DNA Damage Response

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Cold Spring Harbor Perspectives in Biology
www.cshperspectives.org

Structural changes to DNA severely affect its functions, such as replication and transcription, and play a major role in age-related diseases and cancer. A complicated and entangled network of DNA damage response (DDR) mechanisms, including multiple DNA repair pathways, damage tolerance processes, and cell-cycle checkpoints safeguard genomic integrity. Like transcription and replication, DDR is a chromatin-associated process that is generally tightly controlled in time and space. As DNA damage can occur at any time on any genomic location, a specialized spatio-temporal orchestration of this defense apparatus is required.

Selective advantage by random mutations in the genetic material has driven evolution of terrestrial life. Despite this obvious advantage for biological diversity, genome instability has in most cases adverse effects on organismal life. Preservation of genomic integrity is a prerequisite for proper cell function and faithful transmission of the genome to progeny. However, environmental factors and the chemical properties of DNA do not guarantee lifelong stability and proper functioning of the genome.

Genomic insults arise from side effects of DNA metabolizing processes, such as replication errors, uncontrolled recombination, off-target mutation induction by somatic hypermutation during antigen production, and inaccurate VDJ recombination (Liu and Schatz 2009; Mahaney et al. 2009). The biggest genomic burden is, however, induced by processes that directly damage DNA. DNA lesions are derived from three main sources (Lindahl 1993; Friedberg et al. 2006): environmental agents such as ultraviolet light, ionizing radiation, and numerous genotoxic chemicals; reactive oxygen species (ROS) generated by respiration and lipid peroxidation; and spontaneous hydrolysis of nucleotide residues, inducing abasic sites and deamination of C, A, G, or 5methyl-C. It is estimated that each cell is confronted with approximately 10^4–10^5 lesions per day, indicating that clearance of genomic injuries constitutes

Editors: Tom Misteli and David L. Spector
Additional Perspectives on The Nucleus available at www.cshperspectives.org

 doi: 10.1101/cshperspect.a000745
Cite this article as *Cold Spring Harb Perspect Biol* 2011;3:a000745

a demanding task to maintain proper genome function.

Essential genome processes, such as transcription and replication, are severely affected by DNA lesions. Replication over damaged DNA induces mutations, which may initiate and propagate carcinogenesis. Acute effects arise when lesions block transcription causing cellular senescence or apoptosis, resulting in damage-induced accelerated aging (Mitchell et al. 2003; Akbari and Krokan 2008; Sinclair and Oberdoerffer 2009).

THE DNA DAMAGE RESPONSE

To deal with the fundamental problem of genomic erosion, a sophisticated network of DNA damage-response (DDR) systems has evolved. These include a set of DNA repair mechanisms, damage tolerance processes, and cell-cycle checkpoint pathways. The biological significance of a functional DDR for human health is clearly illustrated by the severe consequences of inherited defects in DDR factors resulting in various diseases, including immune deficiency, neurological degeneration, premature aging, and severe cancer susceptibility (Hoeijmakers 2001; Hoeijmakers 2009).

DNA Repair Mechanisms

The heart of the cellular defense against DNA injuries is formed by a variety of DNA repair mechanisms (Hoeijmakers 2001; Hoeijmakers 2009), each with their own damage specificity (Table 1). Together, they are able to remove the vast majority of injuries from the genome. The simplest solution that emerged in evolution is the direct reversal of lesions by specialized activities, such as photolyases that selectively

Table 1. Induction of DNA lesions and corresponding repair pathway.

Lesion	Cause	Repair process(es)
CPD, 6-4PP[1]	Sunlight	NER
Bulky adducts[2]	Food, cigarette smoke	NER
Intrastrand crosslinks	Chemotherapy (e.g., Cis-Pt)	NER
8-oxo-dG[3]	ROS[4], respiration	BER
Thymineglycol[3]	ROS[4], respiration	BER
N^7-Alkyl-dG, N^3-Alkyl-dA	Food, pollutants	BER
O^6-Alkyl-dG	Food, pollutants	DR[5], BER?
5-methyl-dC	DNMT[6]	BER/AID-BER/NER?[7]
Uracil, (Hypo)Xanthine	Spontaneous deamination	BER
Abasic site	Spontaneous hydrolysis	BER
Single-strand breaks	Ionizing radiation, ROS	Ligation, BER
Double-strand breaks	Ionizing radiation, ROS, VDJ-rec	HR, NHEJ
Tyrosyl-3′DNA[8]	Topo-I inhibition, ROS	SSBR
Mismatches	Replication errors	MMR
Small insertion/deletions	Replication slippage	MMR
Interstrand crosslinks	Chemotherapy	ICLR/ HR?

1. CPD: cyclobutane pyrimidine dimer; 6-4 PP: 6-4 pyrimidine-pyrimidone photo-product.

2. A large group of chemicals conjugated to bases that cause DNA helix destabilization such as: Benzo[α]pyrene (a polycylic aromatic hydrocarbon); Aflatoxins (present in fungal food contaminations); and Nitrosamines (tobacco smoke).

3. A large group of different oxidation products affecting either the base or the phosphate-sugar backbone of which 8-oxo-dG is the most abundant.

4. ROS: reactive oxygen species, produced as side-product of respiration/metabolism and ionizing radiation.

5. DR: direct reversal, involving the suicide enzyme MGMT.

6. DNMT: DNA methyltransferase, functions in epigenetic gene-expression control (e.g., at CpG islands).

7. The mechanism of 5-Me-C repair/conversion is a matter of debate. Recently, a GADD45a-dependent NER reaction was suggested (Barreto et al. 2007).

8. Proteolytic degradation of conjugated Topo-I to 3′DNA termini creates tyrosyl-3′DNA bonds, resolved by TDP1 (El-Khamisy et al. 2009).

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reverse UV-induced DNA damage (Weber 2005) and the suicide enzyme O^6-methylguanine transferase (MGMT) that transfers the methyl group from DNA by covalently coupling it to an internal cysteine residue of MGMT, thereby destroying the enzymatic activity (Friedberg et al. 2006). Photolyases are not conserved into the mammalian branch and mammals have to rely on a more complex mechanism to remove UV injuries: nucleotide excision repair (NER) (see below).

Base Excision Repair (BER)

Bases with small chemical alterations that do not strongly disturb the DNA double-helix structure are substrates for Base Excision Repair (BER) (Almeida and Sobol 2007; Hegde et al. 2008) (Table 1). These damages, or group of lesions, are targeted by lesion-specific DNA glycosylases that both recognize and remove the damaged base from the sugar-phosphate backbone. The resulting abasic (AP) site is incised by AP-endonucleases and the single nucleotide gap is filled-in by the BER-specific DNA polymerase β and finally sealed by the XRCC1/Ligase III complex. Single strand breaks (SSBs) are repaired by a specialized BER mechanism, designated single-strand break repair (SSBR). The abundant nuclear protein Poly-ADP-Ribose-Polymerase (PARP) is rapidly activated by SSBs and causes auto-poly-ADP-ribosylation, which recruits the XRCC1/ligase III complex as well as end-processing enzymes such as aprataxin (Gueven et al. 2004) and TDP1 (tyrosyl-DNA-phosphodiesterase) to create ligatable DNA ends (Caldecott 2007; El-Khamisy et al. 2009).

Nucleotide Excision Repair (NER)

NER removes a broad spectrum of single-strand lesions that cause local helix-destabilization (Table 1). NER is a complex multi-step process, involving the concerted action of at least 25 different polypeptides (Hoeijmakers 1993; Gillet and Scharer 2006) (Fig. 1). Two different modes of damage detection are operational in NER: transcription-coupled NER (TC-NER), which efficiently removes transcription-stalling lesions and allows quick resumption of transcription (Bohr et al. 1986; Hanawalt 1994), and global genome NER (GG-NER), which localizes lesions anywhere in the genome. In TC-NER, damage sensing is performed by the stalled RNA polymerase, and the Cockayne syndrome factors A and B (CSA and CSB) play essential roles in TC-NER complex assembly (Fousteri et al. 2006; Fousteri and Mullenders 2008). Lesion discrimination in GG-NER is executed by the concerted action of two complexes: XPC/hHR23B (Masutani et al. 1994) and UV-DDB (DDB1 and DDB2/XPE) (Chu and Chang 1988; Keeney et al. 1994; Sugasawa et al. 2009). The subsequent steps of TC-NER and GG-NER converge into a common mechanism in which first the NER/basal transcription factor TFIIH (Egly 2001) is recruited (Yokoi et al. 2000; Volker et al. 2001). The bi-directional helicase of TFIIH opens the damaged DNA segment over a stretch of approximately 30 nucleotides (Sugasawa et al. 2009). The unwound DNA is stabilized by XPA and RPA (Replication Protein A) that also orient (de Laat et al. 1998) the two structure-specific endonucleases XPG (O'Donovan et al. 1994) and the ERCC1-XPF complex (Sijbers et al. 1996), which respectively incise the damaged strand 3′ and 5′ with respect to the lesion. The resulting 25–30 nucleotide single strand gap is filled in by normal DNA replication proteins, including replication factor C (RFC), PCNA, RPA, and the DNA polymerases δ, ε, or κ (Ogi et al. 2010). Finally, the gap is sealed by DNA ligases I or III, dependent on the proliferation status of the cell (Moser et al. 2007) (Fig. 1).

DNA Double-Strand Break Repair (DSBR)

Lesions that are substrates for NER and BER are located in one of the strands of DNA and are removed in a "cut-and-patch"-mechanism. In both cases, the undamaged complementary strand serves as a faithful template for the repair of the damaged strand. Some damaging agents, however, affect both strands, such as ionizing radiation that induces DNA double-strand breaks (DSBs) and agents that produce inter-strand cross-links (ISCLs) (Table 1). These lesions are extremely cytotoxic because they are more

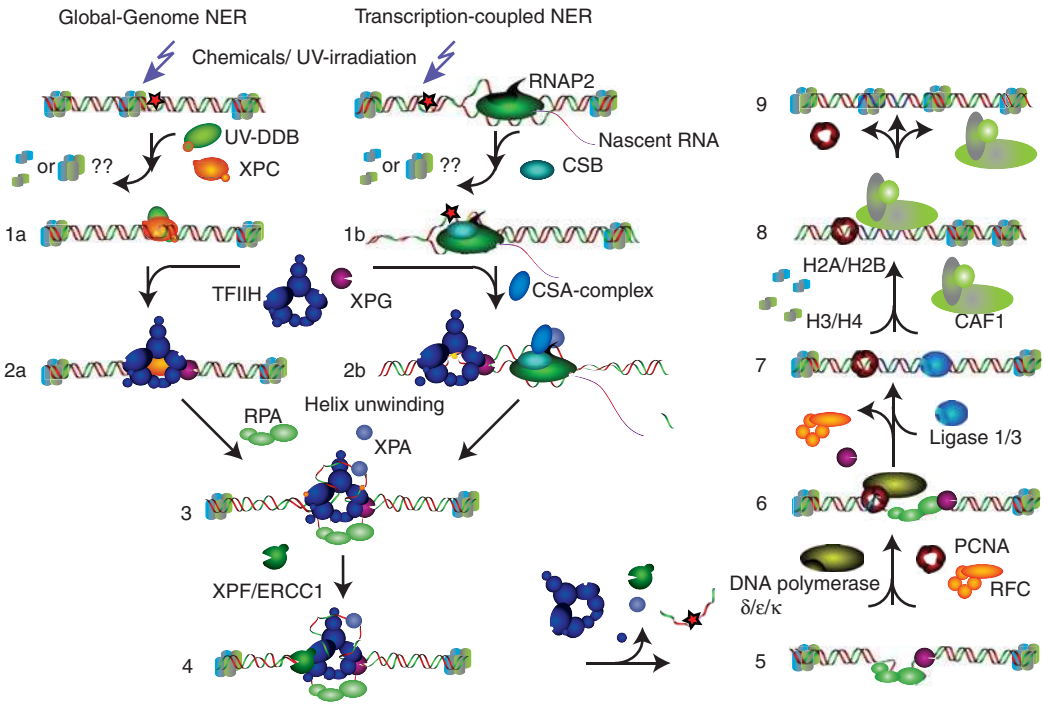

Figure 1. Molecular mechanism of nucleotide excision repair (NER). Bulky DNA lesions (e.g., UV-induced photo-products and chemical conjugates to nucleotides) that destabilize the DNA double-helix are targeted by NER. Damage recognition is performed by transcription-coupled NER (TC-NER) and Global Genome NER (GG-NER). It is suggested that prior to damage recognition, chromatin has to be modified. Lesions in the transcribed strand of active genes are detected by the elongating RNA polymerase II (RNAP2) and stabilize the interaction with CSB (step 1b). Within GG-NER, lesions are recognized by the UV-DDB and the XPC complexes (step 1a). These intermediates load transcription factor TFIIH together with the endonuclease XPG (steps 2a and 2b). In TC-NER, CSA is also recruited to modify and reposition lesion-stalled RNAP2 (step 2b). After the two modes of lesion detection, the two processes merge into a common pathway of NER factor assembly by recruiting XPA and replication protein A (RPA) (step 3). This NER-intermediate loads and properly orients the structure-specific endonuclease ERCC1/XPF complex (step 4). After dual incision by XPG (3′ from the lesion) and ERCC1/XPF (5′ from the lesion), a single-strand of 25–29 nucleotides is created (step 5). XPG is likely involved in recruiting the sliding clamp PCNA, which is loaded by RFC and forms the platform for the gap-filling DNA polymerases δ, ε, or κ (step 6). Each of these polymerases has been found to participate in NER-dependent gap-filling. PCNA or RFC are likely also involved in recruiting the ligases (i.e., Ligase I and Ligase III/XRCC1, depending on the proliferation capacity of the cell) to seal the nick (step 7). PCNA also plays a role in attracting the histone-chaperone CAF1 (step 8) to restore the chromatin structure after repair (stage 9).

difficult to repair as the cell cannot rely on simply copying the information from the undamaged strand. Two distinct pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ), repair DSBs (Cahill et al. 2006; Wyman and Kanaar 2006; Helleday et al. 2007). The division of tasks between these repair mechanisms is mainly determined by the phase of the cell cycle. As HR requires a homologous sister chromatid, it acts exclusively in S- and G2-phase. In contrast, post-mitotic cells and cycling cells in G1 phase have to seal DSBs by NHEJ.

Within NHEJ, breaks are quickly recognized by the Ku70/Ku80 hetero-dimer that activates the PI3-kinase DNA-PK and sets the scene for subsequent recruitment of the Artemis nuclease and the MRE11/Rad50/NBS1 (MRN) protein

complex. These proteins are involved in DNA end-processing, preceding ligation performed by the XRCC4/LigaseIV complex (Weterings and van Gent 2004; Burma et al. 2006; van Gent and van der Burg 2007). During DNA end-processing, loss or changes of a few nucleotides may occur. For this reason, NHEJ, although it very rapidly seals DSBs, is an error-prone repair process.

However, when cells do have a homologous template, as in the S- and G2-phase of the cycle, DSBs can be repaired by HR. Homologous recombination is initiated by binding of the MRN complex to a DSB and functions to hold the broken pieces together (de Jager et al. 2001) and provides the structural bases for the CtIP nuclease. The MRN-CtIP complex catalyzes end resection at the break in concert with exonuclease I (EXO1) (Limbo et al. 2007; Sartori et al. 2007; Takeda et al. 2007). Subsequently, RPA binds to the newly created single-strand region and through a complicated handoff mechanism, the RPA-filament is exchanged into a RAD51 nucleo-protein filament. This RAD51-filament is crucial for strand invasion into the homologous sister, creating a temporarily triplex-DNA structure in which strand exchange occurs (Wyman et al. 2004). The molecular details of these complex transactions are as yet enigmatic, although genetic studies have revealed a whole list of proteins that play an important role in these transactions (Lisby and Rothstein 2009). The biggest challenge within HR-driven DSBR is, however, the question on how homologous regions are identified within the complex nuclear environment.

DNA Damage Tolerance

Persisting lesions not removed by any of the repair mechanism will interfere with DNA replication. Lesion-stalled replication forks can lead to highly cytotoxic DSBs and require a prompt response. At least two DNA damage tolerance mechanisms have evolved: translesion synthesis (TLS) and recombination-dependent daughter-strand gap repair (DSGR) (Scully et al. 2000; Li et al. 2002). These processes do not actually remove lesions, but serve as a temporary solution to overcome stalled DNA replication machines. Upon lesion-induced replication blockage, the regular high-fidelity DNA polymerases (polδ/ε or α) are temporarily exchanged with translesion polymerase (pol ζ-κ) (Friedberg et al. 2005; Lehmann 2006) to synthesize across the lesion. Although TLS can circumvent lesion-induced replication stalling, the reduced fidelity of the alternative polymerases causes generally enhanced mutagenesis.

Damage Signaling

To create an extended time window to allow completion of lesion removal prior to replication or cell division, damage sensing is linked to an intricate signal transduction cascade that induces cell cycle arrest (Bartek et al. 2007; Callegari and Kelly 2007). Depending on the nature of the DNA injury and the phase of the cell cycle in which the lesion is encountered, the cell cycle can be arrested at the G1/S transition, within the S-phase, or at the G2/M transition (Zhou and Elledge 2000). Alternatively, when too many injuries are encountered, apoptosis is triggered in order to protect the organism from potentially harmful cells (Bernstein et al. 2002). The phosphatidylinositol 3-kinase (PI3) ATM (Ataxia Telangiectasia mutated) is directly recruited and activated by the DSB-recognizing protein complex MRN. This initiating kinase transduces phosphorylation to a high number of adapter/transducer proteins, carrying the ATM-consensus sequence (Matsuoka et al. 2007). Finally, downstream effector kinases, such as the checkpoint kinase Chk2, are activated (Falck et al. 2002). Bulky lesions cause replication collapse that induces single-strand DNA by the retraction of the replication fork. RPA binds to ssDNA and recruits ATR (ATM-related) via its association with ATRIP (ATR interacting protein) and activates the checkpoint protein Chk1 (Tibbetts et al. 2000; Chen and Sanchez 2004). RPA covered ssDNA also triggers the Rad17-dependent loading of the RAD9-HUS1-RAD1 (9-1-1 complex), which is an important transducer of checkpoint activation upon DNA damage (Smits et al. 2010). A third PI3 kinase, DNA-PK (DNA-dependent

protein kinase, composed of its catalytic subunit DNA-PKcs and a regulatory Ku70/80 heterodimer), is also activated by IR-induced DSBs. DNA-PKcs is essential for NHEJ in higher eukaryotes (Burma et al. 2006) and additionally functions in telomere maintenance and induction of apoptosis (Burma and Chen 2004). In total, a complicated set of different emergency strategies are called into action when genomic insults are encountered. Although many of the individual players are identified and the downstream signaling cascades have been dissected, their respective interactions and communication is far from resolved.

Intertwined DNA-transacting Processes

The different repair processes are generally considered separate entities. However, in recent years, it has become clear that most of these DDR processes are part of an intricate network with significant overlap, often sharing specific essential components.

Several DDR factors appeared to act in diverse DNA maintenance systems. One typical example is the hetero-dimeric ERCC1/XPF complex. This structure-specific endonuclease was originally identified as the nuclease that incises 5′ of the DNA lesions within NER (Westerveld et al. 1984; Sijbers et al. 1996). Further analysis revealed additional functions for this complex in HR (Adair et al. 2000; Niedernhofer et al. 2001), interstrand cross-link repair (De Silva et al. 2000; Niedernhofer et al. 2004), and telomere maintenance (Zhu et al. 2003).

Besides overlap between distinct repair processes, DDR is also linked to other essential DNA transacting mechanisms, such as transcription and replication. A prime example of such a link is the tight connection between NER and transcription, illustrated by the existence of a specialized transcription-coupled NER pathway (TC-NER) (Bohr et al. 1985; Fousteri and Mullenders 2008). The chromatin remodeling protein Cockayne Syndrome B (CSB) (Citterio et al. 2000) is essential for TC-NER and is implicated in transcription elongation (van den Boom et al. 2004). Moreover, the basal RNA polymerase II transcription factor TFIIH is also a pivotal factor in NER (Fig. 1) (Schaeffer et al. 1993; Drapkin et al. 1994; Egly 2001; Hoogstraten et al. 2002). Finally, the essential replication factor A (RPA) is implicated in basically all DDR mechanisms, including NER, HR, and damage signaling.

Different strategies are used to control the multi-functionality of these factors: (1) distinct spatial organization, (2) incorporation into diverse functional complexes, and/or (3) dynamic sharing of these components. Regulation of pleiotropic functionality of proteins is commonly achieved by distinct posttranslational modifications (PTMs). Within DDR, different PTMs were identified, ranging from phosphorylation, acetylation, methylation, neddylation, mono- and poly-ubiquitylation, and sumoylation to poly-ADP-ribosylation (Harper and Elledge 2007; Huen and Chen 2008). One of the most common PTMs involved in DDR is differential phosphorylation, mainly driven by the ATM, ATR, and DNA-PKcs kinases (Matsuoka et al. 2007). Recent research indicates also that differential ubiquitination plays an important role in DDR regulation (Bergink et al. 2007; Harper and Elledge 2007; Reed and Gillette 2007; Huen and Chen 2008; Alpi and Patel 2009; Panier and Durocher 2009).

STRUCTURAL AND FUNCTIONAL ORGANIZATION OF DDR

Chromatin and DDR

The nucleus is highly structured and functionally compartmentalized in part due to areas of various degrees of chromatin compaction, creating possible obstacles for DDR factor accessibility. Decompaction and subsequent restoration of the starting chromatin structure in conjunction with DDR thus creates another level of complexity in genome maintenance regulation. Chromatin-associated processes such as transcription, replication, and DNA repair are regulated by a complex set of structural changes in chromatin (Groth et al. 2007; Li et al. 2007). Control of chromatin functions and its compaction occurs by at least four known processes: (1) active ATP-consuming

remodeling machines of the SWI/SNF-superfamily of DNA-dependent ATPases (Neves-Costa and Varga-Weisz 2006; Saha et al. 2006) that slide or physically evict core histones or entire nucleosomes from active sites; (2) incorporation of diverse histone variants by histone chaperones (Loyola and Almouzni 2007; Altaf et al. 2009); (3) differential binding of abundant non-core histone proteins, such as the linker histone H1, the family of high mobility group proteins (HMG), or different isoforms of the hetero-chromatin protein 1 (HP1); and (4) covalent modifications or PTMs of the core histones, such as acetylation, methylation, phosphorylation, and ubiquitylation (He and Lehming 2003). Accordingly, recently a large number of chromatin modifications and remodeling events were shown to be linked to DDR (Groth et al. 2007; Dinant et al. 2008; Misteli and Soutoglou 2009; Nag and Smerdon 2009; van Attikum and Gasser 2009).

Access, Repair, and Restore

Despite increasing knowledge of the role of chromatin in DDR, a general mode of action or detailed mechanistic insight is lacking. Already in 1991 a hypothetical three step model for DNA repair in chromatin was postulated (Smerdon 1991), the so-called "ARR-model," for Access, Repair, and Restore, based on analogy to transcription regulation in chromatin. In this model, it was postulated that chromatin remodeling would be required to provide "access" of damage-recognition factors to initiate "DNA repair" and, when the job is finished, "restoration" of the chromatin structure. Since then, clear indications have been found that the H3/H4 chaperone CAF1, likely in conjunction with Asf1 (anti-silencing function 1), is implicated in restoring chromatin after NER (Green and Almouzni 2002; Mello et al. 2002; Polo et al. 2006). Additional studies implicated Asf1 and FACT (facilitating transcription factor) (Chen et al. 2008; Heo et al. 2008) in histone exchange near DSBs. However, less direct evidence for chromatin remodeling factors for the first step (access) was found. Although several chromatin remodelers, such as INO80 (Downs et al. 2000; van Attikum et al. 2004), facilitate DDR factor recruitment by moving histones away from the break, they appear to act after the initial damage recognition. In addition, chromatin modifications in yeast occur after UV-irradiation by Gcn5-induced H3 acetylation and are dependent on the Swi/Snf DNA translocase Rad16/Rad7/Abf1. Despite the more open chromatin structure by hyperacetylation, it is not directly clear whether this modification facilitates the recruitment of the Rad4 (yeast ortholog of XPC) DNA damage recognition protein (Waters et al. 2009).

Phosphorylation of the Histone H2A Variant H2AX

The most prominent DDR-associated covalent histone modification is the phosphorylation of the histone H2A-variant H2AX in response to DNA damage by the checkpoint kinases ATM, ATR, and DNA-PKcs (Rogakou et al. 1998; O'Driscoll et al. 2003; Falck et al. 2005). H2AX is incorporated into approximately 5%–25% of histone octamers, although its phosphorylation (γH2AX) is constrained to microscopically discernable structures, the ionizing irradiation-induced foci (IRIF) (Fig. 2). Phosphorylation of H2AX is a relatively early event after damage, immediately following MRN binding and ATM activation. These γH2AX foci co-localize with most of the DSB-associated DDR factors (see below) and are thought to serve as docking sites for recruiting and retaining DNA repair and signaling factors to DSBs. γ-H2AX spreads over several megabases around DSBs and appears condensed into IRIFs (Rogakou et al. 1999), suggesting a dominant structural role in DSB-DDR. Surprisingly, however, although mice lacking H2AX are radiation-sensitive and exhibit several features associated with defective DDR, they are only partially defective in DSB repair and are not fully compromised in checkpoint activation (Celeste et al. 2002). This notion argues, contrary to expectation, that this impressive structural organization into large molecular assemblies only makes the DDR process more efficient but is not essential for DDR.

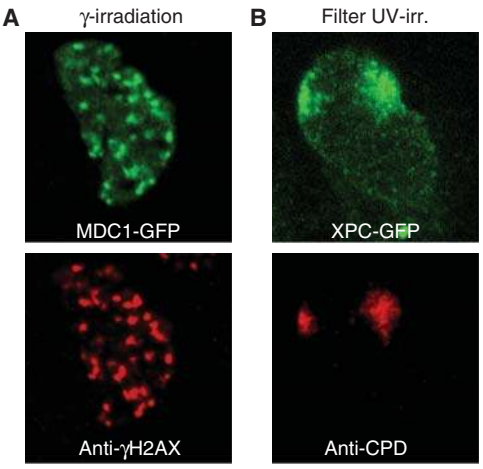

Figure 2. Localization of DSBR and NER factors. Typical examples of subnuclear distributions of MDC1, an important factor involved in an early step of DNA double-strand break repair via homologous recombination (*A*) and XPC, one of the damage-recognizing proteins in NER (*B*). (*A*) GFP signal (upper panel) derived from MDC1-GFP stably expressed in U2OS cells, 2 hours after γ-irradiation and fixed with paraformaldehyde, showing the accumulation of MDC1 in typical ionizing-radiation induced foci (IRIF), counter stained with anti-γ-H2AX antibodies (lower panel). (*B*) NER factors do not accumulate in nuclear foci after DNA damage induction. To allow live cell analysis of NER factor kinetics, local UV-damage infliction through a micro-porous filter is performed (see Fig. 3B,C). XPC-GFP expressed in human fibroblasts (upper panel) accumulates at local UV-damaged sub-nuclear areas as recognized by anti-CPD (the major UV-induced DNA lesion) antibodies (lower panel).

UV-light also induces γ-H2AX, although in this case the modification is homogenously distributed throughout the nucleus, with the exception of cells in S-phase (O'Driscoll et al. 2003; Hanasoge and Ljungman 2007; Stiff et al. 2008). H2AX phosphorylation upon UV in non-S-phase cells depends on ATR and active processing of the lesion by the NER machinery (O'Driscoll et al. 2003; Marti et al. 2006), suggesting that NER-intermediates trigger this response. The notion that γ-H2AX formation occurs in response to NER and that NER is proficient in H2AX-deficient cells, suggests that this modification mainly plays a role in checkpoint activation during UV lesion repair.

DDR in Higher Order Chromatin Structure

Within mammalian cells, chromatin comes in different flavors, classified into compacted and often transcription-silent heterochromatin and the more open transcriptionally active euchromatin. Chromatin compaction is, however, dynamic and varies within different nuclear areas, throughout the different phases of the cell cycle and between different somatic cell types. Obviously, higher order packaging beyond the basic nucleosomal level will raise further accessibility problems. The versatile NER pathway removes lesions throughout the genome, although repair of photo-lesions in nucleosomal templates is repaired slower in vitro compared to naked DNA (Nag and Smerdon 2009). Evidence for an inhibitory effect of higher order chromatin structure on NER in vivo is restricted to yeast studies. Yeast mutants for the histone acetyltransferase Gcn5, which affect chromatin packaging of specific loci, exhibit significant reduced UV-lesion repair on these silenced loci (Waters et al. 2009).

Evidence for reduced repair kinetics of DSBs in compact heterochromatin was recently provided (Goodarzi et al. 2008). Repair in these areas requires the release of the heterochromatin-associated and transcriptional co-repressor protein KAP1 (KAP-associated protein 1) from these compact regions. This release appeared to depend on phosphorylation by ATM, providing evidence for a direct role of this important DSB PI3-kinase in chromatin decompaction to support DDR. ATM-dependent repair in heterochromatin can also be alleviated by depletion of the heterochromatic factor HDAC1/2 and simultaneous depletion of the three isoforms (α, β, and γ) of HP1 (heterochromatin protein 1). The implication of this protein in DDR has recently led to controversy in the DDR literature (Ball and Yokomori 2009), as one group describes a DNA damage-induced release of phosphorylated HP1 from H3K9me (Histon H3, lysine 9 methylation) (Ayoub et al. 2008), while Luijsterburg and co-workers report recruitment of HP1 to damaged sites (Luijsterburg et al. 2009). Ayoub showed an initial release of phosphorylated HP1 followed by a subsequent

spreading to neighboring chromatin. This discrepancy is difficult to explain, besides possible difference in DNA damage induction and slight differences in kinetic measurements. A possible explanation for the apparent initial disappearance prior to the observed accumulation at damaged sites might be the sudden highly localized damage induction. The extreme high local concentration of light might in addition to DNA damage also induce chromatin-protein damage. The next wave of HP1 accumulation reflects then the more physiological response to DNA damage induction. Despite the conflicting data and interpretations, it is clear that compaction of chromatin and HP1 play an as yet not entirely understood role in DDR, as disruption of HP1 orthologs in *C. elegans* induces a diverse spectrum of DNA damage sensitivities (Ball and Yokomori 2009; Luijsterburg et al. 2009).

Tools to Analyze DDR in Living Cells

The dynamic interactions with chromatin and the multiple engagements of DDR factors indicate that analysis of each of the separate processes in vitro is not sufficient to fully uncover mechanistic details, and demands cellular biological approaches. The possibility to genetically tag proteins with the autofluorescent protein GFP has revolutionized cell biology (Tsien and Miyawaki 1998). The simultaneous technological advances in microscopy and development of quantitative fluorescent measurements and sophisticated photo-bleaching procedures (White and Stelzer 1999; Houtsmuller and Vermeulen 2001; Lippincott-Schwartz et al. 2001) have provided spectacular new insights into the regulation and dynamic organization of chromatin-associated processes (Houtsmuller et al. 1999; Phair and Misteli 2000). In particular, the development of several systems to locally introduce DNA damage or immobilize DDR factors in cultured living cells has been beneficial (Figs. 2 and 3): (1) irradiation through a filter or mask that partly shield the cells (Nelms et al. 1998; Katsumi et al. 2001; Mone et al. 2001); (2) micro-beam laser irradiation, with or without photo-sensitizers, at sub-nuclear areas (Cremer et al. 1980; Tashiro et al. 2000; Lukas et al. 2003; Meldrum et al. 2003; Lan et al. 2004; Dinant et al. 2007); (3) guided α-particle and heavy iron radiation (Jakob et al. 2003; Aten et al. 2004; Hauptner et al. 2004); (4) integration of rare-cutting endonucleases (Lisby et al. 2004; Rodrigue et al. 2006; Soutoglou et al. 2007); and (5) DDR protein tethering to specific integrated amplified sequences (Soutoglou and Misteli 2008).

Organization of DNA Double-Strand Break Response

In situ studies revealed that next to γ-H2AX, a high number of DDR proteins relocalize into IRIF foci upon genomic stress (Bekker-Jensen et al. 2006). This is particularly pronounced for proteins implicated in the repair and signaling of DSBs by homologous recombination (HR) (Fig. 2). A systematic analysis of the spatial distribution of DSB-DDR factors using a method to locally introduce DSBs in cultured living cells (Lukas et al. 2003) resulted in a localization classification of DDR factors (Bekker-Jensen et al. 2006). In this procedure, cells were cultured in the presence of photo-sensitizing nucleotide-analogs (Iodo-deoxyuridine) prior to micro-beam laser irradiation with 337 nm that induces DSB in a user-defined sub-nuclear area (Lukas et al. 2003). Several subclasses of repair proteins were found based on their recruitment properties: (1) The major checkpoint mediators, such as Mdc1 (mediator of DNA damage checkpoint protein 1), 53BP (p53 binding protein), BRCA1 (breast cancer protein 1), ATM, and the MRN complex, co-localize in IRIF with γ-H2AX-decorated chromatin, termed "DSB-flanking chromatin". Assembly of these proteins at the DSB-flanking chromatin appeared to occur throughout the cell cycle. It has been estimated that these foci contain several hundred copies of each of the participating DDR factors. (2) Another group of DSB-activated proteins assembles in much smaller ssDNA micro-compartments that are most likely formed by 5′ resections at DSB, an important HR intermediate. These "microfoci" are only formed in S- and G2-phase cells and typically accumulate next to RPA, factors directly involved in HR repair, like

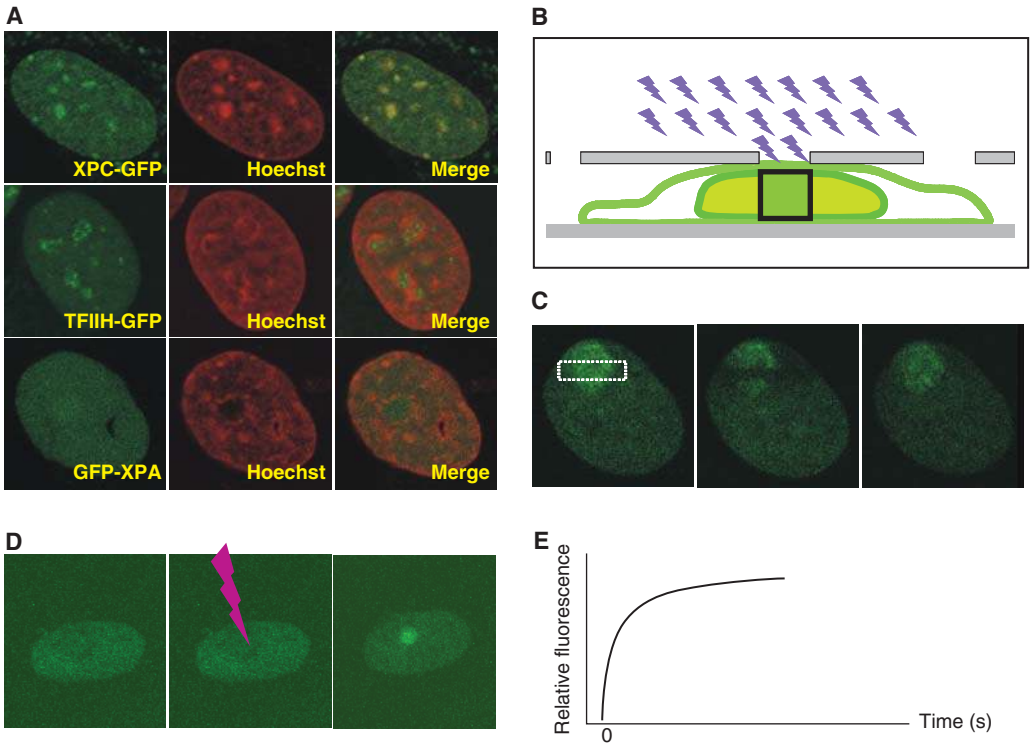

Figure 3. Live cell analysis of NER. (*A*) Distribution of three different NER factors tagged with GFP in living cells; DNA is stained by the DNA stain Hoechst. The damage recognition factor XPC is concentrated in nuclear areas (*top panel*) that also contain high DNA concentrations when the XPC-GFP (Hoogstraten et al. 2008) is expressed in mouse-embryonal fibroblasts that exhibit the species-specific dense DNA-containing areas. This inhomogeneous distribution contrasts to other NER factors such as XPA (Rademakers et al. 2003), which are homogenously distributed (*lower panel*) and the repair/transcription factor TFIIH that is enriched in nuclei (Hoogstraten et al. 2002). (*B*) Schematic cartoon of the procedure to locally inflict UV-damage in living cultured cells by irradiation through a microporous filter (Volker et al. 2001). (*C*) Local accumulation of XPB-GFP (TFIIH subunit) in UV-damaged areas used to determine the dwell time of this NER factor in the damaged area by fluorescence recovery after photo bleaching (FRAP). (*D*) Human fibroblasts expressing XPC-GFP locally damaged at the indicated position (purple flash, middle panel) by UV-C laser (Dinant et al. 2007). The right panel shows a clear accumulation of this protein as soon as 30 seconds after irradiation. (*E*) Schematic representation of the quantification of NER factor time-dependent accumulation at local UV-damage in living cells to determine the assembly kinetics of these factors within the chromatin-bound NER complex.

Rad51, Rad52, BRCA1, and FANCD2 (Fanconi anemia complementation group D2). They also contain the ssDNA-activated checkpoint kinase ATR and the 9-1-1 complex (Warmerdam et al. 2009). Also MRN and BRCA1 were found in these microfoci independent of γ-H2AX or Mdc1, while their recruitment to DSB-flanking chromatin is dependent on these proteins. (3) Several DSB repair factors, particularly those involved in NHEJ, such as DNA-PKcs and Ku70/80, could not be found to re-localize into microscopically discernible foci. It is likely that this process proceeds much faster than HR and that NHEJ factors do not need to be loaded in such large molecular assemblies to execute their function. However, using procedures that introduce high local concentrations of breaks in living cells, with the aid of multi-photon micro-beam laser irradiation, microscopically discernible accumulations of NHEJ could be found (Mari et al. 2006; Uematsu et al. 2007). These accumulations likely reflect high local

concentrations of breaks and repair factors rather than a specific chromatin structure. (4) Other factors implicated in DSB processing do not exhibit discernible accumulation at sites of damage, since these proteins are omnipresent on chromatin and simply get post-translationally modified at or near breaks. One of them is Smc1 (Structural Maintenance of Chromosomes 1), a structural component of the cohesin complex required for sister chromatid cohesion during S-phase and also implicated in DSB repair. Smc1 is phosphorylated on Serine 957 (a canonical ATM target site) by ATM and ATR after exposure to a broad array of stimuli including IR, HU, and UV-light (Kim et al. 2002). (5) While many of the DDR factors are recruited or retained at the site of damage, proteins like the effector kinases Chk1 and Chk2 are released from chromatin in response to DNA damage. Activated checkpoint proteins distribute through to nucleus to activate soluble pan-nuclear targets such as p53 and Cdc25A (Kastan and Bartek 2004). Also, these effectors, crucial for efficient DNA damage-induced gene expression (p53) and cell-cycle arrest (Cdc25A, p53), do not accumulate at DNA damage sites.

Dynamics and Function of IRIF

One obvious question is: What is the function of IRIFs? Although they are certainly associated with DSBs, this seemingly easy question is, however, difficult to answer, and different models can be envisaged; for example, foci may (1) represent sites of active DSB repair or (2) sites refractory or difficult to repair. Determining the dynamics of these structures might shed some light. Dwell time measurements of HR proteins in IRIFs have revealed a highly dynamic interaction of some of the factors (Rad54 and Rad52) with these apparent long-lasting structures (Essers et al. 2002). The more structural protein Rad51 that forms nucleo-protein filaments exhibits much longer residence times in these foci. Real time imaging in living cells of GFP-tagged DSB-DDR protein distribution in response to local damage induction allowed determination of the assembly kinetics of the different factors (Bekker-Jensen et al. 2005). One of the most striking findings in these studies is that assembly occurs in two kinetically separable waves, i.e., an immediate loading of, for example, MRN and MDC1, followed by a second wave of loading of, for example, 53BP1 and BRCA1. This secondary, slower wave has been suggested to retain and concentrate the repair factors near the insult.

It is surprising to note that particularly the DNA repair proteins (Rad51, Rad54, etc.) are only found in the micro-foci, whereas DDR proteins implicated in damage recognition and signaling appear to accumulate in larger structures. Recently, it was shown that H2A and H2AX ubiquitination occurs in response to DSB and that these modified histones as well as the enzymes RNF8, Ubc13, RNF168, and HERC2 accumulate in large foci (Huen et al. 2007; Mailand et al. 2007; Doil et al. 2009; Stewart et al. 2009; Bekker-Jensen et al. 2010). This histone modification appears to play an important role in recruiting the signaling proteins 53BP1 and BRCA1. It seems that the larger structures are particularly important for transducing and amplifying damage signaling.

Despite intensive research, the exact molecular function of IRIFs remains enigmatic. The most popular model is that IRIFs serve to locally concentrate the enzymes required for DSB. If that is indeed their prime function, it is surprising to note that such huge amounts of activities are required. The high number of proteins and the long-lasting presence of foci argue that the reaction catalyzed by these enzymes is inefficient. In light of this reasoning, the option that foci represent breaks refractory or difficult to break remains open. Recently, a hint toward the possible molecular function of the large chromatin depositions was revealed by directly targeting DDR factors to specific artificial genomic positions in the absence of actual lesions (Soutoglou and Misteli 2008). Immobilizing repair factors to chromatin elicits a damage signaling response without the actual presence of DNA damage. These data suggest that prolonged binding of repair factors is sufficient to trigger, sustain, and amplify the DNA damage signaling.

Another interesting debate in the field with respect to structure and nuclear distribution of DSBs is on the choreography of DSBs in the nuclear space and the issue of how ends of different breaks find each other. Chromosomal translocations are initiated by DSBs and it has been shown that translocations between different chromosomes occur in a cell-type specific manner (Meaburn et al. 2007), a phenomenon likely driven by the non-random spatial organization of the genome (Roix et al. 2003; Lanctot et al. 2007; Meaburn and Misteli 2007). These observations favor a so-called "contact-first" model, i.e., that chromosome fibers should be in close proximity to allow translocations, as opposed to a "breakage-first" model in which breaks are mobile and roam the nucleus for interactions. Soutoglou et al. developed an elegant procedure to investigate this enigma by specifically generating a single DSB, using a specific endonuclease site located between two repetitive sequences of distinct repressor binding sites, which can be visualized by different fluorescently-tagged repressors (Soutoglou et al. 2007). With this procedure, very limited movement over time of the DSB was observed, thus supporting the "contact-first" model. In contrast, however, Aten et al. found evidence for the "breakage-first" model, using α-partical tracks to inflict DSB (Aten et al. 2004), in which they observed limited movement and fusion of foci. These contradictory findings might be derived from the different experimental procedures and cell-cycle phase in which the analyses are performed. In addition, both models may not be mutually exclusive and both processes may play a role in the process of chromosomal translocations.

Organization of Nucleotide Excision Repair

In the absence of DNA damage, NER factors are generally homogenously distributed throughout the nucleoplasm (Fig. 3A); however, XPC and TFIIH are exceptions to this rule. XPC appears to co-localize with dense or high DNA concentrations (Hoogstraten et al. 2008) and TFIIH is enriched in the nucleolus (Hoogstraten et al. 2002). Unlike DSB repair, NER factors do not exhibit re-localization into microscopically discernible subnuclear structures upon DNA damage induction, making it difficult to unravel the structural organization of NER-dependent damage response. Despite the absence of microscopically discernable repair foci within NER, live cell studies on NER proteins were, however, the first to reveal the highly dynamic character and mobility of chromatin-transacting proteins in mammalian cells (Houtsmuller et al. 1999). GFP-based studies showed that the NER-specific 5′-endonuclease ERCC1/XPF (Houtsmuller et al. 1999), the damaged DNA binding proteins DDB2 (Luijsterburg et al. 2007) and XPC (Hoogstraten et al. 2008), the damage verification factor XPA (Rademakers et al. 2003), the 3′ endonuclease XPG (Zotter et al. 2006), and the multifunctional TFIIH complex (Hoogstraten et al. 2002; Giglia-Mari et al. 2006) each move with their own unique rate through the nucleus. This notion contrasts to an earlier model, based on isolation of NER factors from cell nuclei, in which it was postulated that an assembly or complex of most NER-factors, i.e., the so-called "nucleotide excision repairosome," forms the functional unit within NER (Svejstrup et al. 1995). Further application of cell lines stably expressing these biologically active GFP-tagged NER factors have allowed detailed analysis of the kinetic properties of each of these factors when actively engaged in NER. The development of a procedure to locally inflict NER-specific DNA damage in mammalian cells at the single cell level, using UV-C light irradiation through a microporous filter (Katsumi et al. 2001; Mone et al. 2001) (Figs. 2 and 3B) and later by the development of UV-C laser microirradiation set-up (Dinant et al. 2007) (Fig. 3D), provided detailed insight into how the different NER factors assemble into NER complexes (Mone et al. 2004; Zotter et al. 2006; Luijsterburg et al. 2007; Alekseev et al. 2008; Hoogstraten et al. 2008; Dinant et al. 2009; Nishi et al. 2009) (Fig. 3E). Additional FRAP studies on a series of NER factors, using different doses of UV (correlating with different concentrations of photo-lesions, which are a prime target for NER), variable repair times and in cell

lines with distinct NER-efficiencies further provided insight into the kinetic framework of NER in living mammalian cells (Politi et al. 2005; Luijsterburg et al. 2010). Most of the NER factors, with the exception of the DNA damage sensor XPC (Hoogstraten et al. 2008; Nishi et al. 2009), freely diffuse through the nuclear space and only assemble into functional repair complexes at the site of the damage. Advanced modeling based on NER kinetic studies favored a model of kinetic-proofreading to achieve high specificity of lesion recognition by proteins with a relatively low discrimination of damaged sites versus non-damaged DNA (Luijsterburg et al. 2010).

NER and Damage Signaling

In spite of detailed knowledge on the NER mechanism, the connection with UV-induced DNA damage signaling is less well characterized. The ATR kinase and loading of the 9-1-1 complex involving the RAD17 clamp-loader certainly play a role in UV-damage signaling (Niida and Nakanishi 2006). However, the confounding effect of UV-induced replication stress (Zou and Elledge 2003; Falck et al. 2005) makes it difficult to disentangle NER-related signaling from replication stress-induced signaling. Nevertheless, a direct relationship between NER and checkpoint signaling was identified (Giannattasio et al. 2004). In addition, NER-dependent ATR activation and H2AX phosphorylation occurred in non S-phase cells (O'Driscoll et al. 2003; Hanasoge and Ljungman 2007), likely caused by ssDNA-containing NER-intermediates (Stiff et al. 2008). NER-processing and ATR are also required for UV-induced H2A ubiquitination (Bergink et al. 2006). A similar chromatin mark was found in response to DSBs (Huen et al. 2007; Ikura et al. 2007; Mailand et al. 2007; Nicassio et al. 2007). Strikingly, the enzymes involved in DSB-induced H2A ubiquitination, such as UBC13 and RNF8, were also responsible for the NER-dependent H2A-ubiquitination (Marteijn et al. 2009). This UV-induced chromatin mark further triggers the recruitment of MDC1, BRCA1, and 53BP1, factors previously known to function in DSB-induced DDR. These findings suggest highly conserved chromatin modification and loading of signaling factors between entirely distinct DDR pathways, DSB repair, and NER. This notion further corroborates the suggestion that large-scale chromatin modifications in response to DNA damage and local concentration of DDR factors play an important function in damage signal maintenance and amplification (Soutoglou and Misteli 2008; Marteijn et al. 2009).

Dynamic Organization in Somatic Cells

It is important to keep in mind that all described live cell studies in DNA repair have been conducted on cultured cells. Cultured cells are under constant stress (e.g., atmospheric oxygen) and usually in a highly replicative status. Moreover, physiological processes critically depend on the cellular context or micro-environment (cell-cell contacts with neighboring cells, extracellular matrix, etc.). Within larger animals, more than 90% of the somatic cells are in a non-proliferative status, thus making extrapolations to the actual in vivo situation even more delicate. To acquire an integral view on DDR in different post-mitotic highly differentiated cells, knock-in mouse models expressing endogenously fluorescently tagged crucial proteins have been generated. In the first example of such a mouse model, the yellow variant of GFP was fused to the XPB subunit of the repair/transcription factor TFIIH, by targeted integration into the endogenous *Xpb* gene locus (Giglia-Mari et al. 2009). Previous studies in cultured cells showed that TFIIH interacts for a few seconds with transcription initiation sites (Hoogstraten et al. 2002). A similar dynamic behavior was observed in highly proliferative cells in mouse tissue, e.g., skin keratinocytes (Giglia-Mari et al. 2009). Surprisingly, transcription-dependent chromatin binding takes on the order of minutes/hours in post-mitotic cells, such as neurons. This suggests that a well-known and extensively studied cellular pathway, such as transcription, can have a completely different dynamic organization in different cells. The mechanistic reason for this dynamic

behavioral change remains enigmatic and will be the next challenge to reveal.

In view of these observations, it remains questionable whether current concepts of DDR functioning are applicable to all cell types and tissues. Is a keratinocyte repairing DNA damage differently than a neuron? Do all DDR factors play similar functions in different cells? Are there also development- and differentiation-driven variations in DDR, and if so, how are they regulated? Intriguingly, differential repair kinetics and damage sensitivities have been found in somatic cultured cells and embryonic stem cells (ESC) (de Waard et al. 2008). Part of these differences can be attributed to a more open chromatin structure in ESC, as further reduction of chromatin compaction by reducing the amount of the linker histone H1 increased the damage response (Murga et al. 2007).

CONCLUSION

With the availability of protein tagging technology and advanced confocal imaging, spectacular novel insight in the dynamic interplay of DDR factors with damaged DNA has been gained. These studies have revealed a general minimal model of freely diffusing constituents that assemble in a stochastic fashion with damaged DNA to create dynamic assemblies of multiple factors at these sites to finally exert their function (Dinant et al. 2009; Luijsterburg et al. 2010). This view of the dynamic organization of complex pathways in the mammalian cell nucleus has challenged the current textbook models that give the impression of stable structures containing large complexes, in which all constituents are present at all times.

Live cells studies on DDR have revealed that pathways intermingle and share components. Controlling this complex interplay requires perfect coordination in time and space of functions to ensure stability and maintenance of functions. But differently from man-made machines, the dynamic organization of nuclear functions is not the result of a predefined master plan, but, fascinatingly, is the result of a long evolution process selecting for a subtle mix of stochastic diffusion and protein affinities for optimal performance.

Dynamic studies in living cells and, recently, in living animals, allow us to study repair mechanisms in action. Together with the current "omics" approaches (proteomic, genomic and transcription arrays, deep-sequencing, etc.) and the emerging systems biological procedures, these new tools and techniques provide tremendous opportunities to reach a full understanding of DDR, the biological consequences of inefficient DDR in patients and in the general population, in cancer protection, and in age-related diseases.

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Cold Spring Harbor Perspectives in Biology

DNA Damage Response

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Cold Spring Harb Perspect Biol 2011; doi: 10.1101/cshperspect.a000745 originally published online October 27, 2010

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