

Formation and Repair of Complex DNA Damage Induced by Ionizing Radiation

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ISBN 978-91-628-8682-0

E-publication: <http://hdl.handle.net/2077/32008>

Printed in Gothenburg, Sweden 2013

Ale Tryckteam

You look at science (or at least talk of it) as some sort of demoralizing invention of man, something apart from real life, and which must be cautiously guarded and kept separate from everyday existence. But science and everyday life cannot and should not be separated. Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment.

Rosalind Franklin

ABSTRACT

DNA is the critical target when cells are exposed to ionizing radiation, a potent stressor with capacity to produce complex DNA damages, thereby increasing the risk of cancer. DNA and associated histones form chromatin, which is an effective protection against ionizing radiation. We have investigated the formation and repair of complex lesions, including double strand breaks (DSB) and clustered damages (two or more lesions within 10-20 base pairs) after exposure to ionizing radiation of different beam qualities, in normal human cells. The biological consequences of clustered lesions are not fully understood.

We present a major influence of chromatin on induction of DSB and oxidized purine- and pyrimidine clusters. For example, sparsely ionizing radiation induces 170 times more clusters in naked DNA, compared with intact cells. For DSB, the same factor was 120. This reflects a pronounced influence of the indirect effect of radiation on clusters, supporting our finding that abolishment of radical scavengers, and suppression of the indirect effect, influence clusters more than DSB. Also, we investigated the repair of complex lesions (i) formed from direct DNA hits, (ii) in cells with hypo- or hyperacetylated chromatin or (iii) in cycling or non-proliferating cultures, conditions assumed to compromise removal of these lesions. We present a fast and efficient repair of clustered damage with no evidence of *de novo* DSB formation due to attempted repair. We observe no large influence of proliferation status. Surprisingly, no major influence of chromatin acetylation was found. Direct DNA hits did not influence repair of clusters but compromised DSB processing. We present that induction of DSB and cell survival is cell cycle dependent for densely ionizing radiation, in contrast to what was previously reported. Compared with sparsely ionizing radiation, α -particles induce more DSB and result in a decrease in cell survival. Also, the repair of DSB was compromised. Surprisingly, clusters induced by α -particles were rapidly repaired.

In conclusion, both DSB and clustered damage, formed by ionizing radiation, are sensitive to the antioxidant level in cells. There are two possible explanations for the observed efficient removal of clusters in normal cells, either the rapid decrease could be due to efficient repair or represent clusters too complex to be assessed in our method.

Keywords: Ionizing radiation, clustered damage, chromatin structure, DSB

ISBN: 978-91-628-8682-0

<http://hdl.handle.net/2077/32008>

LIST OF PAPERS

- I. **K. Magnander**, R. Hultborn, K. Claesson and K. Elmroth, Clustered DNA damage in irradiated human diploid fibroblasts: influence of chromatin organization. *Radiat. Res.* **173**, 272-282 (2010).
- II. K. Claesson*, **K. Magnander***, H. Kahu, S. Lindegren, R. Hultborn and K. Elmroth, RBE of α -particles from ^{211}At for complex DNA damage and cell survival in relation to cell cycle position. *Int. J. Radiat. Biol.* **87**, 372-384 (2011)
*Authors contributed equally to the work
- III. **K. Magnander**, U. Delle, M. Nordén Lyckesvärd, J. Kallin, J. Swanpalmer, A. Morgenstern, F. Bruchertseifer, H. Jensen, S. Lindegren and K. Elmroth. Repair of DSB and clustered damage: A study on influence of direct hits, chromatin acetylation and radiation quality. *Manuscript*.
- IV. **K. Magnander** and K. Elmroth, Biological consequences of formation and repair of complex DNA damage. *Cancer Letters.* **327**, 90-96 (2012)

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ABBREVIATIONS

A	Adenine
¹⁴ C	Carbon-14
8-oxoG	7,8-dihydro-8-oxoguanine
AP	Apurinic/Apyrimidinic
At	Astatine
ATM	Ataxia telangiectasia mutated
ATR	ATM and RAD3 related kinase
BER	Base excision repair
Bi	Bismuth
bp	Base pairs
C	Cytosine
Co	Cobalt
DMF	Dose modifying factor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
E. coli	Escherichia coli
eV	Electronvolt
Fpg	Formamidopyrimidine DNA-glycosylase
G	Guanine

HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HRR	Homologous recombination repair
I	Iodine
LET	Linear energy transfer
MRN	Mre11/Rad51/NBS1
Nfo	Endonuclease IV
NHEJ	Non-homologous end-joining
Nth	Endonuclease III
PFGE	Pulsed field gel electrophoresis
RBE	Relative biological effectiveness
SRIM	The stopping and Range of Ions in Matter
SSB	Single-strand break
T	Thymine
TSA	Trichostatin A
γ H2AX	Phosphorylated histone variant H2AX

1 INTRODUCTION

1.1 From DNA to chromatin

In each cell in our body, all our genetic material is represented in DNA, a two to three meters long helical molecule, packed and arranged in a highly controlled structure forming chromatin. The DNA helix consists of the sugar-phosphate backbone, i.e. two anti-parallel polymers consisting of 2-deoxyribose linked together by phosphate groups, and, between opposing sugar molecules, one of two possible sets of base combinations paired with hydrogen bonds. The double-ringed nucleobases adenine and guanine (A and G purines) pair with single-ringed thymine and cytosine (T and C pyrimidines) with double and triple hydrogen bonds, respectively. The sequence of the nucleobases represents our genetic information since they form genes coding for cellular functions or systems as well as inherited characteristics. Three-nucleobase sequences form codons that are translated into corresponding aminoacids, later combined into a protein.

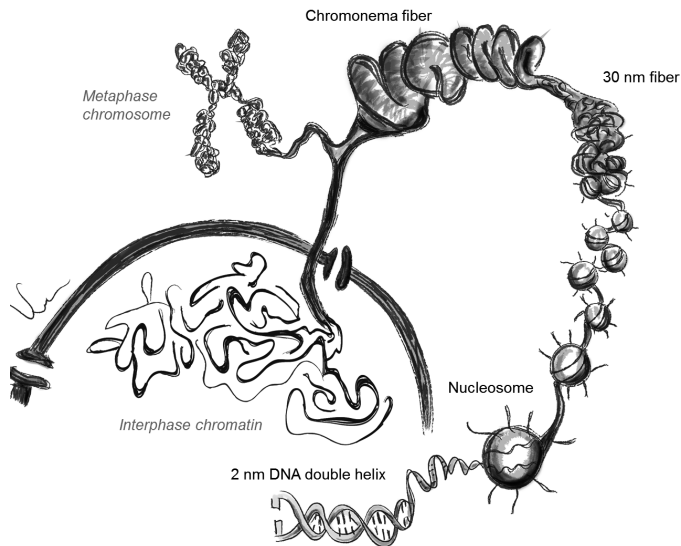


Figure 1. From DNA to chromatin.

In its most common form, the double helix is wrapped nearly two turns around a histone octamer, forming a nucleosome (Figure 1). Two copies of each of the histones H2A, H2B, H3 and H4 build up the octamer and the nucleosomes are linked together with linker DNA and associated histone H1. The nucleosomes and the interstitial linker DNA form a highly dynamic structure usually described as beads on a string, most often packed into 30 nm fiber. In mitosis, the replicated chromosomes pair as sister chromatids to form an extremely condensed structure, only present a very short period of time but certainly the most familiar way to depict cellular DNA (the metaphase chromosome shown in Figure 1). However, in interphase and G₀ cells, the chromatin forms regions of heterochromatin and euchromatin (Figure 2). Heterochromatic domains are located preferentially in the nuclear periphery and consist of densely packed chromatin with low gene transcription activity. In contrast, euchromatin is often lighter packed with high density of genes that are actively transcribed. Modulations of the chromatin conformation facilitate transcription, replication and DNA repair as well as control gene activation and silencing. Chromatin condensation and accessibility of DNA are regulated by posttranslational modifications of histones, such as phosphorylation and acetylation, as well as DNA methylation (1-4).

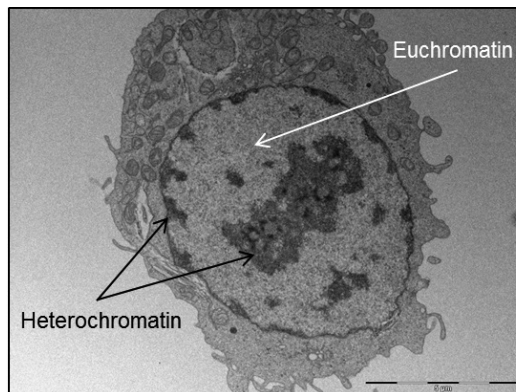


Figure 2. Hetero- and euchromatic regions in the nucleus. Transmission electron microscopic photo of a Burkitt's lymphoma cell.

For example, euchromatic decondensed, accessible regions are associated with acetylated histones governed by histone acetyltransferases (HATs). Methylation of DNA and the binding of methyl-CpG-binding domain proteins lead to recruitment of histone deacetylases (HDACs) that removes acetyl groups and provokes chromatin condensation, i.e. the formation of heterochromatin.

1.2 Genetic integrity under constant threat

Our DNA is constantly subject to external or internal exposure to damaging agents, challenging preservation of the genetic integrity. Reactive oxygen (e.g. peroxides and oxygen ions) and nitrogen (e.g. nitric oxide) species as well as free radicals (e.g. solvated electrons, hydroxyl and hydrogen radicals) are more or less likely to react with the nucleobases as well as with 2-deoxyribose on the DNA strands. These species arise frequently and endogenously in vicinity of DNA during metabolic processes or inflammation. The most important of exogenous genotoxic stressors is ionizing radiation causing damage directly in DNA. It is also likely to produce reactive free radicals when interacting with the water surrounding DNA, i.e. the water radiolysis, preferentially forming hydrogen and hydroxyl radicals as well as solvated electrons. These have the potential to abstract hydrogen atoms from DNA, leading to an unstable DNA bioradical, prone to further unwanted chemical interactions. If not restored by hydrogen donation, the DNA bioradical preferentially reacts with oxygen, if present, fixing the lesion that must then be enzymatically processed. Formation of DNA damages through radical-mediated processes is denoted the indirect effect of ionizing radiation.

1.2.1 Ionizing radiation

As the name indicates, ionizing radiation has enough energy to ionize molecules when traversing for example cells or tissues. In 1895, Wilhelm Conrad Röntgen succeeded in producing and detecting X-rays and the groundbreaking discovery of polonium and radium by Marie Curie followed in 1898. Ever since, the clinical development of ionizing radiation as diagnostic tool and therapeutic agent has been advancing and today, external and internal radiation therapy with different radiation qualities is implemented in cancer therapy programs throughout the world. Ionizing radiation is often categorized into sparsely and densely ionizing radiation, or

synonymously: low-LET and high-LET radiation, respectively. LET, short for linear energy transfer, is defined as mean energy transferred per unit length along the traversed track, usually presented in keV/ μm . Photons and electrons with LET less than a few keV/ μm are categorized as low-LET radiation. Alpha-particles, accelerated ions and low-energy protons with LET-values of up to hundreds of keV/ μm are referred to as high-LET radiation. The dose distribution in target differs. Specifically, the dose depth curve for densely ionizing radiation is initially low with a rapid increase in energy deposition at what is called the Bragg peak, followed by an immediate drop to virtually zero.

Surgery, conventional external photon therapy and systemic chemotherapy are generally unable to cure disseminated cancer and microscopic tumors. Therefore, the physical capacity of high-LET radiation to deposit a massive amount of energy within a much limited region has been an incitement for development of new radiation therapy models. In this regard, α -emitting radionuclides, e.g. ^{211}At and ^{213}Bi , are promising isotopes and recently a phase I study from Gothenburg on α -particle radioimmunotherapy of ovarian cancer was published (5). The potency of one type of radiation quality is often described by the relative biological effectiveness (RBE) and determined by comparison to a standard radiation quality, usually γ -irradiation from ^{60}Co or X-rays of 250 kVp. RBE is then calculated as the quotient between the reference radiation dose and the dose of the radiation of interest, required to obtain the same biological effect. RBE varies with LET and radiation quality and is different for various endpoints. For endpoints with non-linear dose responses, RBE depends also on the dose level.

1.2.2 Radiation-induced DNA damage

There is a wide range of DNA damages that may occur when cells or tissues are exposed to ionizing radiation, either through direct hits or indirectly by attacks from radiation-produced free radicals. The most common single lesions, predominantly induced via the indirect effect but also frequently during endogenous processes, are base lesions such as oxidized purines or pyrimidines and AP sites (apurinic or apyrimidinic) as well as modifications of sugars. If the phosphodiester bond between the sugars is broken a single-strand break (SSB) is formed. Ionizing radiation can also induce more complex DNA damages. If both strands are broken within a region of 10-20 base pairs (bp), neither the hydrogen bonds nor the chromatin can keep the strands together and a double-strand break (DSB) will arise (6). For decades, DSB have been considered the most important of DNA damages. Their

correlation to different radiobiological outcomes, such as chromosomal aberrations and cell death, has been well elucidated, especially after exposure to sparsely ionizing radiation. John Ward postulated early that ionizing radiation induces another type of complex lesions, then called locally multiply damaged sites, now denoted clustered DNA damage, defined as two or more DNA lesions within 10-20 bp (7, 8). Consisting lesions can either be formed on opposite strands, i.e. bistranded, or tandemly induced on the same strand. Also, these types of lesions were suggested in biophysical models (9-11) and it was proposed that enzymatic activity could convert closely spaced lesions into DSB indicating their implication on biological effects (12, 13). Ionizing radiation is one of few agents with the potential to induce clustered DNA damages due to the inhomogeneous energy deposition pattern.

1.3 Defense strategies

During evolution, cells have developed and improved a broad defense for protection against stressors like ionizing radiation and the subsequently produced free radicals. A complex cellular system to handle DNA damages and to minimize or abolish disadvantageous biological consequences thereof has evolved.

1.3.1 Antioxidants

To reduce the risk of free radical attacks described above, a setup of radical scavenging molecules is present in the cellular environment. These can react immediately with the diffusing radicals by donating hydrogen atoms. The antioxidant defense system includes main groups of enzymatic antioxidants such as catalase, superoxide dismutases and glutathione peroxidase. Catalase and superoxide dismutases are both involved in catalyze of superoxides and hydrogen peroxide into less reactive molecules. Vitamin E, ascorbic acid as well as selenium, an important trace element and a component in glutathione peroxidase, have all been shown to strengthen the protection against ionizing radiation (14, 15). Further, cysteine contains thiols and is a constituent in glutathione, one of the most important intrinsic radical scavengers. Thiols are essential in radical scavenging by their capacity to donate hydrogen atoms and thereby become oxidized while forming disulfide groups (16). Dimethyl sulfoxide (DMSO), widely used in preclinical radiobiological studies, is a very potent extrinsic radical scavenger. It is permeable over the cell membrane and if present at sufficient concentrations during irradiation it

effectively abolishes the radical mediated effect, i.e. the indirect effect of ionizing radiation.

1.3.2 Chromatin protects DNA

First, due to its structural conformation, the chromatin organization works as a protection against DNA damages by minimizing the probability of direct hits in DNA. Second, the chromatin excludes water from DNA thereby reducing water radiolysis in direct vicinity of the biomolecule. For example, the hydroxyl radical, frequently produced through the water radiolysis, has an average diffusion distance of 6 nm in the nuclear environment (17). Furthermore, histones and other DNA-bound proteins have a role as radical scavengers and decrease the number of DNA lesions induced through the indirect effect of ionizing radiation by their capacity to donate hydrogen atoms. Besides their role in limiting the formation of DNA lesions, histones and other DNA-bound proteins within chromatin are intimately involved in the regulation of the signaling network and DNA damage response, governed by posttranslational modifications such as phosphorylation, acetylation and methylation, thoroughly reviewed by (18).

1.3.3 Signaling and DNA damage response

A signature for ionizing radiation exposure in a chromatin context is the potential and likeliness of formation of complex DNA damage. A functional DNA damage response is crucial for preservation of the genetic integrity and reduced efficiency in for example activation of cell cycle arrest or DNA damage repair is considered as a hallmark of cancer (19).

The DNA damage signaling system in mammalian cells is a complex process that involves proteins that can be categorized into four groups: sensors, transducers, mediators and effectors. In response to DNA damage, signal amplification and dispersion leading to recruitment of proteins and protein complexes involved in for example cell cycle checkpoint and DNA damage repair are elicited (20, 21). Briefly, immediately after formation of DNA damage, sensors bind to the damaged site, thereby recruiting transducers which help amplifying and maintaining the DNA damage signal. Important factors are sensors like the MRN complex (Mre11/Rad5/NBS1) and Ku70/Ku80, both playing essential roles in the repair of DSB. In addition, ATM (Ataxia telangiectasia mutated), DNA-PKcs and ATR (ATM and

RAD3 related kinase) are transducers and key factors involved in several pathways in the DNA damage response. Due to interplay between transducers and mediator proteins like 53BP1 (tumor protein 53 binding protein 1) and BRCA1, the signal is dispersed all over the cell nucleus and effector kinases (e.g. Chk1 and Chk2) are activated.

As a well-known signature of DSB formation and an early step after occurrence, phosphorylation of the histone variant H2AX (γ H2AX) is triggered over a region of thousands of base pairs around the damage site (22). This rapid response has been suggested as one important but possibly not requisite step in the recognition of DSB, recruitment of repair components and maintenance of checkpoint arrest.

Defects in key factors involved in the DNA damage response lead to elevated sensitivity to agents known to induce DSB, e.g. ionizing radiation or drugs like bleomycin and calicheamicin. For example, the disorder Ataxia telangiectasia (A-T) is due to mutations in the ATM gene, coding for the DNA damage response kinase ATM, and is strongly correlated to increased radiosensitivity and susceptibility to cancer (23).

Homologous recombination repair (HRR)

The processing and ligation of DSB are predominantly performed through one of two main repair pathways: homologous recombination repair (HRR) and non-homologous end-joining (NHEJ). It is likely that crosstalk between them occurs when the choice of DSB repair pathway takes place and indeed, interplay has been demonstrated in the processing of heterochromatic sites as well as in late S phase and in G_2 (24-26). HRR is a slow process and can only take place in late S phase and in G_2 when DNA has been replicated and the sister chromatid is available as undamaged template, promoting high fidelity repair of DSB. HRR involves DNA strand resection and strand invasion followed by DNA synthesis and ligation. Immediately after formation, the MRN complex binds to the DNA ends. MRN then activates ATM, recruits nucleases and is involved in the end-trimming process. One of the key factors in HRR is the tumor suppressor BRCA1 which plays an important role in binding and regulation of several downstream factors (27). For example, BRCA1 is involved in the removal of damaged bases in order to prepare for the homologous recombination and it interacts with Rad51, essential in the search for the homologous sequence in the sister chromatid (27-29). HRR has recently been suggested to be more intimately involved, than was previously assumed, in the processing of DSB induced by high-LET radiation,

supporting the findings correlating functional Rad51 and cell survival after exposure to densely ionizing radiation (24, 30).

Non-homologous end-joining (NHEJ)

NHEJ, in contrast to HRR, is available throughout the whole cell cycle since it does not require a template. Thus it is fast and error prone. Initially, The Ku70/Ku80 heterodimer binds to the DNA ends at the site of DSB and recruits DNA-PKcs which tether the loose ends and forms a bridge (31). The DNA ends are then trimmed, a process involving Artemis, damaged bases are removed to prepare for synthesis and thenceforth ligation is carried out by the LigIV/XRCC4 complex (20).

It has been suggested that in NHEJ deficient or in NHEJ proficient cells but in situations where canonical NHEJ fails to initiate or complete repair of a DSB, an alternative back-up NHEJ pathway is available (32). These two variants of NHEJ are shown to involve different sets of protein complexes.

Cell cycle arrests and apoptosis

Several factors implicated in DNA damage repair play an essential role also in other strategies developed to avoid genomic instability. Some important strategies, activated by ionizing radiation, are cell cycle arrest, apoptosis (programed cell death) and senescence. In proliferating cells, the sensor system regulates cell cycle checkpoints simultaneously as initiating DNA damage repair. Not surprising, ATM plays a central role in cell cycle checkpoint control and in apoptosis as it phosphorylates a number of partaking key factors, such as p53, Chk1, Chk2, BRCA1, H2AX and Artemis (33). Activation of checkpoints at G₁-S and G₂-M transitions arrests cell cycle progression while activation in S phase slows down proliferation to allow sufficient time for repair. If the damage burden is too heavy, certain cell types may be programmed to undergo apoptosis, a cell death process where cellular components are disassembled in a highly organized fashion. Alternatively, in response to ionizing radiation exposure, cells may be permanently arrested in a process called senescence, where cells cease to cycle but maintain other cellular functions. Some organisms or tissues may gain on senescence over apoptosis.

Base excision repair (BER)

Isolated base lesions are processed through the multistep base excision repair pathway in which the damaged base initially is recognized and excised through glycosylase activity generating an AP site. Thenceforth an AP endonuclease cleaves the strand forming a 3' hydroxyl adjoining a 5' deoxyribose phosphate and DNA polymerases can then fill the gap. If only one nucleotide is damaged or lost, short-patch repair processes the gap (single nucleotide repair). Multi-nucleotide gaps (2-8 bases) are processed through long-patch BER. Processing of AP sites and SSB also involve the last steps of BER.

The BER components vary for different substrates. For example, in mammalian cells, the bifunctional glycosylase OGG1 preferentially removes and cleaves at 8-oxoG (7,8-dihydro-8-oxoguanine) sites while NTH1 is mainly responsible for processing of oxidized pyrimidines (34). The *E. coli* homologs, commonly used for detection of clustered lesions in *in vitro* studies, are Fpg (formamidopyrimidine DNA-glycosylase) and Nth (endonuclease III), respectively.

1.4 Clustered DNA damage

The formation of clustered damages is a random process and several factors, such as scavenging condition, radiation quality and cell type influence the composition and complexity of the cluster. Hence, spacing, polarity, type and quantity of the lesions are known to influence the reparability and later biological consequences (35-40). Accordingly, the cellular response to specific types of clustered damages has been difficult to study and therefore simplified systems with well-defined constructed clusters in oligonucleotides or plasmids have been an indispensable tool. It has been found that the composition and complexity as described above directly influences the repair efficiency and the outcome of attempted repair, as discussed in Paper IV.

It is not fully elucidated what factors are involved in the repair of clusters but it has been suggested that BER (41-45) as well as DSB repair pathways play a role in the processing. Accordingly, BRCA1, one of the key-factors in HRR has been shown to affect the repair of clustered damages and deficiency in DNA-PKcs, implemented in NHEJ, gave persistent clustered damages in cells days after irradiation (46-48). Further, MSH2, a key-protein in mismatch repair has been shown to be involved in the repair of clusters (49).

1.5 Biological consequences of complex DNA damage

There are several risks associated with formation and processing of complex lesions. Paper IV is a summary of the current knowledge of the biological consequences associated with DSB and clustered DNA damage.

Formation and repair of prompt DSB are correlated to cytotoxicity, increased risk of mutagenic events and carcinogenesis in several ways. Compromised, insufficient or absent repair of DSB can cause loss of genetic material probably subsequently resulting in cell death. If end trimming is required and new bases are to be synthesized, the absence of an undamaged homologous chromatid as exact template may lead to changes in the genetic sequences. Another risk, associated with ligation is that wrong chromosome ends are ligated resulting in chromosomal rearrangements or translocations. If such chromosomal aberrations are stable and persist through proliferation, damages may be manifested and result in activation of oncogenes or inactivation, or deletion, of tumor suppressor genes, leading to a mutator genotype.

Single DNA lesions, like base damages and SSB, are generally not directly related to cell death but if present during replication a subset of these lesions are more or less mutagenic. For example, one of the most important and well known base damages is 8-oxoG with potential to mispair with both adenine and cytosine and it has been shown that during mismatch repair 8-oxoG:A is likely to result in G → T or A → C transversions (50, 51).

The situation is different if the simple lesions are formed within a cluster. Based on early studies on *E. coli* and mammalian cells and the finding that attempted repair of clustered damages may result in formation of *de novo* DSB (52, 53), experiments on constructs with designed clusters revealed that this was more likely to occur for certain combinations. For example, the processing of two AP sites positioned not too closely on opposite strands is very likely to induce *de novo* DSB and apart from some critical relative positions, an AP site opposite a SSB may also result in DSB formation (54-61). A rapid excision/incision rate was shown to directly correlate to formation of *de novo* DSB (54). Indeed, overexpression of glycosylases and lyases involved in BER has been shown to elevate *de novo* DSB formation resulting in decreased survival and increased mutagenic frequency (52).

However, not all clustered lesions are prone to result in *de novo* DSB. For example, the presence of 8-oxo-G within a cluster has been shown to inhibit the cleavage of adjacent lesions on both strands, thereby increasing the risk for mutagenicity due to persistent lesions (44, 54, 60, 62-66). Also, if constituting damages are formed in very close proximity or flanked by other damaged sites, formation of *de novo* DSB does not take place (56, 59, 60, 63, 67, 68). Accordingly, attempted and compromised repair can, if persistent lesions are present during replication, enhance the risk for miscoding and several studies have shown that compromised cluster processing leads to an increased mutagenic frequency in *E. coli* and eukaryotic cells, a phenomenon important in the carcinogenic process (60, 62, 63, 65, 69-71).

2 AIMS

We have previously shown that DSB induced by sparsely ionizing radiation correlates with the chromatin organization, with an increased yield in relaxed conformations of chromatin (72). With the use of pulsed field gel electrophoresis in combination with fragment analysis, we have confirmed that the formation of DSB, considered the severest of radiation induced damages, and the resulting DNA fragmentation occur in a non-random fashion in cells irradiated with densely ionizing radiation (73, 74). In close collaboration with members of the Targeted Alpha Therapy group at Sahlgrenska Academy, evaluation of cellular radioresponse, toxicity and therapeutic potential of the clinically relevant α -particle emitters ^{211}At and ^{213}Bi has been performed (74-78).

With this assembled knowledge we put up the aim to further investigate the formation and the processing of complex DNA damages, including a newly identified type of lesion, clustered DNA damage, with main focus on radiation quality and chromatin conformation.

Specifically we wanted to investigate:

- The processing of a novel class of complex damage, i.e. oxidized purine- and pyrimidine clusters, induced in normal human cells.
- The influence of structural and functional modifications of the chromatin conformation on induction and repair.
- The reparability of complex lesions formed from direct DNA hits.
- The importance of cell cycle position and proliferation status.
- The formation and repair kinetics of DSB and clustered DNA damages in cells exposed to the clinically relevant α -emitting radionuclides ^{211}At and ^{213}Bi , in comparison to sparsely ionizing radiation qualities.

3 MATERIALS AND METHODS

3.1 Cell lines

In Papers I and III normal human diploid fibroblasts HS2429 were used and in Paper II hamster fibroblasts V79-379A were chosen for synchronization experiments. To study the involvement of polymerase β in repair of complex lesions (Paper III), a murine embryonic fibroblast cell line with deficient polymerase β (M β 19tsA) was used and compared with its polymerase β -proficient parental cell line (M β 16tsA).

In experiments on complex DNA damage, cells were cultured in medium with [2-¹⁴C]Thymidine, prior to irradiation. As described in below, after irradiation and electrophoresis, the ¹⁴C-incorporated activity in DNA is measured and used for quantification of DNA damages.

3.2 Modifications of chromatin

The influence of changes in the chromatin structure and associated scavenging capacity on the radioresponse was studied. To describe the effect of chromatin modulations on the formation of complex lesions, the dose modifying factor, DMF, was calculated as the effect on modified cells divided by the effect in the reference cells at the same radiation dose.

3.2.1 Radical scavenging (Papers I and III)

To abolish the contribution of indirect effect of ionizing radiation and solely investigate the response to direct hits, the potent extrinsic radical scavenger DMSO was present during irradiation (Paper III). To study the radiation response in cells with reduced protection against free radical attacks, all intrinsic soluble scavengers were removed after treatment with a detergent in order to permeabilize the cell membrane (Paper I).

3.2.2 Chromatin condensation (Paper I)

The linker histone H1, as well as the linker DNA, plays a key role in chromatin folding into higher order structure. The presence of cations is essential for the stability in this conformation and hence, reduction of magnesium and sodium cations provokes a relaxation of chromatin. This was used to obtain cells with decondensed chromatin without any further degradation of the structure (Paper I). Nucleoids, histone-free DNA with maintained loop-structure attached to a nuclear protein skeleton, were obtained through high salt denaturation with the addition of a detergent. Also in Paper I, incubation with lysis buffer resulted in the most unshielded structure studied, i.e. naked DNA. In contrast to these gradually stripped off structures, chromatin was condensed into a more compact conformation, relative intact cells, by a moderate high-salt treatment (Paper I).

3.2.3 Cell cycle synchronization (Paper II)

The chromatin changes naturally in cycling cells with regions of exposed open chromatin in S phase and hypercondensed chromatin in the metaphase chromatids in mitosis. In Paper II, the radioresponse was studied after irradiation of cells in different cell cycle phases. Cultured cells were first serum-starved and then synchronized by treatment with mimosine, a drug known to inhibit replication fork elongation in the initiation of S phase. Removal of mimosine allows cells to proceed through S phase as a synchronized population. To collect mitotic cells, this method was combined with mitotic shake-off.

3.2.4 Histone acetylation (Paper III)

Histone acetylation is known to play a key role in the regulation of chromatin condensation and is governed by addition or removal of acetyl groups by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Here, we used the HAT inhibitor garcinol to abate histone acetylation leading to hypoacetylated histone, normally associated with condensed chromatin. In parallel, treatment with the deacetylase inhibitor trichostatin A (TSA) leads to an increase in acetylation of histones, a signature for decondensed chromatin.

3.3 Radionuclides and dosimetry

Cells were exposed to sparsely or densely ionizing radiation. In low-LET experiments in Papers I, II and III, cells were exposed to X-rays (100 kV_p, 8 mA, 1.7 mm Al) and in some experiments presented in Paper III, irradiation was carried out using high voltage photon beam. Both equipments are used for clinical purposes and therefore controlled and calibrated on a regular basis.

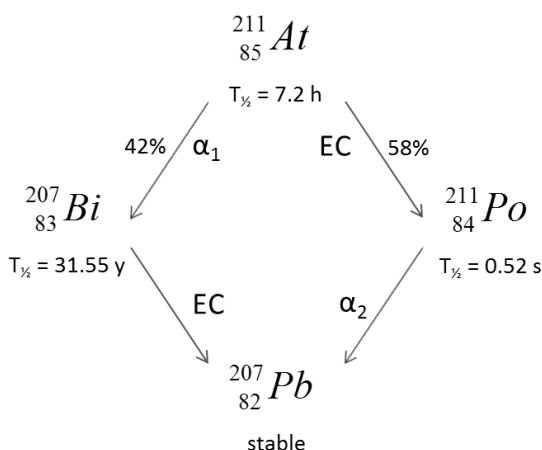


Figure 3. Schematic illustration of the ^{211}At decay.

As high-LET radiation quality, α -particles from ^{211}At (Papers II and III) or ^{213}Bi (Paper III) were used. ^{211}At was isolated after production in a cyclotron through $^{209}\text{Bi}(\alpha, 2n)^{211}\text{At}$ reaction at the PET and Cyclotron Unit, Ringhospitalet, Copenhagen, Denmark (79, 80). Labeling to the non-specific monoclonal antibody MX35 F(ab')₂ was performed by staff at Targeted Alpha Therapy group (TAT group) at the Department of Radiophysics, Gothenburg University, Gothenburg, Sweden according to Lindegren *et al.* (81). ^{211}Bi was produced in a $^{225}\text{Ac}/^{213}\text{Bi}$ generator delivered to the TAT group from the Institute for Transuranium Elements, Karlsruhe, Germany through a procedure described earlier (82, 83).

^{211}At decays with a half-life of 7.21 h in either of two ways as shown in Figure 3. Both possible decay branches result in one α -particle each with the energy 5.87 (α_1) or 7.45 MeV (α_2), respectively. The mean absorbed dose D [Gy], to the total volume of mass m [g], was calculated according to Equation 1. A_0 [Bq] is the activity of the radionuclide at time $t=0$, T [s] is the time of exposure, φ is the absorbed fraction and nE [Gy kg $\text{Bq}^{-1} \text{s}^{-1}$] is the mean energy per transition for each of the two α -particles.

$$D = \frac{A_0 \sum_{i=0}^1 n_i E_i^{\alpha} \times \varphi}{m} \int_0^T e^{-\lambda t} dt \quad (1)$$

$$D = \frac{A_0 (\sum_{i=0}^1 n_i E_i^{\beta} + \sum_{i=0}^1 n_i E_i^{\alpha}) \times \varphi}{m} \int_0^T e^{-\lambda t} dt \quad (2)$$

A simplified picture of the ^{213}Bi decay is presented in Figure 4 and the mean absorbed dose to cells is calculated as according to Equation 2 where the dose contribution from the two α -particles ($E_1=5.87$ MeV, $E_2=8.38$ MeV) as well as the β -particles, β_1 and β_2 , are included. The contribution from the ^{209}Pb decay is considered negligible. Also, because of the very short half-life of ^{213}Po , the emissions of β_1 and α_2 are both included in the decay of ^{213}Bi ($T_{1/2}=45.59$ min).

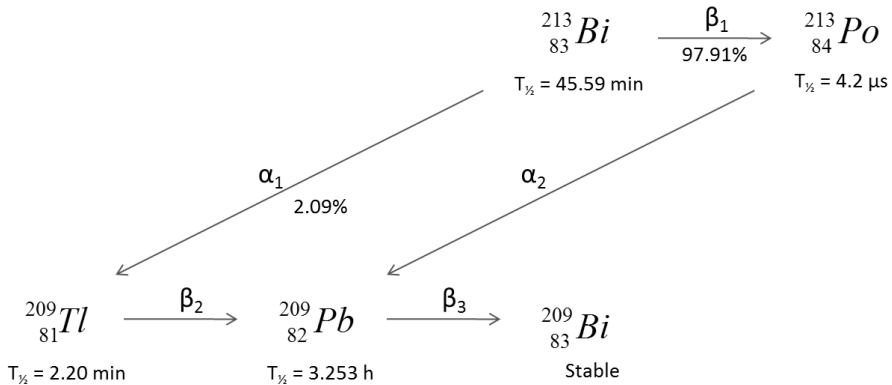


Figure 4. Simplified decay schema for ^{213}Bi .

3.4 Quantification of complex DNA damage

The multistep process in BER including sensing and removal of the damaged base, as well as cutting the DNA strand, thereby introducing a strand break, can be made use of in *in vitro*-studies, early shown in plasmids and bacteriophage DNA (84-86). The use of lesion-specific enzymes was further presented as a tool for converting base lesions situated within a cluster to SSB, thereby introducing new DSB in cellular systems (37, 87). Here, we used Formamidopyrimidine DNA-glycosylase (Fpg) and Endonuclease III (Nth), mainly targeting oxidized purines and pyrimidines, respectively, on DNA from irradiated cells.

To quantify radiation-induced DSB, pulsed field gel electrophoresis was performed on DNA from cells previously labeled with ^{14}C . After electrophoresis, the gel was cut at DNA length standards and the amount of ^{14}C in each corresponding gel piece was measured in a liquid scintillator counter. Depending on experiments, the amount of DSB in each sample was calculated using Blöcher's random breakage formula or by fragment analysis (74, 88, 89). High-LET radiation induces correlated complex lesions resulting in an excess of short DNA fragments and a deficit of long fragments which deviates from the random size distribution found after low-LET irradiation. In fragment analysis, the number of DNA fragments in each size intervals is measured and summarized which gives a more correct quantification of DSB induced by high LET radiation (89). Since post-irradiation treatment with Fpg or Nth cleaves bistranded clusters, these were quantified as the surplus in DSB detected in enzyme-treated cells using the same method as in prompt DSB measurement.

3.5 Cell survival

To investigate the radiosensitivity of cells, the colony forming assay was used in Paper II. The clonogenic survival was calculated as the fraction of irradiated cells that form colonies consisting of at least 50 cells after seeding at low density. Taking into account the plating efficiency found in unirradiated cells, the surviving fraction can be calculated. It is common practice to present cell survival either as the surviving fraction at mean absorbed dose of 2 Gy or as the dose required to reduce cell survival to 37%.

4 RESULTS AND DISCUSSION

The formation of complex DNA damages in cells exposed to sparsely ionizing radiation describes linear dose-response relationships with lesions randomly induced in cellular DNA (Paper I). The linearity found for the DNA damage induction (Figure 5) indicates that one complex lesion is predominantly formed as a consequence of one single event rather than several independent occurrences. Track structure simulations corroborate this, suggesting that one ionization event in the nuclear milieu may produce several free radical pairs with potential, if within diffusion distance, to cause clustered damage in DNA (9, 10).

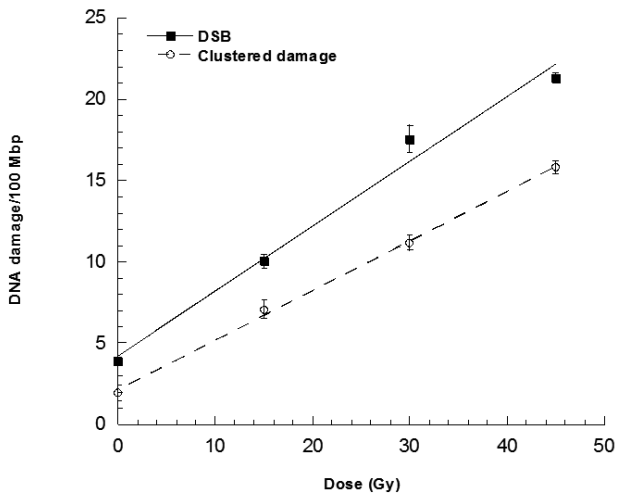


Figure 5. Linear dose-response for complex lesions in intact cells.

Here, we have investigated two large groups of clusters. Bistranded clustered lesions, either containing substrates for Fpg or Nth, are converted into DSB through post-irradiation treatment. According to the manufacturer, some of the main substrates for Fpg are 8-oxoGuanine (8-oxoG), 8-oxoAdenine,

FaPy-Guanine, FaPy-Adenine and similarly, Nth mainly targets urea, thymine glycol and 5,6-dihydroxythymine.

The three types of complex DNA damage, assessed here, is a significant part of the total amount of the complex lesions induced, but for several reasons, not all clusters are included in our method. For example, we do not measure tandem lesions since these are not converted into DSB through enzymatic processing. Further, most AP-clusters are not likely to be cleaved by Fpg or Nth and are therefore not detected in the assay. Indeed, previous data suggest that in human monocytes, γ -rays induce 0.8 AP-clusters/DSB, assessed by post-irradiation treatment with the AP-site specific Endonuclease IV (Nfo) (35). Also, if a cluster is flanked by a DSB, it will not be detected since the resulting DNA fragment is only a few base pairs long and below the limit of resolution in the electrophoretic method.

We present that approximately 20 Fpg-clusters and similar amount of Nth-clusters are induced per cell and Gy in stationary normal human fibroblasts (Papers I and III). This is in the lower range of what has been found by others, even though few studies on normal cells have been performed (35, 37, 46, 47, 90). From induction data on proliferating hamster (Paper II) and human (Paper III) fibroblasts, it appears that fewer clustered lesions are induced in irradiated cycling populations compared with stationary. Also, comparing induction yields for clustered lesions in cells of different origin, it appears that the initial level of clusters varies for different cell types (35, 37, 46, 47, 90). Here we show that low-LET irradiation induces approximately 20-25 DSB per cell and Gy (Papers I-III). This means that in each cell, the total sum of complex damages formed from 1 Gy of sparsely ionizing radiation is at least 60. Accordingly, previous studies suggest that prompt DSB only constitute 20-30% of the total amount of complex DNA damages induced in cells irradiated with sparsely ionizing radiation but the spectrum of lesions has been shown to depend on several factors (38, 87). We present that the number of Fpg- and Nth-clusters induced per DSB is in the range 0.8-0.9 and in cycling cells, the relative number of clusters decreases to 0.5 per each DSB induced (Papers I and III). In the murine cell lines used in Paper III and in the hamster cell line in Paper II these damage ratios are further increased and it can be concluded also from other studies that the damage ratios differ between cells of different origin (37, 38). One could speculate that the decrease in damage ratio in cycling cells compared with stationary cell is because SSB-containing clusters, produced in replicating single stranded DNA, immediately appear as *de novo* DSB. However, this cannot be the only explanation since one then would expect an increased DSB yield in cycling cells compared with stationary cell. On the contrary, we

found no difference in cluster yields between cycling and stationary cells (Paper III). In addition, it is possible that the damage ratios are influenced by differences in the intrinsic level of antioxidants as well as the chromatin conformation and the relative proportion of euchromatin and heterochromatin, respectively. These factors are known to vary between cells of different origin and for example, malignant cells are often associated with an elevated intrinsic oxidative stress. Indeed, in a number of studies, Sutherland and co-workers present that the damage ratios relative DSB fluctuate for clustered damages when irradiation is carried out under different scavenging conditions (36, 38, 86).

4.1 Chromatin conformation – importance for induction

4.1.1 Radical attacks more important for clusters (Papers I and III)

To investigate the contribution of damages induced by free radicals through the indirect effect of radiation, normal human cells were irradiated with photons in the presence or absence of the radical scavenger DMSO. High concentrations of DMSO abolish the radical mediated component and lesions induced are solely due to direct hits in DNA. Results show that 30% of the total amount of DSB and 50% of Fpg- as well as Nth-clusters are induced by radical attacks in normal human stationary fibroblasts (Paper III). This demonstrates that clustered damages are to a greater extent than DSB induced by free radicals produced in close proximity to DNA which corroborates previous findings on clustered damage induction and the influence of the radical scavenger Tris (36). In that study, clusters containing oxidized purines and pyrimidines were equally affected by modifications of the intrinsic scavenging capacity but others have shown, for both low- and high-LET radiation, that the spectrum of lesions varies when the antioxidant level is modulated (38-40). Further, when all soluble scavengers in cells are removed, i.e. the protection against radical attacks is drastically diminished, the same pattern is observed. Accordingly, well over a 3-fold increase in Fpg-cluster formation is detected compared with only 70% extra DSB (Paper I). Consistent with these findings, Nygren *et al.* showed that reduction in the level of intrinsic soluble radical scavengers had a more pronounced effect on

induction of SSB than DSB (91). Similarly, 8-oxo-dG, a frequently formed oxidized base damage, was shown to be affected to a greater extent than strandbreaks in response to diminished antioxidant level (92). Indeed, the correlation of formation of complex lesions and radical scavenging capacity is important since elevated intrinsic oxidized stress which, in conjunction with lower antioxidant capacity, is suggested to be responsible for accumulation of clustered lesions (90, 93, 94). Also, complex damage has been shown to form endogenously at low levels during metabolic processes and in cultured cells and primary cultures, environmental factors such as medium compositions and tobacco use are suggested to influence the DNA damage formation (95-97).

4.1.2 More complex damage in open chromatin structures (Paper I)

One way to study the influence of structural variations in chromatin on complex DNA damage induction is to chemically modify the chromatin compaction in cells before irradiation. By the aid of cations, histone H1 and the linker DNA support chromatin condensation. Reduction of monovalent potassium and divalent magnesium cations leads to decondensation of chromatin without removal of histones (98). Using this strategy, we found that X-irradiation induces over four times more DSB and 11 times more Fpg-specific clusters in normal human cells with a more relaxed chromatin conformation compared with un-treated cells (Table 1). Others have presented similar gain in DSB yield using the same model, and early studies also suggested an increase in strandbreak formation in response to chromatin relaxation (99, 100). Interestingly, it was shown that chromatin decondensation affects SSB and DSB induction yields equally and if this is true also in our experimental setup, the more pronounced effect on Fpg-clusters compared with DSB should be preferentially due to a larger increase in base damage induction (91). Nth-clusters were not assessed in Paper I.

To investigate the role of further chromatin degradation, histone-free DNA attached to cellular matrix (nucleoids) were obtained using salt solution treatment. Furthermore, as the most unshielded structure, induction of complex lesions in naked DNA was studied. Results show an extensive protection against DNA damage by DNA-bound proteins, even more pronounced for clustered damage than for DSB. As presented in Table 1, an increase in Fpg-clusters of 120 and 170 times relative intact cells was measured in nucleoids and naked DNA, respectively. Corresponding dose

modifying factors for DSB are 100 and 120. This dramatic increase in formation of DNA lesions has been shown earlier for both SSB and DSB, and the removal of DNA-bound proteins was shown to affect SSB induction to an even greater extent than DSB, supporting our data (91, 101). Ljungman *et al.* also showed that DNA-bound proteins serve a superior role in the protection against radiation-induced strand breaks compared with soluble intracellular radical scavengers, in line with the tremendous elevation in damage induction found in our experiments on nucleoids and naked DNA, compared with the permeabilization step (101).

In contrast, when the chromatin is condensed using hypertonic medium the induction yields for DSB and Fpg-clusters are slightly but not significantly lower compared with cells with un-modulated conformation (Paper I). Based on earlier studies, this chromatin modification was expected to have larger impact on the DSB induction yield (102). Chromatin is a dynamic structure and the compaction may vary with a factor 10,000, with the hypercondensed chromosomes in mitosis being the most compacted structure. In Paper II we compared the induction of DSB in cells synchronized in different cell cycle phases. X-rays induced 5 times more DSB in mitotic cells compared with cells in G₀-G₁. Interestingly, when analyzing the fragment size distribution it appears that the difference in the level of damage is due to an excess of correlated DSB induced in mitotic cells resulting in a surplus of short DNA fragments, also after low-LET irradiation. It should be emphasized however, that the degree of compaction seen in mitosis is extraordinary and substantially different from what is expected from the hypertonic treatment used in Paper I. More modest chromatin variations occur during S phase and the induction yields presented in Paper II show that the number of DSB induced per unit length of DNA differs as cells progress through the cell cycle. These variations should reflect the influence of chromatin compactness on DNA damage induction, an idea supported by previous studies on synchronized cells irradiated by ¹²⁵I-irradiated cells (103).

4.1.3 Influence of histone acetylation

Histone acetylation is implicated in DNA damage repair and regulated by HATs and HDACs through addition and removal of acetyl groups. Acetylation and deacetylation governs the charge at lysine residues thereby controlling chromatin relaxation and condensation, respectively (18). TSA is a HDAC inhibitor and ideally, incubation with TSA would decondense the chromatin in response to an increased acetylation of histones. In fact,

decondensation of heterochromatic regions after long and short time-incubation with TSA has been visualized in HeLa cells (104).

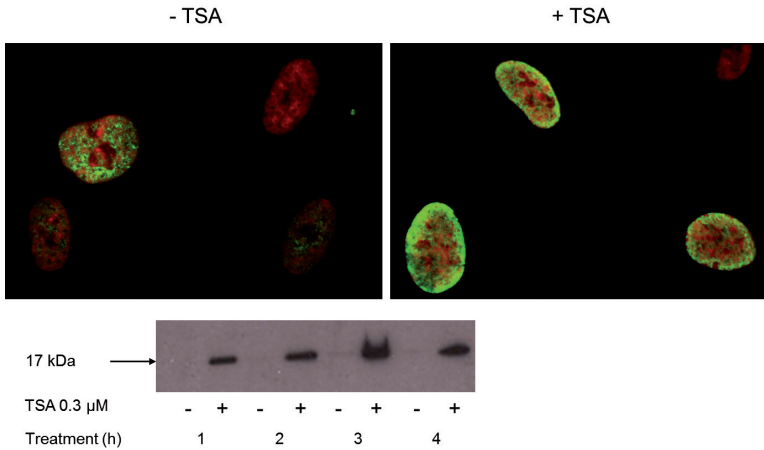


Figure 6. Top: Nuclei from cells treated with (right) or without (left) TSA. Green fluorescence shows hyperacetylated histone H3. Bottom: Western blotting for acetylation of lysine 9 on histone H3.

Indeed, western blotting shows that short time TSA treatment with the concentrations used in Paper III results in acetylation of lysine 9 on histone H3 (Figure 6). Also, using immunohistochemistry, we confirm an enhancement of histone acetylation in TSA treated cells preferentially in the nuclear periphery, regions expected to contain high amount of heterochromatin (Figure 6). However, transmission electron microscopy shows no obvious heterochromatic regions in HS2429 cells and no visible impact on chromatin compactness was seen after TSA-treatment. It is likely that our results on induction and repair of complex lesions describe the influence of histone hyperacetylation only with no contribution due to changes in chromatin compaction.

Indeed, radiosensitizing effects of various HDAC inhibitors on different cancer cell-lines have been documented while no such enhancement was found in normal cell types, as reviewed by (105). For example, the clonogenic survival of low-LET irradiated normal human fibroblasts was not

effected by HDAC inhibitors (106, 107). It is possible that increased acetylation has no effect on our normal fibroblasts.

Table 1. Dose modifying factor (DMF) relative intact stationary normal cells for DSB, Fpg- and Nth-clusters.

Irradiated structure	DMF_{chrom, DSB}	DMF_{chrom, Fpg}	DMF_{chrom, Nth}
Hypercondensed chromatin	0.848 ± 0.094	0.819 ± 0.140	NA
Intact cells	1.00	1.00	NA
Decondensed chromatin	4.75 ± 0.73	11.4 ± 2.2	NA
Nucleoids	103 ± 12	122 ± 20	NA
Naked DNA	123 ± 28	169 ± 28	NA
Acetylation modulation	DMF_{chrom, DSB}	DMF_{chrom, Fpg}	DMF_{chrom, Nth}
Hyperacetylation ^a , stationary cells	1.07	1.00	1.15
Hyperacetylation ^a , cycling cells	0.881	1.43	0.947
Hypoacetylation ^b , stationary	0.985	1.27	1.51

^aTreatment with the HDAC inhibitor TSA gives histone hyperacetylation with assumed association with decondensation of chromatin.

^bTreatment with the HAT-inhibitor garcinol gives histone hypoacetylation with assumed association with condensation of chromatin.

DMF_{chrom} for DSB, Fpg- and Nth-clusters are calculated relative normal untreated fibroblasts irradiated under similar conditions for the same corresponding proliferation status, and are presented in Table 1. Preliminary data shows that TSA-treatment results in little or no effect on induction of complex damage in irradiated stationary cells. In cycling cells however, more than 40% extra Fpg-clusters are induced in TSA-treated cells but too few experiments have been performed to determine significance.

In contrast to HDAC inhibitors such as TSA and the associated histone acetylation, treatment with HAT inhibitors is expected to diminish the degree of acetylation and ideally lead to a more condensed chromatin conformation as a response thereof. We have used the non-specific HAT inhibitor garcinol and its effects on induction of complex lesions are presented as DMF in Table 1. Accordingly, hypoacetylation gives an increase in radiation-induced Fpg- and Nth-clusters with 30 and 50 percent, respectively. This is somewhat unexpected since one would assume a more condensed chromatin structure to serve as an increased protection against mainly the indirect effect of radiation. However, it has been shown that chromatin compaction, induced by inhibitors of protein phosphatases confers increased radiosensitivity in cells (108, 109). Indeed, Price *et al.* showed that the number of total strandbreaks (SSB + DSB) was elevated by a factor 1.6 in chromatin compacted by derivatives of the phosphatase inhibitor cantharidin compounds. In our study, the induction of DSB is unaffected by histone acetylation inhibition by garcinol and the damage ratios for both types of clustered lesions is increased to 1.3 clusters/DSB. It should be noted, however, that maximal chromatin compaction/relaxation is a multi-step process that requires not only posttranslational modifications but other actions as well (110).

4.2 Repair of complex lesions

In normal cells exposed to sparsely ionizing radiation and in similarity to the ligation of DSB, we found that the repair kinetics for clustered damages is biphasic with a rapid decrease in the number of lesions followed by slower rejoining (Papers I and III). The processing of complex lesions was measured for up to 6 h and to study the fast phase of rejoining, the kinetics during the first 30 min was also studied with five-minute intervals in Paper III. This biphasic repair kinetics seen for stationary cells is also found in cycling populations for DSB as well as Fpg- and Nth-specific clusters (Figure 7). As presented in Paper III, there is no statistical difference in processing of

complex lesions between stationary and cycling cells. In fact, our findings are supported by Mayer *et al.* showing no difference in rejoining of SSB or DSB post irradiation due to cell cycle position (111). Similarly, studies on γ H2AX and 53BP1 foci that co-localize at DSB sites, showed no difference in signal decrease between stationary and proliferating populations in low-LET irradiated human epithelial cells (112).

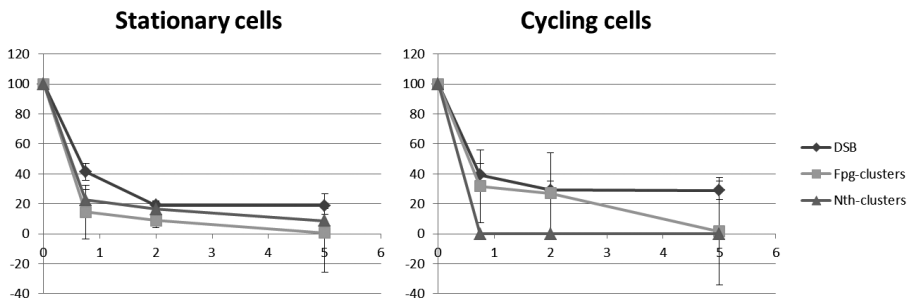


Figure 7. Repair of complex DNA damages of stationary (left) and cycling (right) cells.

There are few studies on the repair of clustered damages in normal cells and those investigating clusters containing oxidized bases, report similar results as demonstrated here with fast and efficient repair (49, 90). Here, we do not find any evidence of formation of *de novo* DSB formed due to enzymatic processing in cells such as presented by others. For example, an increase in DSB formation 30 min post-irradiation in cells overexpressing BER enzymes has been presented, and a correlation between overexpression of glycosylase/AP lyase activity was then suggested (52, 113). Similarly, attempted repair of AP-clusters has been found to result in formation of *de novo* DSB, shown for human monocytes, a few hours after irradiation (114). It is not fully elucidated how clustered damages are processed in human cells but several components involved in the removal of isolated DSB, SSB and base lesions are found to be implicated also in clustered damage repair (41, 45-49). We wanted to further investigate this and assessed the involvement of polymerase β , engaged in BER with both gap-filling and lyase activity as main characters (Paper III). We found that the processing of DSB, Fpg- and Nth-clusters were efficiently repaired in cells with deficient polymerase β

as well as in its parental repair-proficient cell line. In the processing of isolated single base lesions, polymerase λ has been shown to serve as backup when polymerase β is absent (115). Since we found no reduction in the repair efficiency of clusters containing oxidized bases, polymerase β seems not to be indispensable in the processing of clustered damages.

The slow phase in repair of DSB has been suggested to represent the processing of a subgroup of more convoluted lesions or damages formed in heterochromatic regions (26, 116, 117). It has been shown that phosphorylation of H2AX, in response to DSB formation, does not occur to the same extent in heterochromatic regions and that slower repair observed for these lesions is dependent of ATM (117, 118). Further studies have shown that phosphorylation of H2AX also occurs in heterochromatin areas and that, within 20 min after irradiation, the damage sites are re-located to the periphery of heterochromatin, a process that is not dependent on functional ATM (119). However, subsequent repair of these re-located DSB, originally formed in compact chromatin, is dependent on ATM and other downstream mediators in the same pathway (120). At least for DSB, chromatin compaction seems to modify the repair kinetics. Here, we investigated the processing of complex lesions in cells after modification of histone acetylation (Paper III). Also, the reparability of complex damages induced solely by direct hits was detected. It is suggested that NHEJ requires chromatin remodeling and relaxation governed by histone acetylation by HATs (121). Due to this, HAT inhibitors have been suggested to radiosensitize cells and we have used garcinol to investigate the processing in cells with hypoacetylated histones (Paper III). The repair of low-LET radiation-induced complex lesions for times up to 6 hours is presented in Figure 8 and show a minor retardation in the processing of DSB in garcinol-treated cells. After six hours, 40% of initially induced damages are still present in the cells.

Indeed, compromised processing of DSB through NHEJ has been reported after treatment with garcinol concomitant with an increased radiosensitivity of cancer cells (122). In addition, inhibition of HAT co-factors has been shown to suppress recruitment of repair components to DSB sites and thus lead to impaired HRR (123). It is interesting, though, that our preliminary data shows no effect of garcinol on the processing of clustered damages, but rather an increase in repair efficiency compared with un-treated cells. However, data is based on one experiment only and further investigations are needed (Figure 8).

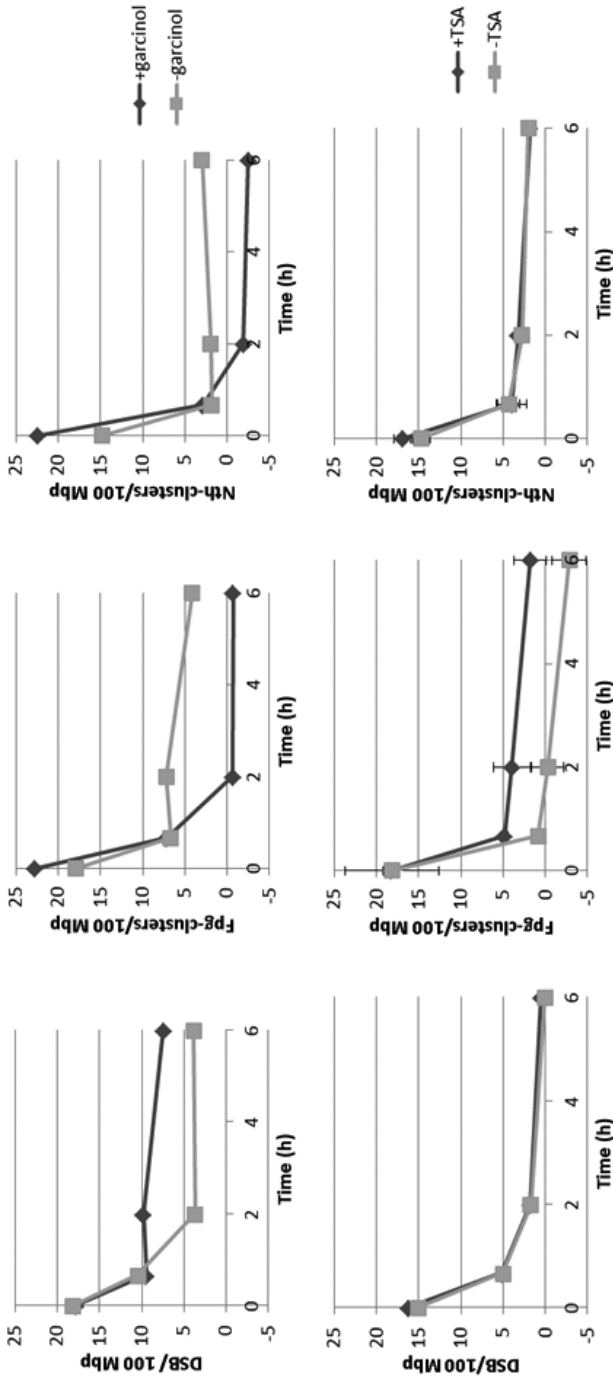


Figure 8. Repair of complex lesions in cells with histone hypoacetylated chromatin (garcinol) and histone hyperacetylated chromatin (TSA).

Also, we investigated the role of enhanced acetylation in the processing of complex lesions and we found, as presented in Figure 8, that TSA does not affect the repair kinetics of DSB or clustered damages. Recently, Manova *et al.* presented data on formation of DSB and the alternative NHEJ pathway in both proliferating and stationary cells (124). In accordance with our results they show that hyperacetylation caused by TSA treatment does affect neither DSB induction nor the processing. Interestingly, and possibly in line with what has been presented here, treatment with HDAC inhibitors has been shown to limit the recruitment of repair components in several different tumor cell types but not in normal cells (105). A lack of effect in our study may reflect a specific influence of HDAC inhibitors on tumor cells only.

For technical reasons, the experiments on processing in cells with hyper- and hypoacetylated histones were performed using different low-LET radiation qualities (X-rays and high energy photons, respectively). Actually, it appears as if DSB induced by X-rays are more efficiently processed than those induced by high voltage photon beam, in line with data from Claesson *et al.* presenting a lower RBE for ^{60}Co γ -rays compared with X-rays (74). High energy photons are expected to produce secondary electrons with a larger energy spectrum contributing relatively more to the total dose than what is expected from X-irradiation. One can speculate that these cause more localized damages challenging the repair process. Interestingly, the damage ratios for both types of clusters are lower after high energy photons compared with X-rays meaning that if this explanation is true, the decrease in repair efficiency should be due to a more pronounced complexity at DSB sites.

The processing of complex lesions induced only by direct hits was studied in cells incubated with the extrinsic radical scavenger DMSO (Paper III). Preliminary data (based on one experiment for long repair times) shows no difference in processing of clustered lesions. For unknown reasons, a pronounced increase in Nth-clusters is observed at 6 hours post irradiation, diverging from corresponding results in similar experiments. Interestingly, preliminary data indicates that DSB induced by direct hits are severer than those formed by radical attacks. One can speculate that direct ionizations cause more “dirty” DSB, i.e. sites flanked by other lesions, suggested to compromise efficient DSB repair due to inhibition of Ku-binding (125).

4.3 Influence of radiation quality

In a chromatin context and due to its energy deposition pattern, high-LET radiation induces correlated DSB with an excess of short DNA fragments responsible for RBE-values higher than unity presented for DSB induction in several studies for different high-LET radiation qualities (74, 89, 99, 126) Table 2 shows the number of complex damages induced by 50 Gy of X-rays, high energy photons or α -particles from ^{211}At and ^{213}Bi , respectively (Papers I-III).

High-LET radiation is proposed to induce dirty DSB and other more complex lesions, also supported by track structure simulations suggesting an increased complexity of DNA damages after high-LET irradiation (127, 128). Due to this, it was surprising when several studies showed RBE-values below unity for induction of clustered damage (40, 84). In Paper II, and in accordance with earlier studies, we found that fewer Fpg-clusters were induced by α -particles than X-rays (RBE=0.59, Table 2). In this study, and in accordance with previously published data, the induction of DSB induced by α -particles increases linearly with dose. We then assumed a linear dose response relationship also for clusters but with poor fit. Rather, it seems as if the induction of clustered lesions is better described by a linear-quadratic relationship for high-LET radiation. Indeed, results in Paper III (Figure 9) present an increase in both Fpg- and Nth-clusters after exposure to α -particles from ^{211}At compared with X-rays (RBE_{Fpg}=1.9, RBE_{Nth}=1.4) as well as high energy photons (RBE_{Fpg}=2.0, RBE_{Nth}=1.3). The difference between the two studies may also be due to cell origin, proliferation status or both. However, it is noteworthy that the amount of DSB induced is identical, representing approximately 65 DSB per Gy in a normal G₀-G₁ cell. In Paper III, we used ^{213}Bi as α -emitting radionuclide primarily for investigations on repair of complex lesions. For unknown reasons, the amount of DSB induced was substantially lower than after exposure to α -particles from ^{211}At measured in the same study (Paper III), in Paper II as well as in a previous study from our group (74). This low level of DSB indicates that cells did not receive the dose of 50 Gy that was calculated for. We can conclude that the deviation seen is most certainly not due to dosimetric mis-calculations or inhomogeneous activity concentration during irradiation procedures. This is because dose calculations have been verified, for example with SRIM simulations, and we know from experimental tests that no intracellular uptake or specific binding of ^{213}Bi to cell membrane occurred, nor binding to plastic surfaces before or during irradiation (data not shown).

It is confusing, however, that the amount of Fpg- and Nth-clusters induced in the ^{213}Bi -experiments is relatively high, and one cannot help but notice the similarity in induction levels and damage ratios between these data and those from low-LET irradiations.

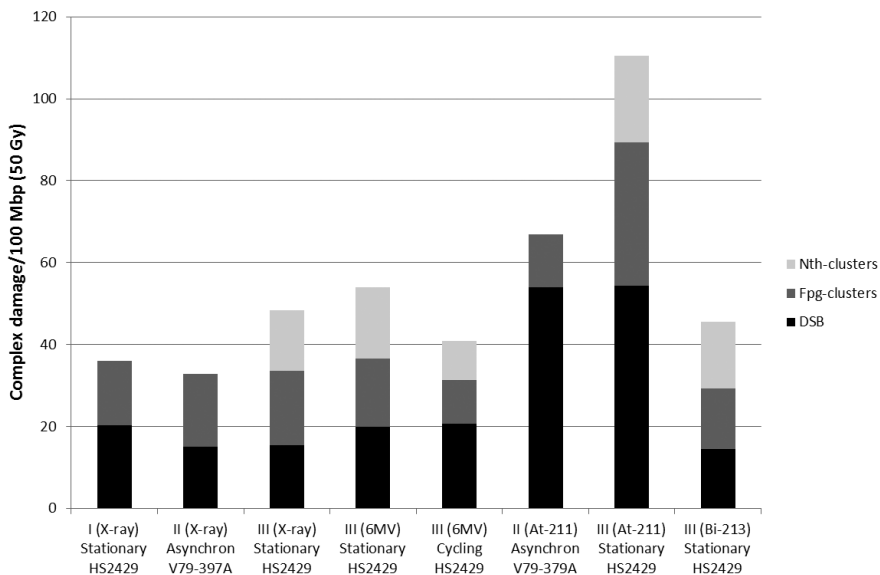


Figure 9. Complex DNA damage induction from Papers I-III for stationary or cycling HS2429 and asynchronous V79-379A for different radiation qualities.

Due to the uncertainties in the dose delivered to cells by ^{213}Bi , we performed identical experiments using ^{211}At which resulted in approximately four times more DSB. These data were used for RBE calculations. Accordingly, α -particles from ^{211}At induce 3.5 times more DSB than X-rays in asynchronous hamster fibroblasts (Paper II) and 2.8 and 3.5 times more DSB than high energy photons and X-rays, respectively, in human normal stationary fibroblasts (Paper III). These RBE-values for DSB induction are in good agreement with our own previous data, comparing ^{211}At with X-rays (RBE=3.1) and γ -rays from ^{60}Co (RBE=2.1) but somewhat higher than RBE

presented by Newman *et al.* for α -particles with similar LET relative X-rays (126).

Table 2. Relative biological effectiveness of α -particles from ^{211}At for induction of DSB, Fpg-clusters, Nth, clusters and 37% cell survival.

	RBE _{DSB}	RBE _{Fpg}	RBE _{Nth}	RBE _{37%}
Stationary ^{1,a}	3.5	1.9	1.4	NA
Stationary ^{1,b}	2.8	2.0	1.3	NA
Asynchronous ^{2,a}	3.5	0.59	NA	8.6
G₀-G₁ ^{2,a}	3.0	NA	NA	6.1
S early ^{2,a}	3.4	NA	NA	7.4
S mid ^{2,a}	3.9	NA	NA	6.1
S late ^{2,a}	2.7	NA	NA	7.9
Mitosis ^{2,a}	1.8	NA	NA	3.1

¹HS2429

²V79-379A

^a α (^{211}At) in relation to X-rays

^b α (^{211}At) in relation to high energy photons

Ever since it was reported 30 years ago that the radioresponse after high-LET irradiation does not differ between cell cycle phases, few studies have been performed to further investigate this (129). In Paper II we present cell cycle dependence for the induction yield of DSB induced by α -particles from ^{211}At . The amount of DSB induced differs significantly from G₀-G₁ in both S phase and mitosis, with approximately twice the lesions in S and nearly 7 times more in mitosis. The influence of cell cycle stage on induction of DSB differs

after low- and high-LET irradiation, and hence, RBE varies between cell cycle phases, and is lowest in mitosis (1.8) and highest in mid S phase (3.9), as presented in Table 2. For cell survival, and in accordance with cell cycle dependence presented for DSB, an increased radiosensitivity is noticed in S phase with the poorest survival in mitotic cells. RBE for 37% survival is lowest in mitosis (3.1) and highest in late S phase (7.9).

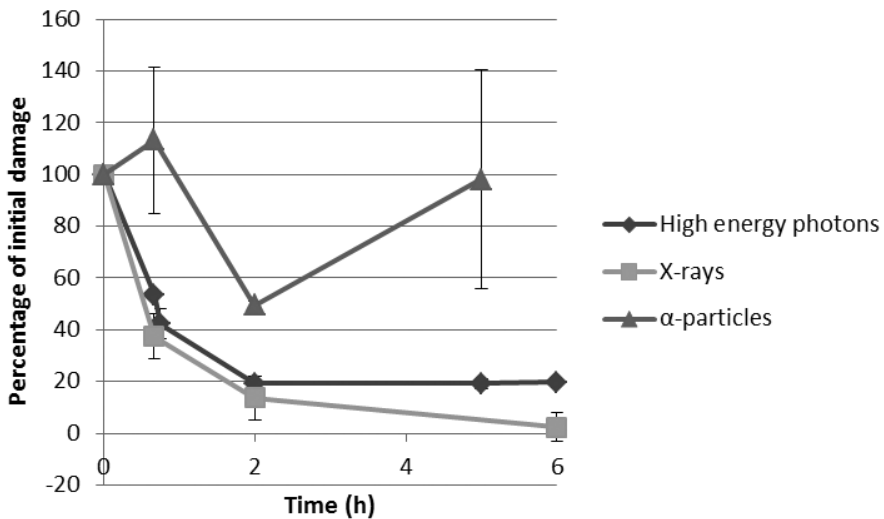


Figure 10. Repair of DSB induced by α -particles, X-rays or high energy photons.

In Paper III, the repair of clustered damages after high-LET radiation was assessed. Normal stationary human fibroblasts were exposed to α -particles from ^{213}Bi and repair kinetics showed efficient processing of clustered lesions. For DSB, approximately the same amount of lesions as initially induced is present in the cell after 6 h (Figure 10). For some reason, at 2 h post irradiation, the level of DSB drops to approximately 50% of initial levels. However, these data should be interpreted with caution since there is some questioning according to ^{213}Bi -irradiations, as discussed above.

5 CONCLUDING REMARKS

It can be concluded that in cells with chemically modulated chromatin structures, complex DNA damages are induced linearly with dose and for each step of chromatin depletion, the induction yields increase demonstrating a stepwise reduction in functional and structural defense against direct and indirect effect of ionizing radiation. Further, it appears as if in some cases the degree of acetylation influences the formation of clustered lesions but not DSB.

It can further be summarized that clustered damages containing oxidized purines or pyrimidines are efficiently processed, also under conditions where repair are expected to be compromised. We found no evidence of formation of de novo DSB due to attempted repair, regardless of radiation quality. Our results imply that clustered damages are not a challenge to the repair machinery. The initial level of complex lesions measured at time zero represents a situation where the cellular repair machinery has not yet been triggered and no partial processing has begun. At later time points when repair of lesions is under progress, the substrates for Fpg and Nth cleavage could be completely different. For example, attempted repair of one or more of the damaged sites within the cluster may inhibit or retard cleavage by our enzymes. Also, recognition and processing by Fpg or Nth do not result in a detectable fragment if the cluster has already been partially repaired. The rapid decrease in cluster levels observed during the first hours of repair could be interpreted as an efficient removal of isolated clusters that do not contribute significantly to mutagenesis or cytotoxicity, or reflecting lesions too complex to be cleaved in post irradiation BER enzyme incubation. The latter interpretation implies that ionizing radiation-induced clusters are repair resistant and hence hazardous to exposed cells.

The biological consequences of persistent damages are not fully elucidated. As reviewed in Paper IV, several lesion combinations may impede or delay the processing of clusters and if present during replication, inefficient repair may result in replication-induced DSB. Repair-resistant clusters can also be mutagenic or cytotoxic thereby contributing to the carcinogenic risks associated with exposure to ionizing radiation.

ACKNOWLEDGEMENTS

This research work has been performed at the Department of Oncology, University of Gothenburg, Sweden and was supported by the King Gustav V Jubilee Clinic Cancer Research Foundation, The Swedish Radiation Authority, Assar Gabrielsson Foundation and The National Board of Health and Welfare, Sweden.

There are several people who in different ways have contributed to this thesis and I want to express my sincere appreciation and gratitude to you all. Especially, I want to thank:

My supervisor **Kecke Elmroth** for introducing me to the interesting field of radiobiology and for sharing your great knowledge in such an inspiring way. Thank you also for your support and for convincingly showing me that you believe in me. It has been a privilege to work with you.

My co-supervisor **Ragnar Hultborn** for interesting scientific discussions. Also, I want to thank you, as head of the Department of Oncology, for giving me the opportunity to do my doctoral studies at the department.

Targeted Alpha Therapy group at the department of Radiophysics for nice collaboration. Especially I would like to thank **Lars Jakobsson** for always taking time for valuable discussions and for sharing your great knowledge in radiation physics and **Sture Lindegren** for technical support and scientific discussions.

All present and former colleagues and friends at the Department of Oncology and Radiation Physics for creating an inspiring and pleasant atmosphere. Especially I would like to thank Elin, Lovisa, Karolina, May, Toshima, Jörgen, Kerstin, Ingegerd, Khalil, Ingela, Anna, Elin, Maria, Nils, Emil and Anders.

Ulla for being such a wonderful colleague, friend and the best technical support one could ever ask for.

Helena for interesting discussions on literature, music, nature and life in general, for laughter and for times in the wild and in the lab.

Madeleine, my co-worker and friend, for always being a helping hand. With you in the group, going to work in the morning this last year has been so much more fun.

Kristina for your support during these years and for all our discussions on scientific and non-scientific issues. I wish you all the luck.

Håkan for your support and for the music and, even if you don't know it, for making me observe those flying creatures. **Niklas** and **Pernilla** for laughter and for introducing me to the most magnificent of animals. And **Jon** for your endless support and patience and for guiding me until I finally came to love them all. Even the silly ones. All four of you, thank you for your unique friendship. You have enriched my life.

Madelene and **Jessica** for all years of friendship. If it wasn't for that silly pink cap...

Linnea, my dear friend and cousin, for always being there.

Karin, my former room-mate and dear friend. Thank you for your support and friendship during these years, making life much easier and more fun.

Nina for being such a lovely friend, for always supporting and helping me out. You have a unique way of making me feel fantastic, and if I am, I am positive that it is partly thanks to you.

Lena and **Janne** for being the most fantastic neighbors. Your generosity and helpfulness is invaluable.

Johanna and **Christian** for being such wonderful friends. For not being family you're very much like family.

My family for your endless support and especially **my parents**, this had not been possible without you. You are truly amazing.

Tobias for being the most wonderful friend and husband, for loving me and for always believing in me. I love you.

Alva, the love of my life. You are the best thing that has ever happened to me. I have missed you so much.

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