Nurturing a revolution in DNA repair and cancer therapy through CRISPR–Cas screens

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Abstract

All organisms possess molecular mechanisms that govern DNA repair and associated DNA-damage response (DDR) processes. Owing to their relevance to human disease, most notably cancer, these mechanisms have been studied extensively, yet new DNA repair and/or DDR factors and functional interactions between them are still being uncovered. The emergence of CRISPR technologies and CRISPR-based genetic screens has enabled genome-scale analyses of gene–gene and gene–drug interactions, thereby providing new insights into cellular processes in distinct DDR-deficiency genetic backgrounds and conditions. In this Review, we discuss the mechanistic basis of CRISPR–Cas genetic screening approaches and describe how they have contributed to our understanding of DNA repair and DDR pathways. We discuss how DNA repair pathways are regulated, and identify and characterise cross-talks between them. We also highlight the impacts of CRISPR-based studies in identifying novel strategies for cancer therapy, and in understanding, overcoming and even exploiting cancer-drug resistance, for example in the contexts of PARP inhibition, homologous recombination deficiencies and/or replication stress. Lastly, we present the DDR CRISPR screen (DDRcs) portal, in which we have collected and reanalysed data from CRISPR screen studies and provide a tool for systematically exploring them.

Introduction

Exposure to exogenous agents, including radiation and certain reactive chemicals, can cause genetic mutations and can give rise to cancer[1]. DNA is also frequently damaged by endogenous agents under physiological conditions[2,3]. Today, we recognise that every single cell in the human body suffers tens to hundreds of thousands of DNA lesions each day[4]. Cellular DNA damage response (DDR) mechanisms have evolved to limit detrimental consequences of DNA damage and safeguard genome integrity (Box 1). The DDR is a complex network of pathways that collectively detect and signal the presence of DNA damage and mediate DNA repair. Additionally, DDR pathways coordinate repair events with other cellular processes such as cell-cycle checkpoint activation, chromatin reorganisation and regulation of gene expression[5]. Acquired or inherited defects in DDR mechanisms can lead to mutations and chromosomal aberrations that undermine cell functions and viability. Accordingly, DNA damage and its aberrant repair underpin myriad human pathologies including cancer, neurodegenerative diseases, infertility, immune dysfunctions and various genetic disorders[5]. Despite major advances in our understanding of genome maintenance over the past decades, our appreciation of the full repertoire of DNA repair and DDR factors is still incomplete. Moreover, our understanding of the functional interactions between these factors and processes operating in physiological conditions and in disease, remains rudimentary.

The ability to assess and modify genetic information is crucial for experimental studies into gene function and DNA repair. Over the past decade, clustered regularly interspaced short palindromic repeat **[G]** (CRISPR) technologies have emerged as revolutionary tools for genome-editing owing to their relatively simple application: the targeting specificity of CRISPR-based approaches relies on base pairing of nucleic acids rather than protein–DNA recognition as in other genome-editing tools such as zinc-finger (ZNF) nucleases and transcription activator-like effector nucleases (TALENs)[6]. In nature, bacteria and archaea employ CRISPR-based mechanisms as adaptive immunity systems to recognise and destroy viruses and other invading genetic elements[7,8]. To date, the most studied and exploited protein components of CRISPR systems are the CRISPR-associated (Cas) endonucleases, which rely on a CRISPR RNA (crRNA) for guidance and target specificity. Additionally, following the target sequence, Cas endonucleases require a defined protospacer adjacent motif (PAM), which dictates the cleavage site. Consequently, site-specific cleavage can be achieved at any target locus containing a PAM site (Figure 1). By taking advantage of these properties and the ease

of generating large lentiviral vector-based libraries of single guide RNAs **[G]** (sgRNAs), various technological applications have been developed, especially CRISPR–Cas tools for high-throughput cell-based loss-of-function screens[9].

Based on the above-described attributes, CRISPR–Cas screens have key advantages for identifying new DDR factors and studying functional relationships between them. In this Review, we discuss recent advances in CRISPR-based screening approaches, describe how these properties have been leveraged for expanding our knowledge of DNA damage and DDR processes, and provide an overview of the main contributions of CRISPR–Cas screens to understanding DNA repair pathways. We also discuss the potential of CRISPR-based technologies for better understanding, monitoring and treating cancer, and describe how knowledge of DDR processes may lead to refinements in CRISPR approaches. In addition, we have generated the DDR CRISPR screen (DDRcs) portal to systematically analyse and compare data obtained from many CRISPR screening studies of the DDR.

[H1] DNA damage repair and cancer therapy

Other than surgery, the most widely used treatments for cancer include radiotherapy and various DNA-damaging chemotherapies. Although factors such as dose-limiting toxicities to non-cancerous cells and intrinsic or acquired resistance in cancer cells limit the impact of such therapies, they are often effective in alleviating and even curing cancers. This efficacy, in part, arises from the higher proliferation rates of cancer cells contributing to them experiencing higher levels of oxidative and replicative stress than normal cells. Radiotherapy generates large amounts of local DNA lesions that limit the proliferation and kill cancer cells, while generally having milder effects on surrounding normal cells[10]. Many standard chemotherapies such as antimetabolites and alkylating agents lead to incorporation of aberrant DNA bases during replication that cause issues during subsequent S-phases. Other widely used cancer therapies that predominantly affect proliferating cells include the platinum-based DNA-crosslinking agents cisplatin, carboplatin and oxaliplatin, the topoisomerase I (TOP1) poison camptothecin and its derivative topotecan, and the TOP2-targeting drug etoposide (reviewed in Refs[11,12]).

Like normal cells, cancer cells can use DNA repair and associated DDR processes. Notably, however, a hallmark of cancer cells is that they often lose or deregulate at least one DNA repair or DDR pathway during their transformation[5,13,14]. This deficiency generally makes the DDR

network of cancer cells less robust than those of normal cells, and consequently cancer cells are often particularly reliant on the repair pathways that they retain. In addition to contributing to the efficacy of conventional DNA-damaging chemotherapeutics, these features provide opportunities for selectively killing cancer cells by targeting their remaining DDR pathways. This potential is highlighted by the widespread use of poly(ADP-ribose) polymerase (PARP) inhibitors, the first marketed compounds targeting a DNA repair enzyme. PARP inhibitors not only inhibit the enzymatic activity of PARP1 and PARP2, but also trap them on DNA, which slows down DNA single-strand break (SSB) repair and generates physical barriers during S phase, leading to stalling and collapse of replication forks with associated formation of DNA double-strand breaks (DSBs)[15]. Whereas such lesions can be successfully resolved by normal cells, this is not the case in cells with certain DNA-repair deficiencies, particularly those defective in homologous recombination (HR) factors such as breast cancer gene 1 (BRCA1) and BRCA2, which display hypersensitivity to PARP inhibitors[15–18]. Prompted by the success of PARP inhibitors in treating ovarian, breast, pancreatic and prostate cancers, inhibitors of other DNA repair and DDR proteins such as ATM, ATR, DNA-PK, CHK1 and WEE1 have been developed and are currently being tested in clinical trials, either as monotherapies or in combination with other agents (reviewed in Refs[19,20]).

[H1] CRISPR screens and their applications

A widely used approach for successful perturbation of gene expression in mammalian cells is based on RNA interference (RNAi), which is mediated by small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs)[9]. Unlike approaches based on ZNF and TALEN nucleases, RNAi enables genome-scale modification of human cells, as perturbation of gene function only requires expression of siRNAs or shRNAs complementary to a target mRNA, thus leading to its degradation. Accordingly, RNAi-based phenotypic screens identified various novel genes implicated in DNA repair and have substantially enhanced our understanding of DDR processes. For example, RNAi-based screens led to the identification of factors affecting hypersensitivity towards PARP inhibitors[21], cisplatin[21,22], mitomycin C[23] or camptothecin[24], and factors involved in the accumulation of the non-homologous end joining (NHEJ)-promoting factor TP53-binding protein 1 (53BP1) and associated proteins in foci flanking sites of DNA damage[25,26]. Furthermore, RNAi-based screens helped elucidate mechanisms regulating the phosphorylation of the histone variant H2AX[27], and factors determining the choice between DSB repair by HR or NHEJ[28,29]. However, issues such as off-target effects and incomplete suppression of target gene expression have posed barriers for RNAi screens and the

interpretation of ensuing results. Mammalian genome-scale screens have also been enabled by use of DNA-damaging chemicals, or by transposon or retrovirus insertions, resulting in random mutagenesis[30–33], although the non-programmable and relatively labour-intensive nature of such methods has so far limited their utility.

The ability of CRISPR–Cas technologies to target DNA sequences in a programmable and highly specific manner (Figure 1) has largely overcome the above-mentioned limitations and has paved the way for establishing various tools for effective genome-wide functional screens[9]. In the context of high-throughput approaches, the key feature of these tools is the relative ease of expressing a Cas protein and a library of CRISPR-sgRNAs to robustly and specifically modulate gene functions (Figure 2). So far, the most widely used screening platform is the generation of CRISPR gene knockouts (CRISPR-KO), where Cas9-induced genomic DSBs are repaired by NHEJ, leading to formation of insertions, deletions and point mutations that in most cases disrupt the function of the targeted gene[34] (Figure 1A).

The first published genome-scale CRISPR screens in mammalian cells demonstrated the feasibility of knocking-out most genes in the genome in a single experiment[35–39]. One study[39] employed mouse embryonic stem cells to screen for resistance to *Clostridium septicum* alpha-toxin or to the anti-cancer drug 6-thioguanine **[G]**, whereas in others[36–38], human cells were screened for resistance to agents including 6-thioguanine and etoposide[38], and for genes essential for cell proliferation or viability[36,37]. Subsequent genome-wide cell "fitness" screens performed in five human cell lines identified a core set of ~1,580 genes that are all essential for proliferation and viability of most cell types, as well as finding additional genes that only affect fitness in certain cell lines[40]. These initial forays thus underscored the potential of using CRISPR-KO approaches to identify genes affecting various cellular mechanisms, including DDR processes in a systematic and unbiased manner.

Building on CRISPR-KO, complementary technologies have been developed and applied, including CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa), which use a catalytically-inactive Cas protein (dCas9) fused to a transcription repressor domain (such as the Krüppel-associated box) or to a transcription activation domain (such as VP64 from herpes simplex virus), respectively. These dCas9 fusion proteins are targeted to promoter regions of genes of interest and affect gene expression (Figure 1B)[29,41–43]. In CRISPRi experiments, bidirectional promoters need to be considered, as genes flanking both sides of the target site can be affected. Nevertheless, CRISPRi appears to have substantially fewer off-target effects than RNAi and can be used to interrogate functions of distal regulatory elements[44,45]. Various

genome-wide libraries for CRISPR-KO, CRISPRi, and CRISPRa have now been made, tested and made widely available[46]. Furthermore, libraries targeting only subsets of genes have been created, offering the potential to study specific aspects of cell biology in more detail[47] (Figure 2A).

To chart functional relationships between genes, including those involved in DDR processes, combinatorial CRISPR-KO screens have been conducted, in which thousands of pairwise genetic interactions have been systematically interrogated using paired-gRNA libraries and Cas9 (Refs[48–51]) or Cas12a[52–54], thereby allowing perturbation of multiple genes simultaneously (Figure 2B). Although Cas9-based methods have been extensively used for single-knockout screening, this enzyme showed less efficiency and was less successful in combinatorial screening compared to Cas12a. This is attributed, in part, to the fact that Cas12a can process multiple gRNAs that are expressed as a single transcript. Similar potential also exists for combinatorial gRNA libraries for CRISPRi[55–57].

Another method used to survey functional interactions between a gene of interest ('anchor' gene) and other genes exploits the use of two orthologous Cas enzymes such as *Streptococcus pyogenes* Cas9 (SpCas9) and *Staphylococcus aureus* Cas9 (SaCas9), each with specific sgRNA targeting requirements. Cell lines are transfected with two vectors, one delivering SpCas9 with a SaCas9-compatible anchor-targeting sgRNA, and one delivering SaCas9 with a SpyoCas9-compatible 'query gene'-targeting sgRNA. Only cells that have been successfully transfected with both vectors will produce DSBs at both anchor and query loci (Figure 2B). This method allows systematic identification of sequence alterations that are synthetic lethal or that display other functional relationships with the anchor gene[58], and is particularly useful when the consequence of anchor-gene alteration cannot be modelled using single-cell-derived isogenic clones owing to the gene being essential, thus providing a tool to uncover genetic interactions that rescue cell viability.

[H1] CRISPR screens of DNA repair pathways

Over several decades, many factors involved in DDR processes were identified through biochemical approaches, their homology with DDR factors identified in other organisms, and by investigation of human genetic syndromes through mutant organisms and cell lines that display hypersensitivity to genotoxins. Despite these efforts, we so far have only a rudimentary understanding of functional relationships and cross-talks between known DDR factors and how DNA repair and associated DDR processes intersect with other aspects of cell physiology. As

we discuss below, CRISPR screens have had major impacts in the field of DDR research (Figure 3). While many of these studies have used genome-wide sgRNA libraries, several DNA repair-related libraries have been designed and effectively deployed. For instance, we designed a dual-guide **[G]** sgRNA library targeting 852 DDR-related genes (as well as non-essential gene controls and sequence-scrambled negative controls)[59]. This library was manually curated to include established DDR components, putative DDR-related interactors and various bioinformatically-predicted DDR factors. Other DDR-focused libraries include the DDR_MKOv4 library, which targets 365 DDR genes plus additional controls[60], and a CRISPRi DDR library targeting 471 genes[61].

[H2] PARP and homologous recombination

Various groups have carried out CRISPR screens to address the nature of PARP activating or trapping lesions and their cellular consequences. The first of these screens[62] employed three different cell lines; neoplastic and non-transformed cells either expressing wildtype or defective BRCA1 protein using the clinically approved PARP inhibitor, olaparib[18]. This work established that loss of genes encoding for different ribonuclease H2 (RNASEH2) proteins sensitised cells to PARP inhibition due to persistence of inappropriately incorporated ribonucleotides in genomic DNA, whose cleavage by TOP1 results in PARP-trapping lesions that disrupt DNA replication[62] (Figure 3). Additionally, it has been found that processing of incorporated modified deoxynucleotides also enhances PARP inhibitor sensitivity. Thus, CRISPR screens in cells lacking MUS81, an endonuclease involved in resolution of DNA Holliday junctions during HR, highlighted how loss of 2'-deoxynucleoside 5'-phosphate N-hydrolase 1 (DNPH1), a sanitizer of cellular nucleotide pools **[G]**, enhances sensitivity to PARP inhibition owing to increased genomic misincorporation of 5-hydroxy-methyl-deoxyuridine (hmdU), which is normally cleared by DNPH1 (Ref.[63]) (Figure 3). These data also suggested potential therapeutic applications for DNPH1 inhibition in enhancing sensitivity to PARP inhibition in HR-deficient cancers. A second screen in MUS81-mutant cells treated with hmdC, a potential precursor of hmdU[64], identified loss of the DNA glycosylase SMUG1 as driving resistance to hmdC and olaparib co-treatment, suggesting that processing of misincorporated hmdU by SMUG1 and ensuing formation of base excision repair (BER) intermediates leads to PARP1 trapping and replication-fork collapse in HR-deficient cells subjected to PARP inhibition[63] (Figure 3).

Release of trapped PARP1 is facilitated by ALC1 (also known as CHD1L), a poly(ADP-ribose)-dependent chromatin remodelling enzyme that enhances chromatin accessibility to promote repair of DNA base damage[65–69]. CRISPR screens showed that ALC1 loss is a key determinant in conferring sensitivity to PARP inhibition in BRCA1 or BRCA2 (BRCA1/2)-mutant cells[65], leading to the hypothesis that ALC1 operates downstream of the DNA glycosylases SMUG1 and MPG in repair of mis-incorporated uracil and alkylated bases, respectively[65,66]. Inability to repair base lesions in the absence of ALC1 is therefore thought to cause accumulation of replication-associated single-stranded DNA (ssDNA) gaps and DSBs that cause toxicity in HR-deficient cells[65,66] (Figure 3). Collectively, these studies revealed novel crosstalk between chromatin remodelling, BER and HR in response to PARP inhibition.

In a complementary study, anchor CRISPR screens were performed employing guides targeting PARP1 (Ref.[58]). As controls for the efficacy of the anchor system, several CRISPR-KO screens were performed in parallel using either an established *PARP1* knock-out cell line, or treatment with the PARP inhibitors olaparib and talazoparib. Collectively, these screens allowed comparison with results generated in a previously mentioned study[62], shedding light on cellular responses to PARP inhibition and the differences between PARP inhibition and *PARP1* knockout. For instance, it has been shown that loss of the BER and SSB repair factor XRCC1 is a strong sensitizer to PARP inhibitors, whereas it promotes cell viability in a *PARP1* knockout background. Similarly, loss of the BER polymerase Polβ sensitizes cells to PARP inhibitors, but not when PARP1 is absent.

CRISPR-KO screens have also been conducted in *BRCA1*/2-deficient cell backgrounds to explore mechanisms underpinning their hypersensitivity to PARP inhibitors[62,65,70–73]. In addition to identifying already-known modulators, such as 53BP1, RIF1 and MAD2L2, two of these studies[72,73] identified loss of components of the shieldin complex (SHLD1, SHLD2 and SHLD3) as drivers of PARP-inhibitor resistance, with these studies then establishing that shieldin acts as a downstream effector of 53BP1–RIF1-mediated DSB end-protection. Additional hits arising from these screens, including factors such as dynein light chain 1, (DYNLL1) and the CTC1-STN1-TEN1 (CST) complex, were subsequently shown in other studies to counteract DNA end resection (and thus HR), explaining how their absence confers resistance to PARP inhibitors[70,71] (Figure 3).

The CST and shieldin complexes have been implicated in telomere biology[74]. Notably, deficiency of the telomere maintenance and protection factor, telomeric repeat-binding factor 2 (TRF2), which normally results in end-to-end chromosome fusions in somatic cells, was

recently found to be inconsequential in mouse embryonic stem cells[75]. CRISPR screens in these TRF2-depleted cells revealed that their survival strongly depends on another telomere protection protein, POT1B, and on the chromatin remodelling factor BRD2[75,112]. Furthermore, a CRISPRi screen in human *POT1*-mutant cells identified lethal effects caused by loss of any of several components of the nuclear pore complex. This study unveiled a role for the nuclear pore complex in resolving replication defects at telomeres upon POT1 dysfunction by preserving the integrity of telomere repeats through relocalization of dysfunctional telomeres to the nuclear periphery[76].

CRISPR screens for synthetic lethal genetic interactions have led to the discovery of hitherto underappreciated sources of endogenous DNA damage that are especially toxic in *BRCA1/2*-deficient cells[77–79]. DNA-(apurinic or apyrimidinic site) endonuclease 2 (APE2; also known as APEX2) was thus identified as being required for viability of BRCA1/2-deficient cells, reflecting its role in repairing 3'-blocked DNA ends[78]. This work also suggested that blocked 3' ends arising from TOP1-mediated processing of genomic ribonucleotides in the absence of APE2 are the main source of lethality in *BRCA1/2*-deficient cells (Figure 3). Of note, APE2 and flap endonuclease 1 (FEN1) were also identified in another study[79] as BRCA1/2 synthetic-lethal partners, and their roles in Polθ-mediated end joining **[G]** (TMEJ) and BER in *BRCA2*-deficient cells were characterised. In accord with this, it was found that TMEJ is a key "backup" process for DSB repair in cells deficient in HR, and that Polθ inhibition in BRCA1/2-deficient cells results in lethality through a mechanism distinct from that evoked in response to PARP inhibition[80,81] (Figure 3). Another CRISPR screen expanded our knowledge of TMEJ by uncovering a considerable number of synthetic-lethal partners of the Polθ encoding gene, *POLQ*, and provided evidence that these relationships reflect a crucial role of Polθ in protecting cells from accumulation of non-productive HR intermediates **[G]** at sites of DSBs associated with DNA replication[82,83].

More recently, the oncoprotein CIP2A was identified as being specifically essential for survival of *BRCA1*-mutated or *BRCA2*-mutated cells[77]. This work went on to show that, in mitosis, CIP2A accumulates at sites of DNA lesions as part of a complex with TOPBP1, in a manner that may serve to hold together broken chromosomes resulting from processing of incompletely replicated DNA in BRCA1/2-deficient cells (Figure 3). Additionally, a recent genome-wide CRISPR screen identified that loss of cyclin C allows cells to survive the loss of BRCA2 and suppresses their hypersensitivity to PARP inhibition, which may be mediated by restoration of replication fork progression during replication stress[84]. Similarly, loss of homologous

recombination OB-fold protein (HROB), which acts in concert with the helicase MCM8–MCM9 to promote HR, sensitized cells to PARP inhibitors and ATR inhibitors[85–87]. Importantly, a recent work revealed that variants of the *HROB* gene are associated with premature ovarian insufficiency, shedding light onto the importance of HR in ovarian function[88].

In line with ATM promoting DNA-end resection to help steer DSBs towards HR, ATM loss entails hypersensitivity to PARP or TOP1 inhibitors, both of which yield one-ended DSBs that need to be repaired by HR. To explore underlying molecular mechanisms, CRISPR–Cas9 screens were performed in ATM-proficient and ATM-deficient mouse embryonic stem cells in the presence of the TOP1 inhibitor, topotecan[89]. This work revealed that loss of the NHEJ factors XRCC4 or ligase IV or of specific components of the BRCA1-A complex **[G]**, confers topotecan and PARP inhibitor resistance in ATM-deficient mouse and human cells (Figure 3). Ensuing investigations established that the hypersensitivity to topotecan or PARP inhibitors observed in ATM-deficient cells is due to delay in HR processes in the absence of ATM, which leads to toxic NHEJ generating chromosomal fusions and other aberrations, mitotic catastrophe and/or apoptosis[89].

[H2] Replication stress

The DNA replication machinery faces various perturbations, arising exogenously and endogenously, that may cause replication stress[90,91]. In eukaryotic cells, the response to replication stress is regulated by the kinase Ataxia telangiectasia and Rad3-related (ATR), which stabilizes stalled replication forks and regulates firing of origins of DNA replication, cell cycle checkpoints and DNA repair[5,92,93] .To gain new insights into cellular responses to replication stress, several CRISPR–Cas9 screens have been performed with ATR inhibitors[85,94–97]. Indeed, one of the first CRISPR screens performed in the DDR field showed that premature entry into mitosis, which occurs upon ATR inhibition, is avoided by loss of CDC25A, and that ATR-inhibitor toxicity can be restored in this background by the inhibition of WEE1, which enforces mitotic entry[96]. Cyclin C and CDK8 — components of the RNA polymerase II (Pol II)-associated Mediator complex — were also identified in genome-wide CRISPR screens as factors whose loss imparted strong resistance to ATR inhibition in both *ATM*-proficient and *ATM*-deficient cells[94]. Mechanistically, this work showed that this ATR-inhibitor resistance is mediated by loss of cyclin C or CDK8 leading to decreased levels of DNA–RNA hybrids during S phase. Loss of cyclin C or CDK8 is associated with reduced

transcription and ensuing occurrence of transcription–replication conflicts and micronuclei formation caused by ATR inhibition, thus highlighting transcription-associated replication stress as a predominant driver of ATR-inhibition-induced cell death[94] (Figure 3). Furthermore, another study found that the loss of another Mediator component, MED12, confers resistance to ATR inhibitors by activating the TGF-beta pathway[97]. Conversely, CRISPR screens have also identified factors whose loss confers hypersensitivity to ATR inhibition in various cell lines[85,95]. We discuss this below, in the context of potential cancer treatments.

Notably, CRISPR screens using agents that induce replication-stress have been harnessed to identify mechanisms of actions of other drugs. In this context, similar genetic profiles produced by CRISPR screens in the presence of the replication inhibitors hydroxyurea and resveratrol revealed that inhibition of replication fork progression and induction of replication stress are main sources of resveratrol-mediated inhibition of cell proliferation[98]. Other studies have provided insights into cellular responses to DNA inter-strand crosslinks (ICLs) and ensuing replication stress [99–102]. For instance, a CRISPR screen for factors affecting cisplatin sensitivity showed the ssDNA-binding protein suppressor of cancer cell invasion (SCAI) is required for promoting ICL repair[100]. The authors established that SCAI and REV3 (also known as DNA polymerase zeta catalytic subunit) form the protexin complex, which maintains genome stability by protecting replication forks from excessive resection (Figure 3). Replication-fork resection is partially mediated by EXO1 following fork reversal, which is mediated by the translocase activity of the ICL repair factor Fanconi anaemia group M protein (FANCM)[100]. Based on the involvement of REV3, the authors suggested that protexin acts by promoting resynthesis of resected DNA, using RNA synthesised by Pol II as the putative primer. Similarly, a CRISPR–Cas9 screen in the presence of the DNA-crosslinking agent mitomycin C revealed that SCAI fosters accurate replication-coupled ICL repair[103]. This work further demonstrated that SCAI interacts with polymerase zeta (Polζ) by binding to REV3, thereby promoting TLS and preventing DSB ligation through TMEJ (Figure 3). Furthermore, DNA polymerase iota (Polι) was found to resume replication following replication fork stalling in cells deficient in the FA pathway[99]. Loss of FA factors was also shown to cause hypersensitivity to ATM inhibition[102], at least in part due to increased toxic NHEJ and decreased HR. This work and another study[89] supported roles[89] for ATM in favouring HR over other DNA repair pathways in repairing replication-stress-related DNA damage and re-establishing replication forks, and highlighted the potential for using ATM inhibitors in cancers deficient in HR and/or the FA pathway. CRISPR screens performed with the crosslinking agent formaldehyde

uncovered additional pathways, such as mTOR signalling, involved in tolerance to DNA crosslinks, suggesting a model in which loss of mTOR components may increase autophagy and enhance cell survival and resistance to formaldehyde-induced stress[101].

[H2] ***DNA damage responses and the cell cycle***

CRISPR screens have been instrumental in identifying cross-talks between DDR pathways and cell cycle control. For example, it was recently reported that replication stress can be caused by loss of AMBRA1 (Ref.[104]). A genome-wide CRISPR screen showed that AMBRA1 deficiency leads to resistance to palbociclib, an inhibitor of CDK4 and CDK6, which normally promote progression into S phase and whose constitutive activation is a prevalent driver of cancer-cell proliferation[105] (Figure 3). The authors found that AMBRA1 is a cullin-4B E3 ligase adaptor that mediates ubiquitylation and proteasomal degradation of the CDK4 and CDK6 regulator cyclin D1 (Ref.[105]), thereby controlling G1 to S phase progression and helping to maintain genome integrity during DNA synthesis[104] (Figure 3). Other screens[94,96] also contributed to our understanding of cell cycle and DDR connections, as they identified premature mitotic entry (promoted by CDC25A and inhibited by WEE1 (Ref.[96]) and see above) and S-phase DNA–RNA hybrids as major sources of ATR-inhibitor toxicity[94] (Figure 3).

[H2] Global resources of genetic networks

The findings from pooled CRISPR screens performed with and without drug treatments enable researchers to assemble and interrogate gene–gene and gene–drug interaction networks. To facilitate such analyses, and to allow researchers to compare and contrast various CRISPR screens carried out by research laboratories worldwide, we have created the DDR-CRISPR screens portal, as described in Box 2.

Highlighting the potential for analyses of multiple screens, a recent publication[106] catalogued 31 genome-wide CRISPR screens with 27 different genotoxic agents in human RPE1 cells, collectively charting 890 genes whose loss causes resistance or sensitivity to DNA-damaging agents. Ensuing analyses associated new factors with different DNA repair pathways, including ERCC6L2 in NHEJ and transcription elongation factor 1 homolog (ELOF1) in the response to transcription-blocking drugs, and showed that the DNA G-quadruplex binding ligand, pyridostatin, traps TOP2 on DNA[106]. CRISPR–Cas9 screens have fostered deeper insights into ELOF1, leading the authors of other studies[107,108] to propose a mechanism by which ELOF1

regulates Pol II ubiquitylation, a key step in the transcription-coupled nucleotide excision repair (TC-NER) pathway (Figure 3). Beyond this role, ELOF1 also safeguards genome integrity in S phase by an as-yet undefined mechanism[107–109].

ERCC6L2 was also uncovered in another study[110] that combined chemical-perturbation screens using 36 compounds with CRISPR-KO screens focused on 414 genes connected to DDR processes performed in mouse CH12 B-cells. The screens identified ERCC6L2 as a DSB repair factor that both dictates the proper orientation of end joining and enhances the efficiency of immunoglobulin gene class-switch recombination **[G]** (CSR)[110]. In this regard, a recently reported CRISPR screen elucidated the regulation of uracil-DNA glycosylase 2 (UNG2) activity by FAM72A during CSR and immunoglobulin-locus somatic hypermutation[111]. UNG2 normally replaces deoxyuracil (dU) with deoxycytidine (dC) to prevent mutations. However, dUs are actively generated by activation-induced cytidine deaminase (AID) within immunoglobulin genes to induce somatic hypermutation and CSR. In this screen, FAM72A was shown to reduce UNG2 levels during G1 phase in B cells, which corresponds with the peak of activation-induced deaminase (AID) activity, thus promoting persistence of U:G base mis-pairs in S phase and facilitating AID activity at antibody heavy chain loci to promote CSR[111]. As FAM72A is overexpressed in many cancers, these results suggest that FAM72A could operate by counteracting UNG2 and increasing mutagenesis in cancer cells.

In another study[60], CRISPR screens targeting 365 DDR genes were performed in human HEK293A cells to probe responses to several DDR inhibitors and DNA-damaging agents. These researchers then applied a similar approach with an inhibitor of the kinase CHK1 (an ATR effector) and inhibitors of the PI3-kinase-like kinases ATR, ATM, DNA-PK and mTOR[112]. Among other findings, they uncovered resistance to ATM inhibition afforded by loss of KLHL15, an E3 ubiquitin ligase that may regulate levels of the HR-promoting DSB-resection factor CtIP. This work also suggested that loss of YWHAE (also known as 14-3-3 protein epsilon) leads to hypersensitivity to CHK1 inhibition due to abnormal activation of CDK2 (Figure 3). A further study systematically characterised functions of 629 ubiquitin ligases and 56 deubiquitylating enzymes in the presence of 41 compounds targeting different pathways including cell cycle progression, genome stability, metabolism and vesicular transport[113]. The results of this work provide a resource for further studies and, for instance, established that loss of F-box only protein 42 (FBXO42) caused hypersensitivity specifically to inhibitors of mitosis and that loss of the F-box protein FBXW7 yielded hypersensitivity or resistance to 16 of the 41 drugs[113]. Accordingly, CRISPR screens by other research groups

confirmed that FBXW7 deficiency leads to multidrug resistance[114,115]. One of these studies[114] showed that this multidrug resistance is associated with mitochondrial functions and that targeting of mitochondria pathways is particularly toxic for *FBXW7*-deficient cells. FBXW7 is a highly mutated tumour suppressor with a role in DNA repair and genome stability[116], shedding further insight into the importance of DNA repair to cancer therapy.

[H2] CRISPR base editor screens

CRISPR base editing harnesses natural or modified DNA base editing enzymes to generate specific mutations in the genome, or in cell-based reporter constructs (such as fluorescent proteins). The potential of CRISPR base editor screens (Figure 1A) to identify and analyse the effects of DDR-gene nucleotide variants in a high-throughput manner, was demonstrated by recent studies[117,118]. One of these used BE3-FNLS, a codon-optimised cytosine deaminase base editor, to mutagenize 86 DDR-associated genes[118]. Among more than 1750 variants that showed altered cellular fitness in response to DNA-damaging agents, the authors found loss-of-function (LOF) and gain-of-function (GOF) mutations in the Tudor domain of 53BP1, leading them to explore how this domain regulates the interaction between 53BP1 and the deubiquitylase USP28. This work also uncovered a domain in the E3 ubiquitin ligase TRAIP, whose mutation rendered cells resistant to camptothecin, and rare ATM variants providing increased genome stability. Several other works used adenine and cytosine base editors to functionally profile known LOF variants in *BRCA1* and *BRCA2* and uncover novel variants affecting protein function and drug responses[117,119,120]. One of these studies identified a GOF mutation in *MCL1* (encoding a protein involved in apoptosis versus cell survival regulation) causing resistance to a BCL2L1 inhibitor, and *PARP1* mutations that conferred either resistance or sensitivity to multiple PARP inhibitors[117]. In an ensuing experiment, a sgRNA library was used to study 52,034 ClinVar natural variants in 3,584 genes for effects in cells in response to cisplatin or hygromycin (an antibiotic and inhibitor of ribosome function). Upon cisplatin treatment, hits were enriched for genes involved in DNA repair and/or chromatin organisation, suggesting that this library could be useful in additional studies into mechanisms and factors connected to cancer and other diseases[117].

To help optimise base editing screens, a recent study reported a comparison between variants of SpCas9, finding that both Cas9-NG and SpG show mitigated off-target effects, and PAM flexibility that increases the targeting range of SpCas9 (Ref.[121]). For base editing screens, the

authors recommend use of SpG, as it shows increased activity at more PAM sites than the other Cas9 variants tested (the authors identified 24%, 71% and 75% potential residues available for missense or nonsense mutations in the *BRCA1* gene using wild-type Cas9, Cas9-NG and SpG, respectively).

[H2] Sequencing of repair outcomes

CRISPR screening technologies can also be coupled with profiling of DNA repair events to further elucidate functions of DDR genes and their effects on DNA repair accuracy. A recently developed CRISPR technology termed Repair-seq couples CRISPRi screening with sequencing of repair outcomes of DSBs generated by Cas9 or Cas12 nucleases in the presence or absence of oligonucleotides as templates for homology-directed repair (HDR)[61]. Repair-seq revealed that the type of DNA lesion determines the functional dependencies driving repair outcomes. For instance, the results suggested that the NHEJ factors Ku70, Ku80 and DNA-PKcs suppress fill-in by Polλ of the 5' overhangs occasionally generated by Cas9, to favour direct rejoining of compatible ends. In addition to short insertions, integration of genomic fragments of ~75 bases were found to be stimulated by the loss of the helicase/nuclease DNA2 or of MCM10. Additionally, clustering of genes with similar repair-outcome signatures, identified correlations of the end/joining factor *POLQ* with other genes, such as the ATR-checkpoint effector *RAD17*, that are not associated directly with end joining[61]. Another notable insight from this work was the identification of different pathways involved in error-free incorporation of HDR templates. Repair-seq has also been applied recently to identify pathways affecting base editing [122]and prime editing[123] (Box 3).

[H1] Relevance of CRISPR screening for cancer therapy

Because the molecular characteristics of tumour cells affect clinical responses, understanding these characteristics may pave the way towards more effectively developing and deploying cancer therapies. The advent of CRISPR–Cas9 screening methodologies has provided powerful tools for identifying genetic factors affecting sensitivity to conventional and new anti-cancer agents, and has fostered the discovery of genes required for cancer-cell proliferation as potential therapeutic targets. Such targets include those displaying synthetic lethality with cancer-associated genetic changes, wherein inactivation of the target gene or inhibition of its product are particularly toxic to cancer cells (Table 1). For example, the A and B subunits of the RNase H2 complex were identified as dropout hits **[G]** in the context of PARP inhibition[62]. Given that genes encoding these factors are frequently mutated in human cancers, and that

these cancers display intact HR, this work suggested opportunities to extend the clinical utility of PARP inhibitors beyond HR-deficient cancers. Similarly, CRISPR screens have highlighted how loss of TIGAR[124] or ALC1 (Ref.[65,66]) induces hypersensitivity to PARP inhibition. RNase H2 components A, B and C were also validated dropouts in CRISPR screens conducted in the context of ATR inhibition[95], along with DNA polymerase epsilon subunit 3 (Polε3) and Polε4 (Ref.[85]). Again, these findings are of potential clinical relevance, as they suggest a therapeutic strategy for cancers exhibiting downregulation of or mutations in these factors, such as prostate carcinomas or chronic lymphocytic leukaemia, in which RNase H2B is downregulated[62]. Base editing, saturation genome editing **[G]** and prime editing CRISPR screens have been employed to assess the effects of *BRCA1/2* single-nucleotide variants that are currently classified as variants of unknown significance on cellular sensitivities to PARP inhibitors, TOP1 inhibitors and other DNA damaging treatments[116–118,124,125]. Furthermore, base editing screens have been recently exploited to characterise cancer-associated mutations in a pooled format[125].

Building on the clinical success of PARP inhibitors and the fact that PARP loss — and to a greater degree, PARP1 inhibition and associated trapping on DNA[126] — is selectively toxic to HR-deficient cells, various CRISPR-screen studies have focused on identifying new synthetic lethal relationships in HR-deficient cells. Thus, *CIP2A*, *APE2*, *FEN1* and *POLQ* have been identified as novel *BRCA1/2* synthetic-lethal partners with potential for being targeted by new therapies[77,79–82,128,129]. Aiming to systematically explore synthetic lethal relationships, one study conducted CRISPR-screens in three different cell lines using a paired-sgRNA library of 1191 gene pairs, including paralogues and known and predicted synthetic-lethal interactions[127]. These screens revealed 105 gene pairs that, when inactivated together (but not individually), negatively affected cellular fitness. More systematically, another study[128] performed genome-wide-sgRNA CRISPR-KO screens across 324 cancer cell lines from 30 different cancer types in the Cancer Dependency Map (DepMap) project. Analyses of these screening data revealed core-fitness genes and cancer-type specific fitness genes, and identified many genes whose products are candidates for drug development[128]. This phenomenon of context-dependent fitness genes supports the idea that certain non-essential genes can become essential in certain cancer-specific genetic-backgrounds. As a proof of concept, the authors of the above work[128] validated the ATP-dependent helicase WRN as a therapeutic target for cancers displaying microsatellite instability[129]. Beyond using DepMap to search for common or specific essential genes for a particular cell line, this resource can be exploited to uncover genes in the same cell-survival pathway as genes of interest, based on similar patterns of essentiality among cell types.

Another recent publication contains a database that incorporates not only cell survival readouts but also expands into other phenotypes, including protein expression, virus responses and drug treatment responses[130]. Yet another dataset, based on results from genome-wide CRISPR screens performed with a panel of 12 tumour suppressor-single knockouts, has shed light onto new synthetic-lethal interactions and essential paralog gene pairs[131]. To identify potential therapeutic vulnerabilities in acute myeloid leukaemia (AML), whole-genome CRISPR dropout screens were performed in five AML cell lines[132]. The authors of this work found that inhibition of lysine acetyltransferase 2 (KAT2A) induces myeloid cell differentiation and apoptosis, and arrests the growth of primary AML cells, but not normal progenitors, suggesting targeting KAT2A as a novel therapeutic strategy for AML. One more recent CRISPR screen revealed that cyclin E1 (*CCNE1*) upregulation creates a specific vulnerability to the loss of PKMYT1 (Ref.[133]), a kinase that negatively regulates CDK1. Prompted by this discovery, the authors developed a PKMYT1 inhibitor, RP-6306, that activates CDK1 in a cell cycle-independent manner specifically in *CCNE1*-overexpressing cells, and in combination with the chemotherapy drug gemcitabine, promotes tumour regression in mouse models of *CCNE1* amplification.

In addition to identifying synthetic-lethal partners, pathway reliances and drug vulnerabilities of cancer cells with specific genetic backgrounds, CRISPR–Cas9 screens are being leveraged to identify genes potentially involved in resistance mechanisms towards particular therapeutic agents. In this context, it has been observed that a substantial proportion of cancers with *BRCA1/2* mutations do not respond well to PARP inhibitor treatments, either due to drug-induced or pre-existing resistance mutations[134,135]. Apart from scenarios where HR functionality is restored by compensatory mutations in *BRCA1/2* genes themselves[136], CRISPR–Cas9 mutagenesis screens have shown that PARP1 point mutations can evade the trapping activity of PARP inhibitors[126] and also unveiled other potentially clinically relevant mechanisms of PARP inhibitor resistance. As mentioned in previous sections in the context of BRCA1/2 deficiency, these include loss of components of the shieldin complex[72,73] and of the CST complex[71], DYNLL1 (Ref.[70]), cyclin C[84], the ubiquitin ligase HUWE1 (Ref.[137]) and KAT5 (Ref.[137]). Interestingly, although these mutations can cause PARP-inhibitor resistance and evidence from patient-derived xenograft models has confirmed their relevance to patient treatment[72], they could also confer vulnerabilities to therapeutic approaches other than PARP inhibition. Highlighting this potential, loss of certain shieldin components sensitises *BRCA1*-

mutant cells to ionising radiation and cisplatin, suggesting there is scope for new treatment strategies if these findings are confirmed in clinical settings[72].

PARP inhibitors are also being explored for their potential in other arenas, including in ATM-deficient cancers. As we describe above, genome-wide CRISPR screens demonstrated that loss of the NHEJ factors XRCC4, ligase IV or XRCC4-like factor (XLF; also known as NHEJ1) suppress the hypersensitivity of ATM-deficient cells to PARP1 inhibition and TOP1 inhibition[89]. Notably, the double-mutant cells were found to be even more sensitive to ionising radiation than cells lacking ATM alone, suggesting that if both mutations arise in patient cells, PARP-inhibitor resistance might be overcome by use of radiotherapy or radiomimetic chemotherapeutics[89].

Cancer cells experiencing high levels of replication stress or that are deficient in DDR factors such as ATM, are particularly reliant on the functions of the kinases ATR and its effector CHK1. Various CRISPR screens have shed light on the mechanisms of resistance to ATR inhibitors in different genetic backgrounds. As we mention above, loss of CDC25A was identified as imparting resistance to ATR inhibition[96]. Given that CDC25A is a well-known oncogene, ATR inhibitors and CHK1 inhibitors might thus be especially useful in CDC25A-overexpressing cancers. Similarly, CRISPR screens have found Mediator complex subunits CDK8, cyclin C[94,96 97]and MED12 (Ref.[97]) to confer ATR-inhibitor resistance, and CDK8 and cyclin C to also confer CHK1-inhibitor resistance. In line with the potential of combinatorial mutations in these genes as biomarkers of drug sensitivity or resistance, high expression of cyclin C and MED12 in breast cancer has been associated with poor prognosis[97]. It will thus be of interest to explore whether mutations in Mediator components produce hypersensitivity to existing or novel therapeutic agents.

[H1] Conclusions and future perspectives

The application of CRISPR tools in eukaryotic cells has greatly facilitated our ability to generate cell-based genetic models and probe gene functions, and has provided new insights into cellular processes and their underlying molecular mechanisms, some with high medical and clinical relevance. To optimise this potential, particularly as the number of published CRISPR studies in the DDR field increases, it will be crucial to compare data across screens and integrate this information through readily accessible platforms, such as the interactive, online resource DDR-CRISPR screens portal that we have generated (Box 2).

Over the coming years, it is likely that data provided by CRISPR screens will be further expanded through readouts other than cell proliferation and viability, such as ones based on microscopy combined with in-situ sequencing or fluorescence-activated cell sorting[9], to assess parameters such as gene expression[138], protein localization and interactions between biomolecules (extensively reviewed in[9]). Furthermore, CRISPR technologies combined with single-cell RNA sequencing[139–142], single-cell transposase-accessible chromatin-sequencing[143,144], Repair-seq[61] and other techniques will deepen our understanding of relationships between DDR processes, gene expression, chromatin accessibility and mutagenesis.

Knowledge of DNA repair factors and processes has already provided opportunities for refining gene-editing technologies, for example by identifying ways to modulate cellular responses to CRISPR-programmed DNA lesions and alter repair outcomes[57] (Box 3). These opportunities will likely be enhanced further by new knowledge of DNA repair mechanisms and the generation of novel tools such as DNA-repair enzyme inhibitors. It seems likely, however, that modulation of no single DDR factor will be universally applicable to all cell types and organisms. Therefore, understanding and profiling tissue-selective or specific DNA repair processes could be of key importance for modulating and optimising the efficacy of CRISPR systems[145], especially in the context of clinical applications, such as gene therapies for individuals with genetic disorders.

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Acknowledgements

We thank Dr Rimma Belotserkovskaya and Dr Giuseppina D'Alessandro for critical reading of the manuscript, Dr Kate Dry for editorial assistance, and other members of the SPJ laboratory for advice and discussions. The authors apologize to authors whose work they did not cite owing to space constraints. SWA is supported by the Mark Foundation for Cancer Research and was a recipient of an Outstanding Postdoctoral Women Fellowship from the Israeli Council for Higher Education. ABS and JCT are supported by ERC Synergy grant no 855741 (DDREAMM). JCT and VG were supported by Wellcome Investigator Award (206388/Z/17/Z). The SPJ lab is supported by Cancer Research UK and the European Research Council (ERC).

Competing interests
The authors declare no competing interests.

Author contributions
S.P.J, S.W.A. and A.S.-B. researched data for the article, substantially contributed to discussion of the content, wrote the article, and reviewed the manuscript before submission. J.C.T. and V.G. researched data for the article and substantially contributed to discussion of the content.

Peer review information

Nature Reviews Molecular Cell Biology thanks Daniel Durocher and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Table 1. Hits of CRISPR screens with potential in cancer therapy

Depletion of	Loss-of-function phenotype			Refs
	PARP inhibition	ATR inhibition	Other phenotypes referenced in this article	
RNASEH2	Sensitivity	Sensitivity	Unknown	62,95
TIGAR	Sensitivity	Unknown	Unknown	124
ALC1	Sensitivity	Unknown	Unknown	65,66
CIP2A	Sensitivity	Sensitivity	SL with *BRCA1,2* loss	77,85
APE2	Sensitivity	Sensitivity	SL with *BRCA1,2* loss	78,79,85,96
FEN1	Sensitivity	Unknown	SL with *BRCA1,2* loss	79
POLQ	Sensitivity	Sensitivity	SL with *BRCA1,2* loss	82
Shieldin complex subunits	Resistance in *BRCA*-deficient cells	Unknown	Sensitivity to IR and potentially ICL agents	72,73
CST complex subunits	Resistance in *BRCA*-deficient cells	Unknown	Unknown	71
DYNLL1	Resistance in *BRCA*-deficient cells	Unknown	Unknown	70
HUWE1	Resistance in *BRCA*-deficient cells	Unknown	Unknown	137
KAT5	Resistance in *BRCA*-deficient cells	Unknown	Unknown	137
XRCC4	Resistance in *ATM*-deficient cells	Unknown	Sensitivity to IR	89
LIG IV	Resistance in *ATM*-deficient cells	Unknown	Sensitivity to IR	89
XLF	Resistance in *ATM*-deficient cells	Unknown	Sensitivity to IR	89
BRCA1A-complex	Resistance in *ATM*-deficient cells	Unknown	Unknown	89
POLE3,4	Unknown	Sensitivity	Unknown	85
CDC25	Unknown	Resistance	Unknown	96
CDK8	Unknown	Resistance	Unknown	94

CCNC	Resistance in *BRCA*-deficient cells	Resistance	Unknown	[84,94]
MED12	Unknown	Resistance	Unknown	[97]
WRN	Unknown	Unknown	SL with MSI-cancer	[129]
PKMYT1	Unknown	Sensitivity	SL with *CCNE1* overexpression	[85,133]

Examples of CRISPR screening outputs of potential use in cancer therapy. Most phenotypes relate to responses to PARP inhibitors that are in clinical use as approved drugs, or to ATR inhibitors that are currently in clinical trials. ALC1, amplified in liver cancer 1; APE2, apurinic/apyrimidinic endonuclease 2; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related protein; BRCA1, breast cancer susceptibility protein 1; CCNC, cyclin C; CDC25, cell division cycle 25; CDK8, cyclin dependent kinase 8; CIP2A, cellular inhibitor of protein phosphatase 2A; CST, CTC1–STN1–TEN1 complex; DYNLL1, dynein light chain LC8-Type 1; FEN1, flap structure-specific endonuclease 1; HUWE1, HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1; ICL, inter-strand crosslinks; IR, ionizing radiation; KAT5, lysine acetyltransferase 5; LIG IV, DNA ligase 4; MED12, mediator complex subunit 12; MSI, microsatellite instability; PARP, poly(ADP-Ribose) polymerase; PKYMT1, protein kinase, membrane associated tyrosine/threonine 1; POLQ, polymeraseθ (theta); RNASEH2, ribonuclease H2; SL, synthetic lethality; TIGAR, TP53-induced glycolysis regulatory phosphatase; WRN, werner syndrome RecQ like helicase; XLF, XRCC4-like factor; XRCC4, X-ray repair cross complementing 4.

Figure 1. Overview of CRISPR–Cas strategies and tools.

A. Genome editing tools. **Aa.** Of the CRISPR-associated (Cas) endonucleases, Cas9 and Cas12a are the most frequently used in loss-of–function experiments. When co-expressed with a single guide RNA (sgRNA), Cas9 and Cas12a induce a DNA double-strand break (DSB) at the target site. Whereas Cas9 generates a blunt DSB, Cas12a generates a DSB with 5'-end overhangs. Cas12a may thus be advantageous for integrating a donor template into the genome in a preferred orientation (see below). Although DSB repair by non-homologous end joining (NHEJ) can reconstitute the original sequence, recurrent cleavages by the endonucleases result in NHEJ-generated short insertions and/or deletions (indels) and gene disruption. Use of an exogenous donor template enables the introduction of defined changes through homology-directed repair (HDR)[34]. **Ab.** Base editors introduce genomic nucleotide variations rather than DSBs. They comprise an enzyme that chemically modifies DNA bases at specific sites (cytosine or adenine base editors) fused to either a catalytically inactive (dead) Cas9 (dCas9) or a nickase Cas9 (nCas9), which either do not cleave the DNA or nick one strand, respectively. When fused to inhibitors of base excision repair, use of base editors results in targeted single nucleotide convertions[146,147]. For example, in conjunction with cytidine deaminases, uracil glycosylase inhibitors (UGI) promote C•G to T•A conversions and uracil DNA N-glycosylases (UNG) facilitate C•G to G•C conversions. **Ac.** In prime editing, a fusion of nCas9 and reverse transcriptase is expressed together with a prime editing guide RNA (pegRNA). The pegRNA targets a genomic site and contains a template for reverse transcriptase with the desired edit that is subsequently incorporated at the target site[148]. **B.** CRISPR technology can be used for gene regulation without genome editing. **Ba.** CRISPR interference (CRISPRi) leverages fusion of dCas9 with transcription repressor domains such as from the Krüppel-associated box (KRAB) protein, to repress the expression of specific genes[41]. **Bb.** CRISPR activation (CRISPRa) activates gene expression through dCas9 fused to transcription activator domains such as

VP64 (Ref.[149]). **Bc.** Inactive Cas9 can be bound to an epigenetic modifier to modulate epigenetic marks at specific loci. Illustrated is the dCas9 fusion to methylcytosine dioxygenase TET1, which demethylates the DNA of targeted promoters, leading to increased transcription[150].

Figure 2. High-throughput CRISPR–Cas9 screening approaches. A. A schematic workflow of a conventional CRISPR–Cas9 genetic screen. A target population of cells is transduced with a lentivirus-based library; the Cas9 nuclease is either stably expressed in the cells prior to library transduction or integrated into the library. The library is transduced at a multiplicity-of-infection (MOI) of ~0.2–0.3, to ensure that only a small proportion of cells integrates more than one virus into its genome. Typical libraries target either the whole protein-encoding genome or a selected subset of genes (generally with 2–10 different sgRNAs per gene to ensure robust statistical parameters in downstream bioinformatics analyses). The library is transduced in batches (pools) and the cells are selected for vector integration. To maximise the number of selected cells available for accurate quantitation of hits, the library is represented at least 200-fold, and sometimes over 1000-fold, in the selected cell population. This population is then divided and further propagated in parallel under control and test conditions (for example in the absence or presence of a DNA-damaging drug), during which time cells edited in genes conferring resistance or sensitivity towards the test condition will become depleted or enriched, respectively, compared to others. At appropriate times, cell samples are taken, genomic DNA prepared and subjected to multiplex PCRs with primers flanking sequences encoding the sgRNAs. These "barcoded" DNA molecules are next subjected to next-generation DNA sequencing (NGS), and analysis of these data allows quantifications of the relative abundances of each sgRNA construct within each condition, thereby enabling inference of genes whose functional disruption results in a particular phenotype[151]. **B**. Types of sgRNA plasmid libraries. Libraries can be generated of single sgRNA expression plasmids, where one sgRNA is expressed from one plasmid, or as a combinatorial library, which combines two or more sgRNAs in the same expression plasmid. The 'anchor' (gene of interest) system allows interrogation of gene interactions by combining two orthologous Cas enzymes with specific sgRNA targeting requirements, such as *Streptococcus pyogenes* Cas9 (SpCas9) and *Staphylococcus aureus* Cas9 (SaCas9). For example, cells are transfected with two vectors, one delivering SpCas9 with a SaCas9-compatible anchor-targeting sgRNA, and one delivering SaCas9 with a SpCas9-compatible 'query gene'-targeting sgRNA. Only cells with both vectors will produce DSBs at both the anchor and query loci. FDR, false discovery rate.

Figure 3. Overview of novel DNA repair factors and associations discovered using CRISPR screens. Cyclin D levels increase in G1 to activate CDK4 and CDK6, which inactivate the retinoblastoma (RB) protein, thereby allowing cells to enter S phase. The E3 ligase AMBRA1 regulates cyclin D1 stability, and loss of AMBRA1 increases cyclin D1 levels and enhances cell proliferation[105]. YWHAE drives CDK2 activation through CHK1 inhibition and the transition from G1 to S. In S phase, various replication-associated lesions can be produced, for example through incorporation of cytotoxic nucleotides such as hmdU[63], or misincorporation of ribonucleotides[62]. Repair of these involves the recruitment of various factors such as SMUG1, RNASEH2, APE2 or TDP1, which act in response to trapped TOP1. Moreover, single-strand breaks (SSB) are generated, leading to PARP1 activation. ALC1, a chromatin remodeller, helps remove PARP1 from chromatin[65,66]. Inter-strand crosslinks (ICLs) can disrupt replication fork progression and stability. The protexin subunits REV3 and SCAI protect FANCM-reversed forks by counteracting excessive resection of DNA ends (in part through re-synthesis by the polymerase REV3), which are partially generated by EXO1 (Refs.[100,103]), thereby enabling fork restoration through effective HR. DNA–RNA hybrids and transcription–replication conflicts are also sources of replication stress, and are regulated by cyclin C (CCNC)–CDK8 (Ref.[94]). Replication-

associated lesions, if not properly repaired, activate the ATR-CHK1 cascade to foster checkpoint activation by inhibiting CDC25, thus inhibiting WEE1-dependent mitotic entry[96]. PARP inhibition leads to accumulation of single-strand DNA breaks, which, during DNA replication can be converted to double-strand breaks (DSBs), whose repair relies on HR (and suppression of NHEJ). CRISPR screens have shown that hypersensitivity of BRCA1-deficient cells to PARP inhibition is suppressed by loss of the 53BP1-RIF1-shieldin complex[72,73], CST complex[71] and DYNLL1 (Ref.[70]), which inhibit DNA end resection and promote NHEJ. ATM favours HR in this context by counteracting the BRCA1-A complex and the NHEJ factors XRCC4 and LIG4 (Ref.[89]). An alternative DSB repair pathway in BRCA-deficient cells is TMEJ, which is regulated by Polθ and FEN1 (Ref.[79–81]), which were identified as synthetic lethal partners of BRCA1 or BRCA2. BRCA-deficient cells accumulate under-replicated DNA and transmit it into mitosis, where CIP2A and TOPBP1 (Ref.[77]) prevent missegregation of acentric chromosomes and cell death. CRISPR screens have also identified novel factors associated with transcription and transcription-coupled nucleotide excision repair (TC-NER), such as ELOF1 and STK19, and shown that cytotoxicity of pyridostatin, a G-quadruplex stabiliser, involves trapping of TOP2 on DNA[106].

Box1. Cellular responses to DNA damage

Environmental mutagens and by-products of intrinsic metabolic processes can produce different types of DNA lesions, ranging from alkylation and oxidation of DNA bases, ultraviolet (UV)-light induced 6-4 photoproducts and pyrimidine dimers, DNA inter-strand crosslinks (ICLs), DNA–protein crosslinks and hydrolysis of the DNA phosphodiester backbone to yield DNA single-strand breaks (SSBs) and double-strand breaks (DSBs)[152–156.] To deal with these heterogeneous lesions, various DNA damage repair pathways have evolved and have been extensively studied (see the Figur). Of these, mismatch repair (MMR) is responsible for resolving DNA mismatches and small insertion-deletions that can arise from errors during DNA replication[157], and nucleotide excision repair (NER) deals with bulky, helix-distorting DNA base damage caused by UV radiation and certain chemicals[158]. Based on where it operates, NER is categorised as global genome NER (GG-NER) or transcription coupled NER (TC-NER). Whereas some base lesions can be repaired through direct protein-mediated reversal[5], base excision repair (BER) is a multi-step pathway that removes chemically-modified bases, resulting in downstream processes that entail SSB generation and subsequent repair. SSB repair (SSBR) – which can be mediated by two partially-overlapping mechanisms termed short-patch BER and long-patch BER – is facilitated by the DNA-end binding protein, poly(ADP-ribose) polymerase 1 (PARP1) and, to a lesser extent, by the related protein, PARP2 (Refs.[159,160]). When activated by binding to SSBs or DSBs, PARP1 undergoes auto poly(ADP-ribosyl)ation, and also produces poly(ADP-ribose) chains on histones and other DDR-relevant proteins to facilitate the recruitment of various factors required for efficient DNA-break repair.

DSBs are widely considered to be the most detrimental type of DNA damage, and although they occur relatively infrequently[4], as little as one unrepaired or inaccurately repaired DSB can lead to mutations, chromosomal abnormalities, genome instability and cell death. DSB formation leads to activation of the PI3-kinase-like kinases DNA-PK, ATM and ATR, which phosphorylate histone H2AX and other DDR proteins to signal the presence of DSBs, thus altering chromatin structure and facilitating the recruitment and activation of various DNA-repair effectors and regulators[92]. The two major DSB repair pathways are "classical" non-homologous end joining **[G]** (NHEJ) and homologous recombination **[G]** (HR)[5,92]. Whereas NHEJ detects and repairs DSB ends without the need for a homologous template, HR generally requires an undamaged homologous template to restore any sequence lost at the DSB site. A key step during HR is DNA end resection, in which 3' single-stranded DNA overhangs are generated. These short overhangs invade the homologous molecule (usually the sister chromatid), leading to their extension by DNA polymerase and eventually repair of the DSB. This process is facilitated by various nucleases and helicases, including the MRE11–RAD50–NBS1 (MRN) complex, CtIP (also known as RBBP8), exonuclease 1 (EXO1), BLM and DNA2 (Ref.[5,161]) and by other crucial HR proteins including RAD51, breast cancer gene 1 (BRCA1) and BRCA2 (Ref.[5]). Although interphase mammalian cells are generally diploid, HR rarely uses the homologous chromosome as template for DSB repair[162]. Accordingly, HR is usually restricted to late S and G2 phases of the cell cycle, when a sister chromatid is available. By contrast, NHEJ can operate in any phase of the cell cycle and is the most frequently used DSB repair pathway in higher eukaryotes. Perhaps reflecting the serious threats DSBs pose to genome stability, other alternative repair pathways, such as Polθ-mediated end joining (TMEJ), microhomology-mediated end joining (MMEJ) and single-stranded annealing (SSA), have evolved presumably to mediate repair when NHEJ and HR are not able to function effectively[163,164]. Of note, NHEJ processes often introduce DNA insertions or deletions, a characteristic exploited widely for generating mutations via CRISPR-mediated genome editing. Like DSBs, ICLs are also very cytotoxic, as the covalent linking of two DNA strands blocks replication and transcription. The repair of ICLs during DNA replication is mediated mainly by the Fanconi anaemia (FA) pathway through polymerase ζ (Polζ)-mediated translesion DNA synthesis (TLS) and HR[165,166].

Finally, when a protein is covalently linked to DNA (i.e., a DNA–protein cross-link (DPC)), it may block DNA transactions. These lesions are induced by a variety of endogenous and exogenous agents such as aldehydes, ionizing radiation, UV, and chemotherapeutic agents.

Given this heterogeneity, there is a wide variety of mechanisms to repair these lesions. These comprise the direct cleavage of DNA by nucleases from NER and HR pathways, the hydrolysis of the covalent bond by specific enzymes and the proteolysis-dependent removal of the covalently bound proteins by the proteasome and proteases[167,168]. CSA, Cockayne syndrome group A; CtIP, CtBP-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; ERCC8, excision repair cross-complementation group 8; FAM111A, family with sequence similarity 111 member A; FN, Fanconi anaemia; FAN1, Fanconi anaemia associated nuclease 1; FANCD2, Fanconi anaemia group D2 protein; KU70, 70 KDa subunit of Ku antigen; MLH1, MutL homolog 1; MSH2, MutS homolog 2; PALB2, Partner and localizer of BRCA2; PMS2, postmeiotic segregation increased 2; RAD51, RecA-like protein; SLX4, structure-specific endonuclease subunit; SPRTN, sprt-like N-terminal domain; TDP1, tyrosyl-DNA phosphodiesterase 1; TFIIH, transcription factor IIH; TRAIP, TRAF-interacting protein; UVSSA, UV-stimulated scaffold protein A; XPD, xeroderma pigmentosum group D.

Box 2. Platforms for exploration of DDR CRISPR screen data

We set out to build a platform for the exploration of published CRISPR screen data by collating available CRISPR screens in the field of DNA repair. Guide abundances were obtained (either provided as a table by authors, or determined from FASTQ files), and screens were reanalysed with DrugZ and MAGeCK algorithms[169,170] using consistent parameters to allow comparison. This platform is available at https://stevejacksonlab.org/ddrcs. The website provides gene-focused and analysis-focused portals. The "Query Genes" page allows users to identify genes whose CRISPR single guide RNA barcodes were significantly enriched or depleted in any experiment within the database. These results can be filtered by false discovery rate (FDR) threshold and by parameters such as drug treatment or cell line used. References for hits identified are provided. The "Explore Screens" page allows users to select an experiment from those included in the database, filter results by the parameters of the experiment and view all gene results for that experiment. More screens will be added to the platform over time, as well as quality control measures and different analysis algorithms.

Other collections of CRISPR screen data have been assembled and made available online previously. BioGRID ORCS[171] collates published analyses of CRISPR screens, where the authors have made these available. It is frequently updated and has an interface that enables the user to find all the results for a screen. Reliance on author analyses means they were performed using different algorithms with inconsistent thresholds for significance. Often

authors only supply guide counts or raw sequencing files, and so these experiments are missing from the collection, but they will be included in our DDRcs database. The DepMap Portal[128] provides access to a large number of screens in cancer cell lines performed by members of the DepMap consortium and affiliates, and an interface for performing various analyses of these data. The DepMap data will not be included in DDRcs, and DDRcs should be considered complementary to DepMap. DDRcs shares some features with CRISPR-view[130], which enables users to make gene-based queries against the included CRISPR screens, but it has not been updated since 2019 and only includes 13 of the papers referenced in this Review.

Box 3. Harnessing of the DNA damage response in CRISPR technologies

As CRISPR-based genome editing relies on the generation of DNA lesions and their repair, the accumulated knowledge of DNA repair mechanisms has boosted genome editing efficiency and has been used to direct genome-editing outcomes. Indeed, a summary of molecules and approaches used to control CRISPR-mediated gene editing outcomes by manipulating DNA repair is reviewed in[57,172]. For example, controlling the cellular choice of DSB repair through suppression of Ku70–Ku80 or inhibition of DNA-PKcs or DNA ligase 4, which are all core components of the non-homologous end joining (NHEJ) pathway, have been shown to enhance genome editing by homology-directed repair (reviewed in[57,172]) (Figure 1). As Cas9 generates DSBs to initiate genome editing, editing completion relies on DSB-repair pathways. Therefore, we reason that cells lacking certain NHEJ factors may be refractory to efficient Cas9-mediated genetic perturbation, meaning that in such contexts other technologies, which are independent of DSB-generation, such as CRISPRi, would be more appropriate for screening.

CRISPR screens have been leveraged to find factors that could be useful for promoting specific genome editing profiles. In the context of cytosine base editing, it was reported that uracil DNA N-glycosylase overexpression, deletion or inhibition can have marked effects on editing outcomes, depending on the desired editing profile (Figure 1A)[122,146,173–178]. One of these studies harnessed the Repair-seq approach targeting DNA repair genes to identify factors affecting CRISPR cytosine base editing outcomes[122]. Together, these studies[122,173,174] have led to the development of various fusion proteins containing base deaminases and Cas proteins fused to DNA repair factors to engineer new base editors with promising C to G editing profiles. Furthermore, two recent screens have been performed to uncover DNA repair factors affecting prime editing outcomes[123,179]. Both these studies found that specific mismatch repair (MMR) genes strongly suppress prime editing efficiency and promote formation of insertions

and deletions. Consequently, ablation of MMR by depletion of the MMR protein MLH1 or by expression of a dominant-negative MLH1, increased the efficiency and fidelity of prime editing. This result suggests that MLH1-deficient cells, such as the HCT116 cell line, might be particularly suited to prime editing.

GLOSSARY

BRCA1-A complex: A protein complex (RCC45–ABRAXAS–MERIT40–RAP80–BRCC36) that has been reported to modify DSB resection dynamics and limit HR repair, somehow counteracting ATM function.

Class-switch recombination: A DNA recombination process that occurs in B cells to switch between the production of immunoglobulin (Ig) isotypes.

Clustered regularly interspaced short palindromic repeat (CRISPR): A form of immunity against viruses in prokaryotes, comprising genomic loci of short repeats interspersed with DNA sequences of viral origin called “spacers”, and the Cas family of nucleases.

Dropout hits: Genes that, when depleted or lost, affect a specific condition measured in a screen.

Dual-guide: A construct including two sgRNA sequences targeting two regions of the same gene.

Homologous recombination: A conserved type of DNA repair process that relies on use of an extensively homologous template from a sister chromatid, another homologous sequence elsewhere in the genome, or an experimentally-delivered DNA molecule.

Non-homologous end joining: The primary pathway in higher eukaryotes that repairs DSBs by directly tethering the break ends without use of a homologous template, and after potential modification of the ends, ligating them in a way that often introduces mutations.

Non-productive HR intermediates: Aberrant intermediates observed in response to unsuccessful HR (e.g., in *POLQ/53BP1* double-knockout cells), which are often associated with larger than usual RAD51 foci.

Polθ microhomology-mediated end joining (TMEJ): A DSB repair mechanism that entails exposure of microhomology sequences internal to the DSB ends before ligation, leading to deletion of the sequence flanking the DSB; sometimes associated with chromosomal rearrangements.

Sanitizer of cellular nucleotide pools: An enzyme involved in preventing the incorporation of aberrant nucleotides into genomic DNA.

Saturation genome editing: CRISPR–Cas9 genome editing aimed at introducing all possible single nucleotide variants into a targeted genomic region.

Single guide RNA: An artificial fusion of the CRISPR RNA (crRNA), which recognizes the target sequence in DNA, and the scaffold trans-activating crRNA (tracrRNA), which includes secondary structures crucial for its loading onto Cas9.

6-thioguanine: (6-TG) A nucleotide (guanine) analogue that, when incorporated into DNA, is toxic to MMR-proficient cells, which are unable to effectively repair 6-TG-induced lesions.