Cellular Radiosensitivity: How much better do we understand it?

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Abstract

Purpose: Ionizing radiation exposure gives rise to a variety of lesions in DNA that result in genetic instability and potentially tumorigenesis or cell death. Radiation extends its effects on DNA by direct interaction or by radiolysis of H_2O that generates free radicals or aqueous electrons capable of interacting with and causing indirect damage to DNA. While the various lesions arising in DNA after radiation exposure can contribute to the mutagenising effects of this agent, the potentially most damaging lesion is the DNA double strand break (DSB) that contributes to genome instability and/or cell death. Thus in many cases failure to recognise and/or repair this lesion determines the radiosensitivity status of the cell. DNA repair mechanisms including homologous recombination (HR) and non-homologous end-joining (NHEJ) have evolved to protect cells against DNA DSB. Mutations in proteins that constitute these repair pathways are characterised by radiosensitivity and genome instability. Defects in a number of these proteins also give rise to genetic disorders that feature not only genetic instability but also immunodeficiency, cancer predisposition, neurodegeneration and other pathologies.

Conclusions: In the past fifty years our understanding of the cellular response to radiation damage has advanced enormously with insight being gained from a wide range of approaches extending from more basic early studies to the sophisticated approaches used today. In this review we discuss our current understanding of the impact of radiation on the cell and the organism gained from the array of past and present studies and attempt to provide an explanation for what it is that determines the response to radiation.

A historical perspective.

The damaging effects of X-rays became quickly evident after the description of this form of radiation by Roentgen in 1895. A number of reports described the acute effects that included dry itchy skin, swollen limbs and fingers, peeling of the skin and severe dermatitis. This was followed by reports of delayed effects including carcinoma and birth defects (Goldstein and Murphy 1929, Brown et al. 1936). These delayed effects were confirmed on a much broader scale after exposure to the atomic bombs in Japan and a series of accidental exposures extending to the latter part of the 20th century (Awa et al. 1987, Neel et al. 1977, Miller 1995) Exposure to whole body radiation doses (>1Gy) leads to acute radiation syndrome that affects all organs with the gastrointestinal system (GI syndrome), the brain and the haematopoietic system being particularly vulnerable (Yoshimoto et al. 1981, Yoshimaru et al. 1995, Otake and Schull 1984). GI syndrome in manifested by dehydration, diarrhea, infection and in severe cases septic shock and death (Potten 1990) The susceptible cells are stem cells close to the base of the crypt (Booth and Potten 2000). It seems likely that the primary pathway causing dysfunction of these stem cells is microvascular endothelial cell apoptosis (Davis et al. 2001). The biological effects of radiation have been extensively studied throughout the 20th century.

The nature of the lesions in DNA and other macromolecules are relatively well described; survival dose-response curves have been thoroughly analysed with different cell types; the relationship between DNA damage and mutation induction is well established and the carcinogenicity of radiation exposure is well accepted (Teoule 1987, Miller 1995, Wolf 1992). What remains controversial is the risk from exposure to low dose radiation and the shape of the kinetic curves below the range of accidental exposure where effects are more evident (Wall et al. 2006, Thierry-Chef et al. 2007, Strzelczyk et al. 2007). Board on Radiation Effects Research (BEIR) VII-Phase 2 Health Risks from Exposure to Low Levels of Ionizing Radiation supports a linear no threshold model as the most practical approach to determining radiation risk. This relies on epidemiological data from atomic bomb survivors, occupational exposure, exposure after release of radioactive materials into the environment and population-based studies from diagnostic and therapeutic exposure (Royal 2008). The latter type of exposure represents a major ongoing investigation into risk from low dose exposure.

Not surprisingly a greater appreciation of the teratogenic, mutagenic and carcinogenic effects of radiation brought with it the realisation that radiation might be

employed as a beneficial tool. The advent of the linear accelerator together with computer-assisted tomographic/magnetic resonance imaging-based computerised treatment planning increased the quality and precision for use of radiotherapy in the treatment of cancer (Goffman et al. 1990). Radiotherapy is an efficient and widely used modality for the treatment of cancer and relies on directing an optimal and effective radiation dose to the tumour while minimising the exposure to surrounding normal tissue. Greater benefit is derived if the tumour is more radiosensitive than the surrounding tissue but more often the emphasis is on protecting more sensitive normal tissue, for which the treatment for head and neck squamous cell carcinomas and glioblastomas provide good examples (Peters et al. 1988, Robins et al. 2007). More precise delivery of radiation to the tumour volume using 3-dimensional conformal radiation therapy has improved tumour control and decreased treatment-related toxicity (Purdy 2008). Intensity modulation of the radiation beam during therapy with the application of intensity modulated radiation therapy (IMRT) has further enhanced treatment (Woo et al. 1994).

Away from the context of radiotherapeutic treatment of tumours, exposure to radiation, at low dose, has been argued to be beneficial (Macklis and Beresford 1991). Reports from the Soviet Union in the 1950s of a "stimulatory" effect of radiation gave way to a more precise definition as radiation hormesis which in effect points to a protective effect of low dose radiation (Wolf 1989a). Hormesis suggests a negative association between low dose exposure and health consequences. It supports non-linearity at low doses as opposed to the BEIR VII findings and an argument in favour of evolution to the "fittest state" in a background of low radiation (Parsons 2006). Experiments with both bacteria and mammalian cells demonstrated that, when exposed to low dose radiation, these cells become refractory to the killing by subsequent exposure to higher doses of radiation (Samson and Cairns 1977, Wolff et al. 1989b) This was subsequently called the adaptive response (Wolff 1992). How this adaptive response functions to protect cells remains unclear, but it seems likely that groups of genes are involved and that protein synthesis and degradation contribute to the process (Tapio and Jacob 2007). Whether this is related to the proposed hormesis effect is still unclear. (Radford 2002) suggests that the diminished fixation of DNA double strand breaks (DSB) in transcription factories may provide at least part of the explanation. Adaptation is not confined to the damaged cell but may also be extended to neighbouring cells. This is referred to as the "bystander" effect that operates by the

release of diffusible signalling molecules or through gap-junction intercellular communication (Morgan and Sowa 2007).

From this brief introduction, it is evident that enormous insight has been gained into understanding how radiation damages cells and how it can be exploited as a therapeutic tool. In this review, we aim to overview the significant advances made in fifty years of studying radiosensitivity. We will highlight seminal findings, discuss how important observations made in the early days of radiation research can now be understood in molecular terms and finally consider the further important questions to be addressed.

Radiation induced DNA damage and its impact within the cell.

While population-based studies are of the foremost importance in understanding risk associated with radiation exposure, they do not provide quantitative data on radiosensitivity. This is addressed to some extent in animal studies where death is the endpoint (Goldman 1982). However, the majority of such experiments are designed to investigate tumorigenesis or induction of other disease states rather than radiosenstivity *per se* (Loken 1983). Notwithstanding the limitations imposed by *in vitro* cell culture studies, the great bulk of our knowledge on cellular radiosensitivity comes from such investigations. Exposure of cells to ionizing radiation (X-rays, γ-rays, high linear energy transfer (LET) radiation) results in cell cycle arrest prior to DNA repair, mutation induction, transformation and cell death. The focus of this review will be on DNA damage, its -repair and radiation-induced cell killing. DNA is the major target for cell killing by radiation (Ward 1981). However, ionization events are not confined to DNA and its immediate environment. Free radicals generated by radiation can also alter membrane proteins and lipids (Wallach 1972). The oxidation state of sulphydryl groups is also altered by radiation and the products of radiolysis lead to lipid hydroperoxides (Agrawal and Kale 2001, Zhao et al. 2001). Nevertheless, radiation doses that alter the permeability properties of membranes are significantly higher than those that damage DNA (Kankura et al. 1969). Hence, damage to DNA and processes responding to that damage that serve to maintain DNA integrity lie at the core of the cellular response to radiation. DNA integrity is affected by a variety of lesions induced by ionizing radiation arising as a consequence of "direct" and "indirect" damage. In the former case radiation interacts directly with DNA generating charged particles or electrons that carry the kinetic energy of photons (X-

rays, γ-rays) causing breaks in the phosphodiester backbone (van der Schans et al. 1973). This represents approximately 30% of the damage to DNA (Chapman et al. 1973). The remainder arises from indirect effects due to ionization of H_2O molecules that generate hydrated electrons, H atoms and hydroxyl radicals (.OH) (von Sonntag 1987). Use of radical scavengers indicates that radicals of this type contribute as much as 70% of DNA damage induced by radiation (Chapman et al. 1973). These radicals damage DNA by both addition and abstraction reactions resulting in base and sugar-derived products; single and double strand breaks as well as DNA-protein cross-links (Dizdaroglu et al. 1991, Teoule and Cadet 1978). Of these lesions it is now evident that the DNA DSB has the greatest potential for cell killing (Ward 1975). Indeed, exposure of murine cells to radiation under conditions that altered the relative amounts of different types of DNA damage showed that the extent of cell killing was directly related to the yield of DNA DSB (Radford 1985).

Earlier models relied on "hits" and "targets" to explain the shapes of cell survival curves in response to radiation damage (Lockart et al. 1961). Without a clear knowledge of the actual target, it was suggested that the shoulder on the survival curve observed after Low-LET radiation could be explained either by the requirement for single hits on more than one target or by multiple hits on a single target. (Goodhead 1985) invoked "saturable repair" to explain the kinetics of cell killing with increasing radiation dose. This model did not require a sublethal damage event which was an inherent part of the Curtis et al. model that relied on irreparable (lethal) and repairable (potentially lethal) lesions (Curtis 1986). He identified these as being DNA DSB of different severity, which can now be explained by clustered or complex DNA damage (Georgakilas 2008). The frequency and complexity of clustered damage depends on the LET value of the radiation with as much as 70% of DNA DSB being of the complex type after high LET radiation exposure (Nikjoo et al. 1998). For low LET radiation, the shoulder on the survival curve can be explained by the action of DNA repair at lower doses, giving way to more lethal hits with increasing dose. The effect on survival does not appear to be explained by the repair mechanisms reaching saturation but rather because of a reduced capacity overall to cope with the damage.

While the majority of DNA repair pathways are constitutively active, there are examples of inducibility. Such inducibility is manifested by increased resistance to radiation when a priming or conditioning dose is applied prior to a higher dose, and is known as the adaptive response. The adaptive response does not require direct

damage to the nucleus *per se* and is observed as a reduced damaging effect by a challenging dose of radiation subsequent to exposure of the same cells to a low priming dose (Tapio and Jacob 2007). The reduced damaging effect is determined by the extent of cell killing; induced chromosome aberrations; mutation induction, capacity for DNA repair or radiosensitivity (Wolff 1998). Non-targeted effects also apply to cells in the vicinity that are not directly traversed by the damaging agent (Preston 2005). This is referred to as the bystander response and can be mediated by cell-to-cell contact on or by transfer of soluble factors (Morgan 2003). In the case of radiation exposure where an α-particle traverses a single cell the effects can also be observed in non-exposed cells (Azzam and Little 2004). The bystander response is seen at the tissue level and is similar to a generalised stress response (Mothersill and Seymour 2004). These phenomena have important implications for cancer initiation and other pathologies arising from cell types only indirectly affected by the damaging agent. Inducibility is also associated with the low dose hyper-radiosensitivity effect (Joiner et al. 1996). This refers to the effect in which cells show elevated sensitivity to small single doses of radiation but become more resistant (per unit dose) to large single doses (Joiner et al 2001;Marples et al. 2004), which is manifested by a "dip" and recovery in the shoulder of a survival curve as the dose increases over a low dose range (20-30cGy). This hypersensitivity is a response specific to G2 phase cells and is directly linked to the failure to activate the (ataxia-telangiectasia mutated) ATM-dependent early G2/M checkpoint at these low doses (Krueger et al 2007;Marples and Collis 2008)). The defect is at the level of cell cycle checkpoint activation rather than DSB recognition or repair (Wykes et al. 2006). As the dose increases, checkpoint arrest is activated, allowing more time for repair prior to progression into mitosis and hence increased survival.

Cell cycle phase can also impact upon radiosensitivity in a manner distinct to the low dose hypersensitivity discussed above. Generally, increased resistance to radiation is observed in late S/G2 phase, which might correlate with the doubling in DNA content following replication (Terasimaand Tolmach 1961).. It is also possible that the ability to exploit two distinct DSB repair mechanisms in late S/G2 phase (see below) enhances the radioresistance in these cell cycles phases.

Methods to monitor repair of radiation damage.

As discussed above, studies undertaken from 1960 to 1980 exposed the concept that DNA damage lay at the root of radiation sensitivity. By the mid 1970s, it was appreciated that, although single strand binding protein (SSB) arise more frequently than DSB, the latter represent the most significant lethal lesion (Ho 1975). Central to these studies were emerging methods to monitor SSB and DSB induction and repair. An early technique was sucrose gradient sedimentation, which separated DNA fragments based on size. The analysis was carried out under alkaline or neutral conditions to detect SSB or DSB, respectively. Radiation was shown to reduce the sedimentation rate of DNA, which, following incubation, returned to that observed in unirradiated cells. Strikingly, Rad52 yeast mutants, whilst exhibiting the same sedimentation profile as control yeast immediately following radiation, failed to recover (Ho 1975). Thus, it was appreciated that an unrepaired chromosome break could lead to reproductive cell death. The alkaline and neutral DNA elution technique and the DNA unwinding technique were additional approaches exploited to measure SSB and DSB induction and repair (Iliakis et al. 1991a). Subsequently, pulsed field gel electrophoresis emerged as an even more sensitive methodology (Iliakis et al. 1991b, Sutherland et al. 1987).

These methods have limitations however. Firstly, they necessitate the use of non-physiological radiation doses, usually > 10 Gy, precluding an examination of DNA repair following physiologically relevant doses. Secondly, the techniques are not readily able to detect subtle repair defects, which may arise if the defect lies in a subset of DSB or a specific cell cycle phase. Thirdly, apoptosis also causes DNA breakage and the techniques do not readily facilitate a distinction between breakage arising as a consequence of radiation-induced apoptosis versus directly induced DNA breaks. Although apoptotic-induced breaks in DNA usually arise at a later stage, they can be difficult to distinguish from a persistent subfraction of unrepaired DSB. Finally, all the techniques employ neutral conditions to monitor DSB repair. Recent findings have suggested that even under these conditions, labile sites which might not generate DSB in vivo can be converted to DSB during analysis, resulting in an over-estimation of directly induced DSB numbers (Ratnayake et al. 2005). Notwithstanding these limitations, the methods have yielded highly significant findings, which in general are consistent with more sensitive methods employed today.

The development of more sensitive methods for monitoring repair of DNA SSB has occurred as our understanding of the mechanisms involved has increased. While SSB arise as a consequence of different forms of damage to DNA there is considerable redundancy in the enzymes involved in their repair. Direct SSB such as those generated by radiation or oxidative stress can either be repaired by DNA ligase or after DNA end processing followed by gap-filling by DNA polymerase and then ligation (Lindahl et al. 1995). Breaks can also arise during base excision repair (BER) of apurinic/apyrimidinic sites by the AP endonuclease or a DNA glycosylase. The mechanism of repair of these breaks is outlined in Fig 1. Clearly the repair of these DNA SSB can be determined by the methods described above. A more recent assay of choice for SSB repair is the alkaline comet assay, a sensitive assay for use with single cells (Singh et al. 1989). Using the comet assay the bulk of DNA repair occurs in the first 15 min after DNA damage and is largely complete by 2h. Basic protocols for the use of this assay have been reported more recently (Breslin et al. 2006; Olive and Banath 2006). Breslin and his colleagues also employed the assay to demonstrate that cells from patients with spinocerebellar ataxia with axonal neuropathy-1 (SCAN1), defective in tryosyl phosphodiesterase 1 (TDP1), have a defect in the repair of chromosomal SSB arising independently of DNA replication from abortive Topoisomerase I (Top1) activity or oxidative stress (El-Khamisy et al. 2005). It seems likely that this defect contributes to the neurodegeneration characteristic of SCAN1 and stresses the importance of SSB repair in maintaining the integrity of DNA in post-mitotic neurons. Poly (ADP-ribose) polymerase (PARP1) plays a key role in recognising DNA SSB where it is activated to poly (ADP-ribosylate) itself and other proteins (D'Amours et al. 1999). During this process NAD(P)H and ATP are depleted. This depletion of NAD(P)H and subsequent recovery has been used as the basis for the detection of an imbalance of DNA repair in X-ray repair cross-complementing group 1 (XRCC1) deficient cells (Nakamura et al. 2003). In this assay a water soluble tetrazolium salt is reduced to a yellow coloured water soluble formazan dye which is dependent on the amount of NAD(P)H present. While the assay does not directly measure DNA SSB it avoids extraction and alkaline conditions and is rapid. Host cell reactivation of plasmids containing oxidative damage to DNA has also been employed to measure DNA SSB repair (Spivak and Hanawald 2006). Cockayne syndrome patient cells showed defective recovery of expression of plasmid indicative of a defect in SSB repair. Use of cell-free extracts has had a major impact

in unravelling the mechanism for both nucleotide excision repair and base excision repair (Wood and Coverley 1991, Dianor et al. 2001). This approach has not only assisted in identifying the order of specific steps and the enzymes involved but has also provided a read-out for the efficiency of DNA repair in extracts from a variety of mammalian mutant cells.

Strikingly, our current understanding of molecular steps involved in the detection and signalling ofDSB has provided the knowledge underlying the development of a recent, highly sensitive technique to monitor DSB formation and repair. It is now known that an early step in the DNA damage response is the phosphorylation of H2AX, a variant form of the histone H2A, generating γ-H2AX (Paull et al. 2000). H2AX phosphorylation extends several megabase pairs from the site of the DSB and can be visualised as discrete foci by indirect immunofluorescence using phosphopeptide-specific antibodies, ie α-γ-H2AX. The number of foci visualised closely correlates with current estimates of DSB formation and their rate of loss closely parallels the rate of DSB repair monitored by the methods described above (Rothkamm et al. 2003). Thus, γ-H2AX foci analysis is an exquisitely sensitive technique to monitor DSB repair, amenable for use with very low doses (Rothkamm and Lobrich 2003). There are limitations to the technique, however. γ-H2AX phosphorylation can also arise from single stranded regions of DNA generated following replication fork stalling or during the processing of bulky lesions (Ward and Chen 2001); it is an indirect method that monitors the consequence of the lesion rather than the lesion itself; and finally, there may be a delay between DSB repair and loss of γ-H2AX phosphorylation or circumstances (eg when cells are in mitosis) when γ-H2AX loss does not occur (Kato et al. 2008). Nonetheless, the technique has allowed a dissection of events that was previously impossible, and facilitated the direct demonstration of a DSB repair defect in Ataxia-telangiectasia (A-T) cells (Riballo et al. 2004) (see further discussion below).The comet assay has also been carried out under neutral conditions to detect the presence of DNA DSB independent of the presence of DNA SSB (Olive et al 1991). The comets prepared under these conditions have a ‘halo’ of DNA loops which distinguishes them from those prepared under alkaline conditions.

It is also noteworthy that, whilst in yeast, the physical methods monitoring DSB repair measure the process of HR, since this represents the major DSB repair

mechanism, in mammalian cells, they primarily monitor NHEJ. Currently, HR is primarily assessed using constructs, either integrated in the host genome or as plasmids, carrying an I-Sce1 site (Moynahan et al. 2001). Whilst this is a useful assay, it does not allow an assessment of the repair of radiation induced DSB by HR.

Finally, a further important aspect of DSB repair is an assessment of its fidelity. An extremely elegant modification of the pulsed-field gel electrophoresis (PFGE) technique was developed to assess the accuracy of repair (Rothkamm et al. 2001). Rare cutting restriction enzymes were employed together with defined probes to allow an assessment of whether repair within the defined fragment could result in the generation of larger sized fragments, which could only arise as a consequence of misrepair. Such an analysis suggested that misrepair occurred more frequently when multiple DSB where present. Further development of techniques to monitor the fidelity of repair is urgently needed.

Radiation sensitive rodent mutants: their contribution to an understanding of radiation sensitivity.

Following the realisation that cellular characteristics, including sensitivity to DNA damaging agents, could be inherited, the isolation and study of radiation sensitive cell lines followed (Timeline 1).

The identification and characterisation of radiation sensitive *E. coli* and yeast mutants progressed from 1969/1970 with, most significantly, the isolation of Recombination Protein A (RecA) *E.coli* mutants and Rad52 yeast mutants (Game and Mortimer 1974, Ho and Mortimer 1975, Ho 1975, Resnick 1969,Willetts and Mount 1969). Subsequently, during the 1980s, it was realised that cultured mammalian cells, like bacteria and yeast, could also be exploited for selection and screening of mutants (Jeggo 1990, Zdzienicka and Simons 1987). Since most mutations conferring radiation sensitivity are recessive, the diploid nature of mammalian cells severely decreases the frequency of mutation induction even following heavy mutagenesis due to the necessity to inactivate both alleles. However, in the late 1970s, it was realised that certain cultured cell lines, including the rodent CHO cell line, had significant regions of functional hemizygosity, allowing mutants to be isolated, at least in a subset of genes, at reasonable frequencies. Indeed, by exploiting different rodent cell lines such as V79, AA8 or CHO cells, which have different regions of hemizygosity, a considerable number of radiation sensitive mutants were identified (see for example

Stamato and Hohmann 1975). The screening and selection procedures that were successfully used to isolate mutants from lower organisms were applied to rodent cell lines and mutants covering a range of radiation sensitivity were obtained. These mutants included XR-1 and xrs1-6, which proved to be mutated in XRCC4 and Ku80, respectively (see Thompson and Jeggo 1995, Zdzienicka 1995 for reviews). Further, the SCID mouse, which was identified via its defect in V(D)J recombination, proved to display radiosensitivity and was subsequently shown to be defective in DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Bosma et al. 1983, Blunt et al. 1995). An important approach that facilitated the exploitation of these cell lines was their classification into complementation groups. This was achieved by examining the radiation sensitivity in hybrids made by pairwise fusion of the cell lines. Hybrids which were complemented for radiosensitivity were considered to be derived from lines with distinct genetic defects whilst non-complementing hybrids were considered to be derived from lines mutated in the same gene.

The cellular analysis of these cell lines consolidated the notion that DSB were the most significant lethal lesion induced by ionizing radiation (IR). Further, they revealed that whereas radiosensitive yeast mutants mainly showed dramatic defects in homologous recombination (HR), demonstrating the important role of HR in repairing radiation induced DSB, the mammalian mutants appeared to be defective in a distinct process. Hence, the realisation that mammalian cells utilise a distinct DSB repair process, subsequently shown to be DNA non-homologous end-joining (NHEJ), emerged. The rodent mutants together with the SCID mouse also facilitated the important discovery that V(D)J recombination, a critical step during development of the immune response, that exploits the process of NHEJ to rearrange and rejoin the V, D or J segments during immune development (Taccioli et al. 1993).

Whilst the cellular characterisation of the rodent mutants provided important insight into DNA damage response processes, their major contribution has been as a tool to facilitate the identification of genes critical for radiation resistance. Initial approaches involved mapping a complementing region in hybrids followed by localisation of the correcting gene. Later, as molecular techniques to transfect exogenous cDNAs into cells improved, the cell lines were exploited for cloning studies by introducing cDNA libraries followed by selection for radioresistance. The introduction of yeast artificial chromosomes (YACs) was particularly important for the identification of DNA-PKcs, due to its large size (Blunt et al. 1995), (see (Jeggo

1998) for a review). Via these and additional approaches, Ku80, Ku70, DNA-PKcs, XRCC4 and subsequently Rad51 and its paralogues where identified.

More recently, the ability to knock out these genes in mice and the use of increasingly efficient interference RNA (siRNA) approaches has diminished the benefits of the rodent cell lines. Nonetheless, a large body of literature on radiation sensitive yeast, rodent and human cell lines has proved to be highly informative and its value should not be forgotten, (Timelines 1 + 2).

Radiation sensitive human patients.

A seminal finding in 1975 was the discovery that A-T represents a radiation sensitive human disorder (Taylor et al. 1975, Timeline 2).

Although clinical radiosensitivity following radiotherapy had been observed in a few A-T patients, and elevated induction of chromosome aberrations by IR was reported in A-T lymphocytes, the finding that fibroblast and lymphoblastoid cell lines derived from A-T patients displayed radiosensitivity was seminal (Taylor et al. 1975, Chen et al. 1978). Strikingly, A-T cells appeared in these early studies to be largely proficient in both SSB and DSB repair, although the presence of a subtle DNA repair defect was described in A-T using cytogenetic approaches as early as 1985 (Cornforth and Bedford 1985). Viewed retrospectively, the complex phenotype of A-T was evident from an early stage. The inability of A-T cell lines to arrest DNA synthesis, conferring the radioresistant DNA synthesis (RDS) phenotype, which we now know to be a consequence of an S phase checkpoint defect, was characterised in 1980 (Houldsworth and Lavin 1980, Painter and Young 1980) . Some eight years later the gene defective in A-T was mapped to chromosome 11q22.23 (Gatti et al. 1988). Via an amazing tour de force in positional cloning, ATM was identified as the gene defective in A-T patients, a particularly difficult challenge due to the large size of the gene (Savitsky et al. 1995). A-T represents the paradigm for a series of syndromes defective in the recognition and/or repair of DNA damage (Table 1)

Nijmegen Breakage Syndrome (NBS) and subsequently, A-T like disorder, (ATLD), were also recognised as A-T like, radiation sensitivity syndromes, which, in the case of NBS, was perhaps surprising considering the markedly distinct clinical features of NBS and A-T patients (Weemaes et al. 1981, Stewart et al. 1999). Nonetheless, cell lines from such patients display a surprising degree of overlapping features.

The relationship between NHEJ and V(D)J recombination deficiency raised the possibility that defects in NHEJ genes might contribute to human immunodeficiency. Subsequent screening for radiosensitivity in patients with compromised immune function has revealed ligase 4 (LIG4), Artemis deficient and XLF-Cernunnos (XLF) deficient syndromes proving that human "mutants" can also be identified by genetic screening (O'Driscoll and Jeggo 2006). The genetic defect in the latter two disorders emerged from mapping studies of immunodeficient, radiosensitive individuals Radiosensitive severe combined immunodeficiency (RS-SCID patients) (Nicolas et al. 1998). These studies have provided insight into the role played by damage response genes during development. Strikingly, both LIG4 and XLF-deficient patients have developmental and growth delay, and characteristic facial features demonstrating a role for NHEJ proteins during neuronal development.

In contrast to the observations made for disorders with defects in repair of DNA DSB there are no syndromes described with marked radiosensitivity as a consequence of defective SSB repair. Cells from patients with ataxia oculomotor apraxia type 1 (AOA1) exhibit either mild sensitivity to radiation (Clements et al. 2004) or normal sensitivity (Gueven et al. 2004). AOA1 cells are also sensitive to a variety of other agents that cause SSB in DNA including H_2O_2 methylmethane sulfonate and camptothecin. While there is no evidence for a defect in repair of radiation-induced SSB in AOA1 cells there is evidence for a defect in SSB repair after exposure to H_2O_2 and agents that cause oxidative stress (Hirano et al. 2007, Gueven et al. 2007). The protein defective in AOA1, aprataxin, resolves abortive DNA ligation intermediates (Ahel et al. 2006). This enzyme catalyses the removal of adenylate groups covalently attached to 5′-phosphate termini at single strand nicks or gaps. This then allows DNA ligation of adjacent 5′ phosphate and 3′ hydroxyl termini to proceed. Extracts from AOA1 patients are defective in this reaction. Cells from patients with spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) are defective in TDP1, a DNA end-processing protein that repairs Topoisomerase 1-induced SSB (Takashima et al. 2002). Camptothecin, which increases the half-life of the Top1 cleavage complex increases the number of Top1-strand breaks (Pourquier et al. 1997). Top1 is also capable of forming complexes with damage arising from different agents including ionizing radiation (Pourquier et al. 2001). In human cells TDP1 is responsible for the repair of SSB arising independently of DNA replication from abortive Top1 activity or oxidative stress (El-Khamisy et al. 2005). This protein is part of a multi-protein

SSB repair complex, through its direct interaction with DNA ligase IIIα, and the complex is catalytically inactive in SCAN1 cells. El-Khamisy et al. 2007 subsequently showed that TDP1 facilitated the repair of ionizing radiation-induced DNA SSB. Notwithstanding these observations lymphoblastoid cells from SCAN1 patients exhibit only a very small degree of radiosensitivity (Zhou et al. 2005). Thus the defect in SSB repair is not associated with radiosensitization.

The contribution of cytogenetic techniques; past history and emerging technology.

Cytogenetics has enormously contributed to our understanding of radiation sensitivity. Work by Sax in the late 1930s demonstrated that radiation induces chromosome breaks, which are subsequently rejoined, misrejoined or remain unrepaired and models based on the nature of the aberrant events are still valid today (eg (Sax, 1938). Several excellent reviews have discussed the contribution of cytogenetics to our current understanding of radiosensitivity and will not be considered in detail here (Bailey and Bedford 2006, Cornforth 2006). Instead, we will consider the seminal findings unearthed by cytogenetic approaches. Cytogenetics remains a valuable technology and we will consider how its further exploitation can continue to contribute to radiation biology.

A critical observation emerging from the early studies was that cells irradiated in G0/G1 phase predominantly form chromosome-type aberrations, characterised by breakage at the same point on both chromatids at the first mitosis (Bender et al, 1974). In contrast, chromatid-type aberrations, where the break or exchange is only observed on one chromatid, are the major aberration observed in irradiated S or G2 phase cells. Other DNA damaging agents that induce base damage or SSB rather than direct DSB do not show this phenotype, yielding chromatid type aberrations even when irradiated in G0/G1 phase, and then only following exposure to high doses. In other words, a characteristic of radiation exposure is the formation of chromosome type aberrations following low dose irradiation of G0/G1 cells. The explanation for these findings is that replication past a direct (or prompt) DSB leads to DSB at the same site on both daughter chromosomes, which rejoin to generate chromosome-type aberrations, such as ring chromosomes or dicentrics (Bailey and Bedford 2006). Although DSB repair defective cell lines showed elevated chromosome breakage and misrejoining events,

they do not adhere to these rules and form both chromosome and chromatid-type aberrations following irradiation in G0/G1 with both occurring in the same cell (Kemp and Jeggo 1986, Bailey and Bedford 2006). This demonstrates that cells with DSB generated in G0/G1 can traverse S phase and progress to the first mitosis. The explanation for why NHEJ mutants display their distinct phenotype is currently unclear but suggests that in the absence of NHEJ, events leading to chromatid type aberrations dominate. Furthermore, this phenotype is also observed in A-T cells. Again, a defined explanation is unclear, although the checkpoint defect of A-T cells may function to diminish the opportunity for repair (Cornforth and Bedford 1987). These important findings should not be neglected since they have the potential to provide insight into events that occur following replication past a DSB.

Another significant observation is that irradiation of G0/G1 cells causes prompt chromatin fragmentation visualised by premature chromosome condensation (PCC) (Cornforth and Bedford 1985). Few other agents except those that induce direct DSB generate PCC breaks in G0 cells. Indeed, this observation provided the first demonstration that A-T cells harbour a DSB repair defect since, although A-T cells had the same level of initial breakage assessed by PCC fragments as control cells, the residual numbers of excess PCC fragments at 24 and 48 h post irradiation was much higher (Cornforth and Bedford 1985). This is somewhat complicated by a more recent study that provided evidence that the initial amount of damage was greater in A-T cells after radiation exposure (Pandita and Hittelman 1992). They suggested that chromatin organisation may play a role in the observed radiosensitivity of A-T cells.

Cytogenetic approaches have also been informative in considering the impact on nuclear organization and chromatin structure on DSB repair, a currently topical component influencing DSB repair. It had been long known that more rapid repair of photoproducts occurs in transcriptionally active regions (Madhani et al. 1986). Prompted by these findings, cytogeneticists examined whether the breakpoints of translocations predominated in G-light band regions of chromosomes, which were considered to be regions of high transcriptional activity. One study found that a transcriptionally activate region of mosquito articifical chromosomes was hypersensitive for the induction of radiation-induced deletions compared to several other sites (Muhlmann-Diaz and Bedford 1994). Further, the frequency of X-chromosome deletions or tranlocations in patients with multiple X chromosomes, which are normally inactivated (Klinefelter syndrome), provided evidence for reduced

radiation induced exchanges (Bailey and Bedford 2006). Recent studies using molecular techniques have suggested that heterochromatin is a barrier to DSB repair and that DSB located within heterochromatin are repaired more slowly than euchromatic DSB and have a preferential requirement for ATM (Goodarzi et al. 2008). The cytogenetic studies are perhaps not what might be expected from the more recent studies, but suggest that although being repaired more slowly than euchromatic DSB, they may have a diminished capacity to participate in exchange type aberrations.

Biomonitoring of radiation exposure is of increasing importance and the assessment of chromatid aberrations has been the gold standard technique for multiple years. Although the analysis of γ-H2AX formation has the potential to be useful, the cytogenetic assays has proven efficacy and strengths. A further hallmark of radiation exposure is the formation of balanced translocations (Anderson et al. 2003). Although such aberrations are low in number, they are long lived. There are powerful techniques, such as Spectral karyotyping (SKY) and multi colour Fluorescent *in situ* hybridization (FISH), currently available that merge cytogenetics with molecular biology and it is crucial to further develop these techniques. It is noteworthy that these techniques also have the potential to monitor misrepair events *in vivo* and can potentially be exploited to address the issue of the fidelity of DSB repair.

Assays to monitor radiosensitivity.

Cells can die after radiation exposure by the induction of apoptosis, by loss of reproductive capacity, by the onset of permanent cell cycle checkpoint arrest or by the onset of premature senescence. These end results may not be distinct or mutually exclusive; for example premature senescence may arise as a consequence of prolonged checkpoint arrest and loss of reproductive capacity may be a consequence of permanent cell cycle arrest. A striking feature of radiation exposure is that cells can remain in a non-replicating but viable state for prolonged periods post-irradiation, a characteristic which is central to the use of irradiated cells as feeder layers to enhance cloning efficiency. This feature, however, has limited the utility of methods that monitor viability markers (such as the 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) and Trypan blue assays) for assessment of radiation survival levels. The most reliable assay for monitoring radiation survival is

clonogenic survival, most frequently carried out using skin fibroblasts, but these are time consuming and not useful for cell types that display low cloning efficiency.

Using such assays, cell lines lacking NHEJ proteins show exquisite radiosensitivity attesting to the importance of NHEJ in repairing radiation induced DSB whilst homologous recombination (HR) deficient cell lines show less sensitivity, which differs in magnitude between cell types. Our current knowledge of DSB repair provides an explanation for this: primary fibroblasts, the cell type -used predominantly for clonogenic survival analysis, are predominantly in G0/G1 phase and traverse slowly to S phase. Hence, NHEJ, the major DSB repair process in G0/G1 phase predominates. HR, in contrast, functions in late S/G2 phase since resection, a key step in HR, requires Cyclin-dependent kinases (CDK) activation and is down regulated in G1 phase (Jazayeri et al. 2006). Radiosensitivity is only observed in HR-deficient cell types that are rapidly replicating (eg CHO cells) where there is a greater percentage of S/G2 phase cells. Thus, HR does contribute to survival post-irradiation, the magnitude of which depends on cell cycle phase.

Our current understanding of DNA damage response (DDR) pathways.

Since DSB are the most significant lethal lesion following radiation exposure, we will first consider the DNA damage response mechanisms (DDRs) to DSB. DSB can undergo repair as well as activate a signal transduction process that leads to cell cycle checkpoint arrest, the onset of apoptosis and influences the repair process (Jackson 2001). NHEJ, as discussed above, represents the major DSB repair pathway whilst HR also contributes in late S/G2 phase (Wyman and Kanaar 2006). ATM lies at the centre of the DSB signal transduction response (Kurz and Lees-Miller 2004). Strikingly, NHEJ and ATM-dependent signalling function largely independently and as an array of signalling proteins, accumulate non-competitively at DSB sites (Jeggo and Lobrich 2006).

Six core proteins required for NHEJ have been identified, Ku70, Ku80, DNA-PKcs, XRCC4, XLF and DNA ligase IV, which are assembled as two discrete complexes (Jeggo and Lobrich 2006, Fig 2). Firstly, the Ku heterodimer rapidly binds to double stranded DNA ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), causing assembly of the DNA-PK complex. This activates DNA-PK kinase activity, whose function appears to be predominantly to regulate NHEJ to co-ordinate end processing with rejoining. The assembled DNA-PK

complex recruits a ligation complex involving XRCC4, XLF- Cernunnos and DNA ligase IV. XRCC4 and DNA ligase IV (LX) are tightly co-associated and whether LX is recruited first to the DSB where it co-associates with XLF is currently unclear. However, optimal DSB repair requires all three proteins (Fig 2). Additional proteins are required for end-processing including polynucleotide kinase and fill-in polymerases, polμ and polε. Artemis, an endonuclease, is required for a subset of DSB repair (Riballo et al. 2004) (see below). NHEJ represents the major DSB repair pathway in G0/G1 phase while HR functions in late S/G2 phase when a sister homologue is available.

DSB also rapidly activate ATM-dependent signalling. Current evidence suggests that the Mre11/Rad50/NBS1 (MRN) complex represents the primary DSB sensor serving to recruit ATM (Lavin,2007, Uziel et al. 2003, Fig. 3). In addition, a number of BRCA1 C-Terminus (BRCT)-containing mediator proteins, including DNA damage checkpoint protein 1 MDC1, p53-binding protein 1 (53BP1), BRCA1 and the MRN complex, function to retain ATM at the DSB site although being dispensable for initial ATM activation (Lavin 2007). While full details of the series of events occurring immediately after the appearance of DNA DSB have not been established, a clearer picture is now emerging. The MRN complex is rapidly localized to nuclear foci at sites of DNA damage in response to radiation (Maser et al. 1997, Nelms et al. 1998). Meiotic recombination 11 (Mre11) / Rad50 binds to DNA as a heterotetramer to tether free ends (de Jager et al. 2001). The complex is completed by the association of a molecule of Nbs1 (Riballo et al. 2004). Sensing DNA DSB by the MRN complex does not require ATM (Mirzoeva and Petrini 2003). Binding is achieved through DNA binding domains on Mre11 in association with the Walker A and B ATPase domains of Rad50 (van den Bosch et al. 2003, Hopfner et al. 2002). Williams et al (2008) have provided additional detail demonstrating that the Mre11 dimer adopts a four-lobed, U-shaped structure critical for complex assembly and for binding and aligning DNA ends. Association with Rad50 stimulates both the exonuclease and endonuclease activities for Mre11 (Paull and Gellert 1998) and Nbs1 stimulates endonuclease activity (Paull and Gellert 1999). Mutations causing loss of Mre11 nuclease activity impair DNA repair and survival. Other nucleases including Artemis also play a role in DNA DSB repair processing. Evidence for a role for MRN upstream of ATM is derived from studies with NBS and ATLD cells, during viral infection where the MRN complex is depleted and with in vitro *Xenopus laevis*

extracts reconstituted for DNA-damage signalling. ATM activation is defective in both NBS and ATLD cells in response to DNA DSB (Uziel et al. 2003). An NBS1 construct, NbFR5, which retained the Mre11-binding site, stimulated ATM activation (Cerosaletti and Concannon 2004). On the other hand NBS cells that express Nb FR5Δ ATM, which lacks the ATM-binding site, had dramatically reduced levels of ATM activation (Cerosaletti et al. 2006). The accumulation of the MRN complex and its retention on chromatin is dependent on the mediator of DNA-damage checkpoint protein-1 (MDC1) adaptor protein (Fig. 3). This retention by MDC1 increases the local concentration of the MRN complex at the sites of DNA DSB. ATM also arrives early at the damaged site, initially associating with DNA regions that flank the break, before associating with the MRN complex at the break site through the C-terminus of NBS1 (Falck et al 2005, You et al. 2007). Interaction of MDC1 through its Forkhead associated (FHA) domain with ATM regulates the accumulation of ATM at damaged sites. MDC1 also mediates the interaction between ATM and γH2AX. ATM is at least partially activated adjacent to DNA DSB (Berkovich et al. 2007), probably due to the initial relaxation of chromatin structure by the break. The activation of ATM by chloroquine, histone deacetylase inhibitors or hypotonic buffer supports this hypothesis (Bakkenist and Kastan 2003). However, ATM that is activated by these factors does not localise to nuclear foci and fails to phosphorylate H2AX, but it is capable of phosphorylating p53, which suggests that ATM needs to be localized to the break for complete activation (Fig. 3). Full activation of ATM and localization to DNA DSB is facilitated by the MRN complex (Berkovich et al .2007).

ATM undergoes autophosphorylation on at least 3 sites (ser 367; ser 1893 and ser 1981) at least one of which appears to be instrumental in the monomerization and activation of ATM (Bakkenist and Kastan 2003, Kozlov et al. 2006, Lavin 2008). The activity of 3 phosphatases protein phosphatase 2A (PP2A), Wild-type p53-induced phosphatase (WiP1) and Protein phosphatase 5 (PP5) are also implicated in ATM activation. The two former enzymes are thought to maintain low basal levels of ATM activity while PP5 appears to remove inhibitory phosphorylations (Goodarzi et al. 2004, Shreeram et al. 2006, Ali et al 2004). Acetylation has also been shown to alter ATM activity. Sun et al. (2005) showed that DNA DSB induce the acetylation of ATM in parallel to Ser1981 autophosphorylation. Overexpression of a dominant-negative form of HIV-1 TAT-interactive protein 60 (Tip60) acetyltransferase reduced levels of both acetylation and autophosphorylation of ATM, reduced ATM-kinase

activity and sensitized cells to radiation. A single acetylation site was identified as Lys3016, adjacent to the kinase domain of ATM (Sun et al. 2007). Mutation at this site prevented the upregulation of ATM activity of DNA damage, inhibited monomerization of the inactive ATM dimer and prevented ATM-dependent phosphorylation of p53 and checkpoint kinase-2 (CHK2).

An early phosphorylation target of ATM is H2AX, the variant form of the histone H2 (Paull et al. 2000) which leads to the generation of γ-H2AX foci. H2AX is required for retention of the mediator proteins, and hence ATM, as well as additional damage response proteins at the DSB site (Paull et al. 2000). A further target of ATM is the checkpoint kinase, Chk2, which is important for the regulation of cell cycle checkpoint arrest via its subsequent phosphorylation of the Cdc25 phosphatases (Kurz and Lees-Miller 2004). In addition to Chk2, Chk1 is indirectly activated in G2 phase following DSB resection and activation of ataxia-telangiectasia and Rad50-like protein (ATR) (Jazayeri et al. 2006). ATM also phosphorylates p53, which acts primarily in G1 phase cells, regulating the transcription of p21, a Cdk inhibitor, and hence causing G1/S checkpoint arrest (Kurz and Lees-Miller 2004). Collectively, ATM regulates checkpoint arrest at G1/S, intra-S and G2/M phase checkpoints. ATM can also regulate apoptosis via p53. Finally, but of important significance for radiosensitivity, ATM is required for the repair of approximately 15 % of the DSB induced by IR. ATM-dependent DSB repair requires Artemis as well as the mediator proteins and the MRN complex (Riballo et al. 2004). Current evidence suggests that these may represent DSB located within heterochromatin and that ATM signalling via phosphorylation of KRAB-associated protein 1 (Kap1) overcomes a barrier to DSB repair posed by heterochromatin (see below) (Ziv et al, 2006, Goodarzi et al. 2008).

The impact of the DDR pathways on radiation sensitivity: repair versus signalling.

Fibroblast cell lines lacking nonhomologous end-joining (NHEJ) proteins exhibit exquisite radiosensitivity demonstrating the important contribution of DSB repair by NHEJ to radiation survival. One patient harbouring a mutational change in DNA ligase IV who received radiotherapy died from radiation morbidity demonstrating that loss of NHEJ capacity confers clinical radiosensitivity. A-T patients and cell lines also show marked clinical and cellular radiosensitivity, respectively. In contrast, it has been argued that cell cycle checkpoint arrest, although important for maintaining

genomic stability post- irradiation, makes a less significant contribution to survival. However, when cell cycle checkpoint defects are combined with defective DSB repair, as observed in A-T cells, the impact is more than additive, consistent with the notion that cell cycle checkpoint arrest enhances the opportunity for DSB repair (Lobrich and Jeggo 2007). Further, cell cycle checkpoint arrest may be particularly important for maintaining genomic stability in the face of DSB formation.

Radiation sensitivity in an individual likely reflects the sensitivity of the most sensitive tissue, and whether or how the DDR pathways are altered in different tissues is currently unclear. There is evidence that some cell types, including certain stem cells and haematopoeitic cells, have a low threshold for activating apoptosis post-irradation and thus display marked radiosensitivity. Indeed, the low sensitivity for activation of apoptosis appears to be a major contributor to the high radiosensitivity of the haematopoietic system. Additionally, the survival of non-replicating cells is normally markedly greater than that of replicating cells. Indeed, unrepaired DSB appear to be well tolerated in most non-replicating differentiated cells.

What have we learnt during 50 years of radiobiology? The current interpretation of classical findings.

The early studies on radiation biology were remarkably insightful and informative in identifying features that influence the survival response to radiation. Our current understanding of the pathways at the molecular level now allows some of these historical findings to be interpreted mechanistically.

The careful, early studies on DSB repair exposed the existence of a fast and slow repair component. Recent studies have now provided evidence that the slow component of DSB repair represents the repair of those DSB located within heterochromatin (Goodarzi et al. 2008). Further, the early DSB repair studies also revealed that the complexity of the DNA damage influences the repair kinetics. Indeed, early studies on radiosensitivity were important in revealing that highly complex DSB induced by e.g. alpha particle irradiation, although undergoing DSB repair, fail to enhance survival, strongly suggesting that they are not repaired accurately. Our current understanding of how radiation impacts upon the complexity of DNA damage, which is not covered in detail in this article, provides an important explanation for many early studies addressing both radiosensitivity and the rate of DSB repair following irradiation of differing LET values (Bedford and Mitchell

1973). Our current knowledge of factors influencing the kinetics of DSB repair, including heterochromatic status, complexity and cell cycle phase, might allow a reinterpretation of previous data.

Arguably the most striking aspect of the early studies relates to the basis underlying the marked radiosensitivity of A-T cells. As early as 1980, it was appreciated that A-T cells were capable of repairing DSB efficiently yet manifested a subtle DSB repair defect evident from cytogenetic analysis. Further, the fact that they fail to respond appropriately to radiation was evident from the characterisation of their RDS phenotype (Houldsworth and Lavin 1980, Painter and Young 1980, Falck et al. 2002). Thus, these early studies recognised A-T as a complex signalling disorder conferring a subtle DSB repair defect. Later studies described A-T as a cell cycle checkpoint disorder but there was intense unease that this fully explained the magnitude of A-T radiosensitivity. Our current appreciation that ATM lies at the core of a signalling response provides insight into the complex A-T phenotype. Importantly, it is now understood that A-T's radiosensitivity can be largely attributed to a subtle DSB repair defect that, at least in part, is due to its inability to phosphorylate Kap1, a heterochromatic building factor, and hence repair DSB located within heterochromatin (Goodarzi et al. 2008). Further, ATM's ability to regulate a wide range of responses via phosphorylation, which impact upon transcriptional changes, chromatin structure and checkpoint responses, is becoming increasingly understood. NBS was another disorder subjected to intense early study. We now know that Nbs1, the gene defective in NBS, functions in the ATM pathway accounting for the strong overlapping cellular phenotype between A-T and NBS cell lines (Kitagawa et al. 2004). However, NBS patients display very distinct clinical features to A-T patients, and whilst our current understanding of the role of Nbs1 and the MRN complex in replication fork stability, provide some explanation, there is still much to be learnt.

The repair of potentially lethal damage (RPLD or PLDR) represents another classical radiobiology phenomenon which can now be interpreted in the light of our current knowledge. PLDR represents the elevated survival that is observed when cells are held in G0 phase post irradiation (Little 1969). NHEJ deficient mutants and A-T cell lines show little PLDR (Iliakis and Okayasu 1990, Thacker and Stretch 1985). Our current understanding of the fast and slow component of DSB repair has suggested that a period of "holding" prior to triggering cell cycle progression enhances the time

allowed for repair of the slow DSB repair component. The fact that A-T cells were unable to carry out PLDR was a mystery since they appeared to- be repair proficient. The explanation is now provided by the fact that A-T cells have a specific defect in repairing the slow component of DSB. However, it is still surprising that control cells, which manifest a sensitive G1/S checkpoint arrest, benefit from "holding" prior to plating. Further insight into the sensitivity and regulation of the G1/S checkpoint may shed light on this enigma. Another phenomenon is the "sparing effect", which represents the enhanced survival of cells when they are exposed to a defined dose under chronic versus acute exposure conditions. Although all the parameters that impact upon this effect are still unclear, this phenomenon likely represents the fact that cells can efficiently repair DSB when only a few are present whilst misrejoining can occur when multiple DSB are present within a cells (see (Bedford 1991) for a discussion of these effects). This important phenomenon underlies the use of fractionation during radiotherapy. The efficacy of this is currently being re-evaluated and clinical trials are in progress to assess whether there any real benefit is derived.

Future Questions.

Given the dramatic strides we have taken in understanding the basis underlying radiosensitivity in the past fifty years, it is difficult to envisage how much we will learn in the next fifty years. We can, however, consider the important questions to be addressed. Our studies to date have focused predominantly on cells in culture. Questions are now addressing how the microenvironment might influence the response to radiation, including such impacts as intercellular signalling and its influence on the bystander effect and genome instability. Whether stem cells have distinct damage responses also needs to be evaluated, including an understanding of the radiation response of the cancer stem cell. Although we have identified a number of highly radiation sensitive syndromes, we have little understanding of the genetic basis underlying the more subtle distinction in radiation sensitivity between individuals. These may, nonetheless, be highly important in response to radiotherapy

and potentially in responding to acute low dose radiation. Indeed, an urgent question to be addressed is how individuals respond to low doses of radiation and the threats imposed by, for example, CT scanning.

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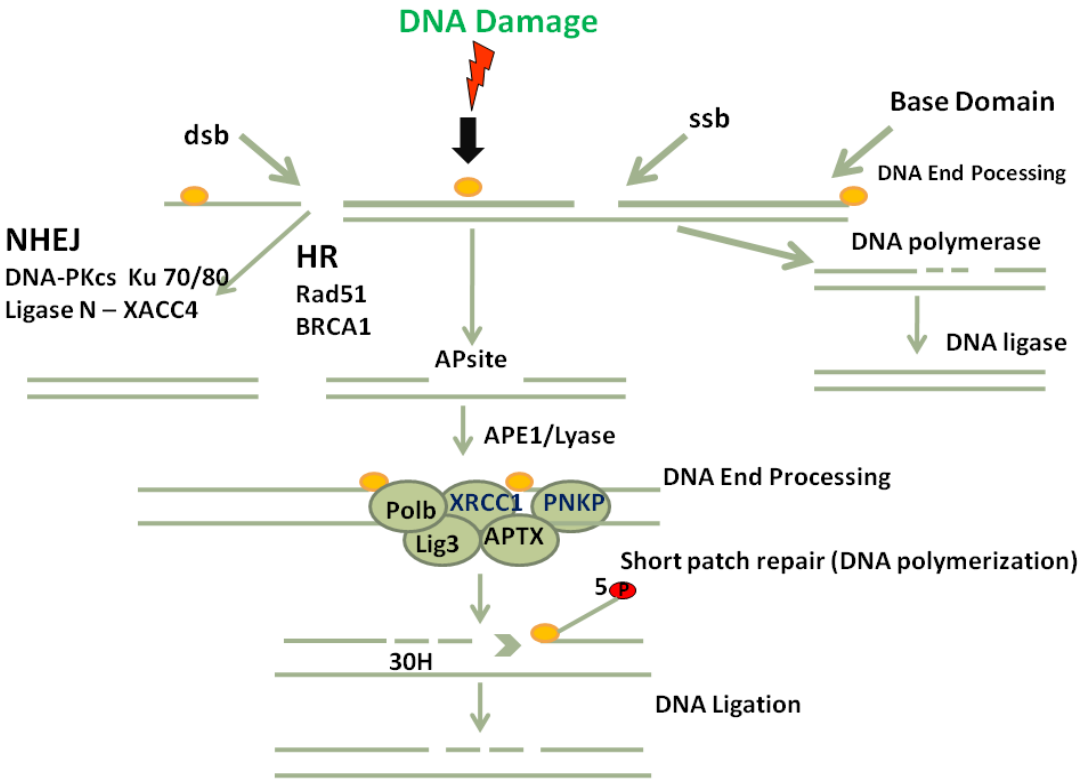
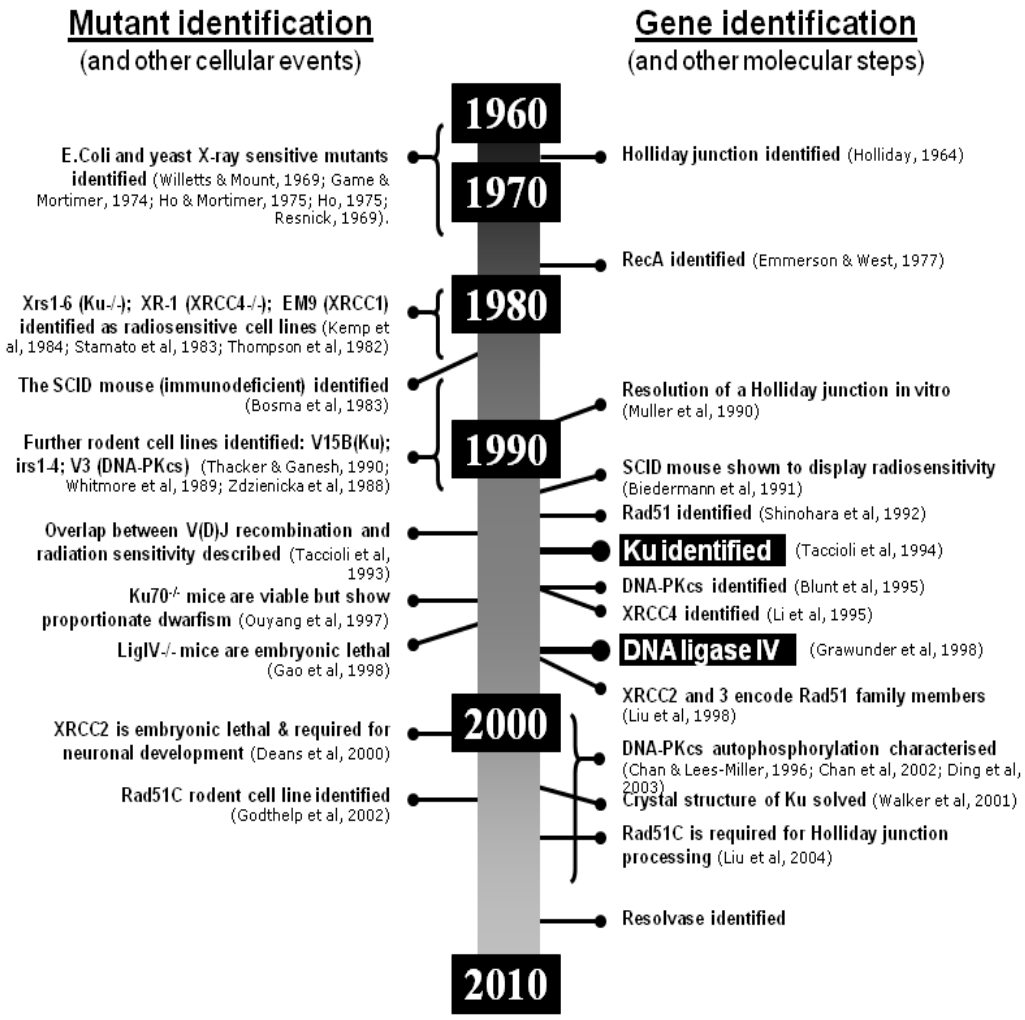


Fig 1. DNA Repair following ionizing radiation exposure. Exposure of cells to ionizing radiation gives rise to DNA DSB, SSB and base modification. DSB are repaired by NHEJ in G1 phase or HR in late S/G2 phases. Direct single strand breaks are repaired by DNA ligation if adjacent 3'0H and 5'phosphate groups are generated. Damage at either end of the break requires end processing and gap filling by DNA polymerase β prior to end joining by DNA ligase 3. Modified bases may give rise to apurinic / apyrmidinic sites which are cleared by anti-human apurinic/apyrimidinic endonuclease (Ape1/lyase). Using XRCC1 as a scaffold a series of SSB repair proteins are recruited for end processing, gap filling and DNA ligation.

Timeline 1


Mutant identification
(and other cellular events)
Gene identification
(and other molecular steps)
1960
1970
1980
1990
2000
2010
E.Coli and yeast X-ray sensitive mutants identified (Willetts & Mount, 1969; Game & Mortimer, 1974; Ho & Mortimer, 1975; Ho, 1975; Resnick, 1969).
Xrs1-6 (Ku-/-); XR-1 (XRCC4-/-); EM9 (XRCC1) identified as radiosensitive cell lines (Kemp et al, 1984; Stamato et al, 1983; Thompson et al, 1982)
The SCID mouse (immunodeficient) identified (Bosma et al, 1983)
Further rodent cell lines identified: V15B(Ku); irs1-4; V3 (DNA-PKcs) (Thacker & Ganesh, 1990; Whitmore et al, 1989; Zdzienicka et al, 1988)
Overlap between V(D)J recombination and radiation sensitivity described (Taccioli et al, 1993)
Ku70-/- mice are viable but show proportionate dwarfism (Ouyang et al, 1997)
LigIV-/- mice are embryonic lethal (Gao et al, 1998)
XRCC2 is embryonic lethal & required for neuronal development (Deans et al, 2000)
Rad51C rodent cell line identified (Godthelp et al, 2002)
Holliday junction identified (Holliday, 1964)
RecA identified (Emmerson & West, 1977)
Resolution of a Holliday junction in vitro (Muller et al, 1990)
SCID mouse shown to display radiosensitivity (Biedermann et al, 1991)
Rad51 identified (Shinohara et al, 1992)
Ku identified (Taccioli et al, 1994)
DNA-PKcs identified (Blunt et al, 1995)
XRCC4 identified (Li et al, 1995)
DNA ligase IV (Grawunder et al, 1998)
XRCC2 and 3 encode Rad51 family members (Liu et al, 1998)
DNA-PKcs autophosphorylation characterised (Chan & Lees-Miller, 1996; Chan et al, 2002; Ding et al, 2003)
Crystal structure of Ku solved (Walker et al, 2001)
Rad51C is required for Holliday junction processing (Liu et al, 2004)
Resolvase identified

Timeline 2

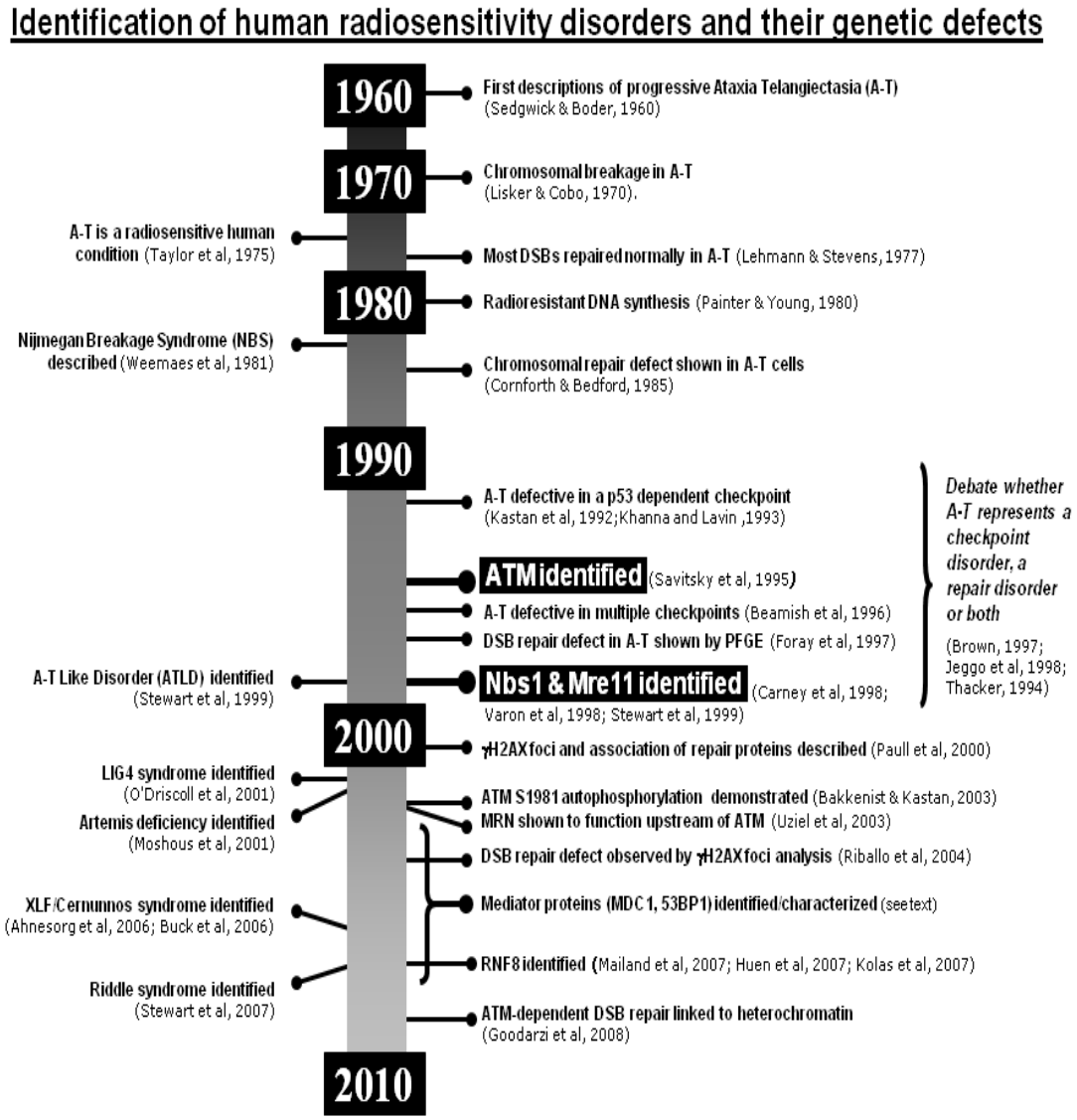

Identification of human radiosensitivity disorders and their genetic defects
1960
First descriptions of progressive Ataxia Telangiectasia (A-T) (Sedgwick & Boder, 1960)
1970
Chromosomal breakage in A-T (Lisker & Cobo, 1970).
A-T is a radiosensitive human condition (Taylor et al, 1975)
Most DSBs repaired normally in A-T (Lehmann & Stevens, 1977)
1980
Radioresistant DNA synthesis (Painter & Young, 1980)
Nijmegan Breakage Syndrome (NBS) described (Weemaes et al, 1981)
Chromosomal repair defect shown in A-T cells (Cornforth & Bedford, 1985)
1990
A-T defective in a p53 dependent checkpoint (Kastan et al, 1992;Khanna and Lavin ,1993)
ATM identified (Savitsky et al, 1995)
A-T defective in multiple checkpoints (Beamish et al, 1996)
DSB repair defect in A-T shown by PFGE (Foray et al, 1997)
A-T Like Disorder (ATLD) identified (Stewart et al, 1999)
Nbs1 & Mre11 identified (Carney et al, 1998; Varon et al, 1998; Stewart et al, 1999)
Debate whether A-T represents a checkpoint disorder, a repair disorder or both (Brown, 1997; Jeggo et al, 1998; Thacker, 1994)
2000
γH2AX foci and association of repair proteins described (Paull et al, 2000)
LIG4 syndrome identified (O'Driscoll et al, 2001)
ATM S1981 autophosphorylation demonstrated (Bakkenist & Kastan, 2003)
Artemis deficiency identified (Moshous et al, 2001)
MRN shown to function upstream of ATM (Uziel et al, 2003)
DSB repair defect observed by γH2AX foci analysis (Riballo et al, 2004)
XLF/Cernunnos syndrome identified (Ahnesorg et al, 2006; Buck et al, 2006)
Mediator proteins (MDC1, 53BP1) identified/characterized (seetext)
RNF8 identified (Mailand et al, 2007; Huen et al, 2007; Kolas et al, 2007)
Riddle syndrome identified (Stewart et al, 2007)
ATM-dependent DSB repair linked to heterochromatin (Goodarzi et al, 2008)
2010

Table 1

Defective DNA damage recognition/repair syndromes

Syndrome	Protein	DNA Damage Recognition/ Repair Defect
Ataxia-Telangiectasia (A-T)	ATM	DNA DSB
A-T like Disorder (ATLD)	Mre11	DNA DSB
Nijmegen Breakage Syndrome (NBS)	Nbs1	DNA DSB
Rad50 defective patient*	Rad50	DNA DSB
Ataxia oculomotor apraxia type 1 (AOA1)	Aprataxin	SSB/ Resolving abortive DNA ligation intermediates
LIG4 syndrome	Ligase 4	DNS DSB
RS-SCID	Artemis	DNA DSB
Ataxia oculomotor apraxia type 2	Senataxin	DNA DSB arising from oxidative stress
Spinocerebellar ataxia with axonal neuropathy (SCAN1)	TDP-1	Top1-induced SSB

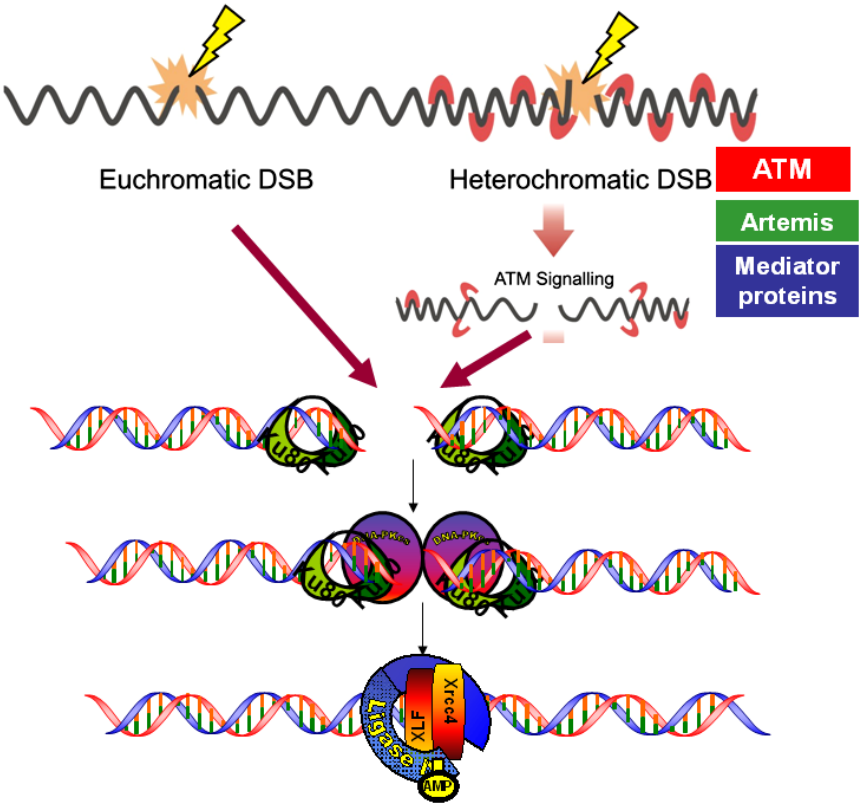


Fig 2. Repair of DNA Double Strand Breaks by NHEJ.
DNA double strand breaks (DSB) located within heterochromatin require additional factors for their repair prior to rejoining by DNA non-homologous end-joining (NHEJ). For heterochromatic DSB, ATM phosphorylation of Kap1 allows localisation chromatin relaxation facilitating repair by NHEJ. This process requires Artemis and the mediator proteins in addition to ATM. The first step of NHEJ is the recruitment of the heterodimeric Ku protein, which encircles the DNA. Ku-bound DNA promotes the recruitment of DNA-PKcs generating the DNA-PK complex. This activates DNA-PK activity. DNA-PK activity regulates the process and also likely promotes end-processing prior to rejoining. The DNA-PK complex may translocate inwards to allow the recruitment of a rejoining complex involving DNA ligase IV, Xrcc4 and Xlf. The stoichiometry of the rejoining is still unclear but one model is the one Ku heterodimer binds each end and recruits a single ligation complex which can undergo two ligation events following *in situ* readenylation. DNA-PK may also have a synapsis function helping to maintain the two ends in proximity.

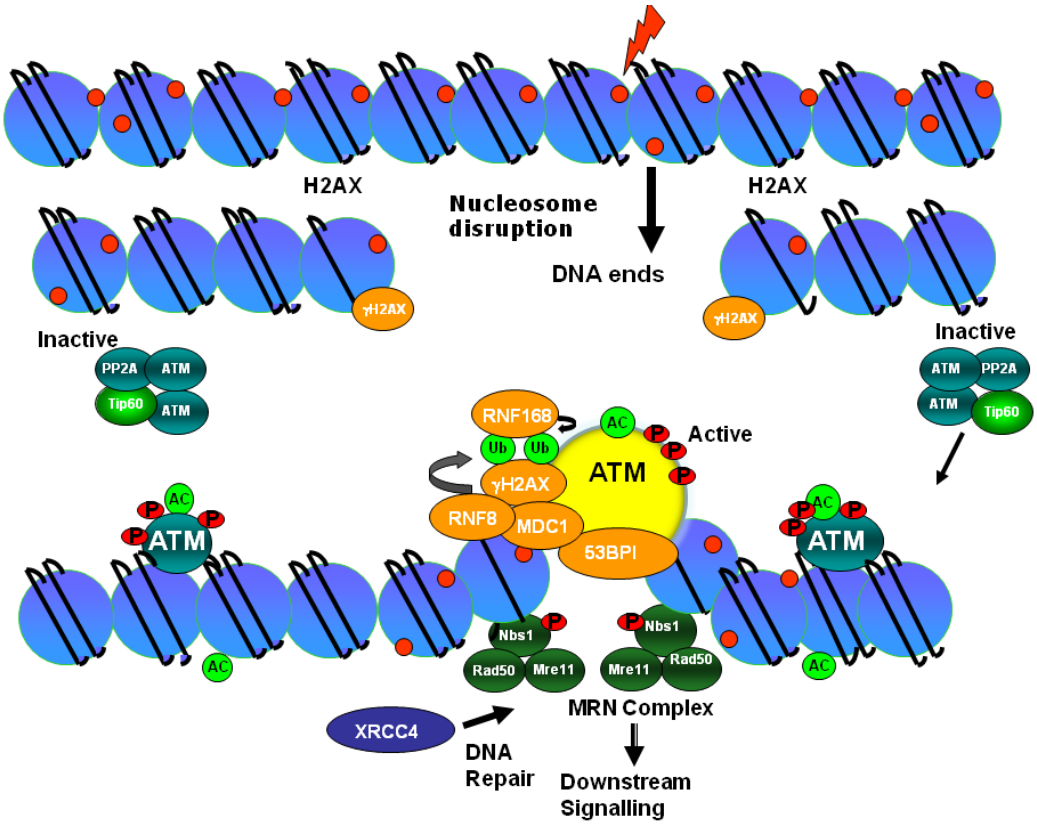


Fig 3. Activation of ATM.
Activation of ataxia-telangiectasia mutated (ATM) is a complex process that involves the relaxation of chromatin as a consequence of a DNA double-strand break (DSB), involves the recruitment of the Mre11-RAd50-Nbs1 (MRN) complex to the break and also the recruitment of ATM to regions that flank the break. In these flanking regions, ATM is partially activated and phosphorylates p53 and possibly other substrates. ATM is then recruited to the site of the break by the MRN complex and phosphorylates members of the complex and other downstream substrates. The MRN complex is not essential for this signalling, but in the presence of hypomorphic mutations in members of the complex, signalling is delayed and/or reduced. An inactive ATM dimer is monomerised in response to DNA DSB, and concomitantly transphosphorylation (autophosphorylation) occurs on at least three sites: Ser367, Ser1893 and Ser1981. Phosphatases also regulate ATM, presumably to ensure that it is not inappropriately activated by autophosphorylation. In the presence of DNA DSB, PP2A dissociates from ATM and loses its activity, therefore minimising the risk of competition between phosphorylation and phosphatise activities. The phosphatise WIP1 is also capable of removing phosphates from all three autophosphorylation sites. PP5 removes phosphates from ATM as part of the process of activation. Acetylation (Ac) also contributes to the process of activation. The acetyltransferase TIP60 is constitutively associated with ATM, and in the presence of a DNA DSB it becomes activated and acetylates ATM as Lys 3016 within the C-terminal FATC domain. Lys3016 mutants fail to upregulate ATM activity after DNA damage,

prevent monomerization of ATM and inhibit downstream signalling through p53 and checkpoint kinase-2 (CHK2). XRCC4, the requisite cofactor of DNA ligase 4 and non-homologous end joining (NHEJ) is detected at the break site after ATM recruitment. MDC1, mediator of DNA-damage checkpoint protein-1 plays a central role in protein assembly at the DSB. It binds γH2AXm, NbS1 and RNF8 a ubiquitin ligase that catalyzes ubiquitylation of γH2AXm and possibly other histone proteins. This helps to enhance the assembly of the DNA damage response proteins. 53BPI, p53 binding protein acts downstream of these events binding to modified histone proteins. Finally the newly described RNF168 also a ubiquitin ligase targets histone H2A and γH2AXm amplifies ubiquitylation to stabilize the DNA damage response protein complex (Doil et al 2009; Stewart et al 2009).