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Paths from DNA damage and signaling to genome rearrangements via homologous recombination

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Abstract

DNA damage is a constant threat to genome integrity. DNA repair and damage signaling networks play a central role maintaining genome stability, suppress tumorigenesis, and determine tumor response to common cancer chemotherapeutic agents and radiotherapy. DNA double-strand breaks (DSBs) are critical lesions induced by ionizing radiation and when replication forks encounter damage. DSBs can result in mutations and large-scale genome rearrangements reflecting mis-repair by non-homologous end joining or homologous recombination. Ionizing radiation induces genetic change immediately, and it also triggers delayed events weeks or even years after exposure, long after the initial damage has been repaired or diluted through cell division. This review covers DNA damage signaling and repair pathways and cell fate following genotoxic insult, including immediate and delayed genome instability and cell survival/cell death pathways.

1. Introduction

DNA is subject to constant threat of damage from endogenous and exogenous genotoxic agents, such as reactive oxygen species (ROS) arising during normal cellular metabolism, and ionizing radiation (IR) which causes DNA damage directly, and indirectly through ROS production. IR creates a variety of DNA lesions including base damage, and single- and double-strand breaks (DSBs) with immediate effects such as point mutations, chromosomal aberrations, homologous recombination (HR) and cell death. IR also triggers delayed effects, apparent many cell generations or even years after the initial exposure (Fig. 1). In general, immediate effects are due to targeted DNA damage, whereas delayed effects are most likely non-targeted, akin to bystander effects (see article by T. Hei in this issue). DNA damage triggers signaling (checkpoint) pathways that are critical for genome stability and are frequently defective in cancer. Nearly all DNA lesions block DNA replication, and stalled replication forks can be processed to DSBs if not restarted in timely manner [1–3]. DNA lesions are processed by a variety of DNA repair mechanisms, some of which restore the chemical integrity of damaged DNA in a relatively error-free manner, which maintains genome integrity. Other repair mechanisms restore the chemical integrity of DNA, but not the genetic information, causing mutations of various types, and thus destabilize the genome. Mutations range from the smallest single-base changes to large alterations

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including chromosome translocation, deletion, insertion, inversion, and amplification events. Some large-scale genetic alterations arise from very complex mechanisms that involve interactions among multiple chromosomes [4–6].

This review focuses on DNA damage signaling and DNA repair processes that regulate genome stability, with an emphasis on homologous recombination repair. The DNA damage response (DDR) comprises interacting networks of DNA damage signaling and DNA repair pathways [7–12]. DNA damage signaling is initiated by proteins that detect and bind to DNA lesions (sensors), triggering signaling pathways mediated by post-translational modifications of downstream proteins (signal transducers), including phosphorylation by protein kinases, PARsylation by PARP, and other modifications [13, 14]. These pathways activate cell cycle checkpoint (effector) proteins that mediate cell cycle arrest and regulate DNA repair and programmed cell death and senescence pathways [15–22]. Here we explore both the spatial and temporal aspects of genetic changes observed in irradiated cells, and relate these topics to cancer etiology and therapy. Dr. William F. ‘Bill’ Morgan played an important pioneering role in the genome instability field, making critical contributions over more than two decades. I was very fortunate to meet Dr. Morgan when he visited Los Alamos National Laboratory while I was completing my postdoctoral training in the late 1980s. I developed a strong personal and professional relationship with him over nearly three decades, and we performed several collaborative projects that led to new insights in the delayed genome instability field. Dr. Morgan’s vast knowledge of radiobiology and cancer biology had a tremendous impact on my career, and the careers of many investigators world-wide. It is certain that Dr. Morgan’s impressive body of work will have a major impact in the radiation sciences far into the future.

Delayed radiation effects on chromosomes were first reported in the 1950s [23, 24], but the vast majority of radiobiological studies, beginning with the pioneering work of Muller nearly 90 years ago [25] focused on immediate effects of radiation on various endpoints including mutagenesis, chromosome aberrations, and cell death. Studies in the 1950s and 1960s indicated that cells exposed to a lethal dose of radiation could divide several times before cell division terminated [26, 27], and that lethal mutations could arise several generations after irradiation [28]. In the early 1990s the Little lab demonstrated that specific (*HPRT*) mutations can arise up to 7 cell generations after irradiation, and that non-specific, lethal mutations can arise up to 50 cell generations after irradiation, revealed as reduced cell cloning efficiency and termed “delayed reproductive death” [29, 30]. By this time, the Morgan laboratory had made significant contributions to the genome instability field, and in 1993 published the first report describing delayed chromosomal instability (DCI) revealed through advanced cytogenetic analysis of hamster cells carrying a single human chromosome [31]. This set the stage for more than 20 years of progress by Dr. Morgan’s group elucidating the molecular mechanisms of DCI. The present review focuses delayed genome instability arising via HR, and includes a discussion of DNA damage response (DDR) pathways that are well-known regulators of immediate cell responses to radiation, and are also likely to be involved in delayed responses to radiation. DDR pathways have gained significant attention in recent years as targets for cancer therapy, either in mono-therapy or as adjunct to traditional chemo- and radiotherapy strategies [9, 32]. Genome instability, in its many forms, is a potent factor in cancer etiology, and the idea that radiation

and other genotoxic agents can induce genome instability many generations after the initial genotoxic insult, has significant implications with respect to risk assessment for populations exposed to radiation from environmental sources, in the workplace, and during medical procedures. A major concern is secondary tumor induction as a result of genotoxic cancer chemotherapy and radiotherapy, particularly in light of delayed or persistent induction of genome instability by radiation.

2. DNA damage signaling

Cells respond to DNA damage by activating checkpoint pathways mediated by protein kinases that arrest cell cycle progression, stimulate repair, promote survival and genome stability, and suppress cancer (Fig. 2). Two major checkpoint signaling pathways include one centered on ATM that responds to DSBs leading to Chk2 activation and p53 stabilization, and one centered on ATR that activates Chk1 in response to single-strand breaks and gaps (i.e., at replication forks). RPA bound to single-stranded DNA (ssDNA) recruits ATRIP-ATR, activating ATR. A third signaling pathway involves ATM, p38MAPK and MK2 kinases, and converges on similar cell cycle regulation targets as Chk1/2 [8, 33]. Checkpoint and repair pathways display substantial crosstalk, through protein-protein interactions and phosphorylation, methylation, acetylation, etc.). Many proteins originally defined for their roles in DNA repair, such as BLM, DNA-PK, MRE11, RAD51, and RAD52, or for their roles in DNA damage checkpoints, such as ATM and ATR play key roles in stabilizing the replisome when DNA lesions are encountered during DNA replication, and these proteins also promote restart of stalled or collapsed replication forks [34]. Through these functions, these DNA repair/checkpoint/fork restart proteins maintain genome stability in response to endogenous DNA damage arising during normal cellular metabolism (reflecting chemical lability of DNA or attack by ROS) or from exogenous genotoxins including the majority of cancer chemotherapeutics and radiation. DDR pathways operate in redundant fashion, and a major goal in the field is to identify synthetic (genetic) lethal interactions to exploit in cancer therapy [35].

DDR pathways are not “on or off” but show graded responses depending on the level of damage. DDR thresholds are genetically regulated [36, 37], and thresholds may vary for each checkpoint [38]. With minimal damage, cells may activate repair but not arrest. At higher levels of damage, cells may arrest in G1 or S to prevent replication fork encounters with lesions [39, 40], in G2 to prevent mitotic catastrophe [41], and still more damage can cause cells to enter one of several programmed death pathways (see below).

An early step in DNA damage signaling is recruitment of the Ku70/Ku80 heterodimer and MRE11/RAD50/NBS1 (MRN) complex to DSBs; these proteins are early DNA damage sensors (Fig. 2). ATR, ATM, and DNA-PKcs are PI3-like kinases (PIKs) that play central roles in DDR signaling (Fig. 2, bold font). Although early studies suggested compartmentalized functions for ATM and ATR in response to frank DSBs and replication stress, respectively, this is an oversimplification as PIKs show considerable functional overlap and crosstalk in the DDR (Fig. 2). For example, DNA-PKcs (bound to DNA end-bound Ku) and ATM are both activated by DSBs, and they phosphorylate at least six shared targets including H2AX, RPA, and c-abl, and ATR/ATM regulate DNA-PKcs via

phosphorylation [42–48]. ATR was originally thought to be primarily responsible for RPA phosphorylation during replication stress, but there is now clear evidence that DNA-PKcs phosphorylates RPA32 Ser4/Ser8 during replication stress [49–58]. These results account for the observation that DNA-PKcs defects sensitize cells to replication stress agents [48, 59, 60]. RPA has important roles in HR, and mutations in either DNA-PKcs or the Ser4/Ser8 phosphorylation targets in RPA32 confer similar phenotypes: in response to replication stress, both types of mutants show defects in replication checkpoint arrest, accelerated replication fork restart upon release from stress, defective suppression of late origin firing, hyper-recombination, and increased genome instability (mitotic catastrophe) [58].

DSBs can be induced by direct action of IR, but they also arise indirectly when other lesions such single-strand breaks and base damage block replication forks. DSBs also arise when stalled forks regress to “chicken foot” structures, or when they are cleaved by the structure-specific endonucleases MUS81, EEPD1, and Metnase [34, 61–68]. Broken DNA ends at DSBs are subject to 5’ to 3’ resection which creates 3’ single-stranded DNA (ssDNA) tails. Resection proceeds in stages, with limited resection initiated by CtIP and Mre11 nucleases, and more extensive resection catalyzed by Exo1 and Dna2 in collaboration with BLM and other accessory factors [63, 68–72]. The extent of end-resection is a key determinant of DSB repair pathway choice [63, 69, 73]. Resection is initially suppressed by 53BP1 and RIF1 bound to DNA ends; when CDK1 phosphorylates CtIP in S/G2 phase, phospho-CtIP collaborates with BRCA1 to dissociate 53BP1/RIF1 from ends allowing resection to proceed [69, 70, 74, 75].

The 3’ ssDNA tails at resected ends are rapidly coated with the abundant, heterotrimeric ssDNA binding protein RPA. ssDNA bound by RPA is a major signal for fork repair and replication checkpoint activation [39, 40, 61]. ssDNA coated with RPA recruits ATRIP and ATR, leading to ATR phosphorylation/activation by a mechanism that involves TopBP1, Claspin, RAD17-RFC, and the RAD9-RAD1-HUS1 (9-1-1) complex, via a RAD9-RPA interaction, and a host of other proteins [8, 76–78] (Fig. 2). Recently a RAD17-indepenent, NBS1-dependent ATR activation pathway was elucidated that depends on an NBS1-RPA interaction [79]. Once activated, ATM, ATR, and DNA-PKcs phosphorylate many targets including the RPA32 subunit of RPA, and Chk1 and Chk2 checkpoint effector kinases that in turn phosphorylate proteins that function in DNA repair (e.g., BLM, H2AX, RAD51, FANCD2, 53BP1, Ku70,RAD51 paralogs, and Metnase) [80–88]; regulate cell cycle progression, checkpoint arrest, and cell death pathways (e.g., p53, p21, RB, CDC25) [12, 33, 89–91]; stabilize or repair stalled/collapsed forks; and prevent late origin firing - presumably to prevent further encounters of forks with DNA damage [8, 92]. The PIK targets noted above represent a tiny fraction of the total: it has been reported that ATM and ATR phosphorylate at least 900 targets on 700 different proteins [7], and this is certainly an underestimate. Chk1 roles in checkpoint and repair responses make it an attractive target for enhancing chemotherapy and radiotherapy [93, 94].

In addition to enhancing DNA repair and regulating the cell cycle, PIK-Chk1-Chk2 signaling also plays a major role in death pathway activation (Section 3). By enhancing DNA repair and regulating cell cycle progression, DDR signaling through PIK pathways

plays a major role in maintaining genome stability in the face of DNA damage and thus suppressing tumorigenesis [95].

As noted above, IR and other genotoxic agents induce lesions in DNA, nearly all of which block replicative polymerases. Replisomes are stabilized at stalled forks by many proteins with roles in DNA repair and DNA damage checkpoints, including RPA, ATR-ATRIP, ATM, BLM, and INO80 [40, 96–98]. In some cases, lesions can be bypassed translesion synthesis (TLS) polymerases, which are error-prone and not highly processive, so TLS creates mutations in nascent DNA strand opposite, and in the immediate vicinity of, DNA lesions [99–101]. Persistent stalling at blocking lesions can lead to fork collapse and one-ended DSBs ("double-strand ends" - DSEs), and replication fork encounters with single-strand breaks (induced by IR far more frequently than DSBs) may cause direct fork collapse to a DSE. As with frank DSBs, ATM and ATR phosphorylate histone H2AX (γ-H2AX) in the vicinity of DSEs [87], activating checkpoint and repair processes [88, 95]. While intra-S checkpoint arrest serves to minimize replication fork encounters with DNA lesions induced by IR, it is likely that once the checkpoint is released and replication resumes, residual damage may remain, triggering replication stress at later times (~8 h) after irradiation [102]. It is difficult to determine the relative contributions of immediate (mis)repair events vs. later replication stress to genome destabilization. As discussed in Section 5, genome stability is also threatened at much later times (weeks to years) in a fraction of cells that survive low to moderate doses of IR. We have a limited understanding of the mechanisms responsible for these delayed effects.

3. Regulation of cell fate after DNA damage

Cell fate following genotoxic insult can be divided into cell survival vs. death, and among survivors, cells may retain a stable genome or succumb to genome instability. Low to moderate levels of genome instability can be tolerated (yet trigger tumorigenesis and/or tumor progression to more aggressive stages) but extreme genome instability is incompatible with cell viability, because of gross gene expression imbalance due to numerical changes in chromosome content due to segregation defects, or from lethal mutations (induced or uncovered via loss of heterozygosity. Regulated cell death pathways guard against massive genome instability and cancer by eliminating highly damaged cells. Apoptosis is a set of well-characterized programmed (regulated) cell death pathways [21, 103]. There are two other programmed death pathways, autophagy and necrosis, as well as senescence, an anti-proliferation pathway in which cells remain metabolically active. From the standpoint of cancer therapy, inducing any of these death/senescence pathways serves to eradicate tumors or control tumor growth. Autophagy ("self-eating") is a stress response pathway conserved from yeast to humans [17, 103] mediated by catabolic processes that degrade proteins and organelles, including mitochondria which helps maintain metabolic homeostasis. Autophagy is inhibited by oncogenic proteins that activate mTOR (e.g., Ras and AKT), and autophagy is stimulated by tumor suppressor proteins that inhibit mTOR (e.g., LKB1, PTEN, and AMPK), thus autophagy operates as a tumor suppressor pathway in normal cells [104, 105]. Consistent with this view, direct chemical or genetic inhibition of autophagy increases tumorigenesis which is associated with genome instability and increased reactive oxygen species. However, once a tumor is established, autophagy can actually protect tumor cells

from endogenous stress common in tumors (nutrient deprivation, hypoxia, “oncogenic stress”), and exogenous stress associated with chemo- or radiotherapy. Once considered a passive death pathway, necrosis is instead a genetically regulated pathway, which has given rise to the term “necroptosis” [18, 19]. Similar to autophagy, necroptosis can influence both tumorigenesis and tumor responses to therapy [106]. Senescence was originally defined as growth arrest due to telomere shortening, which can be bypassed by telomerase in stem cells and cancer stem cells. However, an alternative senescence pathway of permanent checkpoint arrest blocks tumor re-growth after radiotherapy [20]. It is possible to define death pathway spectra for various stress conditions by using pathway-specific markers: caspase-3 cleavage, annexin-V, TUNEL and others for apoptosis [107], LC3-II for autophagy [108, 109], secreted HMGB1 for necrosis [110, 111], and checkpoint/SA-β-Gal expression (senescence) [112].

Some types of DNA damage and defects in chromosome decatenation or segregation cause cell death via mitotic catastrophe, which is revealed as giant cells, nuclear dysmorphism, multinucleate cells, micronuclei, and anaphase bridges [20]. Mitotic catastrophe is not programmed, per se, but it is regulated in the sense that it is suppressed by the DDR, and it may trigger cell death by other programmed death pathways including apoptosis and necrosis [41].

ATM phosphorylates/activates Chk2 kinase, which then phosphorylates and stabilizes p53, altering p53 transcriptional activity, and promoting apoptosis [113]. There are also p53-independent apoptotic pathways [114]. Chk1 also phosphorylates p53 on both shared and distinct residues targeted by Chk2. Chk1 appears to balance Chk2 by suppressing apoptosis, and Chk1 and Chk2 also regulate apoptosis through phosphorylation of the ubiquitin ligase Mdm4/X which promotes p53 degradation [113].

Apoptosis has long been considered a desirable outcome in radio- and chemotherapy, but this paradigm has recently shifted in light of studies demonstrating that a common upstream apoptotic event, caspase 3 activation, stimulates release of the paracrine growth factor prostaglandin E2 (PGE2) which enhances proliferation of nearby surviving cells. This pathway, termed “Phoenix Rising” is akin to wound healing [115]. Phoenix Rising is has clinical importance as patients with caspase 3-defective breast and head/neck cancers survive longer than those with functional caspase 3 [115]. Caspases act early in apoptosis [116] and Phoenix Rising depends on caspase-3 activation (cleavage) which activates the iPLA2-Cox1/2-PGE2 pathway [115]. Phoenix Rising can be blocked at many steps along the pathway from caspase-3 cleavage to PGE2 production/receptor binding [115, 117]. Although blocking apoptosis might enhance cell survival, sufficient DNA damage will likely induce death by another pathway. Inducing cell death without activating Phoenix Rising may be key to improved local tumor control. Although much is known about DDR proteins and their roles in cell survival and genome stability, and there are clear connections between the DDR and apoptosis, virtually nothing is known about how the DDR regulates other cell death pathways. Given the connection between apoptosis and Phoenix Rising [115, 118], it is critical to gain a better understanding of DDR regulation of all death pathways to simultaneously optimize tumor cell killing while preventing accelerated repopulation via Phoenix Rising and thus enhance local tumor control by radiotherapy. To summarize,

cell fate depends on the level and complexity of damage, DDR signaling, DNA repair, programmed cell death pathway choices, and bystander effects like Phoenix Rising in which damaged or dying cells influence growth or genome stability of nearby cells. All of these factors contribute to normal tissue and tumor responses to IR.

4. DNA repair and genome instability

Genome instability takes many forms and arises from mis-repair of endogenous or exogenous DNA damage. Defects in DNA repair pathways can dramatically increase genome instability, with specific mutational spectra dependent on the types of damage and repair outcomes. All DNA repair pathways can restore the proper chemical structure of DNA, but they vary widely in their ability to restore the original genetic sequence, hence the term “mis-repair” denotes restoration of DNA chemical structure without restoration of the genetic sequence. Such errors span the spectrum from single-base changes and small insertion/deletion mutations, to gene-level changes (gene duplication, deletion, amplification, and rearrangement), to chromosome-level changes including translocations, loss of large regions of chromosome arms, and gain or loss of whole chromosomes. Defects in base excision repair and nucleotide excision repair increase point mutagenesis and are associated with many cancers including those of the gastrointestinal tract, breast, bladder, and skin [119]. Defects in mismatch repair greatly increase point mutagenesis including frameshift mutations arising from replication slippage in homo-polynucleotide runs, and slippage at triplet repeats, predisposing to colon cancer and other cancers [120].

Defects in DSB repair may reflect problems with NHEJ or HR (Fig. 3). Although cNHEJ is inherently error-prone, introducing short deletions or insertions at joints, there are greater risks associated with cNHEJ defects because the back-up aNHEJ pathway creates larger deletions and mediates translocations [121–128]. The Jasin lab showed that defects in core cNHEJ factors Ku, XRCC4, and Lig4 suppress translocations stimulated by simultaneous DSBs introduced into two chromosomes [125, 127]. Similarly, translocations were suppressed when cNHEJ was enhanced by overexpression of Metnase [129]. These results appear counterintuitive given that at least some translocations arise via end-joining, but can be explained by a model in which cNHEJ promotes rapid DSB repair, and when cNHEJ is defective DNA ends have a greater propensity to migrate through the nucleus and join with ends from other chromosomes to create translocations. This model gained additional support when it was determined that translocations frequently result from aNHEJ, which is mediated by MRE11 and CtIP (to effect limited resection), NBS1, PARP1, Lig3 and FEN1 [130, 131]. The enhanced genome instability with cNHEJ defects can also be understood in terms of repair accuracy, as aNHEJ requires microhomology and therefore typically creates larger deletions than cNHEJ.

In contrast to error-prone cNHEJ/aNHEJ, HR is often described as an error-free DSB repair pathway. Extensively resected broken ends are first bound by RPA, as discussed above, and RPA is subsequently replaced by RAD51 with assistance from “mediator” proteins including BRCA2 and RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3). The RAD51-ssDNA nucleoprotein filament seeks and invades homologous duplexes elsewhere in the genome which serve as (relatively) accurate repair templates. HR is largely suppressed

in G1 phase and upregulated in S and G2, when sister chromatids can serve as highly accurate (and proximal) repair templates. However, homologous sequences anywhere in the genome may serve as a repair template, including homologous chromosomes and repetitive elements (Alu, MIRs, SINEs, LINEs, etc.) which are extremely common in higher eukaryotes, comprising >50% of human genomic DNA [132]. Because non-sister homologous sequences are often not 100% identical, HR repair from such templates can transfer divergent sequence information to the broken chromosome, a process termed gene conversion [133]. HR between non-sister sequences can also lead to significant structural changes in the genome when HR intermediates are resolved with a reciprocal exchange or crossover, including gene deletions, duplications, inversions, large-scale loss of heterozygosity, and translocations [63, 126, 133–136]. For this reason, mitotic crossovers are suppressed [137–140] and defects in proteins that suppress crossovers, like BLM, cause massive genome instability and cancer predisposition [141]. Thus, some HR events cause genome instability [126, 142], drive tumor evolution, and cause other diseases [136, 143, 144]. Although HR has the potential to destabilize the genome, instability is far more pronounced in HR defective cells, as seen with defects in BRCA1, BRCA2, and FANC proteins, which predispose to breast and other cancers [145–148]. Thus, HR is tightly regulated, and mutations that dysregulate HR, causing hyper-recombination or hypo-recombination phenotypes, generally have detrimental effects on genome stability.

HR plays a critical role in maintaining genome stability in the face of replication stress [3, 34, 40]. When replication forks encounter damage, they can assume a variety of branched structures including 5’ and 3’ flaps, single-strand gaps, and 4-way junctions akin to Holliday junctions in homologous recombination (HR) intermediates [64]. These branched structures are cleaved by structure-specific nucleases (Mus81-Eme2, EEPD1, Metnase) [66–68, 149–151], creating DSEs that are extensively resected to long 3’ ssDNA tails by the combined action of Mre11/CtiP, Exo1, and Dna2 (in association with BLM) to effect fork repair/restart via HR [64, 152]. Unlike DSBs, DSEs have no proximal end with which to join via NHEJ, thus if DSEs were to be subject to repair by NHEJ the outcome would be large-scale structural changes to the genome (deletions and translocations). This explains why HR is of utmost importance in replication fork restart: the alternatives are simply too risky. It also explains why severe HR defects, such as RAD51 null mutations, are cell lethal and cause embryonic lethality in mice [153, 154].

5. Radiation-induced delayed HR

As discussed in Section 1, IR induces both immediate and delayed effects, including mutations, chromosomal aberrations, homologous recombination, and cell death. Between 1996–2003 the Morgan laboratory led the field in defining mechanistic aspects of radiation-induced delayed chromosomal instability, offering insights into the types of DNA damage capable of inducing the effect; chromosomal structures involved (e.g., telomeres); its relationship to other delayed effects such as mutation and cell death; dose/dose rate, and radiation quality effects; the relevant target and target size; and early studies demonstrating that whole genome transcription profiles were similar in stable vs. chromosomally unstable cells [155–168]. This impressive body of work was a direct precursor to collaborative studies between the Morgan and Nickoloff laboratories, initiated in the early 2000s and

focused on a different type of radiation-induced delayed genomic instability, namely delayed homologous recombination (DHR; also termed delayed hyper-recombination). In particular, we were interested in whether IR induced DHR and if so, whether DHR correlated with other delayed effects such as DCI and cell death [169]. To address these questions we constructed derivatives of RKO (human colon carcinoma) and GM10115 (hamster cells with a single human chromosome), each carrying a single integrated copy of a GFP direct repeat HR substrate. RKO cells were chosen because they show normal p53 and p21 induction by IR and have a near-diploid chromosome complement, and GM10115 cells were chosen because they are p53 mutant and had been extensively used in past DCI studies. In each case, parent cells are GFP^- because neither of the two GFP genes were functional, but HR can convert these to GFP^+ (Fig. 4A). We found that X-ray doses from 1–10 Gy induced immediate HR, evidenced as fully GFP^+ colonies, as well as mixed $GFP^{+/-}$ colonies which by definition were evidence of DHR (Fig. 4B). Surprisingly, DHR was induced at very high frequencies: nearly 10% of surviving cells displayed this phenotype (Fig. 4C). DCI was monitored in the same exposed cell population, and consistent with prior studies, DCI was also induced at frequencies approaching 10% and was correlated with delayed death. Interestingly, cells that expressed DHR did not display DCI, and vice versa. Moreover, cells that expressed DHR showed no evidence of delayed death. Thus, although DCI and DHR and both induced by IR at similar, high frequencies, DCI and DHR are mechanistically distinct [169]. Dysregulation of HR is observed as hypo-HR, reflecting defects in HR proteins [170–177], or as hyper-HR, reflecting defects in HR regulatory systems such as in BLM-mutant cells [98, 178, 179]. Hyper-HR can also more generally reflect DNA repair defects or metabolic disorders that increase ROS, which increase HR associated with replication stress due to more frequent encounters of replication forks with DNA lesions [2, 3, 34]. Genome instability is associated with both hypo- and hyper-HR, thus, it is important for cells to maintain appropriate levels of HR in order to maintain genome stability. The relatively high efficiency by which radiation induces DHR suggests that DHR poses significant risks to genome stability at late times after low- to moderate-doses of IR. This risk is further exacerbated by the fact that the DHR phenotype correlates with high viability, as opposed to DCI, in which cells are often sick and prone to delayed death [169].

The next question was whether DHR is induced by non-ionizing ultraviolet light (UV) radiation. The RKO-GFP cells were exposed to either UV-B, or the shorter wavelength and more damaging/more lethal UV-C and DHR was again scored as mixed $GFP^{+/-}$ colonies. As with IR, moderate doses of UV-C (5 J/m^2, yielding ~20% survival) induced DHR in ~15% of cells; in contrast, no induction of DHR was observed with equitoxic doses of UV-B, or higher UV-B doses [180]. Thus, both IR and non-ionizing radiation induce DHR at very high frequencies, and in both cases, the DHR phenotype was not associated with delayed death [180].

As noted above, IR triggers several delayed effects including DCI, DHR, mutation, and cell death. RKO cells are derived from a male donor and carry a single, functional X-linked *HPRT* gene that allows facile detection of *hprt* mutants. As expected, *hprt* mutant frequencies were low in parental (non-exposed) cells. Mutant frequencies increased several-fold among UV-C survivors in which HR was induced immediately (full GFP^+ colonies), but there was a dramatic, ~100-fold increase in *hprt* mutations among all DHR ($GFP^{+/-}$)

colonies tested (n=9). Note that all of the *hprt* mutations scored in this study arose after UV-C (or mock) exposure, hence the modest increase in mutagenesis in immediate HR cells, and the dramatic increase in DHR cells, all reflect delayed mutation. These results indicate that UV-C induced DHR and delayed mutation are strongly correlated and mostly likely stem from the same source. Sequencing of individual *hprt* mutant genes yielded yet another striking result. The most common types of spontaneous mutation in *HPRT* are individual point mutations comprising single-based changes or short frameshift mutations, with less frequent large-scale deletions [181, 182], although mutation spectra vary depending on mutator genotype among cancer cell lines [183]. Interestingly, the spectrum of delayed *hprt* mutations arising in immediate HR cells were typical, largely comprising individual point mutations or deletions, yet among 7 delayed *hprt* mutants arising in DHR cells, 5 had compound point mutations, with three displaying 5 or more mutations [180]. The specific types of delayed mutations arising in DHR cells were indicative of mutagenesis resulting oxidative DNA damage, providing a likely link between the UV-C induced DHR and delayed mutation phenotypes.

Having established that moderate doses of IR and non-ionizing radiation efficiently induce DHR, the next question was whether DHR was induced by low doses of IR, a topic relevant to risk assessment for low dose medical (e.g., diagnostic) exposures. Three key observations were made in this study. First, DHR was indeed stimulated by low dose IR, with significant increases seen with doses as low as 1 cGy (Fig. 4D). This is in the upper range for CT scans [184]. The fact that DHR is induced at high frequencies with extremely low IR doses indicates that the target is very large, either the nucleus or the entire cell, or perhaps that DHR may actually be a non-targeted (bystander) effect. Second, low dose exposures of several cGy showed roughly the same induction of DHR as doses 100-fold higher (compare Fig. 4C and 4D). Although the results did suggest that DHR increased with dose, the dose response was highly non-linear, raising the possibility of a threshold effect. Finally, low dose IR exposures of 1–5 cGy suppressed the greater induction of DHR by a 500 cGy dose delivered 4 h later, relative to a single 500 cGy dose, providing evidence that IR-induced DHR is subject to an adaptive response (Fig. 4D) [185]. Interestingly, these RKO-GFP cells did not show an adaptive response with respect to cell killing, that is, low dose pre-exposures did not reduce the cytotoxic effect of the subsequent challenge dose [185]. Clearly DHR and cell death are independently regulated, and it is also likely that these endpoints will differentially respond to modifications to the DDR, i.e., with PIK inhibitors commonly used as radiosensitizing agents.

Despite the fact that IR-induced DCI is associated with delayed cell death, DCI can persist for many years [166]. We recently undertook a project to determine whether DHR is similarly induced by low LET X-rays and high LET carbon ion radiation, and the length of time that DHR persisted in each case. As with DHR induced by X-rays, DHR was induced to high frequencies by carbon ions, and both showed atypical dose responses, although very low dose carbon ion exposures have not yet been tested. Importantly, DHR was found to persist for 2 weeks before resolving to background levels by the third week after irradiation, and this was true for both X-ray and carbon ion exposures (Allen, Hirakawa, Nakajima, Moore, Nie, Sugiura, Hoki, Araki, Abe, Okayasu, Fujimori, and Nickoloff, manuscript in preparation). These results indicate that if there is indeed a risk of genome instability and/or

cancer associated with DHR, this risk appears more time-limited than that from DCI, and that there is no significant difference in risks associated with low vs. high LET exposures.

6. Future perspectives

Our knowledge of DNA repair and DDR networks has greatly expanded in recent years, yet as we tease out the details through traditional reductionist approaches, the “omics” revolution is providing new insights into the vast complexity of these critical cellular processes. DNA repair and DDR signaling systems are capable of sensing and processing a wide range of lesion types caused by different types of endogenous and exogenous genotoxic agents, and while it is clear that these response systems are fundamental to triggering the various cell fates of stressed cells, the astonishing genetic heterogeneity of tumors poses significant challenges to harnessing our knowledge to create better targeted and more effective, and safe, cancer therapies. Genome instability takes many forms, and often reflects defects in DNA repair and/or DDR signaling pathways. It has long been known that cancer cells harbor unstable genomes, but for many years it was unclear whether instability was a cause or an effect of the tumorigenic phenotype. It has been argued that genome instability is an “enabling characteristic” of cancer [186], and it is clear that instability can precede neoplastic transformation [187–190]. Delayed mutation, DCI, and DHR are still relatively poorly understood mechanistically, but the fact that moderate radiation doses, and in some cases very low doses, trigger these types of instability is certainly a concern.

Many questions remain in the delayed genome instability field, including the most basic question: what is the ultimate trigger? While it is clear that the target is as large as the nucleus and may be larger [166, 191, 192], the persistence of delayed effects may reflect persistent high levels of ROS [193, 194], at least in part reflecting mitochondrial dysfunction [195, 196]. This suggested paths to mitigate these effects through radical scavengers, as shown for IR-induced DCI [197], but parallel studies with DHR have not been performed. Moreover, there is little to no information about how modulating DNA repair or the DDR might influence, positively or negatively, various delayed genome instability phenotypes. Such information is critical as there is growing interest in targeting DNA repair and/or DDR proteins as mono-therapy to treat cancer, or as adjuncts to chemo- or radiotherapy [9, 10, 35, 91, 120, 124, 198–204].

Finally, a understanding the fundamentals that underlie IR-induced delayed genome instability, particularly those induced with high efficiency at low doses, is important with regard to radiation risk assessment. This is particularly true for low dose medical exposures during diagnostic imaging given the frequent use of such procedures. Among cancer patients there are also potential risks associated with genome instability triggered by moderate to high radiation doses delivered to normal and cancer tissues during radiotherapy, including progression of rare surviving tumor cells to a more aggressive state, and secondary cancer induction. Whether chronic low dose (e.g., environmental) exposures pose similar or different risks of delayed effects is poorly understood, but should be explored and factored into risk assessments [205–211]. Dr. Bill Morgan had much to say about these and many

other topics in the radiobiology and radiation risk assessment spheres. We would do well to heed his messages as we drive these fields forward.

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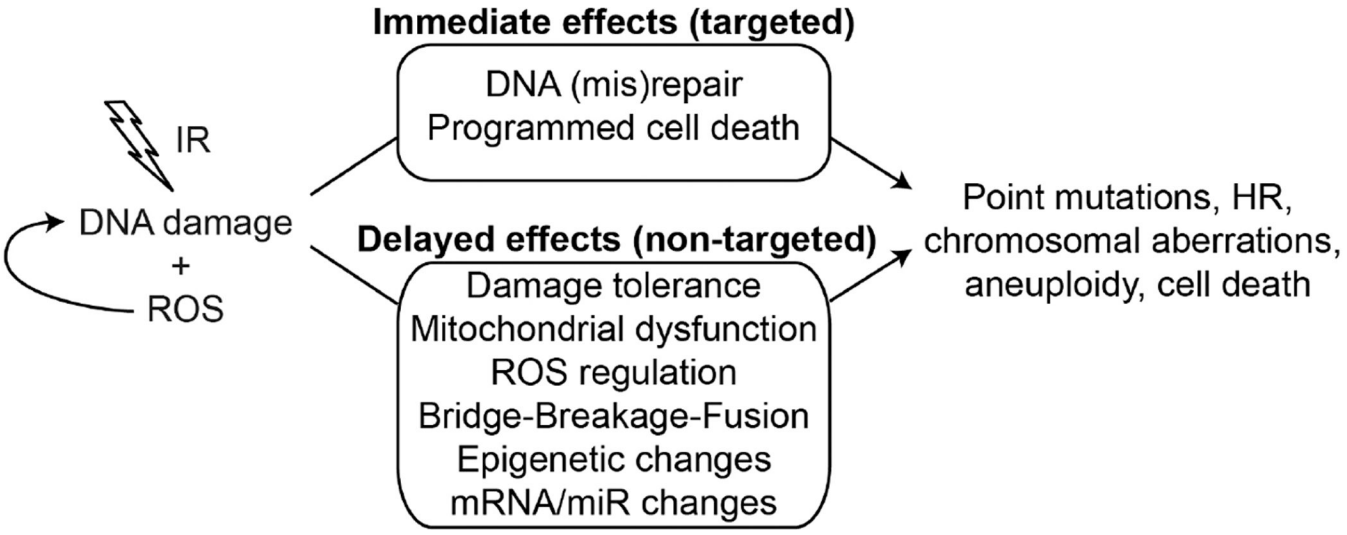

Fig. 1.

IR causes DNA damage directly and through ROS production, causing a wide variety of genetic changes. Some changes appear immediately after exposure and are most likely targeted effects. IR also induces delayed effects that can lead to the same types of genetic changes, but are likely non-targeted effects and may reflect changes in specific organelles or processes that have broad impact on genome stability.

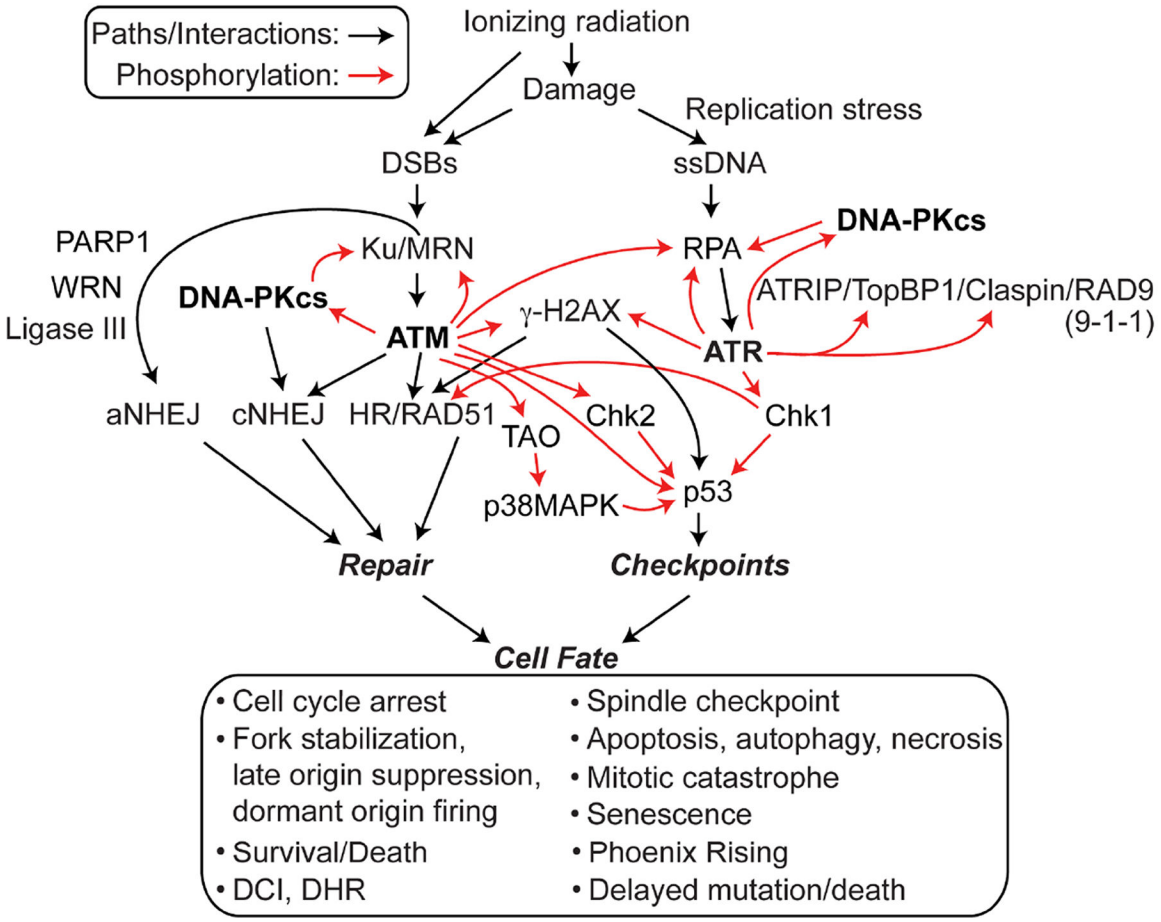

Fig. 2.

Core factors in DNA repair and DDR networks. Ionizing radiation causes DNA damage that activates PIKs (bold) which transmit signals to both downstream and upstream targets that regulate DNA repair by aNHEJ, cNHEJ, and HR, and activate checkpoint response pathways that arrest the cell cycle and trigger programmed cell death pathways, all of which regulate cell fate.

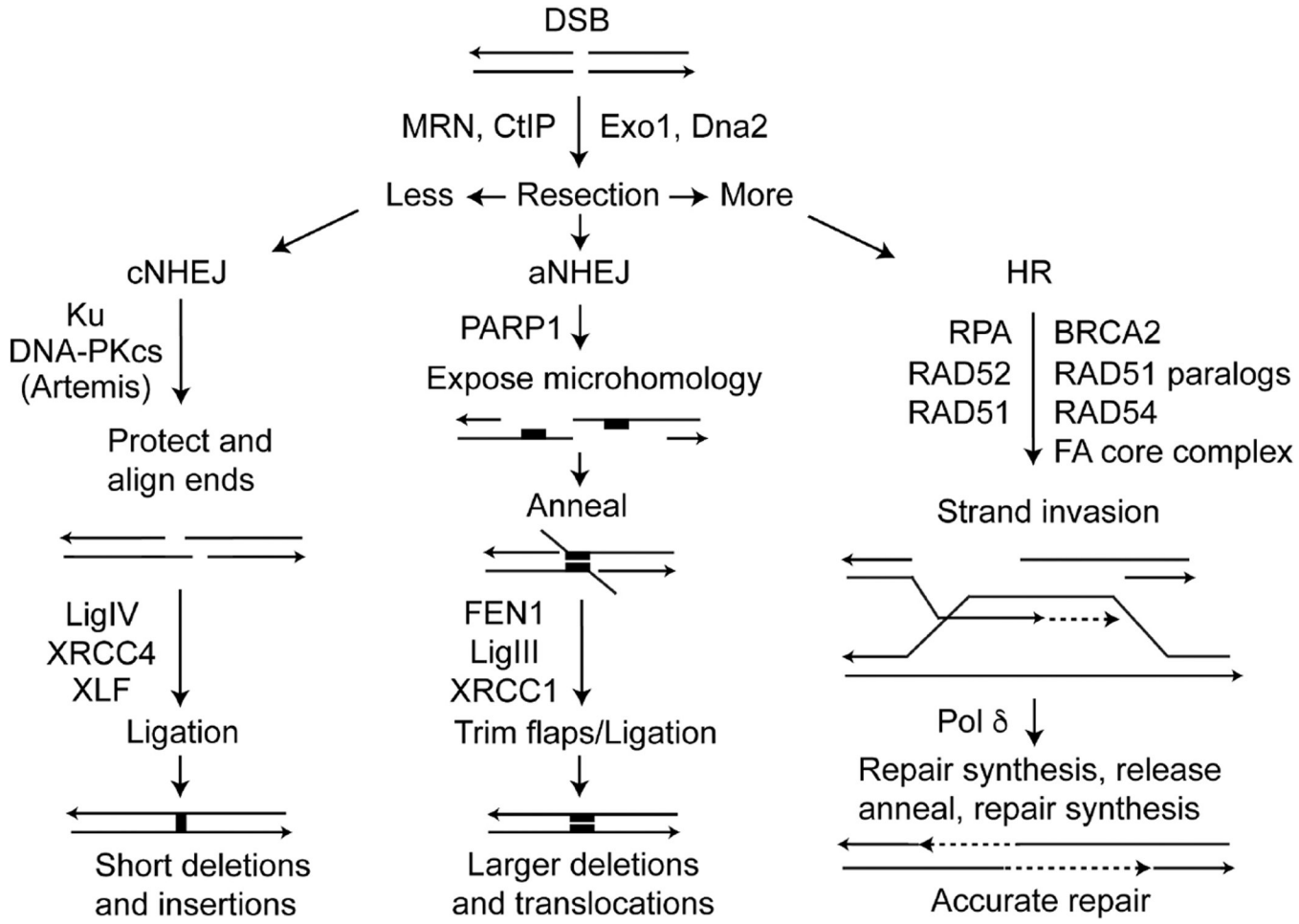

Fig. 3.

DSB repair pathways. DSB repair pathway choice is controlled by resection, mediated by several nucleases. cNHEJ involves little or no resection, aNHEJ limited resection to expose microhomologies near the DSB (black rectangles), and HR involves extensive resection creating long, 3' single-stranded tails that invade homologous sequences (typically sister chromatids) that serve as accurate repair templates.

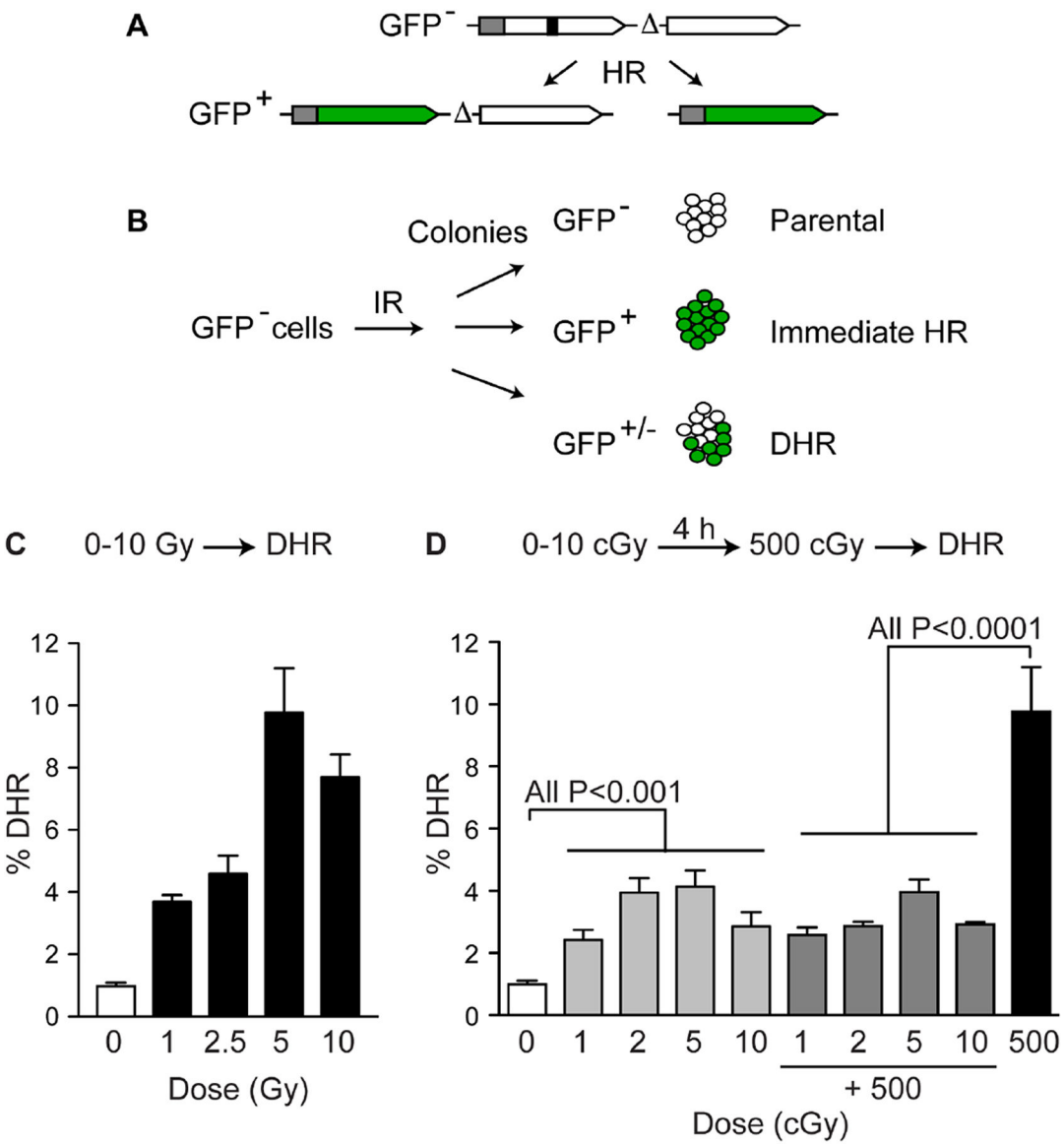

Fig. 4.

IR-induced DHR. (A) Cells carry a single copy of a direct repeat HR substrate with two inactive copies of GFP (GFP⁻). HR creates GFP⁺ products that retain both copies or delete one of the copies via crossover or single-strand annealing. (B) GFP⁻ cells treated with IR produce colonies that are GFP⁻ (parental, non-recombinant), fully GFP⁺ (immediate HR (C) Moderate to high doses of X-rays stimulate DHR at high frequencies. (D) Low (cGy) doses of X-rays stimulate DHR at high frequencies and induce an adaptive response to a later challenge dose of 500 cGy. Data compiled from refs. [169, 185].