Cellular Responses to DNA Damage: One Signal, Multiple Choices

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Abstract

DNA double-strand breaks (DSBs) produce a number of cellular responses, some mutually exclusive. Depending on where on the chromosome it occurs, a DSB may become preserved inside a telomere or eliminated by repair. A cell may arrest division via checkpoint activation to fix DSBs or commit suicide by apoptosis. What determines the outcome: to bury, fix, or succumb to DNA DSBs? With this question in mind, we review recent data on cellular responses to DSBs.

Contents

INTRODUCTION

Extracellular and intracellular insults such as reactive oxygen species, ionizing radiation, and radiomimetic drugs can induce DSBs (double-strand breaks) in DNA. Of the many cellular responses to DSBs, regulation of cell division by checkpoints received the most attention initially. Historically, checkpoints refer to mechanisms that arrest the cell division cycle in response to intracellular conditions such as damaged or incompletely replicated DNA. Signals that activate cell cycle checkpoints were later found to activate additional cellular responses such as DNA repair and apoptosis (**Figure 1**). Thus, currently, these responses, along with cell cycle regulation by checkpoints, are collectively referred to as DNA damage response pathways (110).

As with other signal transduction pathways, DNA damage responses are composed of core components: the signal, sensors of the signal, transducers, and effectors. Recent studies have clarified the contribution of another class of molecules, mediators (also called adaptors), which lack catalytic activity but facilitate signaling by promoting physical interaction between other proteins. We briefly define each component of DNA damage response pathways before delving into recent advances. Although data from a variety of experimental models are considered, studies in metazoan models are emphasized at the expense of studies in unicellular eukaryotes (see Harrison & Haber, this volume).

THE CAST OF CHARACTERS

The concept of checkpoints arose from studies in budding yeast where progression through the cell cycle is clearly visible as an increase in bud size. Checkpoint activation was obvious as arrest at a particular stage in the cell cycle. The arrest phenotype, coupled with molecular knowledge of cell cycle regulation, helped identify effectors of checkpoint signaling, which are integral components of the cell cycle machinery (76, 110).

Cyclin-dependent kinases promote cell cycle transitions, and it is their activity that is ultimately inhibited by checkpoint activation. Cdk1 activity in the cell is a product of activating and inhibitory events. Modulation of one regulator can therefore indirectly affect the potency of a checkpoint, even if this regulator is not a target of the checkpoint.

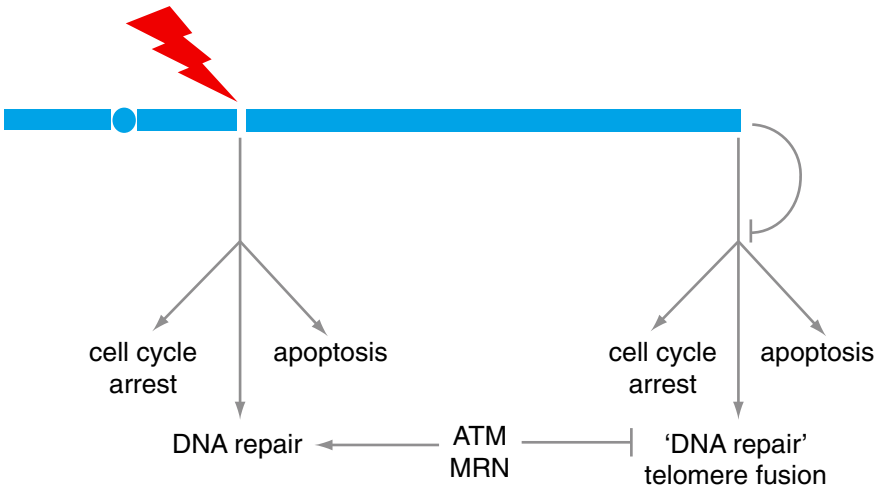

Figure 1

Cellular responses to DNA double-strand breaks include cell cycle arrest by checkpoints, DNA repair, and apoptosis. Ends of linear chromosomes are also capable of generating these responses, but are normally prevented from doing so. ATM and MRN proteins, which normally promote repair of DSBs, prevent joining of chromosome ends via abnormal repair; their absence can lead to telomere fusion and p53-mediated apoptosis. See text for details.

An effector, therefore, is expected not only to influence checkpoint signaling but also to show changes in property (level, phosphorylation status, localization, etc.) that result from checkpoint activation and are necessary for enforcing the checkpoint. Homologs of CDC25 and tumor suppressor p53, for example, fit these criteria (76, 110). The former are phosphorylated and inhibited by checkpoint activation in mammals, Drosophila, Xenopus, and yeast, whereas the latter become phosphorylated upon induction of DSBs (in mammals and Drosophila) and increase in level (in mammals).

Genetic studies in yeast identified several components of checkpoint pathways that were not part of the cell cycle machinery, but are now known to encode signal sensors and transducers. These include a complex of Rad9p, Hus1p, and Rad1p (the so-called 9-1-1 complex), and a complex of Rad17p with four smaller subunits of the replication factor C (RFC2-5) [reviewed in (39, 110)]. The 9-1-1 complex is proposed to resemble PCNA, a donut-like hexamer that is loaded onto DNA by the RFC. Sensor/transducers also include four protein kinases that are conserved from yeast to mammals (76, 110). These are two PI3-Kinase-like Kinases (PIKK): ATM (Ataxia Telangiectasia Mutated), the first checkpoint function to be characterized, and ATR (AT and Rad3 related); Serine/Threonine Kinases Chk1 (Checkpoint Kinase 1) and Chk2 (Checkpoint Kinase 2). ATR is found in complex with ATR Interacting Protein (ATRIP), which is required for ATR function (see **Table 1**). We discuss the functional ordering of sensor/transducers in detail below.

Rad9p of budding yeast, the first checkpoint function to be genetically defined, encodes the archetypical mediator. Orthologs in other species include 53BP1, BRCA1, MDC1, and ToBP1 in vertebrates, and Crb2p in fission yeast. Mediators have in common a BRCT (BRCA1 carboxyl terminal) domain that binds to phosphorylated peptides, the behavior of forming nuclear foci (Ionizing Radiation-Induced Foci or IRIF), and the ability to bind to and promote the interaction

Ionizing radiation: any radiation capable of producing ions by displacing electrons from atoms or molecules. Of these, Y-rays and X-rays can penetrate cells and tissues to cause single and double strand breaks in DNA

DSB: double-strand break

Mediators (or adaptors): proteins that lack catalytic activity but facilitate signaling by promoting physical interaction between other proteins

PIKK: PI3 Kinase-like Kinase

ATM: Ataxia Telangiectasia Mutated

Ataxia telangiectasia: an inherited human disorder characterized by defective muscle coordination, immunodeficiency, defective DNA repair, and an increased risk of cancer

ATR: AT and Rad3 related

BRCT: BRCA1 carboxyl terminal

Ionizing Radiation-Induced Foci: punctate dots of proteins in the nuclei of irradiated cells, usually visible by indirect immunofluoresence

Table 1 Proteins that function in cellular responses to DSBs in DNA in *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), Drosophila (Dm) and vertebrates

Role	Sc	Sp	Dm	Vertebrates	Type
Sensor	Ddc1	Rad9		RAD9	PCNA-like
Sensor	Rad17	Rad1		RAD1	PCNA-like
Sensor	Mec3	Hus1		HUS1	PCNA-like
Sensor	Rad24	Rad17		RAD17	RFC1-like
DNA synthesis/sensor	RFC2-5	RFC3		RFC2-5	RFC component
DNA synthesis/sensor	RFA			RPA	ssDNA-binding
Mediator				BRCA1	BRCT-domain
Mediator				TOBP1	BRCT-domain
Mediator				MDC1	BRCT-domain
Mediator	Rad9	Crb2/Rhp9			BRCT-domain
Mediator	Dpb11	Cut5			BRCT-domain
Sensor/transducer	Mec1	Rad3	Mei-41	ATR	PI3K-like kinase
Sensor/transducer	Ddc2	Rad26	Mus304	ATRIP	ATR-binding
Sensor/transducer	Tel1	Tel1	ATM	ATM	PI3K-like kinase
Transducer	Chk1	Chk1	Grapes	Chk1	Kinase
Transducer	Rad53	Cds1	Chk2	Chk2	Kinase
Multiple	Mre11	Rad32	Mre11	MRE11	Nuclease
Multiple	Rad50	Rad50	Rad50	RAD50	
Multiple	Xrs2	nbs1	Nbs1	NBS1	
Effector			Dp53	p53	Transcription factor
Effector			String	Cdc25A-C	Phosphatase
Effector	Pds1				APC-inhibitor
Effector	Cdc28	Cdc2	Cdk1	Cdk1	Kinase

between multiple components of DNA damage response pathways.

By far the most elusive component of DNA damage responses has been the signal. What feature(s) of a DSB allows it to be recognized as such and start the signal transduction pathway? Indeed, the most exciting advances in the past five years have been in our understanding of the molecular nature of the DSB signal and how it is amplified once generated. Controversies and questions remain, however, as discussed below.

IRIF: IR induced foci

GFP: Green Fluoresent Protein

DNA DAMAGE SIGNALS

Three consequences of DSBs in DNA initiate downstream events: chromatin modification, binding to DNA of MRN protein complexes (MRX in budding yeast) that is composed of Meiotic Recombination 11 (Mre11), Rad50, and Nbs1 (Xrs2p in budding yeast); and resection of the double strand to expose single-stranded DNA.

Histone Modification and Changes in Chromatin

Several lines of evidence support a model wherein changes in chromatin structure result from DSBs and lead to activation of downstream events. Induction of DSBs is followed by chromatin decondensation in yeast and mammals. These, in mouse embryo fibroblasts (MEFs), are energy-dependent and include localized reduction in nuclear density and expansion of photo-bleached Histone-GFP signal (52). In budding yeast, nuclease-mediated DSBs result in localized

loss of core histones from chromatin (99). Changes in chromatin are likely to be of functional importance because treatments that prevented chromatin changes interfered with accumulation of DNA repair enzymes at subnuclear sites of damage in mammals and prevented a timely recruitment of Rad51p DNA repair proteins to DSBs in yeast.

Chromatin decondensation is expected to expose modifications on Histones such as methylation of Histone H3-Lys79 in mammals and budding yeast and on Histone H4-Lys20 in fission yeast (78). Lys79 methylation increases the affinity of 53BP1, a mediator, to Histone H3 in vitro, whereas depletion of a methyltransferase that modifies Lys79 interfered with the formation of 53BP1 nuclear foci after induction of DSBs in human U2OS cells (42). Mutation of an implicated methyltransferase or Lys79 itself on H3 increased the radiation sensitivity of budding yeast (34). Similarly, Set9p methyltransferase that modifies H4-Lys20 is required for phosphorylation and recruitment of Crb2p (an ortholog of 53BP1) to nuclear foci in fission yeast (78). Cells without Set9p or mutated Lys20 are able to initiate but not maintain a cell cycle arrest after exposure to IR and are more sensitive to killing by UV and IR. The level of Lys79 or Lys20 methylation does not change after induction of DNA damage, and for this reason their increased accessibility, brought about by chromatin decondensation, is implicated in DNA damage responses.

Treatment of mammalian cells with hypotonic solutions decondenses chromatin. Such treatments, even in the absence of DNA damage, have consequences similar to those of DSBs in DNA. These include reduced mobility of nuclear 53BP1 in U2OS cells, which is interpreted as increased binding of 53BP1 to chromatin, and activation of ATM in human fibroblasts, via an unknown mechanism, as assayed by autophosphorylation on Ser1981 (3, 42). Thus, chromatin decondensation is likely to be one primary outcome of DSBs that signals to downstream events. Exactly how DSBs cause changes in chromatin is unclear, but MRN proteins and chromatin remodeling factors are required in yeast (99).

γH2AX in DNA Damage Signaling

Unlike methylation of Histones H3 and H4 that occurs constitutively, phosphorylation of Histone H2AX is induced by DSBs in yeast, Drosophila, Xenopus, and mammals, and is implicated in amplifying the DNA damage signal [reviewed in (72)]. Phosphorylation of H2AX on a conserved Serine, Ser139 in mammals and Ser129 in budding yeast, is referred to as γH2AX and occurs within minutes after induction of DSBs [e.g., 3 min after exposure of human breast cancer cells to 0.6 Gray (Gy) of γ−rays (75)]. All three PIKKs can generate γH2AX, but ATM homologs appear to make a more substantial contribution in yeast and mammals, as discussed below (7).

γH2AX induced by IR is not homogeneous within the nucleus but forms foci (IRIF), in approximately the same numbers as those of DSBs in mammals [reviewed in (72)]. γH2AX foci colocalize with 53BP1 foci, which form with similar kinetics, and with foci of BRCA1 and MRN proteins, which form several hours after irradiation. Fibroblasts and B cells from H2AX knockout mice fail to retain rapidly forming 53BP1 foci and fail to form foci of BRCA1 and MRN following exposure to IR (11, 12). Remarkably, however, BRCA1, 53BP1, and NBS1 (of the MRN complex) are still recruited to sites of localized DSB induced with a laser scissor (11). Similarly, ATM$^{-/-}$ MEF or human AT$^{-/-}$ fibroblasts that are deficient in generation of γH2AX still recruit MRN complex proteins to laser scissor cuts. These results suggest that γH2AX is not needed for initial recruitment of BRCT-domain proteins and MRN complexes, but is needed to maintain them at sites of DSB and to allow their accumulation into visible foci.

IRIF as Sites of Signal Amplification

Real-time imaging of GFP-tagged components shows that IRIF remain discrete over

IR: ionizing radiation

Gray (Gy): a unit of radiation. One Gy corresponds to absorption of one joule of energy by one kilogram of matter

Knockout mice: mice with a genome in which the function of a gene has been disrupted

MEF: mouse embryo fibroblast

HR: homologous recombination

NHEJ: nonhomologous end joining

RPA: Replication Protein A

ssDNA: single-stranded DNA

the course of formation and do not coalesce into larger foci (52). Induction of localized DSBs using a laser scissor traversing the nucleus in a defined line results in localized γH2AX induction, but the signal does not become organized into a focus or foci even several hours after irradiation (11). These results suggest that IRIF are not a result of nuclear reorganization into "DNA repair factories" and that location of DSBs as marked by γH2AX remains relatively fixed within the nucleus. Instead, growth of IRIF over time is thought to reflect the spread of γH2AX over large, megabase-sized chromosomal domains in mammals [reviewed in (72)]. In contrast, the number of DSBs predicted for a given dose of IR is greater than the IRIF in budding yeast, suggesting nuclear reorganization (60). Nonetheless, there is substantial spread of the γH2AX signal in yeast as well, covering approximately 50 kb around a DSB (80).

Concurrent with spread of γH2AX may be the recruitment of proteins that bind to γH2AX. One of these, MDC1, is required for formation of IRIF (5, 87, 106). The BRCT domain of MDC1 binds a γH2AX-containing peptide (89). MDC1 directly interacts with the MRN complex whereas NBS1 (a member of the MRN complex) binds and activates ATM (28, 36, 108). Therefore, the spread of DNA damage response proteins on chromatin may occur via sequential recruitment onto γH2AX of MDC1, MRN, and ATM, which then generates more γH2AX distal to the damage and further recruitment. H2AX mutants that are phosphorylated but cannot bind MDC1 because of additional mutations in the tail region fail to generate IRIF of 53BP1, NBS1, and activated ATM (89). Thus recruitment of MDC1 may be the key contribution of γH2AX to IRIF formation.

In MDC1-deficient human U2OS cells, γH2AX occurs initially but is not maintained (89). This may be because MDC1 binding protects γH2AX from a phosphatase and/or because MDC1 recruits and maintains active ATM in close proximity to γH2AX. 53BP1, on the other hand, can accumulate at sites of damage via methylated histones, as discussed above, in addition to association with γH2AX (78, 103). Thus although proteins of IRIF are still recruited to DSB in the absence of H2AX, their organization into foci appears to require amplification of the γH2AX domain and protein-protein interactions that reinforce each other's presence. Such amplification may be necessary to maximize DNA damage responses when the signal is low, as after exposure to low doses of IR (31).

H2AX knockout mice are viable but show pleiotropic phenotypes including radiation sensitivity and increased cancer incidence. Embryonic stem cells from these animals show decreased efficiency of HR (homologous recombination) as measured by gene targeting, whereas V(D)J recombination in lymphocytes, which occurs via NHEJ, is not significantly affected (12). Therefore, one function of H2AX, and possibly, IRIF is to facilitate efficient HR-mediated DNA repair. Paradoxically, $H2AX^{-/-}$ MEFs still form IRIF of Rad51, an essential protein for HR-mediated repair. Presumably, repair enzymes are still recruited to sites of damage but are somehow less functional.

ssDNA-RPA Complex

Single-stranded DNA is a common intermediate in the processing of many types of damaged DNA, including DSBs, UV-induced thymidine dimers, intrastrand cross-links, and mismatches in base-pairing. The multisubunit Replication Protein A (RPA) complex has high affinity for ssDNA. The ssDNA-RPA complex is proposed to act as the signal for DNA damage based on in vivo and in vitro data from yeast, human, and Xenopus (21, 111, 112). First, RPA mediates the recruitment of ATR/ATRIP, Rad17, and 9-1-1 complexes to ssDNA or gapped DNA structures in vitro, and stimulates the kinase activity of ATR toward Rad17. Second, depletion of RPA impaired an ATR-dependent checkpoint in Xenopus extracts; depletion of a subunit of RPA results in loss of IR-induced

ATR/ATRIP foci in HeLa cells; RFA mutants (budding yeast RPA) are deficient in recruitment of Ddc2p (ATRIP) to DSBs and in checkpoint activation.

These results point to a mechanism conserved in yeast and vertebrates, in which ssDNA-RPA complexes at sites of DSBs recruit Rad17/9-1-1 and ATR/ATRIP to facilitate phosphorylation of the former by the latter. Efficient resection of DSBs to expose ssDNA would be important for signaling, but if and how resection is regulated is poorly understood. The affinity of processing enzymes to broken, bulky, or otherwise aberrant DNA structures may be sufficient to passively recruit them to DSBs. Alternatively, other factors, such as chromatin remodeling to expose broken DNA, may play a rate-limiting role. A recent study points to ATM and the nuclease activity of Mre11 in resection, ATR recruitment, and activation of Chk1 in human cells (46). The role of Mre11 as the main resecting nuclease remains to be clarified, however, because budding yeast harboring nuclease-inactive Mre11p are still able to resect DSBs (62).

Mechanisms of ATM/ATR Activation

In response to DSBs, ATM shows marked changes that include monomerization of dimers/oligomers and intermolecular autophosphorylation on Ser1981. Monomerization of ATM is thought to allow interaction with substrates (3). Treatments that decondense chromatin such as exposure of cells to high salt or to the DNA intercalating agent chloroquine also result in Ser1981 phosphorylation in the absence of DSBs. Therefore, a signal for activation of ATM is proposed to be changes in chromatin structure at sites of DSB.

Activation of ATM in response to DSBs under otherwise normal (i.e., nonhypotonic) conditions in a number of mammalian cell lines requires the MRN complex (9, 69, 96, 102). Recent studies indicate that MRN/MRX complexes activate ATM by independently promoting both monomerization and autophosphorylation. First, MRN proteins mediate monomerization of ATM in vitro; at high concentrations of DSBs, however, ATM can monomerize in the absence of MRN proteins (26, 55). Because MRX proteins in budding yeast can associate with DSBs in vivo and purified MRN complexes can tether DNA molecules in vitro, one role of MRN/X may be to tether DNA and increase the local concentration of DSBs to allow ATM monomerization (2, 19, 59, 70, 80). Second, autophosphorylation of ATM on Ser1981 in vitro was found to require binding to Nbs1 regardless of DSB concentration (26). Thus, the second role of MRN may be to promote ATM kinase activity via binding of NBS1 to ATM. Finally, the ability of MRN/X to associate with sites of DSBs would help target ATM activation to where it is needed.

In contrast to ATM, ATR shows no changes in modification or activity after genotoxic stress. For this reason, relocalization of ATR to sites of damage, via association with ssDNA-RPA complexes (as discussed above), has been the proposed mode of ATR activation. A recent study found, however, that ToBP1 (Topoisomerase II Binding Protein 1), a mediator, binds to and stimulates the kinase activity of Xenopus and human ATR in vitro (53). Given that ToBP1 is a component of IRIF in human U2OS cells, activation of ATR by ToBp1 may also have a role in DNA damage responses (107).

A Multisensor Model for Detecting DSBs

In sum, three "signals" emanate from a DSB and lead to cross-talking downstream events (**Figure 2**). One of these is chromatin decondensation, which leads to ATM activation, chromatin association of 53BP1, and recruitment of the Rad51 repair complex. Another is the association of MRN/X complexes to DSBs, which occurs independently of ATM/ATR and leads to recruitment and

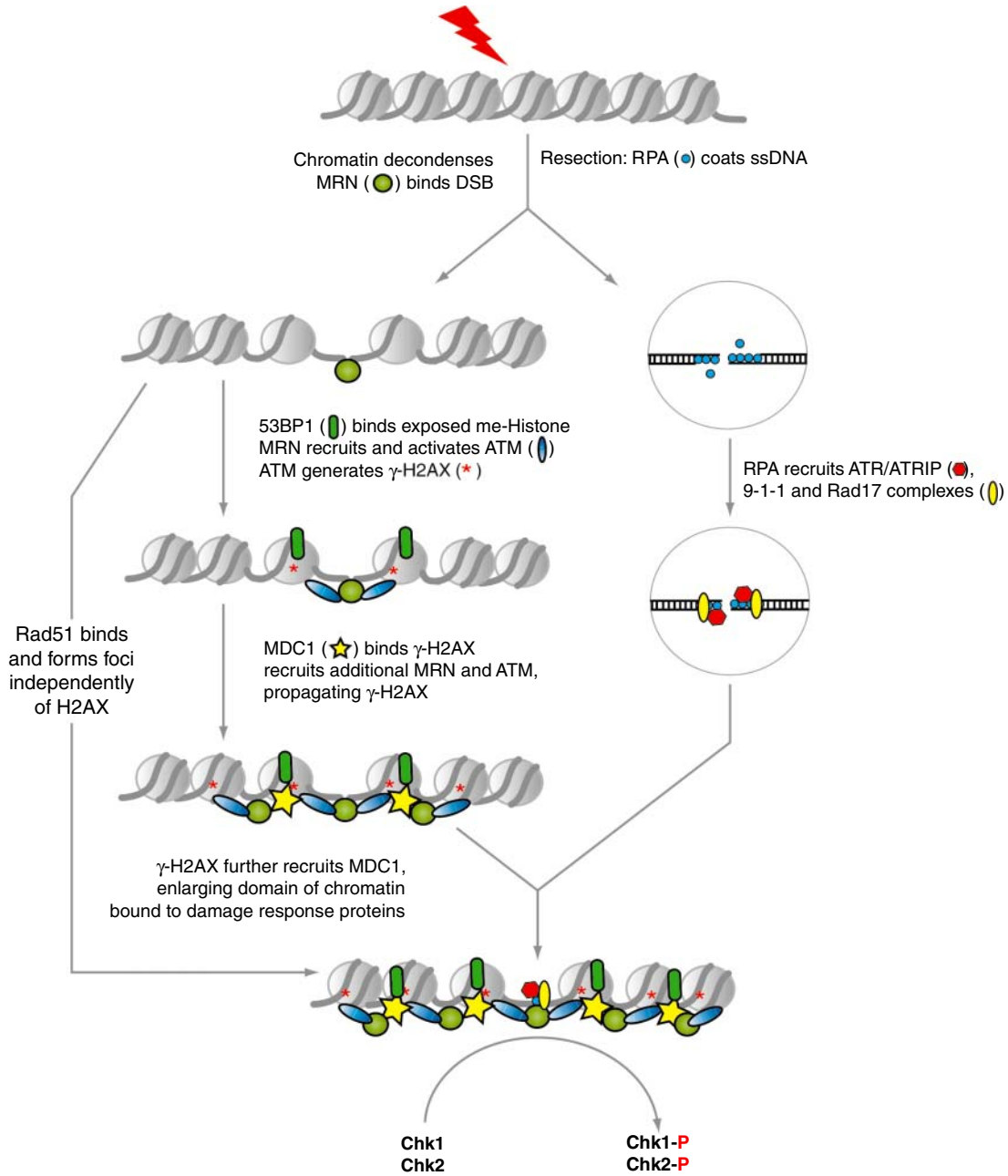

Figure 2

A model showing possible sensor mechanisms for DNA double-strand breaks. At least three initial consequences of a DSB lead to downstream events: chromatin decondensation, binding of MRN proteins, and coating of ssDNA with RPA. Downstream events from these consequences converge in the formation of chromatin domains characterized by modified histones, DNA damage sensors and mediators, repair enzymes, and chromosomal cohesions, and are visible as nuclear foci. The formation of foci appears important for optimal checkpoint signaling that is mediated by signal transducers, Chk1 and Chk2, and for optimal repair of the DSB. See text for details.

activation of ATM. BRCA1 may collaborate with MRN in this regard because it too can associate with damaged DNA independently of ATM activation and can recruit active ATM (51). The third signal is the generation of ssDNA, which leads to binding of RPA and subsequent recruitment of ATR, 9-1-1, and Rad17 complexes. These signaling mechanisms are separable; generation of γH2AX, for example, does not require Rad17. On the other hand, they share components: MRN/X complexes, for example, may help generate ssDNA-RPA complexes.

Events subsequent to sensors strengthen initial interactions, recruit additional proteins to sites of damage, and propagate nucleoprotein complexes along the chromosome. These events include generation of γH2AX by ATM, ATR, or both, which recruits MDC1. MDC1 stabilizes γH2AX, solidifies interaction among ATM, MRN, 53BP1, and BRCA1 and allows the further spread of γH2AX along chromatin. Mediators can also recruit and help activate downstream substrates of ATM/ATR, such as Chk1 and Chk2, to propagate the DNA damage signals. This is exemplified by the role of the Rad9p mediator in facilitating phosphorylation of Rad53p (a Chk2 homolog) by Mec1p (an ATR homolog) in budding yeast (93).

A small (10%–20%) portion each of Chk1 and Chk2 is in an insoluble nuclear fraction, presumably bound to chromatin, in human U2OS and HEK293 cells, respectively (57, 85). Upon induction of DNA damage, both phosphorylation and solubility of these proteins increase; phosphorylation also renders Chk1 and Chk2 more soluble in vitro. Tethering Chk1 to chromatin via fusion with a histone impairs checkpoint signaling in IR-exposed HeLa cells (85). These results suggest a model in which chromatin-associated Chk1 and Chk2 become phosphorylated and activated, and are released to interact with soluble substrates. Unphosphorylated versions would then replace released proteins, leading to signal amplification.

Division of Labor Among Sensor/Transducers

If data across species are considered, homologs of both ATM and ATR appear capable of sensing and transducing the DSB signal. Yet, usually one plays a more substantial role than the other depending on the organism, cell type, or cell cycle stage. The reasons underlying such a division of labor are poorly understood.

DNAPK: DNA-dependent Protein Kinase

In budding yeast, both Mec1p and Tel1p contribute to γH2AX in asynchronous cultures; however, Tel1p (ATM) plays a more substantial role in G1-arrested cells (80). In vertebrates, all three PIKKs, ATM, ATR, and DNA-dependent Protein Kinase (DNAPK), can generate γH2AX; and the contribution from ATM is most substantial after exposure to IR, with DNAPK filling in only when ATM is absent [reviewed in (90)]. ATM and ATR also share substrates such as Rad17 (4, 97). Phosphorylation of Rad 17 after exposure to IR, however, relies more on ATM than on ATR (4). *AT*$^{-/-}$ cells that lack ATM are highly sensitive to IR, consistent with a major role for ATM in DNA damage responses. ATR, meanwhile, acts in checkpoints that respond to UV and monitors ongoing DNA synthesis to arrest mitosis as necessary (71). *ATR*$^{-/-}$ mice are embryonic lethal, as expected from failure to coordinate S phase and mitosis during normal cell proliferation. Mei-41, the ATR homolog in Drosophila, is needed for cell cycle checkpoints at different stages in development and for repair after exposure to IR (38, 45). Mei-41 mutants are highly sensitive to killing by IR, indicating that ATR in Drosophila makes a major contribution to DNA damage responses (54). Mei-41 mutants of Drosophila also die from the failure to coordinate S phase and mitosis during embryonic divisions, much like ATR knockout mice (81). Thus the ATR homolog in Drosophila appears to function in both DNA replication and DNA damage checkpoints. Drosophila ATM, encoded by the *telomere fusion* gene, on the other hand, is essential for the protection of telomeres and

has only a minor role in DNA damage responses [reviewed in (73)].

The potential for plasticity in the DNA damage response extends down the signal transduction pathway to include Chk1 and Chk2 homologs. Work in Drosophila places Chk1 (Grapes), but not Chk2, downstream of ATR (Mei-41) in the DNA damage response (22). In budding yeast, both Chk1p and Rad53p (Chk2) enforce mitotic arrest after DNA damage. In contrast, the vertebrate ATR/Chk1 axis is thought to act primarily in the DNA replication checkpoint whereas the ATM/Chk2 axis has a primary role in DNA damage responses (71). Given the possibility to use ATM or ATR, and Chk1 or Chk2, why does an organism or a cell type prefer one axis over the other in responding to DNA damage? The answer is still elusive but may help us understand tissue specificity of diseases that result from mutations in DNA damage response genes. For example, the role of mediator BRCA1 (Breast Cancer 1) in safeguarding the genome is clearly more important for mammary than for other tissue, even though it forms IRIF in many cell types [for example, osteosarcoma-derived U2OS cells (87)].

Fanconi's anemia: a rare and often fatal inherited human disorder in which the bone marrow fails to produce red and white blood cells, platelets, or a combination of these. The disease may develop into myelodysplastic syndrome or leukemia

THE LINK TO DNA REPAIR

Rad51 Group

Recombinational repair of DSB requires homologs of proteins encoded by members of the *RAD51* epistasis group in budding yeast: Rad51p, Rad52p, Rad54p, and Rad55p. The Rad51 group of proteins are recruited in a sequential and interdependent manner to sites of DSB in budding yeast, with Rad51p being the first to arrive at the site (104). Homologs of Rad51 group of proteins also form IRIF in mammalian cells, but can do so in the absence of H2AX [reviewed in (72)]. In budding yeast, recruitment of Rad51p depends on Cdk1 activity, MRX, and the INO80 chromatin remodeling complex (43, 99). In mammalian cells, recruitment of RAD51 to nuclear foci may rely on at least eight (A, C, D2, E, F, and G) proteins mutated in Fanconi's anemia, because cells from FA patients show reduced recruitment of RAD51 into IRIF, and higher sensitivity to IR (23). One FA protein, FANCD2, is phosphorylated in an ATM-dependent manner after IR exposure and is incorporated into foci that contain RAD51 and BRCA1 (35). Understanding the exact mechanism by which the RAD51 group of repair proteins is recruited to sites of damage should be an important future goal.

H2AX and DNA Repair

Phospho-acceptor mutants of Histone H2A are deficient in repair of DSBs via NHEJ, a major repair pathway in budding yeast (24). The phenotype of $H2AX^{-/-}$ mice and murine cells suggests that γH2AX is required for efficient repair of DSBs (12). RAD51, needed for HR-mediated repair, still forms IRIF efficiently in the absence H2AX (12). What might account for the role of H2AX in DNA repair? One possibility is the failure to organize MRN proteins into IRIF; both MRN and RAD51 complexes may perhaps need to be present for optimal repair. Another possibility is suggested by the finding that H2AX is needed to recruit PP2A(C), a member of the Protein Phosphatase 2A complex, to IRIF in MEFs (17). Cells depleted of PP2A(C) repair DSBs inefficiently, but the exact reason is not known. In budding yeast, Pph3 phosphatase-deficient mutants cannot remove γH2AX, but can still perform HR-mediated repair (49), suggesting that dephosphorylation of H2AX is not required. If this is also the case in MEFs, it may be another target of PP2A that must be dephosphorylated for efficient repair.

Transcriptional and Posttranslational Regulation

Transcriptional induction including that of DNA repair enzymes is a common DNA damage response facilitated, in metazoa, by p53 homologs (110). Genes associated with DNA repair that are induced by IR in a p53-dependent manner include p53R2, DDB2,

XPC, PCNA, BTG2, and MSH2 in mammals and Ku70, Ku80, Mre11, and Rad50 in Drosophila (6, 98). Increased expression of DNA repair factors may promote repair, although this connection remains to be tested.

In addition to increased expression, several proteins involved in DNA repair are modified in response to IR. For example, RAD51 is phosphorylated on tyrosines after IR exposure in a c-Abl- and ATM-dependent manner; phosphorylation of RAD51 by c-Abl enhances the binding of RAD51 to RAD52 in vitro (14). ATM-dependent modification of cohesins, members of large protein complexes that hold sister chromatids together, appear to play a role in DNA repair. Human cohesins are recruited to laser-induced DNA damage (50). Budding yeast cohesins are recruited to a 100-kb region surrounding the DSB via γH2AX and Mre11 (88, 100). Murine cells harboring mutant SMC1 proteins that cannot be phosphorylated by ATM show defects in checkpoint activation, decreased survival, and increased chromosome aberrations following exposure to IR (51). In budding yeast, cohesin enrichment at DSB precedes the completion of repair, mediates cohesion between sister chromatids, and promotes efficient repair (88, 100). The ability of cohesins to facilitate interchromosomal interactions needed for homology-mediated repair may account for their importance in DNA damage responses.

CELL CYCLE CHECKPOINTS

Cell cycle regulation was the first DNA damage response to receive wide scrutiny. It is relatively well understood, has been covered in multiple reviews, and is therefore discussed only generally here, with salient points illustrated by one or two examples [for example, see (58, 63, 71, 76, 110)].

Canonical Cell Cycles

In mammalian cell cycles that contain G1, S, G2, and M phases, exposure to IR arrests the cell cycle at multiple transitions, using multiple mechanisms at each point. In general, arrest before or during S phase (G1-S and intra-S checkpoints, respectively) occurs via inhibition of Cdk2 activity, which is needed for S phase, either by binding of a Cdk inhibitor or by reduction of CDC25 phosphatase activity. ATM, Chk1-, and/or Chk2-mediated phosphorylation, and subsequent degradation of Cdc25A, an activator of Cdk2, contribute to both G1-S and intra S checkpoints [for example, see (20, 29, 47, 64)]. IR can also induce p53-mediated transcriptional activation of p21 that binds and inhibits Cdk2/Cyclin E complexes. Arrest of G2-M transition that occurs in response to DSBs also targets the mitotic cdk, Cdk1, directly or via Cdc25 homologs [reviewed in (110)]. IR induces Chk1- and Chk2-mediated phosphorylation of Cdc25 homologs on a conserved Serine. This modification results in binding to 14-3-3 proteins and inhibition of phosphatase activity. Additionally, p53 mediates the transcriptional activation of 14-3-3σ that binds to and excludes Cdk1/Cyclin B complexes from the nucleus, and is implicated in maintaining the G2-M arrest in human colon cancer cells (13).

Variant Cell Cycles

Not all cell cycles conform to the canonical G1-S-G2-M arrangement. In systems where G2-M regulation is attenuated normally, checkpoints arrest mitosis at different stages via different effector proteins (**Figure 3**). This is well established in budding yeast. Certain events that are associated with mitosis in other systems such as the duplication and separation of the microtubule organizing centers occur during S phase in budding yeast. There is not a clearly defined G2 period, and the cell cycle phase between S and G1 is referred to as G2/M. Instead of an arrest in G2, irradiated budding yeast arrests mitosis at metaphase-anaphase transition via Mec1p- and Chk1p-dependent phosphorylation of Pds1p (an inhibitor of APC) and via Rad53p-dependent

cdk: cyclin-dependent kinase

APC: Anaphase Promoting Complex

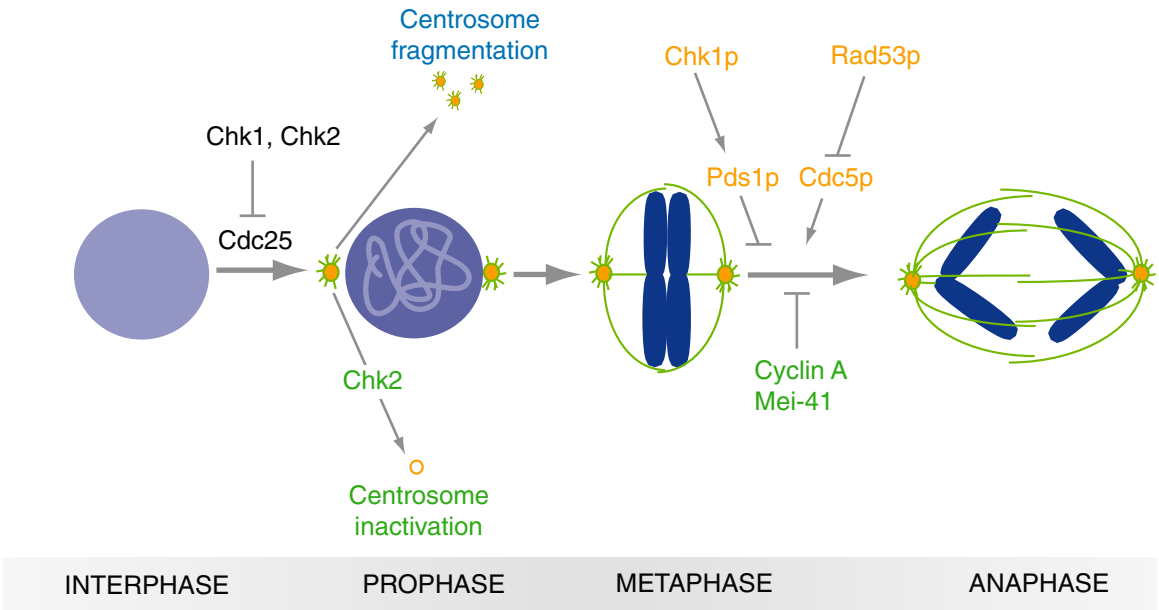

Figure 3

Multiple mechanisms regulate mitosis in response to DNA double-strand breaks. Regulation of mitotic entry occurs via Chk1-/Chk2-mediated inhibition of Cdc25 homologs in Drosophila, fission yeast, and vertebrates. Mechanisms documented in budding yeast are shown in orange, those in Drosophila are shown in green, and centrosome fragmentation documented in Chinese hamster ovary cells is shown in blue. See text for details.

regulation of Cdc5p (polo-like kinase homolog) (18, 77).

Noncanonical cell cycles are an integral part of metazoan development. In *Drosophila melanogaster*, embryogenesis begins with 13 rapid S-M cycles that lack gap phases. DNA damage or replication block during cycles 10–13 results in a Chk2-mediated loss of γ–tubulin ring complex from the centrosome, and consequent inactivation of the latter (94). Nuclei exit mitosis without successful chromosome segregation. The resulting polyploid nucleus is culled into the yolk mass in a Chk2-dependent manner in a process that may be the embryonic equivalent of apoptosis. After 13 gap-less cycles, a G2 phase is added to cycle 14 of Drosophila embryos via inhibitory phosphorylation of Cdk1. Instead of disrupting progression through mitosis as in previous cycles, IR now induces a G2-M delay that relies on inhibitory phosphorylation of Cdk1. These results indicate that regulation of mitosis in response to IR is plastic even in the same organism, and relies on endogenous cell cycle regulatory mechanisms (92).

IR-induced delay in G2 of cycles 14 and 16 is transient (92). Cells overcome this delay and enter mitosis, only to delay again at the metaphase-anaphase transition (much as in budding yeast). The second delay requires Mei-41 (ATR) and Cyclin A, which can act as an anaphase inhibitor in Drosophila, similar to Pds1p of budding yeast (91). Conservation of a bona fide metaphase-anaphase checkpoint that responds to DNA damage (as opposed to radiation-damaged spindle or kinetochores) remains controversial for vertebrate systems (67, 84). The connection between DNA damage and centrosomes may, however, be conserved in vertebrates because centrosomes become fragmented in Chinese hamster ovary cells that enter mitosis with damaged DNA (41, 109).

DNA DAMAGE AND METAZOAN DEVELOPMENT

Studies in Drosophila embryos also suggest a novel link between DNA damage responses and development. In *grapes* (Chk1) and *mei-41* (ATR) mutants, nuclei in S-M cycles enter mitosis prematurely, presumably because they lack a checkpoint to monitor incomplete DNA replication (32, 81, 82). The onset of zygotic transcriptional program is prevented in these mutants and affects such patterning genes as *runt* and *ftz* (81, 82). Later in embryogenesis, exposure of gastrulating embryos to IR results in the repression of a large number of genes many of which encode transcription factors important for development such as *invected*, *achaete*, and *abrupt*. Their repression requires Chk2 (6). Thus, inhibition of developmental stage appropriate transcriptional program may be a bona fide DNA damage response. Such a response would coordinate cell cycle arrest with a developmental arrest, and may be critical for the survival of metazoa after exposure to DNA damaging agents.

TURNING OFF DNA DAMAGE RESPONSES

Anti-checkpoint Signals at Telomeres

The ends of linear chromosomes resemble a DSB, complete with a ssDNA end. Although ATM and members of the MRN complexes are recruited to chromosome ends, the result is not repair, cell cycle arrest, or apoptosis but incorporation into a nucleoprotein complex to form telomeres. Remarkably, ATM and MRN complex members, the same proteins needed for DSB responses, are required to prevent "repair" of telomeres: loss of ATM, RAD50, or MRE11 homologs leads to telomere fusions in yeast, Drosophila, and mammals [see (73) and references therein]. The DNA damage response functions have a role in telomere protection regardless of how telomeres are constructed, either in a telomerase-dependent manner in yeast and mammals or in a telomerase-independent recombination process in Drosophila.

Anti-checkpoint: a hypothetical mechanism that emanates from telomeres and prevents cellular responses to DNA damage

Why are chromosome ends processed differently from DSBs? One clue comes from a study in budding yeast where ectopically generated telomeres are found to produce a locally acting "anti-checkpoint" signal that abrogates cell cycle checkpoints by reversing phosphorylation on Rad9p and Rad53p (66). The anti-checkpoint mechanism is not well understood but is genetically separable from adaptation in which cells with persistent DSB resume cell proliferation [see below; discussed in detail in (39)]: The former occurs in the presence of a mutation in Cdc5p (a polo-kinase homolog) that prevents adaptation.

Recently, TRF2, a mammalian telomere binding protein, was found to associate with ATM and inhibit autophosphorylation on Ser1981, an event indicative of ATM activity (48). Loss of TRF2 induces foci of 53BP1, γH2AX, RAD17, ATM, and MRE11 at telomeres, suggesting that chromosome ends are now being recognized as DSBs (95). This suggests a model in which TRF2 at telomeres antagonizes signaling by ATM, formation of nuclear foci, and downstream events, essentially acting as an anti-checkpoint function. Inhibitory effects of TRF2 cannot, however, explain how ATM functions positively to prevent telomeric fusion.

In addition to avoiding repair, chromosome ends also do not activate cell cycle checkpoints. ATM and Mre11 were recently shown to become colocalized at telomeric foci in senescent human cells (40). Depletion of ATM caused these cells to re-enter the cell cycle. Thus, in senescent cells, ATM and MRN complexes may be recognizing telomeres as DNA breaks and causing cell cycle arrest (although not repair since chromosome fusions are not reported). The onset of senescence may simply reflect a breakdown of anti-checkpoint such that chromosome ends now induce a persistent checkpoint-mediated

arrest of the cell cycle, i.e., senescence. Modulation of an anticheckpoint, if it exists in metazoa, may delay senescence and extend cellular life span.

Gy: Gray

LD_{50}: the amount of a material, given all at once, which causes the death of 50% of a group of test cells or organisms

Recovery and Adaptation

In addition to localized inactivation by telomeres, checkpoints presumably need to be inactivated during the natural course of recovery from DNA damage. The process of recovery is not well understood, but is likely important for survival of irradiated cells. One contributing factor in recovery is γH2AX that is needed for initial signaling. H2A becomes dephosphorylated during HR repair of a DSB, at a step before Rad54p-mediated strand-invasion of the donor template (49). Budding yeast mutants in phosphatase Pph3 are unable to dephosphorylate H2A or turn off checkpoint signaling as assayed by phosphorylation on Rad53p. A phospho-acceptor mutant of H2A rescues this phenotype, indicating that removal of γH2AX is necessary for recovery. The phenomenon of recovery appears to be distinct from adaptation whereby yeast cells resume proliferation in the presence of persistent DSBs; Pph3 is required for the former but not the latter (39, 49).

CHOOSING THE RESPONSE: ARREST, REPAIR, OR DIE?

At the cellular level, one possible outcome of DSBs is death. For multicellular organisms, selective killing of cells with damaged DNA helps preserve genetic integrity in the whole organism. The efficiency of DSB induction by IR is surprisingly similar among eukaryotes; 1.58×10^{-9}/bp/Gy in hypoxic human carcinoma cells and $0.93–1.07 \times 10^{-9}$/bp/Gy in anoxic budding yeast, for example (33, 75a). However, the killing effect of IR varies widely. Mammalian cells in culture are killed effectively in the 1–10 Gy range, depending on cell type (56, 68). Drosophila S2 cells in culture show very little change in growth characteristics after exposure to 80 Gy of IR (our unpublished observations) whereas the LD_{50} for budding yeast is around 100 Gy (34). For fission yeast, LD_{50} exceeds 500 Gy [for example, see (1, 25)]. Even within a single species, the propensity to die after exposure to IR can vary widely depending on cell type [reviewed in (74)]. What is the basis for such disparity in radiation sensitivity among eukaryotic cells?

p53-Dependent Mechanisms

A key molecular determinant of whether a metazoan cell lives or dies after exposure to IR is p53 [reviewed in (30, 83)]. ATM phosphorylates p53 on Ser15, and MDM2 on Ser395. Chk1 and Chk2 phosphorylate p53 on Ser20 (13a, 16, 30, 65, 79, 110). These modifications prevent p53-MDM2 association that targets the former for proteolysis, thereby allowing p53 levels to rise. The consequences of p53 activation include transcriptional activation of DNA repair activities, as mentioned above, cell cycle inhibitors (p21 and 14-3-3σ) and pro-apoptotic functions (PUMA, Fas/APO1, and Apaf1, for example). p53 is also implicated in activating apoptosis independently of transcription activation, by activating pro-apoptotic Bcl-2 in the cytoplasm. The contribution of cytoplasmic p53 to DNA damage responses remains to be determined (15, 16).

Combinatorial deletion analysis of pro-apoptotic genes in Drosophila suggests a threshold model for induction of apoptosis following irradiation (6). In Drosophila embryos, IR induces Chk2-dependent hyperphosphorylation of p53 without changes in p53 levels. p53 activates the transcription of Smac/Diablo orthologs, Hid, Reaper, and Skl. These proteins, along with another Smac/Diablo ortholog, Grim, compete with caspases for binding to Inhibitor of Apoptosis Protein 1 (DIAP1) in Drosophila. Studies of mutants in which pro-apoptotic genes have been deleted singly or in combination support a model whereby Smac/Diablo orthologs

constitute to a pool of pro-apoptotic activity that must reach a threshold before DIAP1 is sufficiently inhibited and caspases sufficiently activated to induce apoptosis.

Drosophila p53 does not have a role in cell cycle regulation (6). Therefore, the fate to die or not in this system may simply depend on the strength of the p53 signal as proposed by the threshold model. In mammals, p53-mediated transcription activation affects apoptotic DNA repair and cell cycle regulators, and even SLUG, an antagonist of p53 itself (105). It is the combination of these outcomes that determines the final fate of the cell. How might this differ according to cell type or growth conditions? One possibility is that p53-responsive genes respond differently to the same stimulus. For genes that are activated by p53 in the same cell type, the mechanism of activation can differ significantly (27). In human U2OS cells, p53-dependent transcriptional activation of p21, for example, occurs via elongation of preassembled transcription initiation complexes. p53-dependent transcriptional activation of pro-apoptotic Fas/APO1, on the other hand, requires new assembly of initiation complexes. The difference in mechanisms may help explain why p21 is induced before Fas/APO1 after exposure to UVC and doxorubicin. Furthermore, p53-responsive genes show a differential requirement for transcriptional cofactors, which may explain differences in cell type in p53-mediated responses if cofactors are present in some cells but not others (37).

p53-Independent Mechanisms

p53 is not the sole determinant of cell death; certain cell types in $p53^{-/-}$ mice still commit apoptosis after IR exposure. One possible mechanism for p53-independent apoptosis involves another p53 family member, p73. Although p73 has not been shown to induce apoptosis after IR, it is implicated in p53-independent apoptosis that is induced by E2F1 (44). p73 expression is under the control of Chk1- and Chk2-mediated activation of E2F1, suggesting a way to link DNA damage to p73 [(101); reviewed in (86)]. The many transcriptional targets of E2F1 include proteins associated with DNA repair (BRCA1, Msh2 and Msh6, RFC, and PCNA), cell cycle checkpoints (ARF and Chk1), and apoptosis (Caspase 7, Apaf1). Thus, like p53, E2F1 has the potential to make life-or-death decisions. As for p53 and SLUG, E2F1 induces a negative modulator of itself. TopBP1 is a BRCT-domain protein that is induced by E2F1 and can recruit a chromatin remodeling activity to repress E2F1-responsive genes, thereby serving as a modulator of E2F1 activity (61). Understanding the consequences of E2F1- and p53-mediated transcription would be necessary to understand how the choice between cell survival and cell death is made in response to DNA damage.

SUMMARY POINTS

1. Cellular responses to DNA DSBs include cell cycle arrest by checkpoints, DNA repair, and apoptosis. Not fully understood is what causes a cell to meet different fates, arrest/repair, or die.
2. Three consequences of DNA DSBs are chromatin modification, binding to DNA of MRN protein complexes, and resection of the double strand to expose single-stranded DNA. These lead to activation and recruitment of ATM kinase and recruitment of ATR kinase to sites of damage, followed by activation of signal transducers, Chk1 and Chk2 kinases.

3. Large domains of chromatin flanking a DSB bear γH2AX modification and become coated with proteins that participate in DNA damage responses. Binding of mediator MDC1 to γH2AX is a key step in this process. The nucleo-protein domain may serve to amplify the signal and to facilitate efficient DNA repair.
4. Three ways in which DNA repair may be promoted are phosphorylation of repair enzymes, recruitment to sites of damage, and transcriptional activation of repair enzymes. Cohesins are also recruited to sites of damage and facilitate repair, presumably by promoting interchromosomal interactions needed for homology-directed repair.
5. Although homologs of both ATM and ATR are capable of facilitating DNA damage responses, one usually makes a more substantial contribution than the other, depending on the organism or cell type.
6. The ends of linear chromosomes are also capable of generating DNA damage responses, but are normally prevented from so doing via as yet poorly understood mechanisms. Recovery and adaptation are other mechanisms for turning off checkpoints. *Erratum
7. Cell cycle checkpoints can arrest mitosis at multiple points using multiple mechanisms.
8. Transcription factors p53 and E2F1 are capable of activating genes needed for DNA repair, cell cycle arrest, and apoptosis. Combinational output of their activity can translate into life-or-death decisions for the cell.
9. IR remains a treatment of choice for cancer and acts by inducing DSBs. Understanding the basis cellular choices in response to DNA damage is essential for designing treatment regimes that direct diseased cells to apoptosis while sparing their healthy neighbors.

FUTURE DIRECTIONS

IR remains a treatment of choice for cancer. The therapeutic potential of radiation requires the ability to selectively eliminate mutant cancer cells while sparing healthy wild type neighbors. This may be possible if IR-induced DSBs produce different outcomes depending on cellular genotype—apoptosis in cancer cells and DNA repair in wild-type, for example. Although individual responses to ionizing radiation are now better understood, how they are coordinated and selected to produce different outcomes remains a mystery. Understanding the genetic and experimental basis for choices in cellular response to DNA damage would be essential for designing treatment regimes to direct cells of different genotypes into different fates.

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*Erratum (14 Nov. 2006): See online log at http://arjournals.annualreviews.org/errata/genet

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Corroborative data from budding yeast and human cells demonstrate a role for ssDNA-RPA complexes in recruitment and activation of ATR.

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