK2, DNA replication, replication stress, genomic instability, cell cycle, cancer

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INTRODUCTION

Cellular proliferation is controlled by an intricate network of proteins that dictate the order and timing of cell cycle events. Progression through cell cycle phases is regulated by a family of Cyclin-Dependent Kinases (CDKs), which associate with respective Cyclin regulatory subunits. Oscillations in Cyclin levels determine fluctuations in CDK activity, which ultimately control cell cycle phase transitions (Malumbres and Barbacid, 2009; Matthews et al., 2021). In normal mammalian cells, expression of E-type cyclins, named Cyclin E1 and Cyclin E2, is reached as a consequence of RB inactivation and E2F transcription factor release, which is initially caused by Cyclin D/CDK4-6 activation upon mitogenic stimulation during G1. E2F-mediated Cyclin E transcription is followed by Cyclin E protein accumulation that peaks at the G1/S transition, when Cyclin E binds and activates CDK2 to promote S phase entry and progression. Cyclin E/CDK2 complex then phosphorylates numerous substrates to control essential cellular processes, such as progression through the restriction point (R point), initiation of DNA replication, and regulation of histone biosynthesis among others. By the end of S phase, Cyclin E protein levels are completely degraded by the SCF^{FBW7} ubiquitin ligase complex, thus eliminating Cyclin E/CDK2 activity up to the subsequent G1 phase (Hwang and Clurman, 2005; Chu et al., 2021).

Oncogenic activation of Cyclin E/CDK2 complex is frequently observed in human cancers and may be achieved by different genetic events, such as amplification of Cyclin E genes (*CCNE1* or *CCNE2*), disruption of the RB/E2F pathway (leading to increased Cyclin E transcription), and mutation of *FBXW7*

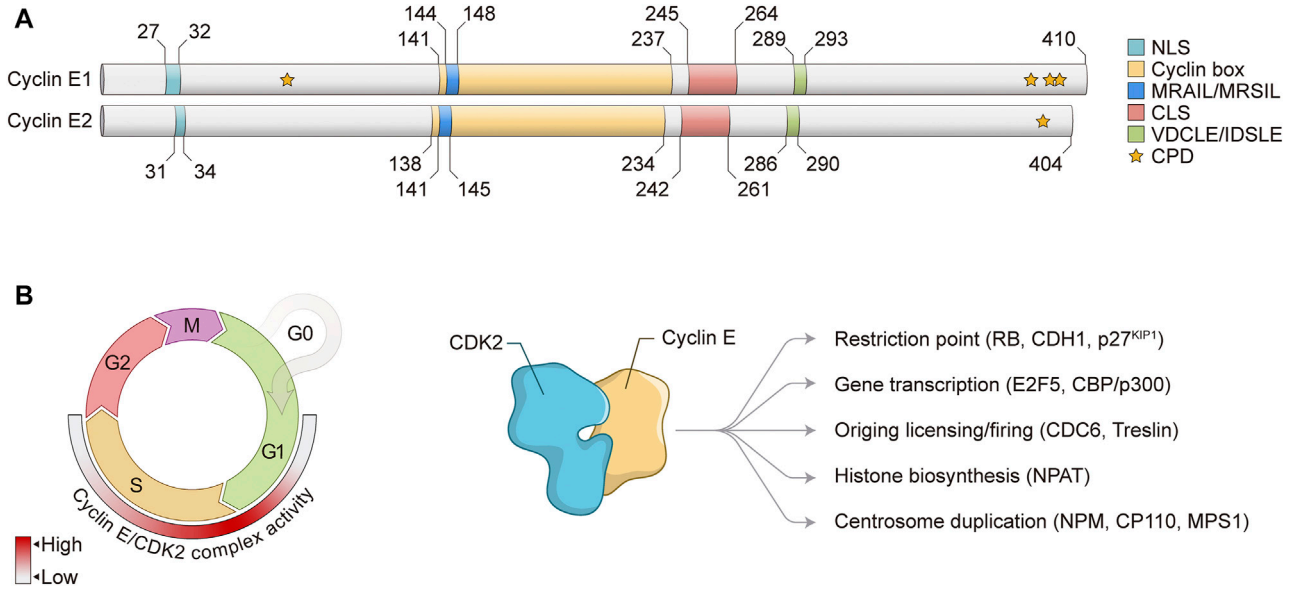

FIGURE 1 | Cyclin E structure and function in the cell cycle. **(A)** Schematic representation of Cyclin E1 and Cyclin E2 protein structures. Numbers represent amino acid positions. NLS, nuclear localization signal; CLS, centrosome localization signal; CPD, CDC phosphodegrons. **(B)** Left: Cyclin E/CDK2 complex controls G1/S phase transition and S phase progression. Heat map indicates Cyclin E/CDK2 complex activity through the cell cycle. Right: Cyclin E/CDK2 complex regulates different cell cycle-related events and substrates. RB, Retinoblastoma; CDH1, CDC20 homolog 1; KIP1, CDK inhibitory protein 1; E2F, E2 promoter-binding factor; CBP, CREB-binding protein; CDC, Cell division cycle; NPAT, Nuclear protein, coactivator of histone transcription; NPM, Nucleophosmin; CP110, Centriolar coiled-coil protein 110; MPS1, Monopolar spindle 1.

ubiquitin ligase (causing accumulation of Cyclin E protein). In fact, high levels of Cyclin E protein and increased CDK2 kinase activity are both independently associated with poor prognosis, reduced survival, and therapy resistance in cancer patients (Hwang and Clurman, 2005). Under the cell cycle perspective, oncogenic activation of Cyclin E/CDK2 complex has been largely demonstrated to impair DNA replication, causing DNA replication stress, which may be defined as the slowing or stalling of replication fork progression during DNA synthesis upon different insults. Hyperactivation of Cyclin E/CDK2 complex directly interferes with DNA replication through several mechanisms, leading to DNA double strand breaks (DSBs) and genomic instability. In fact, specific targeting of oncogenic Cyclin E/CDK2 complex has been proposed as a promising therapy against cancer (Tadesse et al., 2020; Suski et al., 2021).

Regulation of E-type cyclins and the effects of Cyclin E/CDK2 complex in normal physiology and disease states have been extensively reviewed in the literature (Hwang and Clurman, 2005; Caldon and Musgrove, 2010; Siu et al., 2012; Chu et al., 2021). In this review, we focus on the role of Cyclin E/CDK2 complex in DNA replication and the molecular mechanisms by which hyperactivation of Cyclin E/CDK2 complex causes DNA replication stress and genomic instability in human cancer.

CYCLIN E: STRUCTURE AND FUNCTION

Cyclin E1 was the first member of the E-type cyclin family to be identified (Koff et al., 1991; Lew et al., 1991). In humans, *CCNE1* gene localizes at 19q12 region and encodes for a full-length protein of 410 amino acids (**Figure 1A**). Several splice variants and protein isoforms have also been described for *CCNE1* gene. Cyclin E1 protein has been shown to bind and activate CDK2 kinase, creating an active complex that is responsible for S phase entry and progression (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994). In accordance with its role in S phase promotion, the highest levels of Cyclin E1 mRNA are observed during G1/S phase transition, coinciding with maximum activity of Cyclin E1/CDK2 complex (Dulić et al., 1992; Koff et al., 1992). Cyclin E2 was the second member of the E-type cyclin family to be described in humans (Lauper et al., 1998; Zariwala et al., 1998; Gudas et al., 1999). *CCNE2* gene is localized at 8q22.1 region and encodes for a full-length protein of 404 amino acids (**Figure 1A**). Like Cyclin E1, Cyclin E2 protein also binds and activates CDK2, forming an active kinase complex whose activity also peaks during G1/S phase transition (Lauper et al., 1998; Zariwala et al., 1998; Gudas et al., 1999). Cyclin E1 and E2 proteins show approximately 50% of overall sequence identity and are assumed to be functionally redundant. However, several reports have indicated distinct regulation and functions for Cyclin E1 and E2 proteins (Caldon and Musgrove, 2010).

Cyclin E is a nuclear protein that mainly exerts its regulatory functions through interaction with and activation of CDK2 to induce phosphorylation of target proteins. Of note, it has been shown that Cyclin E1 can also bind and activate CDK1 *in vivo* (Aleem et al., 2005), however CDK2 is the main binding partner of Cyclin E. Cyclin E interacts with CDK2 through its Cyclin box with the PSTAIRE helix on CDK2, leading to conformational

changes on the CDK2 T loop (Honda et al., 2005) (**Figure 1A**). Exposure of CDK2 catalytic site allows for activating phosphorylation of CDK2 Thr160 by CDK activating kinase (CAK). Interaction between Cyclin E/CDK2 complex with substrates is mediated by two distinct domains found on Cyclin E: MRAIL and VDCLE (**Figure 1A**). The MRAIL domain is localized at the N-terminal region of the Cyclin box and mediates binding to RLX-containing proteins, such as RB, $p27^{KIP1}$, and CDC6 (Adams et al., 1996; Chen et al., 1996; Schulman et al., 1998; Furstenthal et al., 2001). The VDCLE domain is found on Cyclin E C-terminal portion and regulates the interaction with the pocket protein family members RB1, p107, and p130 (Dowdy et al., 1993; Kelly et al., 1998). Of note, similar sequences are observed in Cyclin E2 protein: MRSIL and IDSLE, respectively (**Figure 1A**). Two additional domains are observed on Cyclin E1 and E2 proteins: nuclear localization signal (NLS) and centrosome localization signal (CLS) (**Figure 1A**). The N-terminal NLS potentially contributes to Cyclin E nuclear localization, however it is clear that other mechanisms also regulate Cyclin E nuclear accumulation in human cells (Jackman et al., 2002; Moore et al., 2002). The CLS targets Cyclin E to the centrosomes, where it is essential for Cyclin E/CDK2-mediated centrosome duplication (Matsumoto and Maller, 2004). Furthermore, E-type cyclins also show CDC phosphodegrons (CPDs) at the N- and C-terminus, which are represented by Serine and Threonine (S/T) residues that are initially phosphorylated by certain kinases and later recognized by the SCF^{FBW7} ubiquitin ligase complex and directed to proteasomal degradation (Clurman et al., 1996; Won and Reed, 1996) (**Figure 1A**). Importantly, Cyclin E/CDK2 activity is negatively regulated by the KIP/CIP family of CDK inhibitors, $p27^{KIP1}$ and $p21^{CIP1}$, which prevent CDK2 activation by CAK phosphorylation and also inhibit Cyclin E/CDK2 complex interaction with substrates (Sherr and Roberts, 2004).

Cyclin E levels are tightly regulated throughout the cell cycle by a timely combination of gene expression and protein degradation. During G1, mitogenic stimulation induces Cyclin D accumulation, which together with CDK4/6 phosphorylates and inactivates RB, releasing activating E2F1-3 transcription factors to induce Cyclin E transcription (Ohtani et al., 1995; Geng et al., 1996). Upon S phase progression, Cyclin E transcription is repressed through the assembly of inhibitory proteins to Cyclin E promoter, including E2F6 and E2F7 transcriptional repressors (Giangrande et al., 2004; Westendorp et al., 2012). Apart from transcriptional repression, protein degradation is mostly responsible for progressive decrease of Cyclin E levels through S phase. Degradation of Cyclin E1 protein is mediated by phosphorylation of several S/T residues (CPDs) observed within the N-terminal (T77, also known as T62) and C-terminal regions (S387, T395, and S399, also known as S372, T380, and S384) of full-length Cyclin E1 protein (Clurman et al., 1996; Won and Reed, 1996; Welcker et al., 2003; Ye et al., 2004) (**Figure 1A**). Phosphorylation of Cyclin E1 CPDs may be achieved by either GSK3 or CDK2 autophosphorylation. Phosphorylated Cyclin E1 is then mostly recognized by the SCF^{FBW7} ubiquitin ligase complex and subsequently marked for ubiquitin-mediated degradation via proteasome (Koepp et al., 2001; Strohmaier et al., 2001). On the other hand, Cyclin E2 protein degradation has not been fully investigated yet, but it is assumed that its proteolysis is similar to Cyclin E1. Cyclin E2 protein C-terminal residue T392 is conserved to Cyclin E1 protein residue T395 and its phosphorylation is also required for protein degradation (Lauper et al., 1998) (**Figure 1A**). Of note, Cyclin E protein may be cleaved by intracellular proteolytic processing, generating low-molecular weight Cyclin E (LMW-E) isoforms. These LMW-E lack the N-terminal NLS, accumulate in the cytoplasm, and have been shown to present increased affinity for CDK2 and resistance to CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (Caruso et al., 2018).

In normal conditions, Cyclin E/CDK2 complex controls several critical biological functions (**Figure 1B**). During G1/S transition, Cyclin E/CDK2 phosphorylates and inactivates RB protein, leading to release of E2F transcription factors and a positive feedback loop for Cyclin E transcription (Harbour and Dean, 2000). Through inactivation of RB and release of activating E2Fs, Cyclin E/CDK2 complex activity induces the expression of a variety of genes that are essential for S phase entry and progression, such as cell division cycle 6 (*CDC6*), chromatin licensing and DNA replication factor 1 (*CDT1*) and members of the minichromosome maintenance (MCM) complex, all three components of pre-replication complex (pre-RC); A-type cyclins; and EMI1, inhibitor of the APC/C^{CDC20} complex (Ishida et al., 2001; Hsu et al., 2002; Polager et al., 2002). Besides phosphorylation of canonical RB protein, Cyclin E/CDK2 complex also phosphorylates other critical targets for cell cycle progression, including $p27^{KIP1}$ CDK inhibitor; E2F5 and CBP/p300 transcription factors; NPAT and HIRA, proteins involved in histone biosynthesis; and NPM, CP110 and MPS1, all involved in centrosome duplication (**Figure 1B**) (Sheaff et al., 1997; Ait-Si-Ali et al., 1998; Ma et al., 2000; Morris et al., 2000; Okuda et al., 2000; Zhao et al., 2000; Fisk and Winey, 2001; Hall et al., 2001; Tokuyama et al., 2001; Chen et al., 2002). High-throughput proteomic screening approaches have been performed to determine the profile of Cyclin E1 interactome, as well as to identify novel CDK2 substrates (Pagliuca et al., 2011; Odajima et al., 2016; Chi et al., 2020). These studies have provided powerful resources for the investigation of Cyclin E/CDK2 regulatory network and function. Furthermore, as CDK2 may be activated by Cyclin E and Cyclin A, these findings are instrumental to the analysis of similarities and differences between Cyclin E- and Cyclin A-associated CDK2 activity, which are essential for timely progression of the cell cycle (Pagliuca et al., 2011; Chi et al., 2020). For the purpose of this review, we will focus on the functions of Cyclin E/CDK2 complex in normal and aberrant DNA replication.

CYCLIN E/CDK2 COMPLEX IN DNA REPLICATION

Eukaryotic cells must ensure accurate chromosome replication in order to maintain genome stability. DNA replication is a multi-step process that is characterized by the chronological assembly of different protein complexes onto DNA replication origins (ORIs), followed by replisome activation and subsequent DNA synthesis. From origin licensing to replication completion, all necessary

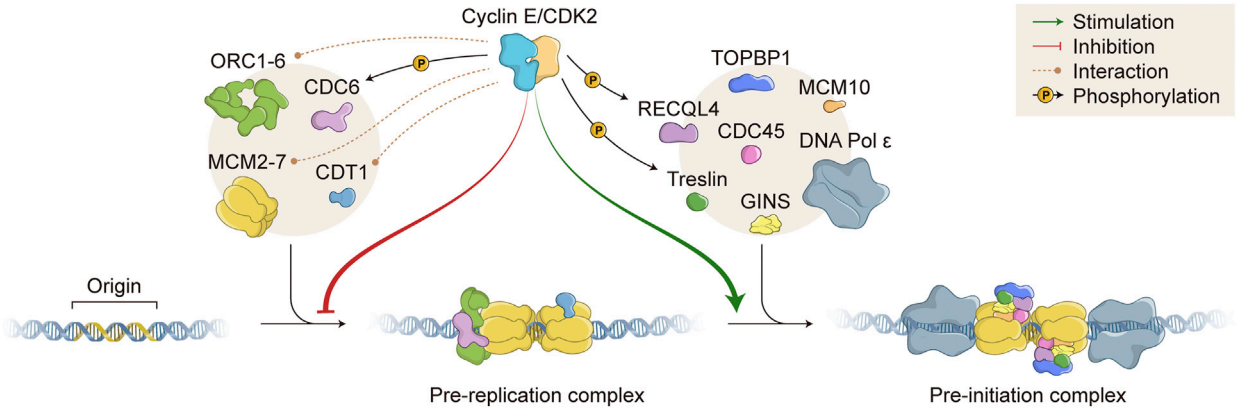

FIGURE 2 | Role of Cyclin E/CDK2 in DNA replication. Cyclin E/CDK2 complex directly interacts with (orange dashed lines) and phosphorylates (black arrows; P, phosphate) several proteins that are essential for DNA replication. Cyclin E/CDK2 activity inhibits pre-replication complex (pre-RC) formation (red arrow) and stimulates pre-initiation complex (pre-IC) formation (green arrow). DNA replication origins (Origin) are recognized by different proteins, which are sequentially recruited to form pre-RC, including ORC1-6 and MCM2-7 complexes (left beige circle). Subsequently, many other proteins are assembled onto DNA replication origins to form pre-IC, including TOPBP1/RECQL4/Treslin and CDC45/GINS (right beige circle). DNA molecule (blue double strand); ORC, Origin recognition complex; CDC, Cell division cycle; MCM, Minichromosome maintenance; CDT1, Chromatin licensing and DNA replication factor 1; CDK, Cyclin dependent kinase; TOPBP1, DNA topoisomerase II binding protein 1; RECQL4, RecQ like helicase 4; GINS, Go-ich-ni-san. Numerous replisome proteins are omitted for simplicity.

steps are tightly regulated to permit appropriate genome replication. Alterations that compromise the function of key proteins involved in DNA replication can lead to progressive accumulation of errors in the DNA molecule and genomic instability (Masai et al., 2010; Bellelli and Boulton, 2021).

In order to accomplish one single round of DNA replication per cell cycle, two fundamental steps that precede DNA synthesis have to be executed in a temporally separated manner: origin licensing and origin firing. Origin licensing takes place during late mitosis and early G1 phase, when cells possess low levels of CDK activity, and involves the assembly of pre-RC onto ORIs. Origin licensing starts with the binding of origin recognition complex subunits 1–6 (ORC1-6) and CDC6 to ORIs, followed by recruitment of CDT1 and MCM2-7 helicase complex, completing pre-RC assembly (**Figure 2**). At this point, ORIs are inactive, though primed for later activation in the cell cycle (Masai et al., 2010; Siddiqui et al., 2013; Fragkos et al., 2015). Origin activation takes place during G1/S transition and requires the recruitment of additional proteins to generate pre-initiation complex (pre-IC) (**Figure 2**). Unlike origin licensing, pre-IC formation requires high kinase activity that results from the combined action of two S phase kinases: CDK2 and CDC7 (also known as DDK, DBF4-dependent kinase), which associate with regulatory subunits Cyclin E/A and DBF4, respectively. Together, these kinases phosphorylate several replication factors and facilitate protein recruitment to ORIs, including the recruitment of CDC45 and GINS to allow formation of the CMG helicase complex (CDC45-MCM-GINS). Helicase activation and subsequent DNA unwinding leads to the recruitment of other additional proteins, including DNA polymerases, and eventually origin firing (Masai et al., 2010; Tanaka and Araki, 2013; Fragkos et al., 2015; Burgers and Kunkel, 2017).

Cyclin E/CDK2 complex has essential and opposing roles in DNA replication (**Figure 2**). As mentioned before, origin licensing occurs when cells experience low CDK environments. Accordingly, hyperactivation of Cyclin E1/CDK2 complex impairs MCM loading and prevents pre-RC assembly, causing DNA replication stress (see below). However, under certain circumstances, normal levels of Cyclin E/CDK2 complex are essential to promote origin licensing. It has been shown that cells deficient for Cyclin E1/E2 or depleted of CDK2 activity are not able to re-enter the cell cycle from quiescence due to failure in MCM loading (Geng et al., 2003; Chuang et al., 2009). In fact, during cell cycle re-entry from quiescence, Cyclin E/CDK2 complex activity is required for accumulation of *CDC6* and *CDC7* mRNA levels, both of which are necessary for MCM loading (Chuang et al., 2009). Cyclin E/CDK2 complex is also able to directly interact with pre-RC components and form a ternary complex with ORC1 and CDC6 proteins, both acting as receptors for Cyclin E/CDK2 binding onto chromatin during origin licensing (Furstenthal et al., 2001; Hossain and Stillman, 2016; Hossain et al., 2021) (**Figure 2**). In agreement, it has been shown that Cyclin E/CDK2 complex and CDC6 protein work synergistically to promote pre-RC assembly and abrogation of Cyclin E-CDC6 interaction leads to failure in DNA replication (Furstenthal et al., 2001; Cook et al., 2002; Coverley et al., 2002). Cyclin E1 has been also shown to physically interact with CDT1 and members of the MCM complex, possibly further facilitating MCM loading onto ORIs (Geng et al., 2007; Odajima et al., 2016) (**Figure 2**). In terms of phosphorylation of pre-RC components, Cyclin E1/CDK2 complex has been shown to directly phosphorylate CDC6 in human cells, protecting CDC6 from APC/C-mediated ubiquitylation and proteolysis. Cyclin E/CDK2-mediated CDC6 phosphorylation potentiates MCM loading onto chromatin and allows for DNA replication (Mailand and Diffley, 2005) (**Figure 2**). Additional reports have suggested that Cyclin E/CDK2 complex is able to phosphorylate other proteins that constitute pre-RCs, such as

CDT1 and MCM complex components, possibly interfering with chromatin loading or protein-protein interaction. However, further definitive work is still necessary to clearly demonstrate and elucidate the effects of Cyclin E/CDK2-mediated phosphorylation of other pre-RC components in human cells.

Apart from regulating pre-RC assembly, Cyclin E/CDK2 complex exerts a positive role during pre-IC formation, which occurs upon high kinase activity (**Figure 2**). It has been shown that Cyclin E/CDK2 directly phosphorylates Treslin (also known as TICRR, TOPBP1 interacting checkpoint and replication regulator), promoting its interaction with TOPBP1 (DNA topoisomerase II binding protein 1) and facilitating recruitment of CDC45, GINS, and DNA polymerases onto chromatin (**Figure 2**). Indeed, interaction of Treslin-TOPBP1 induced by Cyclin E/CDK2 phosphorylation is essential for initiation of DNA replication *in vivo* (Kumagai et al., 2010; Boos et al., 2011; Kumagai et al., 2011; Sansam et al., 2015). Besides Treslin, Cyclin E/CDK2 complex also phosphorylates RECQL4 (RecQ like helicase 4), increasing its helicase activity (**Figure 2**). However, it is still not clear whether Cyclin E/CDK2-dependent RECQL4 phosphorylation favours its chromatin binding and/or pre-IC formation (Lu et al., 2017). Consistently, Sld2 and Sld3, the yeast counterparts of human RECQL4 and Treslin, respectively, have been shown to bind Dpb11 (the yeast counterpart of human TOPBP1) upon CDK phosphorylation. CDK-dependent Sld2 and Sld3 phosphorylations in yeast are essential for the initiation of DNA replication (Tanaka et al., 2007; Zegerman and Diffley, 2007).

ONCOGENIC ACTIVATION OF CYCLIN E/CDK2 MEDIATES REPLICATION STRESS

As discussed above, Cyclin E/CDK2 complex plays a central role in controlling normal DNA replication. Therefore, it is expected that oncogenic activation of Cyclin E/CDK2 interferes with DNA synthesis and causes replication stress. Indeed, it has been shown that Cyclin E1 overexpression impairs replication fork progression, leading to premature termination of replication forks, fork collapse, and DSBs (Bartkova et al., 2005, 2006). Importantly, Cyclin E-mediated replication stress is directly associated with increased CDK2 activity, as a hyperactive *CDK2* allele is sufficient to impair replication fork progression and cause DNA damage (Hughes et al., 2013). In normal cells, aberrant activation of Cyclin E1/CDK2 complex induces the replication stress response (RSR), leading to cell cycle arrest, cell death, and senescence. This is an essential mechanism to prevent tumor progression in normal tissues. However, oncogenic activation of Cyclin E1/CDK2, associated with disruption of the RSR pathway, allows for increased cell proliferation in the presence of sustained replication stress, contributing to genomic instability in early steps of human tumorigenesis (Bartkova et al., 2005, 2006; Teixeira and Reed, 2017).

Oncogenic activation of Cyclin E/CDK2 complex is able to mediate replication stress by several different molecular mechanisms (**Figure 3**). One of the primary mechanisms is interference with origin licensing (**Figure 3**, upper left). As discussed above, pre-RC assembly onto chromatin occurs during late mitosis and early G1, when cells experience low CDK environments (Masai et al., 2010; McIntosh and Blow, 2012). Unscheduled CDK activity during these cell cycle stages is likely to compromise origin licensing. Indeed, it has been demonstrated that overexpression of yeast G1 cyclin Cln2 inhibits pre-RC assembly and leads to gross chromosomal rearrangements (Tanaka and Diffley, 2002). Consistently, high levels of Cyclin E1 at the M/G1 boundary, accompanied by hyperactive CDK2 activity, impair loading of specific MCM helicase components onto chromatin in mammalian cells (Ekholm-Reed et al., 2004). One possible explanation is that oncogenic Cyclin E1/CDK2 activity forces rapid progression through G1 and premature entry into S phase with insufficient pre-RC formation. However, the molecular mechanism by which hyperactive Cyclin E/CDK2 complex interferes with MCM loading and/or distribution onto chromatin is still not understood.

Cyclin E/CDK2-induced replication stress may be caused not only by interference with origin licensing, but also with origin firing (**Figure 3**, lower left). Indeed, Cyclin E1 overexpression has been shown to decrease origin firing (Liberal et al., 2012), consistent with the idea that cells experiencing oncogene-induced replication stress trigger the intra-S-phase checkpoint to prevent new origin activation (Gaillard et al., 2015). On the other hand, in agreement with a positive role of Cyclin E/CDK2 complex in origin activation, hyperactivation of Cyclin E1/CDK2 complex has been shown to aberrantly induce origin firing (Hughes et al., 2013; Jones et al., 2013). High levels of Cyclin E1 are able to interfere with time and location of origin activation, inducing premature, novel origin firing in intragenic regions (Macheret and Halazonetis, 2018). Again, the molecular mechanisms for disturbed origin firing upon oncogenic Cyclin E/CDK2 activation remain to be determined.

Another important mechanism by which Cyclin E/CDK2 hyperactivation mediates replication stress is interference with nucleotide pools (**Figure 3**, upper right). Precise regulation of nucleotide metabolism and biosynthesis is essential for execution of numerous biological processes, including DNA replication and RNA production (Lane and Fan, 2015). Reduction in nucleotide availability directly interferes with DNA replication dynamics (Anglana et al., 2003). It has been shown that Cyclin E1 overexpression induces aberrant activation of the RB/E2F pathway without accompanying increase in nucleotide biosynthesis (Bester et al., 2011). As a consequence, cells are enforced to proliferate with insufficient levels of nucleotides, leading to decreased progression of replication forks and induction of DSBs. Interestingly, supplementation with exogenous nucleosides is able to attenuate Cyclin E1-mediated replication stress and DNA damage (Bester et al., 2011).

Transcription-replication collisions represent another source of replication stress that may be induced by hyperactive Cyclin E/CDK2 complex (**Figure 3**, lower right). Precise regulation of Cyclin E/CDK2 activity is not only essential for normal DNA replication, but also for appropriate RNA production as it represents a critical step in RB inactivation and subsequent E2F-mediated transcription activation (Harbour and Dean, 2000). Initially, it has been shown that inhibition of either replication

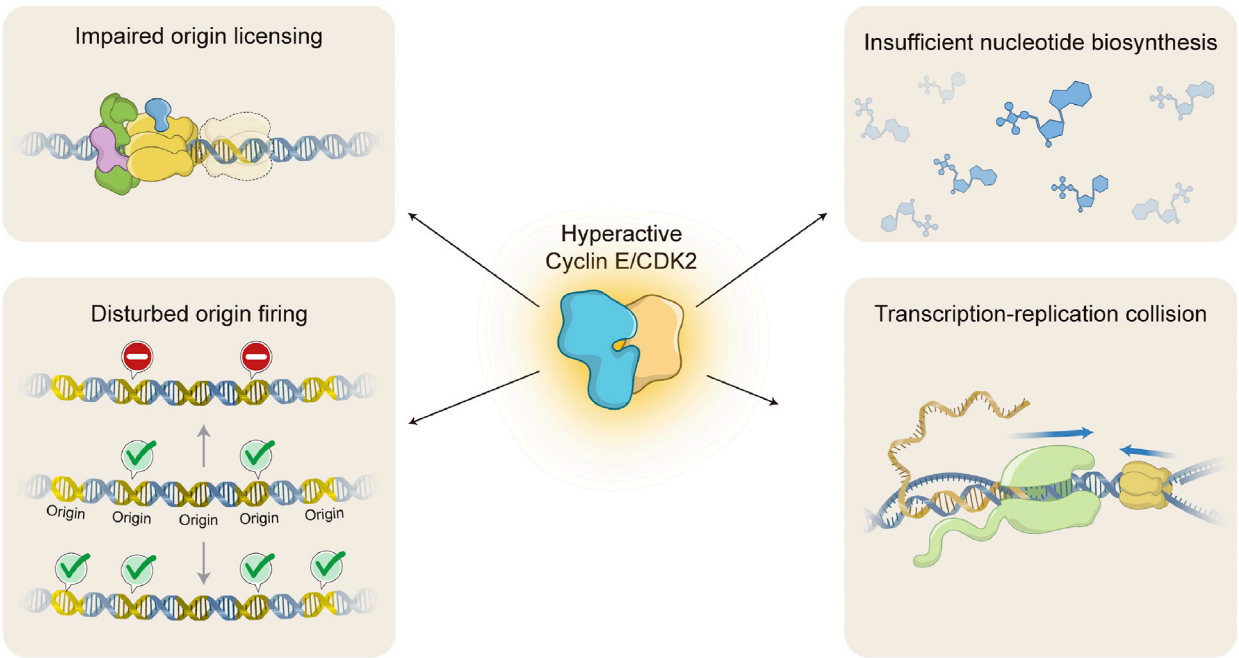

FIGURE 3 | Molecular mechanisms of Cyclin E/CDK2-induced replication stress. Oncogenic activation of Cyclin E/CDK2 interferes with DNA replication through several different molecular mechanisms, causing DNA replication stress. Hyperactive Cyclin E/CDK2 complex impairs origin licensing by interference with pre-replication complex (pre-RC) loading onto chromatin **(upper left)** and origin firing by either decreasing or increasing activation of DNA replication origins (**lower left**; upper or lower DNA molecule, respectively). Hyperactive Cyclin E/CDK2 complex also leads to insufficient nucleotide pools by forcing cell hyperproliferation without accompanying nucleotide biosynthesis **(upper right)**. Finally, hyperactive Cyclin E/CDK2 complex causes transcription-replication collisions due to imbalances in DNA replication and gene transcription processes **(lower right)**. Upper left: DNA molecule (blue double strand); ORC complex (green); CDC6 (purple); MCM complex (yellow); CDT1 (blue); Faded MCM complex represents impaired origin licensing. Lower left: DNA molecule (blue double strand); DNA replication origins (Origin, yellow double strand); Activated origin firing (green check); Impaired origin firing (stop signal). Upper right: chemical structures represent different nucleotides (blue). Lower right: DNA molecule (blue double strand); messenger RNA (yellow single strand); RNA polymerase (light green); MCM complex (yellow); R-loop (RNA-DNA hybrid molecule). Blue arrows indicate direction of transcription and replication progression. Replisome is omitted for simplicity.

initiation or transcription elongation are able to alleviate replication stress induced by Cyclin E1 overexpression (Jones et al., 2013). Later on, it has been demonstrated that high levels of Cyclin E1 induce inappropriate origin firing within intragenic, coding sequences, leading to transcription-replication conflicts, replication fork collapse, DSBs, and chromosomal aberrations (Macheret and Halazonetis, 2018). Transcription-replication collisions may cause DNA topological tension and formation of persistent R-loops (RNA-DNA hybrid structures) (Helmrich et al., 2013). Indeed, it has been shown that Cyclin E1 overexpression induces accumulation of aberrant DNA replication intermediates, such as reversed replication forks (Neelsen et al., 2013). Altogether, hyperactivation of Cyclin E/CDK2 complex clearly interferes with normal DNA replication, causing replication stress and contributing to genomic instability.

CYCLIN E/CDK2-INDUCED REPLICATION STRESS CAUSES GENOMIC INSTABILITY IN HUMAN CANCERS

Genomic instability is a hallmark of cancer and is defined as an increased frequency of genetic alterations during cell divisions. It may be caused by cell extrinsic or intrinsic genotoxic insults, and is observed in human cancers under various forms, such as whole chromosome gains and/or losses, focal copy number alterations, chromosomal rearrangements, and clustered base-pair mutations (Negrini et al., 2010; Aguilera and García-Muse, 2013). Oncogene activation, another hallmark of cancer, may interfere with normal cell proliferation and DNA replication. In fact, certain oncogenes are able to induce replication stress and promote genomic instability in human carcinogenesis (Primo and Teixeira, 2019). One such example is oncogenic Cyclin E/CDK2 complex. Hyperactivation of Cyclin E/CDK2 causes genomic instability in different experimental models and correlates with an increased frequency of genomic alterations in human cancers (Teixeira and Reed, 2017). Furthermore, high levels of Cyclin E and/or increased activity of CDK2 have been observed in numerous cancers, ranging from hematological malignancies to numerous solid tumors, and are associated with poor prognosis and decreased survival in cancer patients (Hwang and Clurman, 2005).

High levels of Cyclin E1 protein have been initially shown to induce chromosome gains and losses, causing aneuploidy in normal human mammary epithelial cells (Spruck et al., 1999). Consistently, either *FBXW7* deletion or *SKP1* silencing, which results in accumulation of Cyclin E protein among other oncoproteins, led to increased micronucleus formation,

multipolar spindles, and chromosome instability in human colorectal cancer cells (Rajagopalan et al., 2004; Thompson et al., 2020). These findings were associated with increased levels of Cyclin E1 protein, as additional *CCNE1* silencing in *FBXW7*- or *SKP1*-deficient cells partially rescued micronucleus formation. Importantly, the effects of high levels of Cyclin E1 on chromosome instability seem to depend on CDK2 activity, as a hyperactive *CDK2* knockin allele was sufficient to induce increased Cyclin E1-associated CDK2 activity and micronucleus formation in human colorectal cancer cells (Hughes et al., 2013). *CCNE1* and *CCNE2* amplification, as well as increased mRNA expression, have been also correlated with whole genome doublings in human cancers (Zack et al., 2013; Lee et al., 2020). One of the proposed mechanisms to explain how hyperactive Cyclin E/CDK2 causes genome doublings is the induction of mitotic failure or endoreduplication, with subsequent formation of polyploid cells. Indeed, high levels of Cyclin E1 have been shown to impair mitotic progression and cause accumulation of cells in early stages of mitosis. This is caused by Cyclin E1/CDK2-mediated phosphorylation and inactivation of the APC/C adaptor protein CDH1, followed by accumulation of Cyclin B1 and Securin, and eventually mitotic failure (Keck et al., 2007). In agreement with these findings, precise regulation of Cyclin E protein is critical to normal endocycles, as *CCNE1/2*-double deficient mice present defects in endoreduplication of megakaryocytes and placental trophoblast giant cells (Geng et al., 2003; Parisi et al., 2003).

Another potential mechanism to explain how oncogenic Cyclin E/CDK2 interferes with normal ploidy and causes genomic instability is centrosome amplification, which induces the formation of merotelic kinetochore-microtubule attachments and ultimately leads to aberrant chromosome segregation (Godinho and Pellman, 2014). As mentioned before, Cyclin E1 localizes to centrosomes and, together with CDK2, is able to phosphorylate several centrosome proteins, such as NPM, CP110, and MPS1, regulating the process of centrosome duplication (Okuda et al., 2000; Fisk and Winey, 2001; Tokuyama et al., 2001; Chen et al., 2002; Matsumoto and Maller, 2004). Even though high levels of Cyclin E1 alone are not sufficient to induce centrosome amplification in human cells (Spruck et al., 1999), it does synergize with *TP53* loss to cause centrosome amplification and chromosome segregation errors (Mussman et al., 2000; Kawamura et al., 2004). Accordingly, a hyperactive *CDK2* knockin allele mouse model was sufficient to induce increased centrosome numbers (Zhao et al., 2012). It is possible that Cyclin E/CDK2 hyperactivation impairs the localization and function of certain centrosome proteins, causing centrosome amplification and subsequent chromosomal gains and/or losses. In fact, oncogenic Cyclin E1/CDK2 aberrantly hyperphosphorylates centromere protein A (CENPA), reducing CENPA localization at centromeres and causing chromosome missegregation and increased micronucleus formation (Takada et al., 2017).

Apart from whole chromosome gains/losses, oncogenic activation of Cyclin E/CDK2 has been shown to cause copy number alterations at specific genomic segments (Costantino et al., 2014; Miron et al., 2015; Teixeira et al., 2015; Menghi et al., 2018; Giraldez et al., 2019; Kok et al., 2020). It has been demonstrated that Cyclin E1/CDK2 hyperactivation impairs S phase progression, allowing cells to enter into mitosis with unreplicated genomic regions. Incompletely replicated chromosomal segments, in turn, lead to segregation abnormalities in mitosis and eventually genomic deletions. In fact, *CCNE1* amplification associates with copy number losses at specific genomic sites in human breast cancers (Teixeira et al., 2015). It has been shown that large genomic deletions may contribute to oncogenesis by promoting loss of heterozygosity at tumor suppressor genes and deletion of fragile sites (Bignell et al., 2010). More complex mechanisms have been also shown to contribute to Cyclin E/CDK2-induced genomic instability. It has been indicated that Cyclin E1-induced replication fork collapse during S phase can be repaired by break-induced replication (BIR), generating segmental tandem duplications as a consequence of BIR (Costantino et al., 2014). Again, *CCNE1* amplification has been associated with tandem duplications in different human cancers, mostly favoring oncogenesis by causing oncogene duplication (Menghi et al., 2018). Besides copy number alterations, *CCNE1* amplification has been also associated with chromosomal breakpoints and rearrangements in human cancers (Macheret and Halazonetis, 2018). Together, these data indicate that oncogenic activation of Cyclin E/CDK2 complex drives numerous forms of genomic instability in human cancers, ranging from whole chromosomal gains and/or losses to focal genomic deletions and/or amplifications, as well as chromosome rearrangements.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Uncontrolled cell proliferation and abnormal activity of cell cycle proteins are at the basis of carcinogenesis. As a result, various cell cycle regulators have been considered as potential targets in cancer therapy (Otto and Sicinski, 2017; Suski et al., 2021). Normal activity of Cyclin E/CDK2 complex is essential for appropriate cell cycle progression and DNA replication. On the other hand, oncogenic activation of Cyclin E/CDK2 complex has been shown to interfere with DNA replication and cause replication stress through several different mechanisms. Hyperactivation of Cyclin E/CDK2 induces genomic instability in human cancers, typified by the increased frequency of chromosomal gains and/or losses and rearrangements. High levels of Cyclin E and/or increased CDK2 activity are associated with poor clinical outcome and decreased survival in cancer patients. Therefore, targeting oncogenic activity of Cyclin E/CDK2 complex (e.g. through Cyclin E-targeted degradation or CDK2-selective inhibition) may represent an attractive therapeutic strategy for specific cancer subtypes, such as breast, uterine, and ovarian cancers (Tadesse et al., 2020; Suski et al., 2021).

Oncogene activation causes numerous alterations in tumor cells. Along the tumorigenic process, oncogenic insults may uncover potential cancer vulnerabilities. In fact, oncogene-induced replication stress leads to activation of the RSR pathway, which has been proposed to represent a promising target in cancer therapy (Ngoi et al., 2021). Due to the excessive level of replication stress

caused by activation of certain oncogenes, cancer cells heavily rely upon RSR pathway activation in order to survive. Indeed, several screening approaches have identified synthetic lethal interactions between oncogene activation and inhibition of replication checkpoint proteins. Cancer cells experiencing high levels of Cyclin E protein and/or high CDK2 activity have shown increased sensitivity to inhibitors of RSR protein kinases, such as ATR, CHK1, and WEE1 (Murga et al., 2011; Toledo et al., 2011; Chen et al., 2018; Kok et al., 2020; Sviderskiy et al., 2020). Combination of Cyclin E/CDK2 hyperactivation and inhibition of such protein kinases leads to irreversible DNA damage and selective death of cancer cells in different models. More recently, *CCNE1* amplification has been also identified to be synthetic lethal with inhibition of PKMYT1 kinase, a negative regulator of CDK1 (Gallo et al., 2021). The results suggest that further activation of CDK1 is not compatible with oncogenic Cyclin E/CDK2 environments, leading to unscheduled mitotic entry and genome instability. Altogether, these data indicate that cancer subtypes with oncogenic activation of Cyclin E/CDK2 complex and subsequent robust activation of RSR may be especially vulnerable and uniquely sensitive to inhibitors of replication checkpoint proteins. Exploiting these therapeutic opportunities will certainly prove beneficial to cancer treatment in the following years.

AUTHOR CONTRIBUTIONS

RF and LT designed and wrote the manuscript. LT supervised and reviewed the manuscript. Both authors approved the submitted version.

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Tools for Decoding Ubiquitin Signaling in DNA Repair

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The maintenance of genome stability requires dedicated DNA repair processes and pathways that are essential for the faithful duplication and propagation of chromosomes. These DNA repair mechanisms counteract the potentially deleterious impact of the frequent genotoxic challenges faced by cells from both exogenous and endogenous agents. Intrinsic to these mechanisms, cells have an arsenal of protein factors that can be utilised to promote repair processes in response to DNA lesions. Orchestration of the protein factors within the various cellular DNA repair pathways is performed, in part, by post-translational modifications, such as phosphorylation, ubiquitin, SUMO and other ubiquitin-like modifiers (UBLs). In this review, we firstly explore recent advances in the tools for identifying factors involved in both DNA repair and ubiquitin signaling pathways. We then expand on this by evaluating the growing repertoire of proteomic, biochemical and structural techniques available to further understand the mechanistic basis by which these complex modifications regulate DNA repair. Together, we provide a snapshot of the range of methods now available to investigate and decode how ubiquitin signaling can promote DNA repair and maintain genome stability in mammalian cells.

Keywords: DNA damage, DNA repair, genome stability, ubiquitin, CRISPR-Cas9 screen, cryo-EM, cross-linking mass spectrometry, proteomics

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INTRODUCTION

Maintenance of genome stability is critically important for cellular fitness and organismal survival. As such, the genome has to be safeguarded by numerous DNA repair pathways, which are collectively termed the DNA damage response (DDR) (Ciccia and Elledge, 2010). Importantly, defects within the DDR lead to various cancers and contribute to the etiology of various chromosomal instability disorders, so understanding the mechanistic basis of DNA repair is of fundamental importance (Hanahan and Weinberg, 2011; Schumacher et al., 2021). At a broad level, the DDR may be viewed as a highly inter-related signal transduction pathway constructed of DNA lesion-specific sensors, transducers, mediators, and effectors, involving both protein and RNA signaling mechanisms (Jackson and Bartek, 2009). The DDR is also integrated within numerous other cellular pathways, such as the cell cycle and the innate immune response, which allows it to dictate cell fate outcomes after DNA damage (Reislander et al., 2020). Recently, our understanding of these two general principles of DDR function, the inter-relatedness of the DNA repair pathways and integration within other cellular pathways, have been brought into focus as they offer great potential to be exploited for therapeutic purposes (Setton et al., 2021).

Orchestration of the DDR signaling network is performed, in part, by post-translational modifications (PTMs), such as ADP-ribosylation, phosphorylation, ubiquitination and SUMOylation (Dantuma and van Attikum, 2016; Blackford and Jackson, 2017; Palazzo and Ahel, 2018). Protein ubiquitination as part of the ubiquitin-proteasome system (UPS) is carried

out by an enzymatic cascade involving E1 ubiquitin-activating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases (Oh et al., 2018). Ubiquitination of protein targets, either by single addition of the ubiquitin molecule or by formation of polymeric ubiquitin chains, provides a multifaceted signaling mechanism to control various aspects of protein function, including localisation, half-life, activation and repression. Ubiquitin signaling is regulated by deubiquitinating enzymes (DUBs), which function to catalyse the removal or trimming of ubiquitin from substrates (Mevissen and Komander, 2017; Clague et al., 2019). In this manner, ubiquitination is a reversible and highly dynamic process within cells, the vast complexities of which we are only beginning to understand (Yau and Rape, 2016). Moreover, given its wide-ranging role in regulating myriad cellular pathways, the ubiquitin system has become a prominent target for drug discovery to treat a range of different pathologies (Rape, 2018; Wu et al., 2020; Duan and Pagano, 2021; Morgan and Crawford, 2021; Tokheim et al., 2021).

The ability of ubiquitin to act in a multitude of processes is largely due to the diverse ubiquitin structures that are formed and then recognised by effector proteins (Swatek and Komander, 2016). The seven internal lysine residues within ubiquitin and the N-terminal methionine can function in polyubiquitin chain formation providing a broad repertoire of ubiquitin chain architectures. Moreover, the existence of heterotypic ubiquitin chains, which can be classified as mixed or branched chain types, further expands the complexity of ubiquitin signaling (French et al., 2021). Ubiquitin can also be modified by ubiquitin-like proteins (UBLs), such as SUMO, or post-translational modifications, including phosphorylation and acetylation (Guzzo and Matunis, 2013; Hendriks and Vertegaal, 2016; Swatek and Komander, 2016; Song and Luo, 2019). Thus, these different mechanisms generate an essentially unlimited number of combinations of ubiquitin chain architectures, referred to as the 'ubiquitin code' (Komander and Rape, 2012). In order to decode this signaling, cellular proteins use a range of ubiquitin binding domains (UBDs) to non-covalently interact with ubiquitin, thereby facilitating the transfer of information from the substrate linked ubiquitin chain architecture to the effector protein containing the UBD (Dikic et al., 2009; Mattern et al., 2019). There are at least 20 distinct UBDs in the human genome, many of which display remarkable specificity for ubiquitin chain linkage types and lengths, though they generally exhibit low affinities for ubiquitin (Husnjak and Dikic, 2012). Multiple mechanisms exist to increase avidity between UBDs and ubiquitin, such as combinations of UBDs within the same protein or protein complex, which may help overcome the low affinities of individual UBDs for ubiquitin in cells (Rahighi and Dikic, 2012). It's possible that the low affinities of UBDs for ubiquitin has prohibited the discovery of a larger repertoire of UBDs in the human genome, with the disconnect between known UBDs and the complexity of the ubiquitin code described as the 'dark matter' of the UPS (Radley et al., 2019).

The complexity of ubiquitin chain architectures poses a technical challenge if we are to understand how this ubiquitin code promotes various cellular processes and how its dysregulation impacts disease. To address this challenge, a number of recent methodological approaches have been developed and utilised to better understand the assembly, structure and cellular function of the ubiquitin code. Given that the role of ubiquitination in the DDR has emerged as a key paradigm in understanding genome stability pathways over the last two decades, these new approaches can provide further insight into the mechanisms of the DDR (Jackson and Durocher, 2013; Garcia-Rodriguez et al., 2016). In this review we discuss a number of recent discoveries in the DDR through the lens of ubiquitin signaling, whilst also pinpointing discoveries in each field that could be used at the intersection of the two. We highlight the methodologies used to make these discoveries, potential limitations, and how these tools can be evolved and used to answer remaining questions. To do this we focus on discoveries in the three broad areas of genetics, proteomics and biochemistry, which have helped illuminate our fundamental understanding of DDR mechanisms and the role that ubiquitin plays within them, as well as how this understanding can be harnessed for therapeutic purposes.

GENETIC APPROACHES TO UNDERSTANDING THE DNA DAMAGE RESPONSE AND UBIQUITIN SIGNALING

Lessons From RNAi Screens

A genetic screen is a powerful tool with which to ascertain gene function in complex biological signaling networks in both unperturbed conditions and in response to stimuli that engage specific cellular pathways, for example, after exogenous addition of DNA damaging agents. Genetic screens can uncover relationships between genes by comparative analysis of wild type and engineered, typically knockout (KO), cells (a synthetic lethal (SL) screen) or by use of a small molecule inhibitor against a desired protein target of choice in a particular genotypic background (a chemogenetic screen). At the beginning of this century, large-scale genetic approaches were mainly used in tractable model systems such as bacteria, flies, yeast, or zebrafish. However, the leveraging of RNAi technologies into genome-wide tools revolutionised mammalian genetic approaches in the mid-2000s (Berns et al., 2004; Paddison et al., 2004; Silva et al., 2005; Root et al., 2006). For the first time, this new technology allowed targeted large-scale loss-of-function screens in mammalian cells in both forward and reverse genetic approaches, which put it in stark contrast to random mutagenesis approaches or time-consuming low-throughput mouse knockout generation. Practically, genome-wide libraries of short interfering RNA (siRNA) or short hairpin RNA (shRNA) were constructed and used on a per well basis (for shRNA and siRNA) or used in a pooled format (shRNA only), whereby all the shRNA expressing lentiviral vectors are combined in one pool, with one shRNA sequence per vector. The shRNA sequence is linked to a DNA barcode which then allows it to be identified and its abundance quantified in a population of cells by high-throughput DNA sequencing. Typically, viability assays, flow cytometry, or microscopy were

used to apply these genome-wide RNAi technologies. However, some major drawbacks to the RNAi-based screening included partial knockdowns and non-specific or off-target effects (Chang et al., 2006; Boutros and Ahringer, 2008). Whilst partial knockdowns may be useful for studying essential genes, the off-target effects require strict awareness of this limitation and requirement for additional validations.

One pertinent example of the off-target effects associated with RNAi came from a screen designed to identify regulators of homologous recombination (HR). Briefly, HR and non-homologous end joining (NHEJ) are the two major DNA repair pathways that respond to double strand breaks (DSBs) in mammalian cells (Scully et al., 2019; Tarsounas and Sung, 2020). Whilst NHEJ promotes the ligation of DSB ends and can operate throughout the cell cycle, HR requires an undamaged donor template from which to perform DNA synthesis, so is active in S/G_2-phase of the cell cycle. A key step in the HR pathway is the formation of single-stranded DNA (ssDNA), which is first bound by the RPA complex. RPA is then displaced by the RAD51 recombinase via the actions of BRCA1 and BRCA2, allowing RAD51 to perform a homology search (Scully et al., 2019; Tarsounas and Sung, 2020). Understanding regulators of RAD51 localisation at DSBs was therefore an important question to address and formed the basis of a microscopy-based genome-wide RNAi screen (Adamson et al., 2012). However, the authors found that RAD51 is a common off-target hit in siRNA screening, creating many false positives, which was particularly challenging when the screen was designed to identify regulators of HR (Adamson et al., 2012).

Nevertheless, despite these issues, RNAi-based screens have made a major contribution to our understanding of the DDR and its ubiquitin-dependent regulation. For example, a focused DUB-based shRNA screen coupled to immunoblotting led to the identification of USP1 as a key DUB in the Fanconi Anemia (FA) DNA repair pathway (Nijman et al., 2005). The FA pathway senses inter-strand crosslinks (ICLs) in DNA and promotes their unhooking, followed by downstream DNA repair steps (Semlow and Walter, 2021). A key step in the activation of the FA pathway is the monoubiquitination of the FANCD2-FANCI, which can then be reversed by USP1 (Nijman et al., 2005). In addition, an shRNA-based genome-wide dropout screen in response to the ICL-inducing drug mitomycin C identified several new factors in the FA pathway, including the ubiquitin-binding FAN1 nuclease (Smogorzewska et al., 2010).

In addition to these discoveries, genome-wide microscopy-based RNAi screens identified the E3 ligases RNF8 and RNF168 as key components of the ubiquitin-dependent response to DSBs (Kolas et al., 2007; Doil et al., 2009; Stewart et al., 2009). RNF8 and RNF168 promote histone ubiquitination in response to DSBs, helping to recruit two protein complexes, BRCA1/BARD1 and 53BP1-RIF1-REV7. These two complexes antagonise each other at DSBs, and promote either HR or NHEJ, respectively, and therefore represents a decision point for DSB repair pathway choice in cells (Tarsounas and Sung, 2020; Becker et al., 2021).

Lastly, a focused DUB-based siRNA library was used in a number of orthogonal assays for DSB repair phenotypes to establish new roles for members of this enzyme family (Nishi et al., 2014). These are just a small selection of important findings demonstrating how RNAi-based screening approaches helped shape and expand our understanding of ubiquitin-dependent regulation of the DDR. Despite their subsequent loss in popularity over recent years, RNAi-based screening approaches set the foundation for the rapid development of the next generation of eukaryotic functional genomics tools by advancing the platforms, tools and pipelines for genome-wide screening.

CRISPR-Cas9 Screens

Engineering of the bacterial CRISPR-Cas9 system for use in eukaryotic cells led to another leap forward for mammalian functional genomics (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013). Soon after these ground-breaking discoveries, the CRISPR-Cas9 system was quickly adapted for genome-wide gene essentiality and drug sensitivity/resistance screens in a variety of cancer cell types (Blomen et al., 2015; Hart et al., 2015; Sanjana et al., 2014; Tzelepis et al., 2016; Wang et al., 2015; Wang et al., 2014). Similar to RNAi-based screening, these gene essentiality approaches employ pooled sgRNA libraries in lentiviral vectors, with multiple sgRNAs targeting each gene and each sgRNA sequence linked to a unique DNA barcode, allowing quantification of abundance by high-throughput DNA sequencing (**Figure 1**). The collective efforts of these monogenic perturbation studies revealed that CRISPR-Cas9 screening identified 3–4 times as many essential genes versus RNAi-based approaches (Hart 2014), with a consensus list emerging of approximately 2000 genes. As expected from RNAi approaches, the UPS ranked highly amongst the essential cellular pathways, as well as checkpoint signaling components of the DDR and components of the DNA replication machinery. However, the majority of human genes can be deleted at no fitness cost to the cell, which in turn presents potential therapeutic opportunities under certain genetic circumstances (Rancati et al., 2018).

The success of CRISPR screening approaches has been underscored by the equitable availability of reagents for performing the screens and the code and software for analysis. However, there are various limitations that need to be considered (Hart et al., 2017; Sanjana, 2017; Doench, 2018). During the assay design of chemogenetic screens, a drug dose is optimised to try to ensure that both sensitisation and resistance effects can be observed in a pooled population of cells for dropout screens. The dose threshold might not reveal more subtle regulators and only identify core nodes of the signaling network of interest. Another limitation is that loss of the sgRNA abundance may reflect some aspect of gene function related to cellular fitness that results in an increased doubling time that, over the time course of the screen, causes the sgRNA to be diluted from the population, but doesn't mean that gene is essential *per se*. Taking samples at regular intervals during the screen may circumvent this issue. A major potential problem is the extent to which the genome editing causes true KOs or whether the mutants are instead hypomorphs, which could lead to false negatives. In addition, the higher the number of off-target effects, and more DSBs

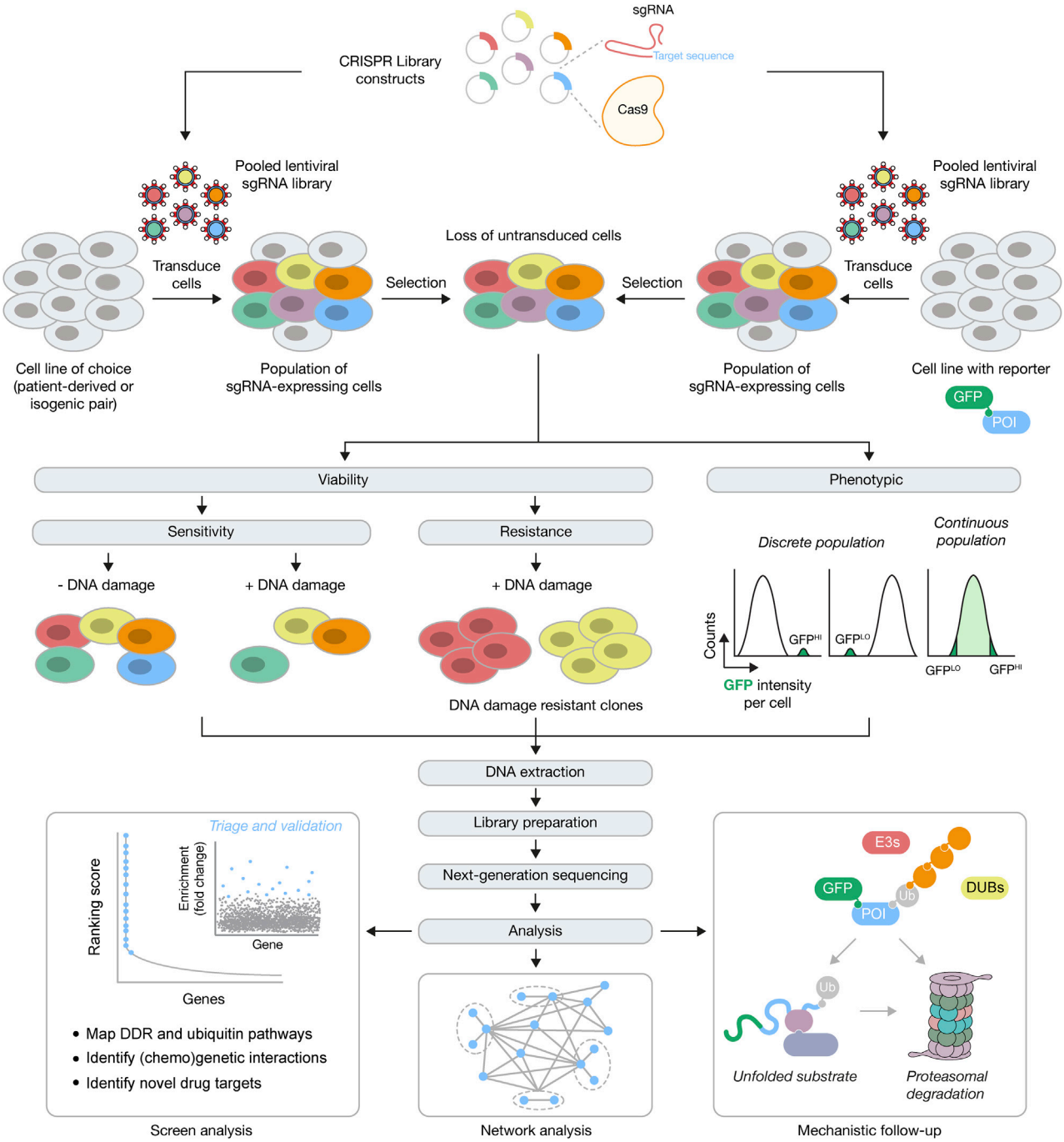

FIGURE 1 | Forward genetics CRISPR-Cas9 screening approaches for the DDR and ubiquitin signaling. *Top*, for genome-wide screens constructs with Cas9 and sgRNAs against every gene are packaged into lentiviral particles, followed by transduction of cells (typically patient-derived, engineered isogenic pairs or cells with integrated fluorescent reporters) at low multiplicity of infection, and then selection for stable integrants. POI, protein of interest. *Middle*, cells with stably integrated constructs expressing Cas9 may now be used in an assay-dependent manner, in viability or phenotypic screens. For viability screens, negative or positive selection can be used to identify genes whose function is essential for survival (e.g., in response to DNA damage) or whose function causes a selective advantage (e.g., resistance in response to DNA damage), respectively. For phenotypic screens, FACS can be used to physically separate the population of cells of choice, depending on the expected population(s) of interest. *Bottom*, after DNA extraction, library preparation and next-generation sequencing, downstream analysis will identify numerous genetic interactors within a DDR or ubiquitin signaling network, that then undergo triage and further validation. After validation, further investigations are needed to understand the mechanistic basis for the genetic interactions. For example, if components of the UPS (E3s, DUBs, protein quality control components and the and proteasome) are found to regulate the GFP-POI, subsequent work will be required to understand the mechanism of this regulation.

produced, due to copy number variations for example, the greater the likelihood that the lethality phenotype is independent of the on-target gene loss (Meyers et al., 2017). sgRNAs are typically designed to target common exons, however, alternative splicing, in-frame deletions, or inefficient degradation of the mRNA might still lead to protein function. Use of multiple sgRNAs per gene seeks to add statistical robustness in screens, but another approach to ensure KO generation is to target sgRNAs to important functional domains. However, unless the protein is fully characterised then it may have other domains that play different roles in different cellular contexts or pathways and the genome editing may thus just create a separation of function mutant (Shi et al., 2015). Lastly, a potential limitation that hasn't been fully addressed is whether KO of individual genes cause compensatory regulation of other gene products (Housden et al., 2017). Recent findings from zebrafish found that mutant mRNA production triggered the transcriptional activation of compensatory genes, suggesting that this could be more widespread than fully appreciated (El-Brolosy et al., 2019). Single-cell RNA sequencing coupled to CRISPR-Cas9 editing, such as Perturb-seq (Adamson et al., 2016; Dixit et al., 2016) or CRISP-seq (Jaitin et al., 2016) hold great promise to combine combinatorial genetic perturbations with transcriptomic profiling. Once these approaches are applied at larger scales, perhaps in parallel to single-cell proteomics, they should soon reveal the extent to which genome editing impacts the re-wiring of transcriptional and proteomic profiles.

Rationale for Targeting the DNA Damage Response

As noted earlier, the DDR is a highly inter-related signaling network, whereby DNA lesions may be channeled from a primary DNA repair pathway to another back-up DNA repair pathway, if the primary DNA repair pathway fails for some reason (e.g., mutation of a particular gene or methylation changes altering gene expression profiles). Thus, in cancers that contain a defect in the primary DNA repair pathway, a back-up DNA repair pathway may repair any DNA lesions that occur, allowing survival of those cancers. However, if the back-up DNA repair pathway is then targeted by small molecule inhibitors, the cancer cell cannot repair the DNA lesions and undergoes apoptosis, effectively targeting and killing the cancer cell. These chemogenetic approaches are therefore a form of synthetic lethality as the inhibition of repair enzymes in the back-up pathways can be viewed as loss-of-function (Setton et al., 2021). This also makes the DDR a highly attractive pathway for identifying therapeutically actionable SL interactions, as healthy cells with both DNA repair pathways can still use the pathway untouched by the small molecule inhibitor, reducing the collateral damage to healthy cells that often occurs in chemotherapies (Lord and Ashworth, 2017). This concept is best illustrated by the identification of a SL interaction between DNA repair enzymes PARP1/PARP2, via small molecule inhibition, and genetic perturbation of the DNA repair genes *BRCA1* and *BRCA2*, which are both mutated in breast and ovarian cancers. BRCA1, an E3 ubiquitin ligase, and BRCA2 function in multiple DNA repair pathways, including HR and fork protection pathways (Tarsounas and Sung, 2020; Qiu et al., 2021; Tye et al., 2021). One major role of BRCA1 and BRCA2 is to facilitate RAD51 loading at DNA lesions, promoting error-free HR DNA repair versus other mutagenic repair pathways. In cancers that contain loss-of-function mutations in *BRCA1* and *BRCA2*, back-up DNA repair pathways exist. One of these pathways is mediated by the PARP family of enzymes, particularly PARP1 and PARP2, which bind to DNA lesions and catalyse the formation ADP-ribosylation signaling to promote DNA repair. Small molecule PARP inhibitors (PARPi) inhibit their ability to produce the ADP-ribose signal, which then traps these enzymes on DNA, as the ADP-ribosylation is also required for their removal from DNA. The PARP trapping causes replication fork collapse upon replisome encounter, which would then require a functional BRCA1/BRCA2 pathway for repair. However, in cancer cells deficient for BRCA1/BRCA2, the lesions resulting from PARP trapping cause irreversible damage that kills the cells (Bryant et al., 2005; Farmer et al., 2005). The seminal findings that inhibition of PARP1/2 in *BRCA1*/*BRCA2* mutated cancer cells selectively kills the cancer cells, but leaves the wild type *BRCA1*/*BRCA2* cells intact, helped define a new epoch in personalised medicine that is already transforming patients' lives. More recently, the last five years have seen the convergence of newly developed specific DDR drugs and CRISPR-Cas9 screening technologies, the result of which has led to rapid progress in mapping the genetic landscape of the mammalian DDR network and identifying novel SL interactions in the DDR.

CRISPR-Cas9 Screens and the DNA Damage Response

The success of the PARP-BRCA SL interaction provided the foundation to investigate whether genetic interactions such as this are rare occurrences, or whether there are other SL interactions that are not only therapeutically attractive but provide novel mechanistic insights into the functionality of the DDR network. Furthermore, it led researchers to ask whether there are other genetic determinants that might enhance the PARP-BRCA SL interaction or cause resistance to it, as resistance is a common occurrence in patients treated with PARPi over extended durations. A major breakthrough came after a genome-wide CRISPR-Cas9 chemogenetic viability screen uncovered additional sensitisers to PARPi in three different cell lines (Zimmermann et al., 2018). A high confidence hit across all cell types was RNase H2, an enzyme that functions within the ribonucleotide excision repair pathway (RER) to remove RNA misincorporated into DNA during DNA synthesis. Mechanistically, loss of RNase H2 within the RER pathway causes lesion processing to channel into a TOP1-dependent pathway. These lesions are then recognised by PARP1/2, which are then subsequently trapped at the lesion by PARPi (Zimmermann et al., 2018). An important lesson from this and other papers is that there are numerous ways the efficacy of PARPi-mediated cell death can be enhanced in cells, with PARP1/2 trapping being a focal point that can be increased both genetically and chemically for maximal cell killing effect.

TABLE 1 | Selected DDR (chemo)genetic interactions.

Screen type	Assay	Genetic background	Genotoxin	Interactor(s)	References(s)
Resistance	Viability	BRCA1 mutant	PARPi	DYNLL1	He et al. (2018)
Resistance	Viability	BRCA1 mutant	PARPi	Shieldin	Dev et al. (2018), Noordermeer et al. (2018)
Sensitisation	Viability	BRCA1 mutant and various	PARPi	RNase H2	Wang et al. (2019), Zimmermann et al. (2018)
Sensitisation	Viability	BRCA1 null BRCA2 null	PARPi	CIP2A	Adam et al. (2021)
Sensitisation	Viability	WT BRCA1 mutant BRCA2 mutant	PARPi	ALC1	Hewitt et al. (2021), Verma et al. (2021)
Sensitisation	Viability	WT	PARPi	HPF1	DeWeirdt et al. (2020), Hewitt et al. (2021)
Sensitisation	Viability	BRCA1 mutant BRCA2 mutant	PARPi	APEX2	Alvarez-Quilon et al. (2020), Mengwasser et al. (2019)
Sensitisation	Viability	Microsatellite instability	-	WRN	Behan et al. (2019), Chan et al. (2019), Lieb et al. (2019)
Sensitisation	Viability	p53	ATRi and MMC	HROB/C17orf53	Hustedt et al. (2019), Wang et al. (2020a)
Sensitisation	Viability	WT	Illudin S and UV	ELOF1	Geijer et al. (2021), van der Weegen et al. (2021)

Another major breakthrough in our understanding of the DDR came with the discovery of the Shieldin complex by multiple groups using various approaches (Dev et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018; Mirman et al., 2018; Noordermeer et al., 2018). Those groups that used genome-wide CRISPR-Cas9 chemogenetic viability screening identified the Shieldin complex with an elegant experimental set-up in which *BRCA1* mutated cancer cell lines or engineered *BRCA1* KO cells, were treated with PARPi at a dose with which the majority of cells are killed (Dev et al., 2018; Noordermeer et al., 2018). However, KO of genes that caused resistance to PARPi would lead to their survival and increased abundance in the population. From here, three previously uncharacterised genes, SHLD1 (C20orf196), SHLD2 (FAM35A), and SHLD3 (CTC-534A2.2), were identified from the CRISPR screens that were then shown to form a complex with REV7, and collectively termed the Shieldin complex. Mechanistically, the Shieldin complex functions downstream of ubiquitin-dependent signaling and the 53BP1-RIF1 axis and binds ssDNA at DSBs via the OB-folds in SHLD2, thereby protecting the DNA ends from BRCA1-mediated resection, RAD51 loading, and engagement of the HR pathway, instead promoting non-homologous end joining (NHEJ) (Dev et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018; Noordermeer et al., 2018). Use of the NHEJ pathway is more error-prone than HR and in BRCA1-deficient cells treated with PARPi causes lethality through erroneous ligation of broken DNA ends. However, upon loss of the Shieldin complex HR is partially restored in BRCA1-deficient cells, promoting cell survival and resistance to PARPi. The partial restoration of HR in BRCA1- and Shieldin-deficient cells was later shown to be dependent on the RNF168 E3 ligase, which recruits the PALB2-BRCA2 complex to load RAD51 at DNA lesions (Zong et al., 2019; Belotserkovskaya et al., 2020; Callen et al., 2020). In addition, microhomology-mediated end joining via POLQ provides an alternative DNA repair pathway in BRCA1- and Shieldin-deficient cells that is crucial for cell survival (Zatreanu et al., 2021).

Expectedly, much effort has been made to fully explore the PARP-BRCA SL interaction for mechanisms of sensitization and resistance, however, a range of other chemogenetic screens have revealed SL interactions in the DDR (**Table 1**). A logical culmination to these chemogenetic screening approaches was presented by the Durocher lab, which performed 31 CRISPR screens using 27 different genotoxic agents (Olivieri et al., 2020). Their results provided the first comprehensive genetic map of the DDR in mammalian cells using monogenic perturbation screens, identifying novel components within a network of around 900 genes that cause sensitivity and/or resistance, further underscoring the inter-relatedness of mammalian DNA repair pathways. Given some of the potential limitations to CRISPR screens noted above, it's likely more factors, especially regulators, of the DDR remain to be uncovered. However, the spectacular progress of this and other chemogenetic screening studies have provided the foundation to move on to combinatorial approaches to dissect gene-gene and gene-gene-drug interactions and beyond.

CRISPR Screens and Ubiquitin Signaling

Beyond the BRCA1-related pathways above, genome-wide CRISPR-Cas9 screens have uncovered novel mechanisms of other ubiquitin-dependent signaling processes, both within the DDR and beyond, with a few selected examples discussed below. The two most common approaches have involved CRISPR-Cas9 screening in viability assays (sensitivity/resistance) or in combination with flow cytometry. Using viability as an endpoint, several groups used chemogenetic screens to identify gene products whose loss caused resistance to centrosome loss via PLK4 inhibition, including TRIM37 and USP28, and therefore identified components of a centrosome surveillance pathway (Fong et al., 2016; Lambrus et al., 2016; Meitinger et al., 2016). Mechanistic follow-ups revealed that this pathway activates p21-dependent cell cycle arrest after centrosome loss or prolonged mitotic progress via USP28-dependent stabilization of p53. These screens also identified the E3 ligase TRIM37 as a hit that functions independently of the above 53BP1-USP28-p53 pathway, with its loss leading to the formation of centrosome-like structures, thereby causing resistance. This finding was then extended to show that PLK4 inhibition is synthetically lethal with TRIM37 amplification, which is found in 17q23-amplified breast cancers

(Meitinger et al., 2020; Yeow et al., 2020). More recently, chemogenetic screens were used to identify regulators of the cellular response to CDK4/6 inhibitors, drugs which are used to treat breast and other cancer types (Chaikovsky et al., 2021; Simoneschi et al., 2021). Both groups identified AMBRA1 as a gene whose loss caused resistance to CDK4/6 inhibition. Further mechanistic investigation revealed that AMBRA1 is part of a Cullin-RING ligase (CRL) four complex, which targets cyclin D for ubiquitination and proteasomal degradation. Thus, in the absence of AMBRA1, cyclin D isn't degraded, promoting cell and tumour growth that is resistant to CDK4/6 inhibition.

In CRISPR-Cas9 screening approaches that have utilised flow cytometry, fluorescent reporters allow the physical separation of cells exhibiting the phenotype of interest. Typically, the fluorescent reporter is linked to a model substrate or a protein of interest, which allows their abundance to be modulated by genetic perturbation of UPS components. This approach has proved powerful for network mapping and identification of new functions for various components of the UPS, including the E3 ligase RNF185 as a novel regulator of a branch of the ERAD pathway (ER-associated degradation) (van de Weijer et al., 2020), USP5 as a positive regulator of m6A deposition by stabilising METTL3 (Sun et al., 2020), and the ubiquitin-conjugating enzyme/ubiquitin ligase BIRC6 as an autophagy regulator (Jia and Bonifacino, 2019). One limitation to this approach is the potential diversity of substrates for a given pathway. However, evidence shows that it's possible to detect distinct and overlapping pathways for substrate degradation using multiple model substrates (Leto et al., 2019).

The UPS system of E2s, E3s, and DUBs, is often targeted in genetic screens using smaller, focused libraries. For example, using mammalian cells expressing a fluorescently linked peptidomic library covering the entire human proteome, Koren et al. employed a small scale CRISPR screen to identify adaptors of the CRL families that regulate the rapid turnover of unstable GFP-peptide fusions (Koren et al., 2018). After identification of the adaptors, sequence analysis revealed that the proteins targeted by these adaptors contain a C-terminal glycine residue, thereby providing evidence for proteasomal degradation via a C-terminal degron. Beyond this example, a focused library of E3s and DUBs was used on a large scale to interrogate the UPS for genes that caused sensitivity or resistance to 41 different compounds, each of which target various cellular pathways (Hundley et al., 2021). An interesting observation from this study was that resistance phenotypes could be assigned as being either truly resistant to a compound or that the compound rescued the slow growing phenotype of the genetic alteration. However, this chemogenetic approach was able to assign new mitotic functions to a range of UPS components such as FBXO42, HUWE1, and UBE3D, and will no doubt provide a rich resource for further mechanistic studies. A potential limitation of using focused UPS libraries is that it relies on all the enzyme families being fully annotated and updated with any recent discoveries. Thus, there may be uncharacterised factors that might be missed through focused screening approaches. Moreover, use of only one cell line may limit discovery of important genetic interactions if UPS components exhibit cell-type specific expression profiles, as was recently shown to be the case for the human DUB family (Pinto-Fernandez et al., 2019).

Genetic Screens and Paralogs Within the Ubiquitin-Proteasome System

Paralogs represent an attractive opportunity to discover new SL targets if the paralogs have maintained some degree of functional relationship. Generally, paralogous genes are less likely to be essential than those genes with no corresponding paralog, suggesting that functional buffering occurs when one of the paralogs is lost, thus paralogs provide 'genetic robustness' (Gu et al., 2003; Koonin, 2005). Combined loss of both paralogs may therefore completely ablate function, providing a rationale for pursuing discovery of paralog specific SL targets. Given that several duplication events occurred during the evolution of the ubiquitin system in eukaryotes that led to the generation of numerous paralogous genes (Burroughs et al., 2012; Koonin and Yutin, 2014; Vlasschaert et al., 2017), it will be important to determine the extent of paralog SL and whether this represents a suitable therapeutic opportunity. Encouragingly, evidence beyond the ubiquitin system suggests that this approach might lead to novel and SL interactions as there are numerous pieces of experimental evidence that have revealed paralog SL interactions (**Table 2**). Furthermore, a computational analysis of over 500 CRISPR screens performed in cancer cell lines suggested that 13–17% of paralog pairs may be SL (De Kegel and Ryan, 2019).

If novel paralog SL interactions are to be discovered in the ubiquitin system, what tools are there to address this? It would be impractical to generate isogenic knockouts of all components of the UPS and perform SL screens in each of them as there are approximately 800 genes. Therefore, combinatorial genetic approaches are required to target at least two loci per cell. Promisingly, a number of these combinatorial approaches have been developed recently, providing scope to address this. The first method, termed CHyMErA, makes use of a Cas9 gRNA and Cas12a (formerly Cpf1) gRNA that are contained within one hybrid gRNA (hgRNA) transcript (Gonatopoulos-Pournatzis et al., 2020). Cells that express both the Cas9 and Cas12a nucleases can cleave the hgRNA due to an inserted Cas12a cleavage site and the RNA cleaving activity of Cas12a. Each gRNA may then direct the corresponding nuclease to its target site. A second approach termed 'anchor screening' uses orthogonal Cas9 enzymes from two different bacterial species, *S. pyogenes* and *S. aureus*, to target two different loci in a two-step method (DeWeirdt et al., 2020). In the first step the *S. aureus* anchor sgRNA is delivered to cells together with *S. pyogenes* Cas9. Next, the *S. aureus* Cas9 is delivered together with the library expressing *S. pyogenes* sgRNAs. Thus, only when both steps have occurred is there any genetic perturbation. This approach also negates the necessity for generating single cell clones before carrying out the screen. Lastly, Cas12a has been optimised for pooled screens with multiplexed libraries, with the ability to target up to three genes (Liu et al., 2019; DeWeirdt et al., 2021). Indeed, CRISPR-Cas12a was recently used to screen 400 potential paralog SL interactions using a

TABLE 2 | Selected paralog genetic interactions.

Identification method	Genetic background	Paralog #1	Paralog #2	References(s)
CRISPR knockout screen	HAP1 (chronic myelogenous leukaemia); PC9 (lung adenocarcinoma); A375, MeWo (both melanoma), and RPE-1 (diploid hTERT immortalised)	ASF1A	ASF1B	Kegel et al. (2021), Parrish et al. (2021), Thompson et al. (2021)
TCGA analysis and hypothesis-driven	HCT 116 (colon cancer); KBM-7 (chronic myelogenous leukaemia) and engineered STAG2 KOs	STAG1	STAG2	Benedetti et al. (2017), van der Lelij et al. (2017), van der Lelij et al. (2020)
Cancer-dependency dataset analysis and CRISPR knockout screen	Cancers with 18q or 16q loss; PC9 (lung adenocarcinoma)	VPS4A	VPS4B	Neggers et al. (2020), Parrish et al. (2021)
CRISPR knockout screen	786-O (renal cell carcinoma), A375, Meljuso (melanoma), A549 (lung adenocarcinoma), HT-29 (colon cancer), OVCAR8 (ovarian cancer)	AKT1	AKT2/3	Najm et al. (2018)
Cancer-dependency dataset analysis	Chromosome 1p loss	MAGOH	MAGOHB	Viswanathan et al. (2018)
shRNA screen	BRG1-deficient cancer cells	SMARCA2	SMARCA4	Hoffman et al. (2014)
CRISPR knockout screen	A549 (lung adenocarcinoma), HT-29 (colon cancer), OVCAR8 (ovarian cancer); A375, MeWo (both melanoma), and RPE-1 (diploid hTERT immortalised)	FAM50A	FAM50B	Dede et al. (2020), Thompson et al. (2021)
CRISPR knockout screen	A549 (lung adenocarcinoma), HT-29 (colon cancer), OVCAR8 (ovarian cancer); Jiyoye (Burkitt's lymphoma), K562 (chronic myelogenous leukaemia), KBM-7 (chronic myelogenous leukaemia), Raji (Burkitts Lymphoma)	RPP25	RPP25L	Dede et al. (2020), Wang et al. (2015)

two-gene approach across 3 cell lines, with 24 SL interactions identified (Dede et al., 2020). Collectively, these combinatorial approaches provide a framework to understand paralog function in mammalian cells. However, when considering paralogs within the UPS, a potential complication is that for most E3 ligases and DUB enzyme classes, there are multiple members, making combinatorial approaches more difficult, but not insurmountable. Specific paralogous pairs to be tested could be stratified and prioritised based on phylogenetic analysis and in combination with various other publicly available datasets.

Interestingly, it's been shown that ubiquitin paralogs are synthetically lethal in high-grade serous ovarian cancer, as well as other uterine and endometrial cancers (Kedves et al., 2017; McDonald et al., 2017). In mammalian cells, ubiquitin is generated from four genes, *UBB*, *UBC*, *RPS27A* and *UBA5*, with *UBB* and *UBC* producing polyubiquitin gene products. The SL interaction between ubiquitin paralogs arises from the high frequency of *UBB* silencing in these gynecological cancers, causing a dependency on *UBC*, despite the presence of *RPS27A* and *UBA52* (Kedves et al., 2017). This finding therefore identifies the *UBC* gene and mRNA as potential therapeutic targets in these cancer types. However, the mechanistic basis for this SL interaction remains to be determined and warrants further investigation – does the loss of *UBC* exert a global impact on cellular processes through exhaustion of the ubiquitin pools, which the authors termed 'ubiquitin catastrophe', or are there other more specific pathways and components whose threshold levels for ubiquitin levels are particularly sensitive and which could be targeted and exploited? Regardless, this reiterates the need for further genetic dissection of the UPS via interrogation of paralog and enzyme class SL interactions.

PROTEOMIC APPROACHES FOR IDENTIFYING DNA DAMAGE RESPONSE AND UBIQUITIN SIGNALING FACTORS

Whilst the developments of CRISPR-Cas9 screening approaches have provided a powerful tool for mapping genetic interactions in both the DDR and other ubiquitin-dependent pathways, advances in proteomic methods have provided the opportunity to further dissect the protein complexes involved in the DDR, together with how these pathways are orchestrated by ubiquitin signaling. Recently, the development of methods including CHROmatin MASS spectrometry (CHROMASS), nascent chromatin capture (NCC), isolation of proteins on nascent DNA (iPOND), and proximity labelling methods have greatly expanded our understanding of the protein complexes involved in the DDR, and provide the potential to understand how ubiquitin signaling shapes repair events at specific lesions.

ChEP and CHROMASS

A number of approaches have been developed in order to capture and analyse chromatin at a proteomic level, also referred to as the "chromatome" (Kustatscher et al., 2014b). Chromatin enrichment for proteomics (ChEP) is a biochemical procedure to enrich interphase chromatin. ChEP uses formaldehyde cross-linking of chromatin proteins to DNA, followed by isolation of cross-linked proteins by centrifugation under denaturing conditions (**Figure 2A**). When coupled with mass spectrometry this approach enables analysis of global chromatin composition (Kustatscher et al., 2014a). An integrated chromatin score, based on over 5,000 proteins, defined as chromatin or non-chromatin associated proteins was used to provide a probability

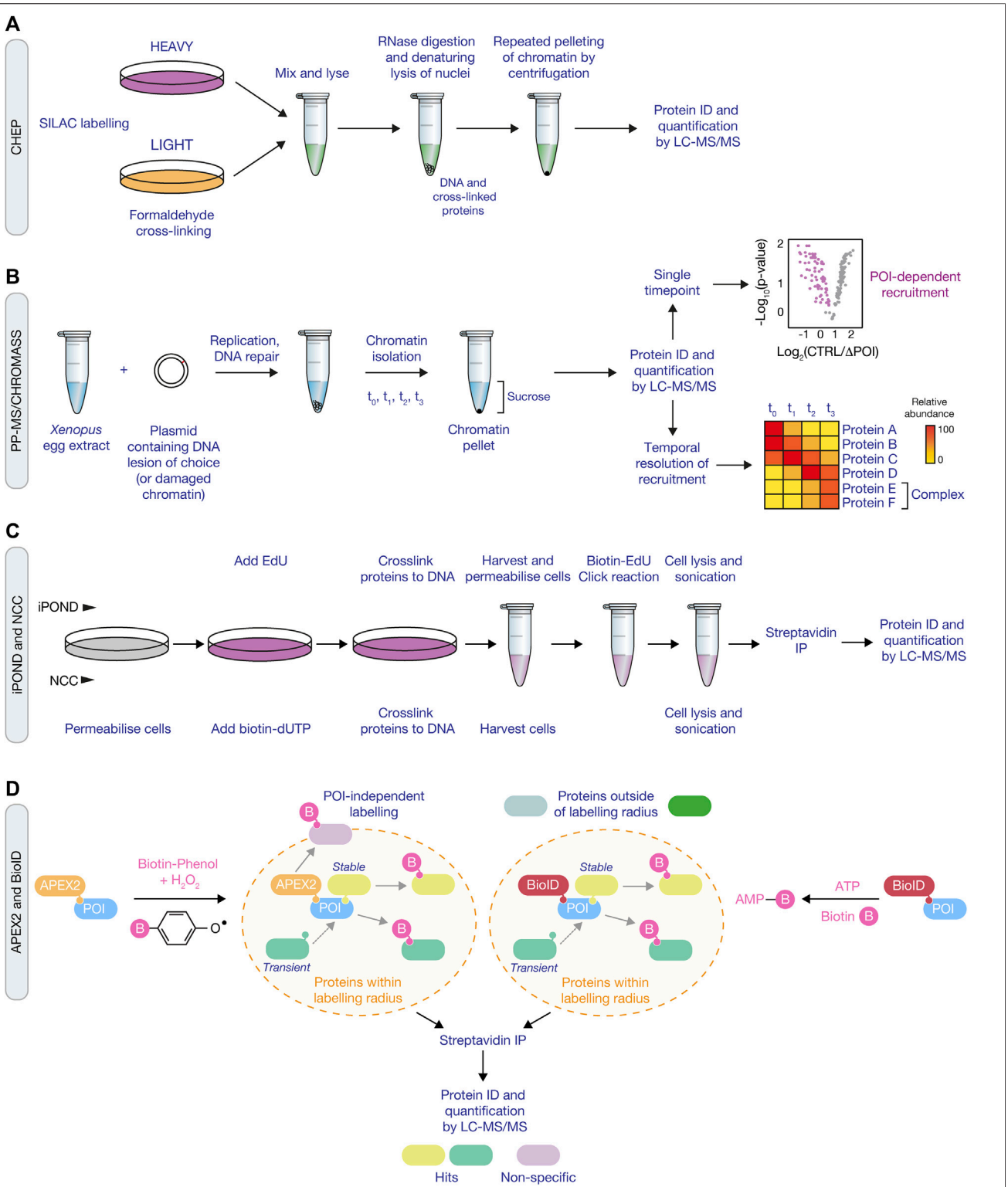

FIGURE 2 | Proteomic approaches for mapping the DDR network and ubiquitin signaling. **(A)** Schematic of the ChEP method, which requires isolation of cross-linked proteins from SILAC-labelled cells to isolate whole chromatin before analysis by LC-MS/MS. **(B)** Overview of the CHROMASS and PP-MS workflows in which damaged chromatin or plasmids with a site-specific DNA lesion, respectively, are incubated with *Xenopus* egg extracts, followed by isolation and subsequent analysis by LC-MS/MS. **(C)** Comparison of iPOND (*top*) and NCC (*bottom*) techniques for identifying factors associated with nascent DNA. iPOND utilises the incorporation of EdU followed by the Click reaction to conjugate biotin to EdU, whilst NCC uses incorporation of biotin-dUTP. **(D)** Schematic of APEX2 ***(left)*** and BioID ***(right)*** proximity

(*Continued*)

FIGURE 2 | labelling methods. APEX2 generates a phenoxyl radical in the presence of biotin-phenol and hydrogen peroxide, resulting in labelling of proximal proteins with biotin. For BioID, biotinylation of proximal proteins uses ATP and is initiated following treatment of cells with biotin to generate biotin-5′AMP to covalently tag proteins. Both approaches use streptavidin pull down to enrich and identify biotyinylated proteins by LC-MS/MS.

score (interphase chromatin probability; ICP) for any given protein to have a chromatin function. ICPs for 7,635 proteins were defined enabling identification of 1840 novel chromatin associated proteins. Whilst ChEP provides a method to globally define chromatin associated proteins, it's limited by DNA damage that induces the recruitment of a small number of molecules to a lesion, thereby making it difficult to ascertain protein complex recruitment above background levels, or if the protein complex relocalises from one chromatin context to another. Moreover, the method doesn't allow locus specific enrichment, which is particularly important for understanding protein dynamics within the DDR as repair events invariably occur within discrete foci, for example, at the replication fork or within ionising radiation-induced foci (IRIF).

An alternative method, termed CHROmatin MASS spectrometry (CHROMASS), was developed to identify proteins that are specifically recruited to damaged chromatin (Raschle et al., 2015). This approach utilises DNA replication and repair competent *Xenopus* egg extracts that are incubated with psoralen crosslinked chromatin, followed by chromatin isolation and label-free mass spectrometry to identify proteins bound to the DNA (**Figure 2B**). To determine recruitment kinetics, chromatin can be isolated at regular intervals to provide a temporal map of the dynamic recruitment of proteins to damaged chromatin. In the first example of its use with chromatin containing psoralen interstrand crosslinks, the authors identified a number of novel DDR factors, including SLF1 and SLF2 (Raschle et al., 2015). Further investigation found that SLF1 and SLF2 form a complex with the ubiquitin E3 ligase RAD18, to promote the ubiquitin-dependent recruitment of the SMC5/6 complex to DNA lesions. CHROMASS has also been used to identify factors recruited to chromatin when DNA replication termination is blocked, including various components of the ubiquitination machinery involved in this process, such as Lrr1 and p97/VCP (Dewar et al., 2017).

The CHROMASS method has since been adapted to investigate protein recruitment dynamics and global analysis of post translational modifications in response to plasmids harboring specific DNA lesions, termed 'plasmid pull-down with quantitative high-resolution mass spectrometry (PP-MS)'. This approach improves the resolution of the temporal dynamics of protein recruitment versus non-specifically damaged chromatin, and allows for the investigation of specific replication-associated events or lesion-specific DNA repair pathways. As an example, PP-MS was used to map protein recruitment dynamics in response to a defined DNA-protein crosslink (DPC), using a plasmid containing a covalently bound DNA methyltransferase (Larsen et al., 2019). The PP-MS approach identified the SPRTN protease and the recruitment of the proteasome to the DPC, the latter being shown through follow-up studies to be dependent upon TRAIP-dependent polyubiquitination of DPCs, underlining the power of PP-MS as a discovery tool upon which further mechanistic studies can be based (Larsen et al., 2019; Wu et al., 2021). Mechanistic follow-ups are underpinned by the ability to specifically deplete the identified factors from *Xenopus* egg extracts using antibodies. This powerful approach will continue to provide novel insights into various DDR pathways and their regulation by ubiquitin signaling. For example, it may help define the factors and signaling involved in a newly described pathway that repairs acetaldehyde-induced DNA lesions (Hodskinson et al., 2020). Furthermore, as genome editing approaches in *Xenopus* become more robust, this previously genetically intractable system will become amenable to targeted reverse genetics. Further discussion on the *Xenopus* system as a biochemical tool is featured in **Section 4** below.

Nascent Chromatin Capture

Nascent chromatin capture (NCC) was developed to analyse changes in the chromatin proteome of mammalian cells by monitoring biotin-dUTP incorporation of replicating DNA (Alabert et al., 2014). For this approach, cells are released from a single thymidine block and labelled with biotin-dUTP in early/mid-S phase for a short time (5 min) before fixation in formaldehyde to capture the nascent chromatin or chased for 2 h before fixation to capture the mature chromatin. Nuclei are then isolated using a sucrose buffer and chromatin is solubilised by sonication followed by enrichment of biotinylated chromatin by streptavidin beads (**Figure 2C**). To quantify the composition of nascent and mature chromatin, NCC was combined with stable isotope labelling using amino acids in cell culture (SILAC) to profile 3,995 proteins, providing a comprehensive analysis of proteins enriched in nascent, mature or both nascent and mature chromatin. Moreover, by combining NCC enrichment with a ChEP chromatin probability score, as described above, a chromatin function for 93 uncharacterised proteins was proposed. In an extension to this, NCC-SILAC was recently used to profile protein recruitment in response to different types of DNA replication stress: fork breakage by camptothecin (CPT) or fork stalling by hydroxyurea (HU) (Nakamura et al., 2021). A comparison of the replication fork-associated proteomes identified three classes of replication fork repair factors, with class I and class II factors recruited only in CPT or HU, respectively, and class III factors enriched with both CPT and HU treatment. Class I included DSB (ATM) and HR (CtIP) factors together with PLK1, and class II included factors with known functions in ubiquitin signaling at DSBs, such as RNF168 and RNF169, and the BRCA1-A, FANCI:FANCD2, and SMC5/6 complexes. These findings highlight that NCC-SILAC is capable of detecting distinct fork protein compositions between broken and stalled forks, uncovering novel DDR factors and signaling mechanisms.

iPOND

Isolation of proteins on nascent DNA (iPOND) is a method that enables purification of newly replicated DNA and its associated proteins from mammalian cells using incorporation and purification of the thymidine analogue 5-ethynyl-2′-deoxyuridine (EdU) (Sirbu et al., 2012). EdU contains an alkyne functional group permitting cycloaddition to a biotin azide by click chemistry, which tethers biotin to the newly synthesised DNA. Following EdU labelling, cells are treated with formaldehyde to cross-link protein–DNA complexes. Cells are then lysed in denaturing buffer and sonicated to fragment the DNA, resulting in solubilised DNA-protein complexes. Biotin enables isolation of DNA-protein complexes using streptavidin affinity purification and detection by immunoblotting or mass spectrometry (**Figure 2C**). In the first application of this method, iPOND identified a number of replisome components including PCNA and CAF-1 after 2.5 min of EdU labelling (Sirbu et al., 2011). The detection of histones H2B and H3 after 5 min, and linker histone H1 at 20 min following EdU labelling demonstrated that the temporal resolution of iPOND is regulated by EdU incorporation time. Therefore, longer labelling times enable analysis of newly deposited chromatin and assembly, whereas short labelling times capture the components at the replication fork. In a subsequent study, iPOND was used to investigate how replication fork associated proteins are dynamically regulated in response to replication stress. For this, cells were treated with HU or HU and ATR (ATR serine/threonine kinase) inhibitor, to assess how the replisome is impacted by the replication checkpoint (Dungrawala et al., 2015). Proteomic analysis revealed that the collapse of stalled forks which trigger checkpoint activation are distinct from the collapse of forks that start from aberrantly fired origins following inhibition of the replication checkpoint. In addition, novel replisome-associated proteins were identified, including ZNF644 which forms a complex with the G9a/GLP methyltransferase at replication forks. Thus, iPOND is another approach capable of detecting distinct fork protein compositions in response to different DNA lesions, helping to reveal novel factors and their temporal dynamics at the replication fork.

The development of NCC and iPOND methods have both made major contributions to our understanding of the composition of DNA replication forks and mature chromatin in mammalian cells, in both unperturbed conditions and in response to replication stress. However, there are some potential pitfalls that offer avenues for further improvement. Both iPOND and NCC approaches utilise incorporation of a modified DNA base with either EdU for iPOND or biotin-dUTP for NCC. Both of these approaches assume that the modified base is not recognised as DNA damage and assume that the modified base does not affect binding of proteins to the DNA. In longer-term assays, incorporation of either biotin-dUTP or EdU may result in decreased proliferation and increased DDR signaling, indicating that the modified base could impact normal protein recruitment dynamics (Cortez, 2017). The development of native iPOND without formaldehyde cross-linking may circumvent detection issues associated with using formaldehyde and potentially help to provide better access to the labelled DNA by improving efficiency of the click reaction. Moreover, improvements in the efficiency of the click reaction for iPOND and capture of the labelled DNA may help to increase retrieval of proteins at the replisome. Probably the biggest issue with these methods is that even a 10 min pulse of EdU or biotin-dUTP will label, at the very least, approximately 10 kb of DNA in mammalian cells, suggesting that a significant amount of purified DNA will derive from post-replicative DNA. Thus, future approaches might seek to remove as much of the post-replicative DNA as possible. In turn, by increasing the sensitivity and specificity of replisome isolation, it should then be possible to couple such an approach with a secondary purification strategy for PTMs such as ubiquitin, which will not only allow identification of replisome components, but also uncover novel replisome-associated ubiquitin signaling events that have so far remained elusive. Lastly, the recent progress of inducible protein degradation systems for mammalian cells, such as the AID or dTAG systems, provide the tools to deplete DDR and ubiquitin factors in minutes to hours, drawing mammalian approaches closer to the power of the *Xenopus* system when combined with the proteomic approaches described here (Nabet et al., 2018; Yesbolatova et al., 2020).

APEX2 and BioID

The approaches discussed above rely on stable associations between protein complexes and DNA, which may preclude identification of proteins that only transiently interact with the replisome or are poorly expressed in the cell. As such, proximity labelling may provide an alternative approach to map factors that only transiently interact with the replication fork. APEX, or the more recently developed APEX2, is an engineered peroxidase derived from plant ascorbate peroxidases that can be targeted to a specific subcellular compartment or to a protein of interest. In the presence of biotin-phenol, APEX generates a reactive phenoxyl radical when treated with a pulse of hydrogen peroxide (**Figure 2D**) (Lam et al., 2015; Hung et al., 2016). This enables the covalent tagging of biotin with nearby nucleophilic electron-rich amino acids such as Tyr (>95%), Trp, His, and Cys of interacting or neighbouring proteins within a small 10–20 nm radius. Biotinylated proteins can then be enriched by streptavidin beads and identified by mass spectrometry. Demonstrating the application of this strategy, Gupta et al. endogenously tagged 53BP1, BRCA1, and MDC1 with APEX2 to generate interaction maps for each of these key DDR factors, which resulted in the identification of the Shieldin complex, the function of which was described above (Gupta et al., 2018).

In addition of the APEX proximity approach, BioID (Biotin IDentification) is a promiscuous mutant of the *E. coli* biotin ligase which can also be used to biotinylate proximal proteins (Roux et al., 2012). In this system, only biotin needs to be supplied to catalyse formation of biotin-5′-AMP anhydride and initiate covalent tagging, preferentially targeting lysine residues (**Figure 2D**). However, slow kinetics require biotin labelling for 18–24 h to produce sufficient biotinylated material for proteomics. As a result, two variants were identified that could reduce labelling times to 10 min, namely TurboID, a 35 kDa

variant with 15 mutations relative to WT BioID, and miniTurbo, a 28 kDa with the N-terminal domain deleted and 13 mutations relative to WT BioID (Branon et al., 2018). Split versions of APEX2 and TurboID have also been developed in which two inactive fragments of the labelling reporters become activated when they physically interact (Han et al., 2019; Cho et al., 2020a; Cho et al., 2020b). Each fragment can be driven together when engineered to detect a specific protein-protein interaction or organelle contact and can provide higher targeted specificity relative to full length enzymes.

Both APEX and BioID strategies are appealing for mapping potential enzyme-substrate interactions involved in ubiquitin signaling, which has proven difficult historically using traditional affinity purification approaches, with a few reports showing promise (Coyaud et al., 2015; Bakos et al., 2018; Dho et al., 2019). However, the use of a 28 or 35 kDa labelling tag may interfere with localisation or function of the bait protein. In addition, bias is generated from the number and accessibility of the targeted amino acid residues of the interacting proteins, and therefore the level of biotinylation does not necessarily correspond to the strength of the association. In addition, the use of hydrogen peroxide at 1 mM for 1 min for proteomic studies to generate the reactive phenoxyl radical by APEX will inactivate DUBs and cause oxidative damage, which could have implications for activation of DNA repair pathways. Despite the improved labelling times (reduced to 10 min) with TurboID and miniTurbo, the reported self-biotinylation of the bait the protein may have some impact and limit accessibility to the full repertoire of interacting proteins (Branon et al., 2018). For both proximity labelling approaches the inclusion of various technical and biological controls, such as cellular spatial references, is essential to determine the specificity of the labelling, as they both suffer from high numbers of false positives from random spatial associations that occur with the bait protein (Lobingier et al., 2017; Go et al., 2021).

MASS SPECTROMETRY AND CHEMICAL APPROACHES FOR DECODING UBIQUITIN SIGNALING

As noted above, further improvements in the ability to purify specific structures from mammalian cells in which DNA repair processes are actively being carried out, such as the replisome or IRIF, will pave the way for better sensitivity and specificity of the factors involved and their temporal changes following DNA damage. Furthermore, combining these approaches with the recent advances in ubiquitin mass spectrometry (MS) techniques and chemical biology approaches described below, will be vital for revealing the deep level of DDR regulation by ubiquitin signaling.

Ubiquitin Site Profiling

Developments in MS methods over the past decade have significantly advanced our understanding of the complex and diverse nature of ubiquitin signaling in cells, as well as the enzymatic machinery responsible (Vere et al., 2020). Prior to these advances the ability to detect ubiquitinated sites relied on expression and enrichment of tagged ubiquitin from cells. For example, expression and enrichment of His-tagged ubiquitin from *S. cerevisiae* allowed MS-based detection of ubiquitinated peptides after identification of the signature di-glycine (K-GG) remnant of ubiquitin, which remains covalently attached to the target lysine after trypsinisation (Peng et al., 2003). In this study, 72 ubiquitinated proteins were identified with 110 ubiquitination sites, including identification of modifications on ubiquitin at lysine residues. Since this study, tagged ubiquitin variants have been used to identify ubiquitinated substrates in mammalian cells, however, they have suffered from an inability to conclusively identify the specific ubiquitination sites, hampering further mechanistic studies from these datasets (Kirkpatrick et al., 2005; Tagwerker et al., 2006; Danielsen et al., 2011; Oshikawa et al., 2012).

A major breakthrough for detecting ubiquitination sites came with the generation of an antibody against the resulting K-GG remnant after tryptic digestion of ubiquitin (**Figure 3A**) (Xu et al., 2010). Utilisation of the K-GG antibody increased the detection of ubiquitinated peptides to 19,000 on approximately 5,000 proteins (Kim et al., 2011). Furthermore, application of this new tool within a DDR context revealed the widespread global extent of DNA damage-driven ubiquitin signaling, as well as leading to novel ubiquitin-dependent repair mechanisms at a single protein level (Povlsen et al., 2012; Elia et al., 2015). Whilst the number of identified ubiquitinated sites is increased by ubiquitin peptide level enrichment relative to protein level enrichment, the K-GG remnant is also present following tryptic digestion of the UBLs NEDD8 and ISG15. In addition, it has been reported that the K-GG antibody has certain amino acid preferences near the modified lysine and also fails to detect N-terminally ubiquitinated proteins (Wagner et al., 2012). In an attempt to circumvent these issues, an antibody was generated that detects the 13 residues at the C-terminus of ubiquitin that remain attached to modified peptides following LysC digestion (Akimov et al., 2018). This approach, termed UbiSite, is specific to ubiquitin and can also detect N-terminal ubiquitination sites. Following sequential LysC and trypsin digestion, UbiSite enabled identification of over 63,000 unique ubiquitination sites on 9,200 proteins in two human cell lines. This approach profiled ubiquitinated proteins of diverse function and localisation and did not show preference for amino acids near the modified lysine, indicating an improved strategy for unbiased identification of ubiquitination sites. Recently, data-independent acquisition (DIA) has been gaining momentum as an alternative approach to extract peptide fragment information from mass spectrometry. DIA continuously acquires both MS1 and MS2 spectra without any bias to precursor ions, unlike data-dependent acquisition (Ludwig et al., 2018). Use of DIA in combination with the K-GG antibody has provided yet further depth in precisely and accurately quantifying ubiquitination sites, highlighting its use as a major future tool in understanding ubiquitin signaling in the DDR at unprecedented detail (Hansen et al., 2021), especially when combined with enrichment strategies discussed above.

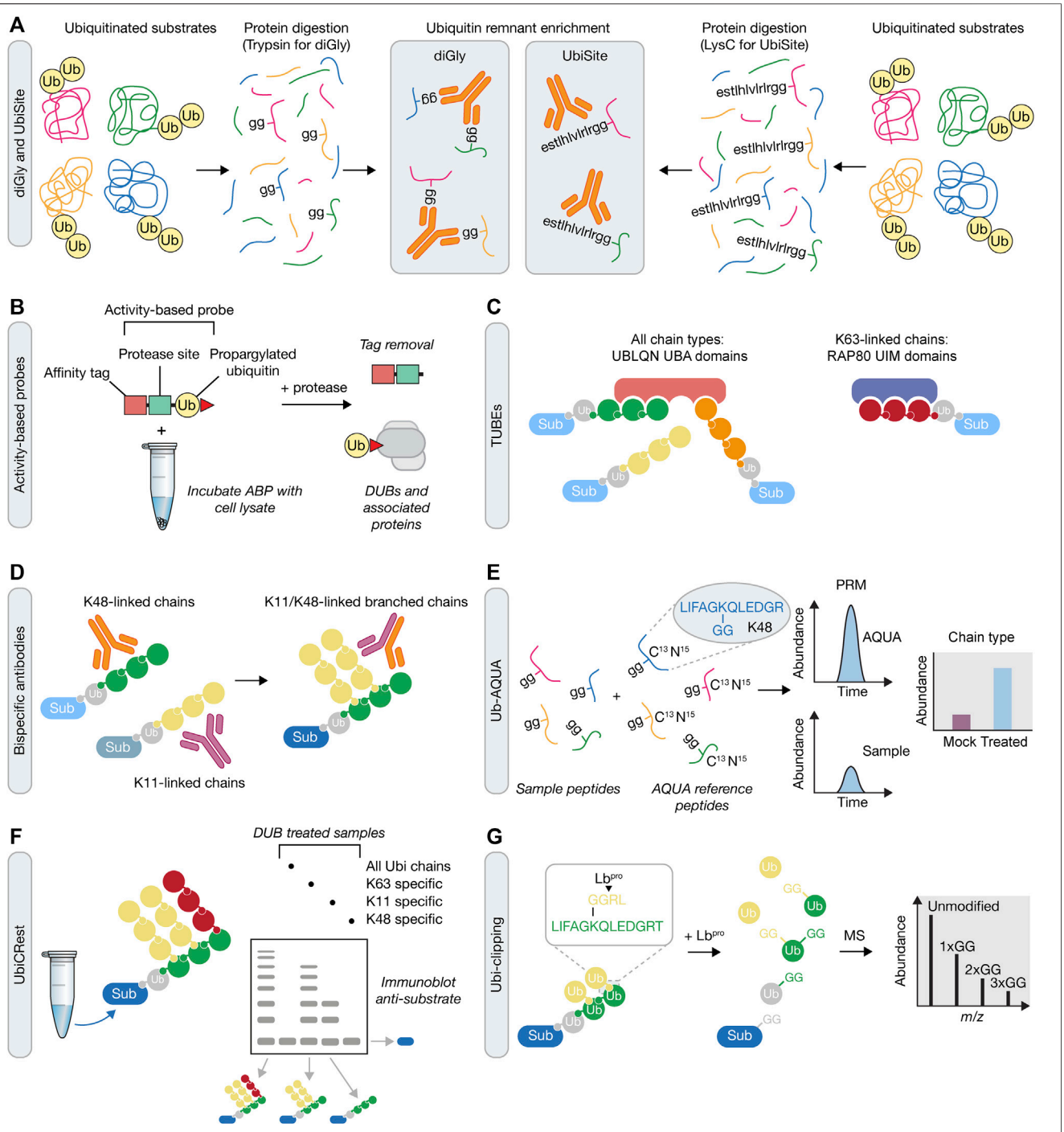

FIGURE 3 | Mass spectrometry approaches for understanding ubiquitin signaling. **(A)** Prior to mass spectrometry, ubiquitin remnants can be enriched following either trypsin or LysC protein digestion. These approaches rely on antibodies that recognise either the di-glycine (diGly) remnant peptide following tryptic digestion of ubiquitin using the K-GG antibody ***(left)***, or a longer sequence that is recognized by the UbiSite antibody ***(right)***. **(B)** Activity-based probe (ABP) tools in the UPS. Irreversible reactivity of the ABP with an enzyme's active site, in this case a DUB, enables purification of the probe-enzyme complex, prior to downstream use, e.g., for mass spectrometry or crystallisation. **(C)** Tandem Ubiquitin Binding Entities (TUBEs) contain a number of specific ubiquitin binding domains that allow for the capture of polyubiquitinated proteins. TUBEs can be designed to capture all ubiquitin chain types (e.g., four UBA domains from UBLQN) or specific ubiquitin chain types (e.g., three UIM domains from RAP80 that bind K63-linked chains). Sub, substrate. **(D)** In contrast to single ubiquitin linkage-specific antibodies ***(left)***, bispecific antibodies ***(right)*** contain arms from each of these two antibodies that can be used to determine formation of branched ubiquitin chains in downstream assays, such as

(*Continued*)

FIGURE 3 | immunofluorescence, immunoblotting or mass spectrometry. **(E)** Ub-AQUA allows for absolute quantification of ubiquitin linkages of trypsin digested samples that have been spiked with heavy-labelled reference peptides and subsequently detected by multiple reaction monitoring or parallel reaction monitoring. **(F)** UbiCREST uses a panel of linkage-specific DUBs to treat ubiquitinated samples to provide a qualitative gel-based method to assess substrate ubiquitin chain architecture. **(G)** Ubi-clipping uses the Lb^{pro} viral protease to cleave ubiquitin after R74 which, when combined with middle-down MS, provides a quantitative approach for detecting branched ubiquitin linkages.

ABPs

In order to investigate the ubiquitin conjugation and removal activities within a cell, chemical-based approaches have been developed, referred to as activity-based protein profiling (ABPP) (Hewings et al., 2017). This method utilises activity-based probes (ABPs) that mimic an enzyme's substrates and which become covalently attached to enzyme active sites. ABPs can therefore help determine enzyme activity, which can be applied to study the biological function of components of the UPS on a global proteome-wide scale (An and Statsyuk, 2016; Byrne et al., 2017; Mulder et al., 2016; Pinto-Fernandez et al., 2019). ABPs generally consist of an epitope tag for isolation of labelled proteins, a recognition substrate such as ubiquitin, and an electrophilic warhead that reacts irreversibly with the catalytic residues of the enzyme (**Figure 3B**). The development of ABPs with different thiol reactive groups has proved particularly valuable in the profiling of DUB activity. Initial profiling of a DUB using an ABP was performed using ubiquitin vinyl sulfone (UbVS), which identified USP14 as a proteasome-associated DUB (Borodovsky et al., 2001). Subsequent ABP designs have demonstrated that the electrophile used imparts reactivity towards different DUBs (Borodovsky et al., 2002). Propargylated ubiquitin (Ub-Prg) can react with cysteine residues in the DUB active site forming a vinyl thioether linkage and providing a selective cysteine DUB ABP (Ekkebus et al., 2013). The application of ABPs in understanding the DDR is highlighted by the recent identification of ZUP1, the founding member of a novel class of DUBs (Hewings et al., 2018; Kwasna et al., 2018). In these studies, Ub-Prg was incubated with mammalian cell lysates followed by mass spectrometry to identify cysteine-based DUBs. ZUP1 was readily modified by Ub-Prg, with subsequent analysis confirming that it as an active DUB with specificity for cleaving K63-linked polyubiquitin and a function in maintaining genomic stability (Haahr et al., 2018; Hermanns et al., 2018; Hewings et al., 2018; Kwasna et al., 2018). With ABPs against E1 (An and Statsyuk, 2016), E2 (Mulder et al., 2016), E3 (Byrne et al., 2017) and DUB (Hewings et al., 2017) enzymes, there is now an extensive toolkit with which to analyse temporal activity changes in response to DNA damage when coupled to mass spectrometry and sample multiplexing methods, such as SILAC and tandem mass tagging (TMT).

TUBEs and Bispecific Antibodies

Often the low stoichiometry of ubiquitination on target proteins makes it difficult to detect the ubiquitinated form from cell lysates. As such, there is a requirement for an enrichment step prior to mass spectrometry or other downstream analytical methods, such as immunoblotting. Coupled to this, there is also a need to purify endogenously ubiquitinated proteins, rather than rely on over-expression of ubiquitin. A tool that addressed both these requirements was the development of Tandem Ubiquitin Binding Entities (TUBEs), which are synthetic constructs that contain multiple UBDs. TUBEs were initially based on the tandem repeated ubiquitin associated (UBA) domains from Ubiquilin and HR23A (Hjerpe et al., 2009). The combination of UBA domains increases the affinity for polyubiquitinated proteins which, when combined with an epitope tag, provides an enrichment strategy to purify ubiquitin chains before MS-based methods (see below) (**Figure 3C**). Design of the TUBEs can be further modified to capture specific polyubiquitin linkages using linkage-specific ubiquitin binding domains, such as the ubiquitin interacting motifs (UIMs) from RAP80 that bind K63-linked ubiquitin chains (Sims et al., 2012; Mattern et al., 2019). A modified form of the TUBE is the trypsin resistant (TR)-TUBE, which can be expressed in cells to prevent the action of DUBs and proteasomal degradation by acting as a 'molecular shield' on the polyubiquitinated chains, providing improved characterisation of the numerous ubiquitination events occurring under steady state conditions (Yoshida et al., 2015). Alternatively, recombinant TR-TUBEs can be used to determine the length and composition of ubiquitin chains purified from cell lysates in combination with MS-based approaches (Ub-AQUA-PRM – see below), in a method termed Ub-ProT (Tsuchiya et al., 2018).

A broader range of linkage-specific TUBEs is limited by several challenges. Perhaps most importantly, for the less well studied atypical and heterotypic chain types, there is a paucity of data about the readers of these chain types and hence the UBDs, or combination of UBDs, that could be leveraged in a TUBE. This difficulty is in part also linked to the ability to distinguish between low affinity and indirect binders of different ubiquitin chain topologies. Thus, by closing these knowledge gaps, the available tools should expand concomitantly, allowing the development of a full repertoire of TUBEs that may then be used to explore the biological function of different ubiquitin chain types and architectures.

Over the last few years, there has been a growing understanding of the functional importance of heterotypic ubiquitin chains in multiple cell processes (Haakonsen and Rape, 2019; French et al., 2021). This understanding has been underpinned by recent developments in strategies for analysing branched ubiquitin chains. One such approach engineered ubiquitin to include a TEV cleavage site after either Gly55 or Glu64, or both (Meyer and Rape, 2014). After substrate modification with these ubiquitin variants, subsequent

incubation with TEV would collapse the modified forms of the substrate if the attached ubiquitin chains were branched. A second approach engineered a cell line in which an R54A ubiquitin mutant is expressed with simultaneous shRNA-based removal of endogenous ubiquitin. The R54A ubiquitin mutant removes a tryptic cleavage site, enabling quantification of unbranched K48 linkages, unbranched K63 linkages, and K48/K63 branched linkages by MS-based approached (Ub-AQUA-PRM). This approach, which may also utilise TUBEs for prior enrichment, provided evidence that heterotypic ubiquitin chain formation is dependent on collaboration between distinct E3 ubiquitin ligases, with K48/K63 chain production being mediated by TRAF6 and HUWE1, or ITCH and UBR5 (Ohtake et al., 2016; Ohtake et al., 2018).

Another breakthrough tool used to analyse heterotypic ubiquitin chain formation was the development of a bispecific antibody that detects K11/K48-linked chains (**Figure 3D**) (Yau et al., 2017). The bispecific antibody was generated from known sequences of K11- and K48-specific antibodies, using knobs-into-holes technology. After extensive validation steps, the authors used the bispecific antibody to analyse the products of the E3 anaphase promoting complex (APC/C) to confirm previous biochemical results demonstrating that it is capable of producing K11/K48-linked chains. The authors then used this bispecific antibody to identify a protein quality control pathway that functions in response to proteotoxic stress, via K11/K48-linked chain formation, with a range of factors identified, including BAG6, UBR5, HUWE1, and p97/VCP (Yau et al., 2017). In the future, production of such bispecific antibodies will be invaluable to help further understand the cellular function of other heterotypic ubiquitin types, such as K48/K63, especially in response to DNA damage, where it could be used to identify both substrates and regulators. Moreover, beyond bispecific antibodies that recognise heterotypic ubiquitin chain types, it may also be possible to generate bispecific antibodies that couple recognition of a specific ubiquitin chain type and recognition of the substrate itself, thereby generating an antibody that recognizes the ubiquitinated form of the substrate.

Ub-AQUA, UbiCRest, UbiChEM and Ubi-clipping

To analyse ubiquitin chain types via bottom-up approaches, the ubiquitin-AQUA (absolute quantification of ubiquitin) method was developed to provide quantitative analysis of ubiquitin chain linkages by mass spectrometry (Kirkpatrick et al., 2006; Phu et al., 2011). This approach uses heavy labelled synthetic internal standard peptides to quantify the abundance of different ubiquitin tryptic peptides using selected reaction monitoring (SRM). More recently, multiple reaction monitoring (MRM) or parallel reaction monitoring (PRM) approaches can be used to improve the sensitivity of ubiquitin chain type detection (Ordureau et al., 2015). The Ub-AQUA method can be integrated into various proteomics workflows, including the prior enrichment of targets or chain types using TUBEs or linkage-specific antibodies. However, Ub-AQUA cannot be used to determine ubiquitin chain length, nor can it be used to quantify the abundance of heterotypic ubiquitin chains.

As an approach to overcome this obstacle, complex ubiquitin chain types can be analysed using linkage specific DUBs in an assay termed Ubiquitin Chain Restriction (UbiCRest) (Hospenthal et al., 2015). By using a panel of DUBs with known linkage specificities, ubiquitinated samples can be subjected to DUB assays and subsequent gel or immunoblot analysis using linkage specific antibodies to provide qualitative information on ubiquitin chain architecture (**Figure 3E**). Moreover, UbiCRest may also be used in conjunction with Ub-AQUA to quantitatively assess the linkage types in the products of the DUB reactions (Harris et al., 2021).

The application of middle-down mass spectrometry approaches in which ubiquitin is subject to restricted trypsin digestion under native conditions has proven applicable to detecting complex ubiquitin chain architectures (Valkevich et al., 2014; Ohtake et al., 2019). This method, termed ubiquitin chain enrichment middle-down mass spectrometry (UbiChEM-MS), is based on the observation that minimal trypsin digestion after position R74 liberates ubiquitin monomers with a GG motif attached at a lysine previously engaged in chain formation. As branching requires the addition of multiple ubiquitin subunits then minimal trypsin digestion will generate two or more GG motifs at lysine residues. Therefore, with chain branching, at least three distinct species would be observed by mass spectrometry, including mono-ubiquitin (Ub) at the ends of a chain, singly modified ubiquitin (^{GG}Ub) within the linear chain, or doubly modified ubiquitin (^{2xGG}Ub) at branch points. When this approach is combined with linkage-specific antibodies, the abundance of branching at the defined linkage can be defined. This approach was used to detect branching using a K11 specific antibody and demonstrated the formation of K11/K48 branches in response to proteasome and DUB inhibition (Rana et al., 2017).

In an alternative strategy to quantify branched ubiquitin chains, a method termed Ub-clipping was recently developed (Swatek et al., 2019). This method took advantage of the observation that the viral protease Lb^{pro} cleaves ubiquitin after R74, leaving ubiquitin with a GG remnant on a target substrate. More than one GG remnant indicates a branchpoint in the ubiquitin chain and can provide information on the polyubiquitin architectures by intact MS analysis (**Figure 3F**). To counter the effect of free unassembled monoubiquitin influencing the chain composition, a TUBE was used to remove monoubiquitin prior to Lb^{pro} treatment. This study quantified 10–20% of ubiquitin polymers existing as branched chains across 3 cell types, indicating that a substantial amount of branched ubiquitin can occur in cells.

In summary, there's now a number of powerful MS-based tools that will provide the opportunity to identify and quantify changes in ubiquitin chain architecture at a much deeper mechanistic level. For the DDR, this promises to uncover novel components of ubiquitin signaling, site-specific changes in response to DNA damage, the dynamic changes of ubiquitin

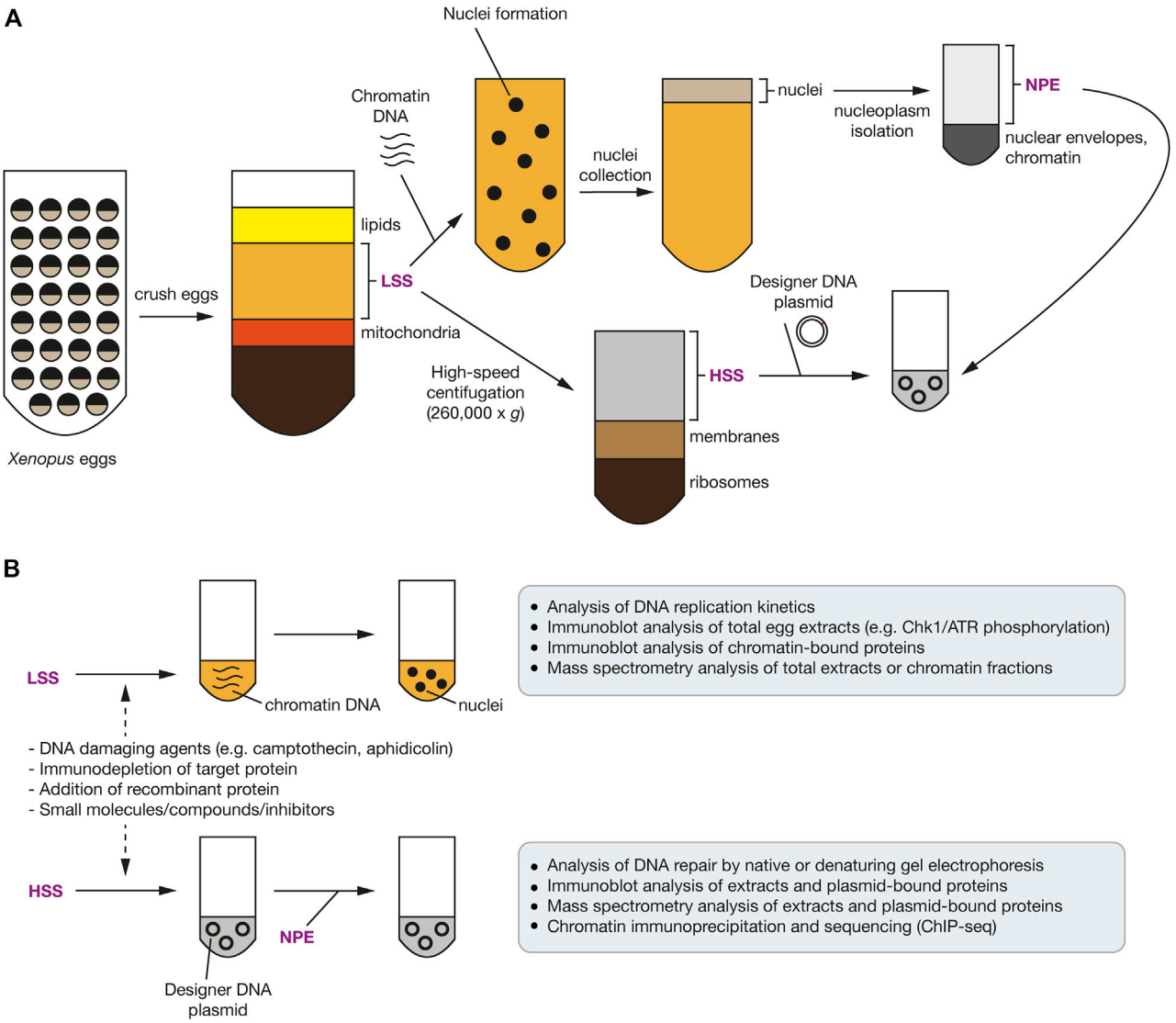

FIGURE 4 | Use of *Xenopus* egg extracts to investigate the DDR and ubiquitin signaling. **(A)** Schematic representation of the common steps involved in preparing *Xenopus* egg extracts. Unfertilised eggs are crushed and a low-speed centrifugation of the crude extract produces cytoplasmic extract including membranes (LSS). High-speed centrifugation produces cytoplasmic extract without membranes or ribosomes (HSS). Incubation of the LSS with sperm chromatin, in the presence of ATP, induces nuclear envelope formation. Nucleoplasm (NPE) can be isolated by centrifugation after isolating these nuclei. **(B)** Upon addition of sperm chromatin DNA to the LSS, nuclei form and chromatin DNA undergoes replication. Addition of DNA damaging agents can be added to activate the DDR. Plasmid DNA with a defined site-specific DNA lesion, such as a replication barrier or ICL, can be added to the HSS, which is further supplemented with the NPE to stimulate DNA replication and repair of the site-specific DNA lesion. In both systems, a protein-of-interest can be immuno-depleted with antibodies and rescued using recombinant wild-type or mutant proteins.

chain architecture, and how chain architecture promotes genome stability.

BIOCHEMICAL AND STRUCTURAL APPROACHES FOR UNDERSTANDING THE DNA DAMAGE RESPONSE AND UBIQUITIN SIGNALING MECHANISMS

A limiting factor for dissecting the precise mechanisms of ubiquitin signaling in DNA repair is the production of physiologically relevant, purified, and uniform components. In contrast, many of the key phosphorylation-dependent signaling components involved in DNA repair such as the PIKK-family kinases of ATM, ATR, and DNA-PK have been thoroughly investigated to reveal their mechanisms of action (Deshpande et al., 2017; Jansma et al., 2020; Chen S. et al., 2021; Chen X. et al., 2021; Chaplin et al., 2021; Hepburn et al., 2021; Tannous et al., 2021). Due to the complexity of ubiquitin signaling and the difficulty in producing specific and uniformly ubiquitinated proteins, there have been discrepancies in assigning the function of particular ubiquitin signals. In recent years, however, the development of techniques for *in vitro*

biochemical reconstitution and the rapid expansion of methods for the structural investigation of these multi-factor assemblies has enabled greater consensus for the function of ubiquitin modifications in genome stability.

Model *In Vitro* Systems

As described in the previous two sections, genetics, cell biology and cellular biochemistry have helped reveal the protein factors involved in the DDR, but the complexity of these systems limits the elucidation of mechanistic details of their activities and the pathways they are involved in. To circumvent this complexity, cell-free or reconstituted systems have been developed to enable greater control and design over the factors present and the types of signaling to occur.

Xenopus egg extracts have been used to study a variety of complex signaling pathways including DNA replication and termination, apoptosis, mitosis, and DNA repair mechanisms (Cupello et al., 2016; De Robertis and Gurdon, 2021; Gillespie et al., 2012; Hoogenboom et al., 2017; Willis et al., 2012). Many of the factors involved in mammalian DNA replication and repair are highly conserved in *Xenopus*, making this an excellent model system. Egg extracts from *Xenopus laevis* contain a high concentration of the factors required for proficient DNA replication and repair without needing the tailored production of all components, with addition of the low-speed supernatant (LSS) to demembranated sperm chromatin resulting in a complete round of DNA replication (**Figure 4A**). The requirement for membrane formation can be limiting in some cases, so alternatively, sequential addition of the high-speed supernatant (HSS) to DNA, such as plasmid DNA, followed by the highly concentrated nucleoplasmic egg extract (NPE) can trigger replication initiation in a synchronous manner. Such fine synchronisation and control over replication timing can be difficult in a cell culture setting. Furthermore, chemical perturbation of DNA replication and repair and ubiquitin signaling can be investigated with the treatment of egg extracts with compounds such as camptothecin (Topoisomerase I inhibitor) or aphidicolin (DNA polymerase α inhibitor) (**Figure 4B**). The control and reproducibility of this system allows experimental design with high spatial and temporal resolution, with typical assay outputs varying from immunoblot analysis of chromatin extracts over a particular time course, targeted enrichment of a particular protein of interest, or mass spectrometry for protein identification and/or analysis of PTMs in response to a particular DNA lesion (Gallina et al., 2021). Moreover, in recent years, this cell-free extract system has also been coupled with single-molecule techniques (Gruszka et al., 2020; Cameron and Yardimci, 2021). Lastly, to more accurately define the DDR and ubiquitin signaling in response to particular DNA lesions, specifically designed DNA plasmid templates can be used with the *Xenopus* egg extracts (Hoogenboom et al., 2017). For example, plasmids may be generated that contain a DNA-protein crosslink (DPC), interstrand crosslink or mimic a terminated DNA replication fork (Duxin et al., 2014; Deng et al., 2018; Larsen et al., 2019; Sparks et al., 2019).

Whilst the *Xenopus* egg extract system does allow a high degree of control over assay design, the ability to deplete a particular factor can be limiting, particularly as genetics approaches are not readily available, as noted above. Depletion from the egg extract requires the production of tailor-made antibodies to specifically immuno-deplete the protein of interest (POI) without targeting other factors involved in the same process as the POI. Moreover, rescue or add-back experiments require recombinant protein (wild-type and mutants) to be added at high concentrations, which can be an obstacle if such reagents cannot be produced or behave differently upon addition to egg extracts. For example, it was recently shown that the RPA complex-interacting E3 ligase RFWD3 ubiquitinates a range of substrates at stalled replication forks in *Xenopus* egg extracts (Gallina et al., 2021). However, difficulty in preparing active and specific recombinant RFWD3 has so far prohibited rescue experiments in this setting, whilst therefore also making reconstitution of this ubiquitin signaling *in vitro* a major challenge.

Beyond the *Xenopus* system, several groups have extended cell-free approaches by producing entirely reconstituted systems for specific cellular processes, with the reconstitution of DNA replication and some DNA repair events being notable major advances (Yeeles et al., 2015; Yeeles et al., 2017; Guilliam and Yeeles, 2020). Whilst this approach allows highly controlled experimental design, it requires that all the components of such a system are known. Thus, it can be difficult to fully reconstitute the dynamic DDR and ubiquitin signaling events found in a mammalian cell or *Xenopus* egg extract.

Recombinant Tools for Investigating Ubiquitin Signaling

Cell-free systems provide a powerful biochemical alternative to more complex genetic and cell biology-based approaches. However, to obtain insights into the mechanistic functionality of DDR proteins and ubiquitin signaling, a more reductionist and purified system is required. Both prokaryotic (e.g., *E. coli*) and eukaryotic (e.g., yeast, insect and mammalian) expression systems have been used to produce recombinant proteins, with developments in multi-component co-expression, such as MultiBac, and endogenous tagging enabling larger protein assemblies to be purified with minimal steps and to high purity and yield (Bieniossek et al., 2012). This has been utilised for many signaling components in DDR, such as the PIKK-family kinases in conjunction with enzymatic assays, biophysical and structural techniques, and microscopy and single-molecule methods (Jansma and Hopfner, 2020).

An additional complication for ubiquitin signaling is the ability to produce uniformly ubiquitinated substrates in high yields, on the physiologically relevant ubiquitin modification site(s), and chain linkages and length. Generally, specific E3 ubiquitin ligases, and subsequent DUB treatment in some cases, can be used to produce ubiquitin chains of particular chain linkage types and lengths (Michel et al., 2018).

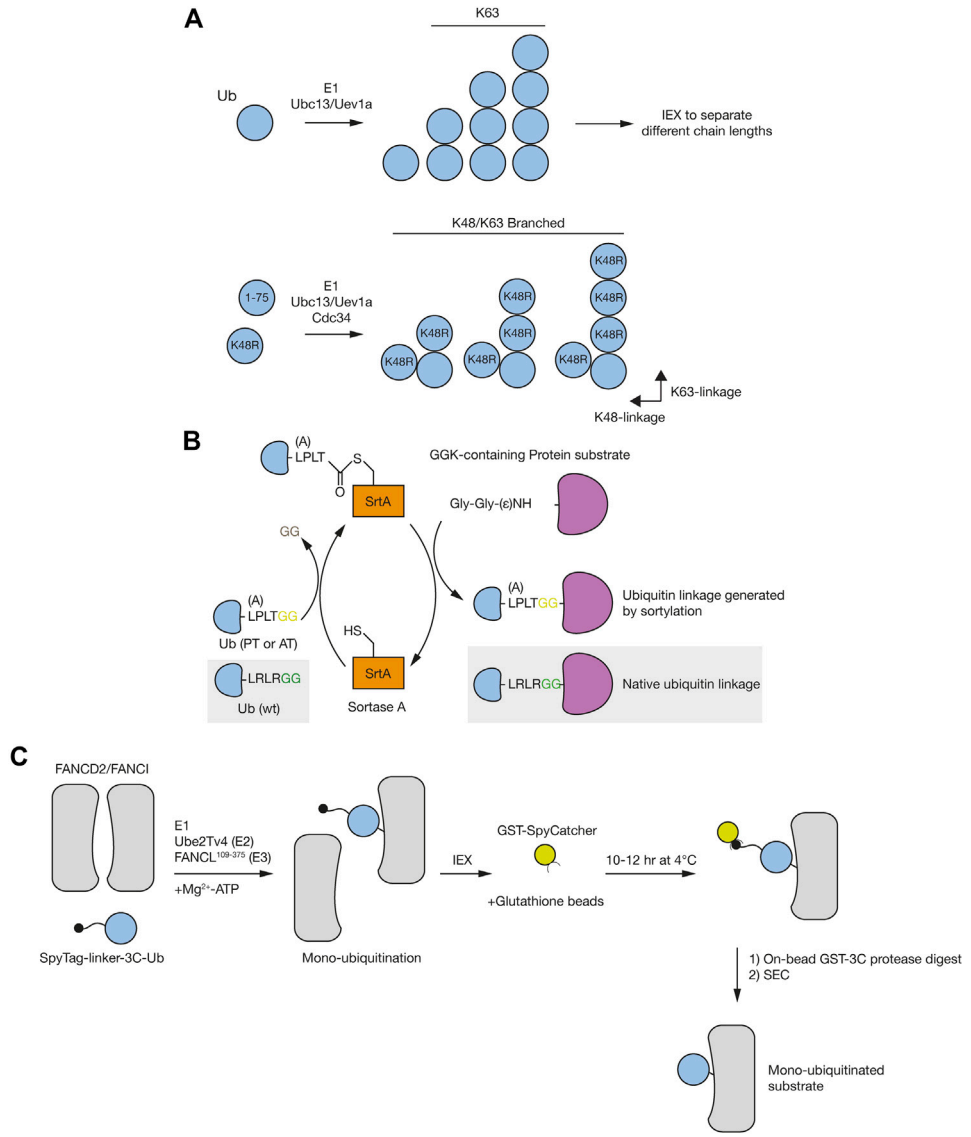

FIGURE 5 | Preparation of designer substrates to investigate the DDR and ubiquitin signaling. **(A)** Using E2 conjugating enzymes and specific E3 ligases and/or DUBs enables the formation defined ubiquitin chain types. Ubiquitin chains of different lengths can be separated by ion-exchange chromatography (IEX). Use of specific ubiquitin mutants enables the formation of branched or more complex species. **(B)** A GGK-containing protein can be prepared by site-specific incorporation of the unnatural amino acid AzGGK using genetic-code expansion. *In vivo* Staudinger reduction converts AzGGK to GGK, which can undergo transpeptidation with a ubiquitin mutant containing a sortase recognition motif (LPLTG or LALTG) via SrtA. The resulting ubiquitinated protein displays a native isopeptide bond with R72P/R72A and R74T point mutations in the linker region. **(C)** Use of engineered E2 and E3 enzymes with tagged ubiquitin enables the efficient formation of specifically ubiquitinated substrates. Subsequent steps such as IEX, affinity purification and size-exclusion chromatography (SEC) enable enrichment of the uniformly ubiquitinated species.

Combining these scalable enzymatic methods with specific Lys-to-Arg or other mutations, such as in the hydrophobic patch or C-terminal Gly-Gly within ubiquitin, enables further control over the types, length and branching of ubiquitin chains (**Figure 5A**). Proteomic approaches such as Ub-AQUA and middle-down mass spectrometry can be used to validate the ubiquitin chain architecture produced (Ohtake et al., 2019). Furthermore, incorporating fluorophores and other functional chemical moieties into ubiquitin by semi-synthetic chemical or enzymatic methods, such as for activity-based probes described above, provides a chemical toolbox for creating a whole suite of ubiquitin-based substrates to investigate the activity of enzymes involved in ubiquitin signaling.

In order to investigate the specific function and mechanisms of ubiquitin signaling in DNA repair more bespoke methods for producing ubiquitinated substrates are required. For example, histone H2A ubiquitination can be produced by several different enzymes: BRCA1/BARD1 (K125/127/129), RNF168 (K13/15) and RING1A/B (K118/119) (Uckelmann and Sixma, 2017). Whilst the extent of ubiquitination can differ widely in these enzymatic-based

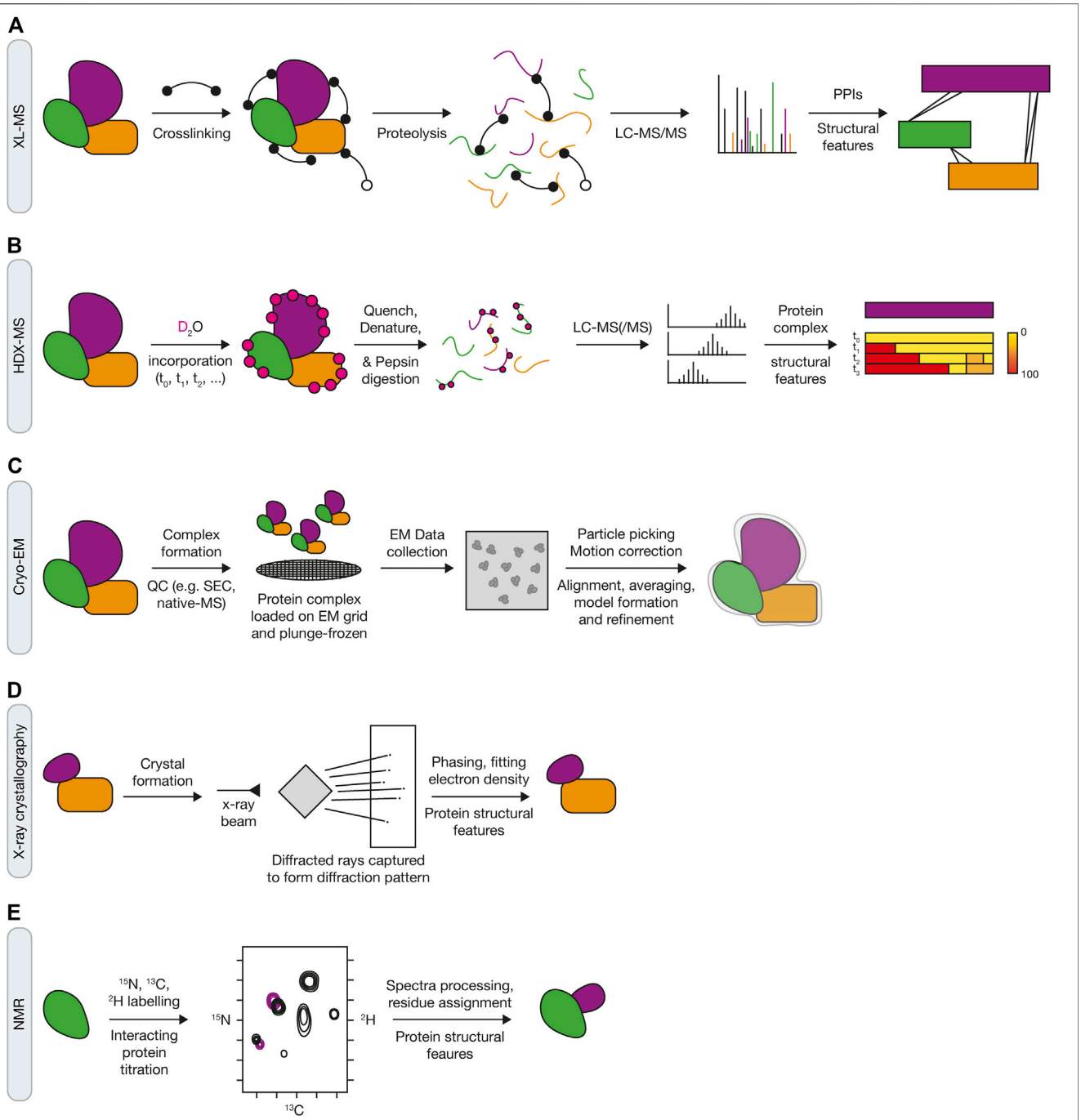

FIGURE 6 | Integrated structural approaches to investigate mechanisms of ubiquitin signaling in the DDR. **(A)** Crosslinking with mass spectrometry (XL-MS) requires the use of chemical crosslinkers to react with amino acid side chains, such as BS3 NHS-ester chemistry for primary amines on lysine residues or N-termini of proteins. Subsequent proteolysis with Trypsin or LysC, tandem mass spectrometry and data analysis using specialised software enables identification of crosslinked peptide species. **(B)** Hydrogen-deuterium mass spectrometry (HDX-MS) relies on the incorporation of ^{2}H into the protein by incubation in deuterated water. The experiment uses a time course of ^{2}H-incorporation followed by a rapid quenching and denaturation step at pH 2.5 before pepsin digestion and mass spectrometry analysis. Specialised data analysis pipelines can assess differences in ^{2}H-incorporation for a protein across experimental conditions. **(C)** Single particle cryo-electron microscopy (cryo-EM) has recently evolved as a technique to get high-resolution structural data of larger multi-protein assemblies. Protein samples go through several stages of quality control (QC) via biochemical and biophysical techniques (e.g., SEC and native mass spectrometry) before loading onto carbon-coated EM grids, plunge-freezing in liquid ethane and data collection using high-power electron microscopes. **(D)** X-ray crystallography and **(E)** nuclear magnetic resonance (NMR) are established techniques to gain atomic-level resolution of protein structures that relies on the formation of crystals and isotopically labelled proteins, respectively.

preparations, the use of tagged-ubiquitin can be used to enrich for the modified form. Furthermore, depending on the preparation of the enzymes, the specificity of the enzyme or the target enzyme complex, the substrates may rarely be uniformly modified, particularly if neighbouring lysine residues can also be modified. In some cases, it has been possible to introduce the specific ubiquitinated site through the use of non-natural amino acids and semi-synthetic chemistry, however, the lack of a non-natural linkage (i.e., not an isopeptide bond) prevents cleavage by DUBs (Virdee et al., 2011). More recently, sortase-based approaches have enabled larger and more complex ubiquitinated proteins to be produced (**Figure 5B**) (Crowe et al., 2016; Fottner et al., 2019; Hofmann et al., 2020). These sortase-based methods allow the ability to produce ubiquitinated proteins both recombinantly and within a cellular environment. However, mutations near the C-terminus of ubiquitin mean the isopeptide linkage is not cleavable by DUBs, which is a substantial limitation when investigating such dynamic signaling events. Efforts to engineer sortase mutants to utilise the natural ubiquitin C-terminus are likely underway.

A recent notable example that demonstrates method developments to produce uniformly ubiquitinated species is the FANCI:FANCD2 complex. Described in more detail elsewhere, this heterodimeric complex is a key component in the ubiquitin-dependent Fanconi Anaemia pathway of DNA repair (Nalepa and Clapp, 2018). A critical junction in the FA pathway is the specific monoubiquitination of FANCD2 and FANCI. However, the function of this sequential multi-monoubiquitination was still unclear, hampered by an inability to produce the monoubiquitinated FANCI:FANCD2 complex at sufficiently high yields. As such, several groups have developed methods to efficiently produce mono-ubiquitinated FANCI:FANCD2 (**Figure 5C**). The Walden group developed a UBE2T variant that enables more efficient ubiquitin transfer to FANCD2 with FANCL alone, whilst maintaining target specificity (Chaugule et al., 2020). This was then combined with the use of the high affinity SpyTag/SpyCatcher system to purify the ubiquitinated species from other reaction constituents and unmodified substrate (Chaugule et al., 2019). Alternatively, the Deans group used an His-Avi-3C-tagged ubiquitin alongside known E2 and E3 enzymes for successful isolation of ubiquitinated species: UBE2T and FA core complex for the FANCI:FANCD2 complex, UBE2D3 (UbcH5c) for PCNA and BRCA1-BARD1 for H2A (Tan et al., 2020a). Thus, the continuing improvement in methods such as these will allow the uniform production of site-specific ubiquitinated substrates that will be key to understanding the mechanisms and function of ubiquitin modifications in the DDR. Whilst techniques to produce free ubiquitin chains are well established (Michel et al., 2018), methods to produce specifically ubiquitinated substrates relevant for DNA repair are still in their infancy. Progress has been made in some instances but more specialised systems and optimised protocols are likely required to be able to fully recapitulate some of these dynamic signaling pathways.

Integrated Structural Techniques

Probably the greatest technical advancement for investigating the mechanisms of ubiquitin signaling in DNA repair is the development of high resolution, single particle cryo-electron microscopy (Cryo-EM, **Figure 6**) (D'Imprima and Kuhlbrandt, 2021; Glaeser, 2019; Kim et al., 2018). The enhancements in microscope design, detection methods and rapid software development has enabled high resolution structures of protein assemblies to be solved that would not have been thought possible little over a decade ago (Scheres, 2012; Fernandez-Leiro and Scheres, 2017; Punjani et al., 2017; Zivanov et al., 2018). Structures have now been solved of large E3 ligases and DUBs with and without their substrates (Rabl et al., 2019; Shakeel et al., 2019; Wang et al., 2021; Witus et al., 2021). Combining these novel structures with further biochemical, proteomic, and biophysical approaches has led to a new era of integrated structural approaches, whereby this structural information can be corroborated with genetics and cell-based approaches.

Recent structural studies of the factors involved in FA pathways, such as the FA core complex, the FANCI:FANCD2 heterodimer and the USP1-UAF1 DUB complex, are prime examples (Li et al., 2020). The ability to produce uniform ubiquitinated substrates in combination with state-of-the-art cryo-EM and mass spectrometry techniques has provided a much deeper insight into the mechanisms of ubiquitin signaling DDR pathway. Improvements in multi-subunit co-expression enabled the FA core complex, the E3 ligase responsible for FANCI:FANCD2 complex monoubiquitination, to be purified in high yields for structural investigation (Shakeel et al., 2019; Wang et al., 2021). In addition to using the described improvements in cryo-EM, native mass spectrometry was used to analyse subunit stoichiometry and complex uniformity. This level of sample quality assurance in conjunction with advanced structural methods provides important information about the protein complex and aids in forming conclusions about the functional significance of solved structures. Further mass spectrometry-based methods, including crosslinking with mass spectrometry (XL-MS, **Figure 6A**) and hydrogen-deuterium exchange mass spectrometry (HDX-MS, **Figure 6B**), also proved invaluable in helping to assign subunit and domain locations within such a large and complex assembly. Indeed, the rise in quality and use of single particle cryo-EM has occurred alongside technological advances in biological and structural mass spectrometry (Chen and Rappsilber, 2019; Mistarz et al., 2016; O'Reilly and Rappsilber, 2018; Walzthoeni et al., 2013). Mass spectrometers are becoming increasingly sensitive and the depth of sequence coverage for proteomics experiments has improved several-fold. This, along with developments in software packages and data analysis pipelines, has vastly enhanced the extraction of robust structural proteomics data and opened up wider access to these types of methods. Despite the advancement in technology for these structural mass spectrometry methods however, there have been inconsistencies in analysing and interpreting the resulting data and as a result, there has been a move to produce a standardised set of parameters in experimental design (Iacobucci et al., 2019; Masson et al., 2019; Leitner et al., 2020).

Improved sample preparation and advances in cryo-EM also proved fruitful in assigning the function of the sequential monoubiquitination of the FANCI:FANCD2 complex. Cryo-EM reconstructions, alongside XL-MS and DNA-binding experiments, suggested a role for the monoubiquitination in transforming FANCI:FANCD2 into a DNA clamp (Alcon et al., 2020; Tan et al., 2020b; Wang R. et al., 2020). In addition to this novel finding, the ubiquitin of one protomer (i.e., FANCD2 or FANCI), binds to the other protomer within the complex, effectively shielding it from a potential role in the recruitment of other DNA repair factors via their ubiquitin binding domains. This clamp role for the ubiquitinated FANCI-D2 has been proposed to protect the underlying DNA during repair of the lesion, with the removal of the modification enabling FANCI:FANCD2 to be released from the site upon repair. Furthermore, the mechanism for the removal of ubiquitin from FANCD2 was also clarified by cryo-EM and crystallography experiments with USP1-UAF1 (Rennie et al., 2021). Crystals of the apo- and ubiquitin-bound form of USP1-UAF1, in conjunction with cryo-EM reconstructions of the enzyme-substrate complex, revealed important details of the specificity and regulation of this reaction. Amino acid residues at the FANCI-UAF1 interface, including those of known ATR phosphorylation sites, were shown to be critical for regulating USP1-mediated removal of the FANCD2-Ub mark, corroborating previous genetic and biochemical data (Tan et al., 2020c). Collectively, these findings show that the recent technical developments in structural biology have led to fundamentally important discoveries of how ubiquitin signaling regulates the DDR.

Recapitulating the DNA Damage Response and Ubiquitin Signaling in a Chromatin Context

A prominent question in the DDR field is how ubiquitin signaling events occur in the context of chromatin. The production of recombinant nucleosomes for investigating chromatin-based signaling mechanisms has been demonstrated within the epigenetics field (Luger et al., 1997; Dyer et al., 2004; Dao et al., 2020; Liu et al., 2020). Furthermore, preparing ubiquitinated nucleosome core particles, similar to the enzymatic and chemical methods noted above, has become increasingly common to investigate the regulatory mechanisms in chromatin processes, including how DDR factors function in the context of chromatinised DNA lesions (McGinty et al., 2014; Nguyen et al., 2014; Worden et al., 2019; Worden et al., 2020). For example, the critical choice between HR and NHEJ has been investigated with structural investigations of how 53BP1 interacts with nucleosomes containing H4K20me2 and H2AK13/15-Ub via its Tandem Tudor domain and ubiquitin-dependent recruitment motif (UDR), respectively (Wilson et al., 2016). Furthermore, a recent cryo-EM structure of BRCA1/BARD1 with UBE2D3 (UbcH5c) on a nucleosome also provided mechanistic details for the specificity of the enzyme for H2AK125/127/129 ubiquitination (Witus et al., 2021). Thus, developments in cryo-EM, such as phase plates and sample preparation, paves the way for further ubiquitin-modified nucleosome-bound complexes to be solved in the context of the DDR (Chua et al., 2016; Chua and Sandin, 2017).

Whilst mono- or di-nucleosome containing structures have been solved, how DNA repair factors and ubiquitin signaling events function in the context of higher order chromatin is still relatively unclear. *In vitro* assembly and subsequent structural reconstruction of chromatin relies on forming unnaturally rigid nucleosome arrays to reduce sample heterogeneity. This is added to the complication of including the relevant PTMs at the correct sites and including all the necessary protein factors within the DNA repair machinery. With increasing capabilities in reagent production and data acquisition by cryo-EM, it might be possible to reconstitute some of these complex ubiquitin signaling pathways and visualise them by time-resolved techniques. However, it is unlikely that the precise dynamics of these reactions in a chromatin context can be recapitulated by structural techniques noted here alone and perhaps single molecule techniques can help to fill these mechanistic gaps alongside other experimental systems.

FUTURE PERSPECTIVES

The development and application of the myriad methods and tools discussed here have helped shape our understanding of how ubiquitin signaling regulates the DDR over the past few years. Whilst the vast complexities of ubiquitin signaling are now starting to be decoded, future work will require integrated multidisciplinary approaches to gain a deeper mechanistic understanding of these processes both *in vitro* and in *in vivo*, with genetics, proteomics and biochemical methods critical to the success of this. Understanding the limitations of these technologies will also lead to innovation and the creation of new tools that can be applied to the DDR.

For genetics, CRISPR-Cas9 has revolutionised biological science over the past decade. This technology has converged with the recent explosion in small molecules that have been designed to target the DDR and UPS, and their associated pathologies. The intersection of these two advances has facilitated transformational gene discovery within the DDR and UPS, uncovered novel sensitisation and resistance mechanisms and revealed new SL interactions. Further genome editing capabilities will continue to drive this progress, such as base editing screens (Cuella-Martin et al., 2021; Hanna et al., 2021). Moreover, whilst most of these screening approaches have been used in forward modalities, reverse genetics screens are coming to the fore with advances in both arrayed and pooled sgRNA libraries for image-based approaches (Feldman et al., 2019; Askary et al., 2020; Wheeler et al., 2020; Chandrasekaran et al., 2021; Kanfer et al., 2021; Lawson and Elf, 2021; Yan et al., 2021).

For proteomic approaches, the past decade has also seen the development of many novel techniques coupled with advances in MS approaches to map proteins at the replication fork and

identify ubiquitinated proteins. The role of ubiquitination at the replication fork and its function in the DDR remains far from complete. The identification of novel ubiquitin-regulating enzymes and factors continues to expand and further our understanding of ubiquitin-mediated signaling. Furthermore, approaches that better interrogate ubiquitin chain architecture on proteins will help to provide insight into the extent of mixed and branched chain types and their role in the DDR. An integrated approach that utilises multiple proteomic methods, including those described here, may help to assign function to these chain types and identify how they are regulated. While not discussed in detail in this review, the interplay between ubiquitin, UBLs, and post-translational modifications provides an additional level of regulation that contributes to the complexity of the ubiquitin code and must also be considered. Further technical developments in MS data acquisition enabling greater detection and profiling of ubiquitin modifications across multiple samples in parallel may also help to achieve better resolution and corroborate findings from large-scale DNA damage screens.

Developments in cryo-EM, structural mass spectrometry, and recombinant tool development described here build on the plethora of data available via tailored biochemical and biophysical data, X-ray crystallography (**Figure 6D**), and NMR approaches (**Figure 6E**). It is becoming increasingly clear that to understand the mechanisms of how ubiquitin modifications function in DNA repair, highly specific reagents and multiple integrated experimental systems need to be utilised. Moreover, although not discussed in detail here, future directions in single molecule techniques and super-resolution microscopy will allow greater resolution of some of these signaling machines within the context of a cellular environment. Already, temporal resolution of protein signaling can be resolved *in vitro* using cryo-EM (Miller et al., 2019). Thus, perhaps we are not too far away from obtaining high spatial and temporal resolution for DDR and ubiquitin-dependent signaling events in real-time.

Collectively, in light of recent technological advances, as well as novel insights from a variety of disciplines, it is conceivable that we are on the precipice of unravelling the complexity of ubiquitin signaling mechanisms in DNA repair through interdisciplinary approaches at an unprecedented level.

AUTHOR CONTRIBUTIONS

BF and MA wrote individual sections of the manuscript and contributed figures; IG-S wrote a section, contributed figures and edited the manuscript and all figures.

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