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# POLYCYCLIC AROMATIC HYDROCARBONS

## DNA DAMAGE AND CELL SIGNALING

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Stockholm 2008

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ISBN 978-91-7409-265-3

*Till Min Familj*

# ABSTRACT

Oxidative stress is a threat to our wellbeing. The formation of reactive oxygen species may result in oxidative lesions in DNA and RNA. When challenging A549 cells with $^{18}$O-labeled hydrogen peroxide, we found that RNA was between 14-25 times more sensitive to [$^{18}$O]-8-oxoGuo formation than [$^{18}$O]-8-oxodGuo formation in DNA. The A549 cells showed slow turnover rates of adducts in RNA and DNA with half-lifes of approximately 12 h for [$^{18}$O]-8-oxoGuo in RNA, and 21 h for [$^{18}$O]-8-oxodGuo in DNA, respectively.

Polycyclic aromatic hydrocarbons (PAHs) are widespread mutagenic and carcinogenic environmental contaminants. They require metabolic activation into electrophilic diol epoxides (DEs) to be able to bind to DNA and elicit their biological activity.

The bay-region DE of benzo[*a*]pyrene (BPDE) is more easily removed by nucleotide excision repair (NER) than the fjord-region DE of dibenzo[*a,l*]pyrene (DBPDE). This could reflect the ability of DBPDE to escape recognition and to investigate this, we studied the affect these DEs have on histone H2AX phosphorylation (γH2AX). Human A549 cells were exposed to the DEs for various time and concentration. The results showed that BPDE induced a transient γH2AX, while DBPDE exposure resulted in a continuously increasing and persistent γH2AX and these data correlated with the known effect on nucleotide excision repair (NER). Thus, the extent of γH2AX formation and the persistence was related to both the number of adducts and their structural feature.

Further, the γH2AX, as well as effects on Mdm2 and p53 were studied in A549 cells in response to the bay-region DEs of chrysene (CDE) and dibenz[*a,h*]anthracene (DBADE), or the fjord-region DEs of benzo[*c*]chrysene (B[*c*]CDE), benzo[*g*]chrysene (B[*g*]CDE) and benzo[*c*]phenanthrene (B[*c*]PhDE). We found that the fjord-region DEs induced a rapid and concentration-dependent response on Mdm2 2A10 phosphorylation, p53 stabilization and phosphorylation, as well as γH2AX, where Mdm2 was the most sensitive marker. The bay-region DEs had less effect on Mdm2 2A10 phosphorylation and induced neither p53 stabilization nor phosphorylation. No γH2AX was detected with Western blot in response to bay-region DEs, however, immunostaining revealed reversible γH2AX. Also here, the variance between bay- and fjord-region DEs most likely reflect their recognition and handling by NER.

PAH contamination of soil at industrial setting constitutes a risk to humans, but the risk is often difficult to estimate due to the complexity of present contaminants. We compared the DNA damage signaling effects in HepG2 cells exposed to PAHs extracted from contaminated soils collected at six different industrial settings in Sweden. Most of the soil extracts induced Mdm2 2A10 phosphorylation at low concentration, which may indicate repairable damage. We found concentration- and time-dependent γH2AX and 53BP1 responses, sustaining up to 48 h indicating persistent damage. Effects on cyclin D1 and p21 indicated cell cycle arrest, and phosphorylation of Mdm2 at Ser166, known to attenuate p53 response, was found and was associated with Erk phosphorylation. The PAH extracts elicited unpredictable DNA damage signaling that differed between the samples, and where also more polar compounds, oxy-PAHs, contributed. We found that established approaches to evaluate carcinogenic potentials of PAH mixtures in contaminated soil are insufficient and call for the development of more sophisticated endpoints.

## LIST OF PUBLICATIONS

I. Hofer T, Badouard C, Bajak E, Ravanat JL, **Mattsson Å**, Cotgreave IA. Hydrogen peroxide causes greater oxidation in cellular RNA than in DNA. *Biol. Chem. 386 (2005) 333-337*

II. **Mattsson Å**, Jernström B, Cotgreave IA, Bajak E. H2AX phosphorylation in A549 cells induced by the bulky and stable DNA adducts of benzo[*a*]pyrene and dibenzo[*a,l*]pyrene diol epoxides. *Chem. Biol. Interact. 2008. In press. DOI: 10.1016/j.cbi.2008.09.015*

III. **Mattsson Å**, Malmlöf M, Seidel A, Stenius U, Jernström B. H2AX, Mdm2 and p53 phosphorylation are differently affected by bay- and fjord-region diol epoxides derived from carcinogenic polycyclic aromatic hydrocarbons. *Polycyclic Aromatic Compounds. 2008. In press.*
*DOI: 10.1080/10406630802374937*

IV. **Mattsson Å**, Lundstedt S, Stenius U. Effects of complex PAH mixtures from contaminated soils on DNA damage signaling in HepG2 cells. *Submitted manuscript*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ATM | Ataxia-telangiectasia mutated |
| ATR | ATM-Rad3-related |
| AhR | Aryl hydrocarbon receptor |
| B[*c*]CDE | Benzo[*c*]chrysene diol epoxide |
| B[*c*]PhDE | Benzo[*c*]phenanthrene diol epoxide |
| B[*g*]CDE | Benzo[*g*]chrysene diol epoxide |
| BP | Benzo[*a*]pyrene |
| BPDE | Benzo[*a*]pyrene diol epoxide |
| CYP | Cytochrome P450 |
| DBADE | Dibenz[*a,h*]anthracene diol epoxide |
| DBP | Dibenzo[*a,l*]pyrene (dibenzo[*def,p*]chrysene) |
| DBPDE | Dibenzo[*a,l*]pyrene diol epoxide |
| DE | Diol epoxide |
| DNA-PK | DNA-dependent protein kinase |
| DSB | Double stand break |
| EH | Epoxide hydrolase |
| GGR | Global genome repair |
| γH2AX | Phosphorylated H2AX |
| Mdm2 | Murine double minute 2 |
| NER | Nucleotide excision repair |
| PAH | Polycyclic aromatic hydrocarbon |
| PIKK | Phosphatidylinositol 3-kinase like protein kinase |
| ROS | Reactive oxygen species |
| TEF | Toxic equivalency factor |
| TEQ | Toxic equivalency quota |
| 53BP1 | p53 binding protein 1 |

# GENERAL BACKGROUND

The importance of genomic stability in preventing carcinogenesis is well known [Hoeijmakers, 2001]. A perplex diversity of DNA lesions may arise from environmental agents (*e.g.* ionizing radiation, ultraviolet light, and genotoxic contaminants), or from byproducts of normal cell metabolism (*e.g.* reactive oxygen species and reactive nitrogen species). These damages on DNA can, if left unrepaired, lead to mutations and initiation of carcinogenesis.

Chemical carcinogens are often divided into mutagenic/genotoxic or non-genotoxic carcinogens [Weisburger and Williams, 1983]. Genotoxic carcinogens may directly interact with DNA, require metabolic activation into DNA interacting intermediates or alter the fidelity of DNA replication. A genotoxic compound act as a tumor initiator and does not have a threshold dose, and a non-genotoxic carcinogens act as a tumor promoter and exhibit threshold tumor dose-response [Melnick *et al*., 1996].

Cancer is a disease that involves dynamic changes in the genome with mutations that activate oncogenes resulting in dominant gain of function, and inactivate tumor suppressor genes associated with recessive loss of function. Human carcinogenesis is a multistep process, and the steps reflect genetic alterations that drive the progressive transformation of normal cells into malignant derivatives [Hanahan and Weinberg, 2000]. Six essential alterations in cell physiology were suggested by Hanahan and Weinberg to manifest cancer, "the hallmarks of cancer". They are; self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, evading apoptosis, and tissue invasion and metastasis. Virtually all cancers must gain the same six hallmarks, but their means of doing so may vary significantly both mechanistically and chronologically. In some tumors, a certain genetic lesion may result in the gaining of several capabilities at the same time, decreasing the number of distinct mutational events needed to complete carcinogenesis. While in other tumors, gaining one capability migth require the collaboration of two or more distinct genetic changes, thereby increasing the total number of events necessary for completion of tumor progression [Hanahan and Weinberg, 2000].

In experimental literature, the carcinogenic process is often divided into three steps; initiation, promotion and progression. Tumor initiation requires that the compound cause changes in DNA, and that the change is fixed as a mutation in DNA after DNA replication. A compound that is capable of directly inducing mutations in DNA is, as stated above, called genotoxic. The tumor initiation is irreversible, unless the cell is removed by cell death (apoptosis). The promotion phase of carcinogenesis involves clonal expansion of initiated cells. This is commonly induced by increased cell proliferation, as well as inhibited apoptosis. Compounds that are tumor promotive do not require interaction with DNA to exert their action, unlike chemical mutagens. The progression is characterized by an increased genomic instability and further development towards malignancy and autonomous cell growth, and is considered irreversible. [Boström *et al*., 2002].

In this thesis, damages in nucleic acids have been studied in human cell systems. The first study involves oxidation of RNA and DNA in response to hydrogen peroxide, where RNA was found to be much more sensitvie to this type of damage than DNA. The study also showed slow turnover rates for the oxidative adducts, with half-lives that was almost the double for the oxidatively damaged DNA compared to RNA.

In the second study, we investigated factors underlying the variance in carcinogenic potency of two polycyclic aromatic hydrocarbon metabolites belonging to different structrual groups. By analysing their effect on a DNA damage recognition protein, we found a difference in the evoked signal between the two metabolites that correlated with previous results on DNA adduct formation and removal.

The third study confirmed the results from study two, in that polycyclic aromatic hydrocarbon metabolites from the two groups affect DNA damage signaling differently. Here, a total of five metabolites were investigated, and their effect on three signaling protein was assessed.

In the fourth study, the effects of complex mixtures of polycyclic aromatic hydrocarbons extracted from contaminated soil on DNA damage signaling were investigated. This study showed that the established approaches to evaluate the risk of exposure to complex mixtures of polycyclic aromatic hydrocarbons are insufficient.

# POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are versatile environmental contaminants of which some have mutagenic and carcinogenic potency [Phillips, 1999]. They are a group of highly lipophilic organic compounds consisting of two or more condensed benzo rings. PAHs are formed during incomplete combustion of organic matter. The main sources are industrial handling of coal and oil, domestic heating and natural forces, *e.g.* forest fires and volcano activity. PAHs can spread over large areas bound to soot particles and the main route of exposure in non-smokers is through the diet. For instance, leafy vegetables may be a significant source of exposure due to deposition of small airborne particles on their surface. Smoked and charcoal cooked meat and fish are other food sources of PAHs [Phillips, 1999]. Life-style factors, such as cigarette smoking, and certain occupations, such as fossil fuel processing, aluminum production, steel and iron foundries, wood impregnation and road paving may greatly increase the exposure [Baird *et al*., 2005].

Benzo[*a*]pyrene (BP) is one of the most well-studied PAHs. Since its isolation from coal tar in the 1930s, and the discovery that BP is a potent carcinogen, it has served as a model compound [Phillips, 1983]. PAHs can be divided into bay- and fjord-region compounds depending on their structure (Figure 1A), where the fjord-region compounds represent non-planar compounds that are more powerful mutagens/carcinogens than the planar bay-region PAHs. The biological activity of PAHs is dependent on the metabolic formation of reactive diol epoxides (DEs) (Figure 1B), which may bind covalently to specific targets in DNA [Szeliga and Dipple, 1998].

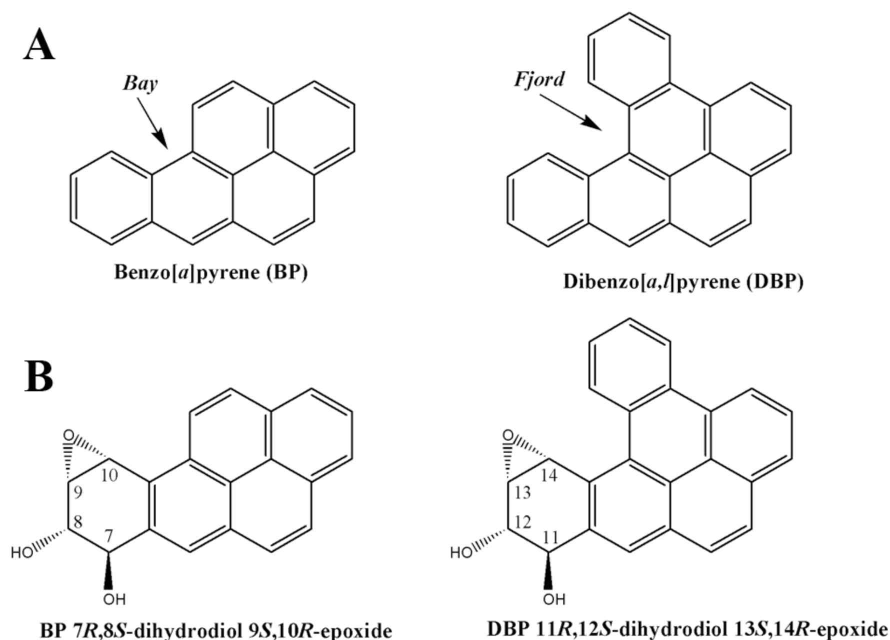


**Figure 1.** In A, the structure of two PAHs, the bay-region benzo[*a*]pyrene and fjord-region dibenzo[*a,l*]pyrene. In B, the ultimate carcinogenic metabolites of the respective PAHs.

## METABOLIC ACTIVATION

Since PAHs are highly lipophilic compounds, they require metabolism into more hydrophilic molecules for our body to be able to excrete them. In the process of making PAHs water-soluble, reactive electrophilic intermediates are formed that, before they are inactivated again, may interact with DNA to form adducts.

The distribution and toxicity of xenobiotics are dependent of the absorption, distribution, metabolism and excretion in the body. The absorption determines the bioavailability to the body, and is affected by compound solubility, chemical stability in the acidic stomach and permeability to the intestine wall. Many compounds enter the body orally, and via the digestive tract enter the bloodstream, from which it may be taken up into target organs or cells. The distribution of xenobiotics into different tissues and organs varies, and is mainly dependent on the lipophilicity of the compound. Barriers, such as the blood-brain barrier affect the distribution of xenobiotics, as well as transporter proteins. The metabolism, or biotransformation, depends on the hydrophilicity of the compound where hydrophilic compounds are easily excreted via urine, while lipophilic compounds either are stored in lipophilic compartments or biotransformed into more water-soluble compounds. The compounds and their metabolites may then be removed from the body by excretion, either via urine through the kidneys, or with the feces through bile.

Biotransformation of PAHs is performed by cytochrome P450s (CYPs), epoxide hydrolase (EH) and detoxicifation enzymes, such as glutathione transferases, uridine diphosphate, glucuronosyl transferases, epoxide hydrolases and sulfo transferases (Figure 2) [Baird *et al*., 2005; Shimada, 2006]. The two most important CYPs in PAH metabolism are CYP1A1 and CYP1B1. They are mono-oxygenases that add an epoxide group to the PAH, which can be converted into a dihydrodiol by EH. This metabolite may be further metabolized by CYPs to form a diol epoxide (DE), which is the ultimate carcinogen of a PAH [Shimada and Fuiji-Kuriyama, 2004]. The most carcinogenic metabolite of BP is the (+)-*anti*-benzo[*a*]pyrene-7*R*,8*S*-diol-9*S*,10*R*-epoxide [(+)-*anti*-BPDE) (Figure 1B), which is one of four possible enantiomer. Subsequently, the (+)-*anti*-BPDE may covalently interact with DNA, preferably with the exocyclic amino-group of 2'-deoxyguanosine in the minor groove through *trans* opening of the epoxide, which, if not repaired, can induce mutations such as G $\rightarrow$ T transversions during DNA replication [Luch, 2005]. Dibenzo[*a,l*]pyrene (DBP) is a fjord-region PAH, and the strongest tumor-initiating PAH identified so far. It is metabolized into the ultimate carcinogenic metabolite (-)-*anti*-dibenzo[*a,l*]pyrene-11*R*,12*S*-diol-13*S*,14*R*-epoxide [(-)-*anti*-DBPDE] (Figure 1B) [Mahadevan *et al*., 2005], which preferably interact with the $N^6$-amino group of deoxyadenosine in the major groove of DNA.

The arylhydrocarbon receptor (AhR) is a ligand activated transcription factor that can up-regulate *CYP* genes. PAHs are ligands for the AhR, and PAHs may thus increase the level of CYPs that are responsible for their metabolism [Nebert *et al*., 2004]. When a PAH binds to the AhR, it results in the activation and translocation of the complex into the nucleus where it heterodimerizes with the AhR nuclear translocator (ARNT). This complex may in turn bind to xenobiotic-responsive elements (XREs) and induce the

expression of a variety of genes involved in carcinogen metabolism, as well as factors involved in cell growth and differentiation.

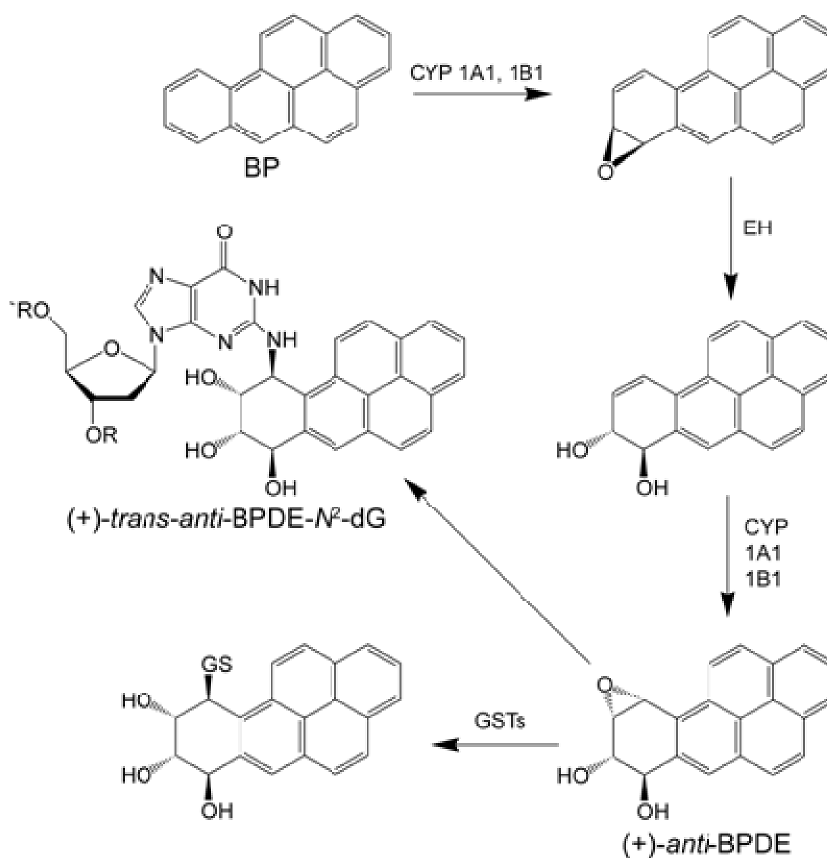


**Figure 2.** Major pathways for metabolic activation and DNA-binding of PAHs, exemplified by BP.

## REMOVAL OF PAH ADDUCTS

Maintenance of DNA integrity is essential for the viability of cells and the health of organisms. The major repair pathway to remove bulky DNA adducts in eukaryotes is via the nucleotide excision repair (NER) [Geacintov *et al*., 2006]. NER has extraordinary wide substrate specificity and is able to recognize and repair a large number of structurally different lesions. It involves recognition of the DNA damage, opening of the DNA helix around the lesion, dual incision on both sides of the damage, excision of the oligonucleotide containing the DNA lesion, and subsequent DNA repair synthesis [Gillet and Schärer, 2006]. Two distinct subpathways of NER has been described; global genome repair (GGR) that may detect and remove lesions throughout the genome, and transcription coupled repair (TCR) that ensures faster repair of lesions located on the transcribed strand of actively transcribed genes.

In GGR (Figure 3), the XPC-hHR23B complex detect the damage distorted DNA, stabilizes the bend and recruit the transcription factor TFIIH to the site. In TCR (Figure 3), the stalled RNA polymerase II is detected by CSB and CSA, which attracts TFIIH

to the site [Hoeijmakers, 2001]. After TFIIH, the two helicases XPB and XPD are recruited and unwind the DNA. Next, the “pre-incision complex” RPA, XPA and XPG bind to the site. RPA is thought to bind to the undamaged strand of the open DNA bubble and allow correct positioning and stimulation of the endonucleases. XPA together with RPA control the proper assembly of the NER preincision complex by confirming the existence of the lesion. XPG is an endonuclease with cleaving specificity at the 3’ incision of the lesion. Subsequently, the ERCC1-XPF endonuclease compelx assembles 5’ of the lesion and a dual incision is made. In the last step, RPA facilitates the transition to DNA repair synthesis by aiding the binding of RFC, which in turn helps with the loading of the polymerase processivity factor PCNA. PCNA slides along DNA and interacts with DNA polymerase δ or ε and DNA ligase I finally seals the nick [Dip *et al*., 2004; Gillet and Schärer, 2006; Thoma and Vasques, 2003]. Recent research also suggests a role for DNA polymerase κ in NER [Ogi and Lehmann, 2006].

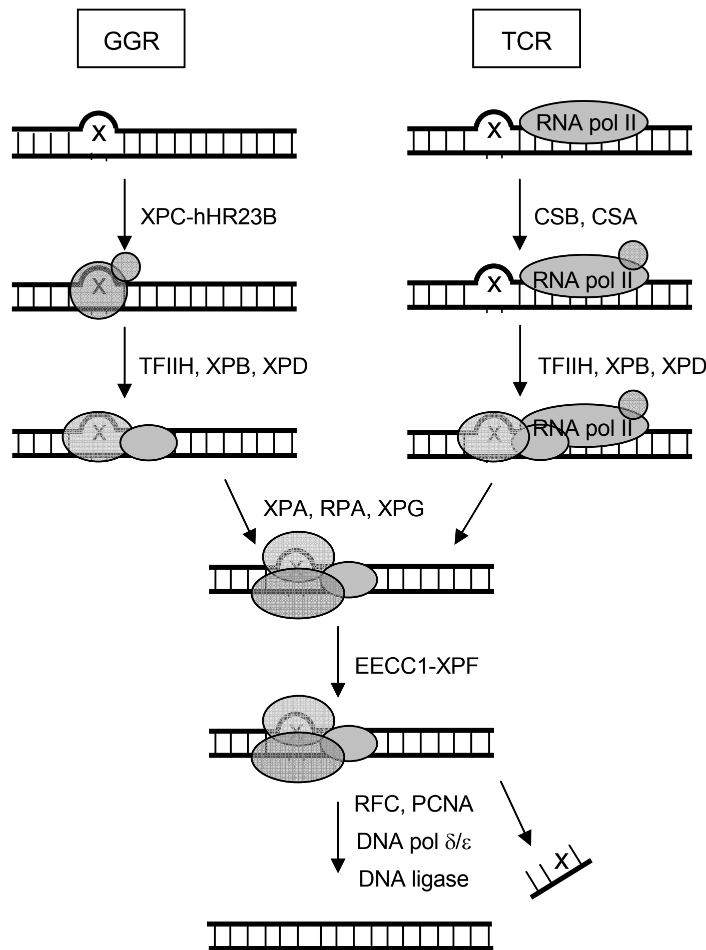


**Figure 3.** Overview of the GGR and TCR nucleotide excision repair pathways.

There are diseases associated with deficiency in NER genes. Patients with xeroderma pigmentosum (XP) are extremely sensitive to sunlight, and have more than a thousand-fold increased risk of developing skin cancer. Individuals with XP also have a higher incidence of internal tumors, and sometimes neurological abnormalities. They are classified into seven repair-deficient complementation groups designated XPA-XPG [Dip *et al*., 2004]. Cockayne syndrome (CS) is a rare disorder caused by mutation in the *CSA* or *CSB* genes, associated with a variety of clinical symptoms including dwarfism, mental retardation, eye abnormalities, photosensitivity and premature ageing, but no enhanced susceptibility to cancer. At the cellular level, CS results in the inability to resume damage inhibited DNA and RNA synthesis after exposure to UV-light and chemical agents that induce bulky DNA adducts. Trichothiodystrophy (TTD) is also associated with NER and TCR, and shares many symptoms with CS, but with the additional hallmarks of brittle hair, nails and scaly skin [Hoeijmakers, 2001]. The effects these diseases have on an individual implicate the importance of these repair systems.

## PAHS AND CANCER

The wide spread of PAHs in the environment and their negative association with human health implicate the importance of the ongoing study of PAHs. The modern research on carcinogenesis induced by PAHs began with the isolation of BP in the 1930s and the finding that BP initiate tumors when repeatedly painted on mouse skin [Baird *et al*., 2005]. Some fifteen years before that, pathologists in Japan showed formation of malignant epithelial tumors when applying coal tar to the ears of rabbits [Luch, 2005], but the active component responsible for the tumors was not isolated from coal tar until 1933, and was found to be BP [Luch, 2005; Phillips, 1983].
In 1974 Sims *et al.* showed that it is the 7,8-diol-9,10-epoxide of BP that is responsible for BP DNA adducts. Bay-region PAHs (*e.g.* BP) has been observed to likely be potent carcinogens, and fjord-region PAHs (*e.g.* DBP) are even more potent carcinogens [Baird *et al*., 2005].

PAHs are procarcinogens since they require metabolic activation into their ultimate carcinogenic form [Luch, 2005]. They may have either mutagenic/genotoxic or non-genotoxic actions, and PAHs with both initiator and promoter activities are considered complete carcinogens [Boström *et al*., 2002]. Studies have revealed a structural requirement for PAHs to be mutagenic and carcinogenic, where alternant PAHs composed of fused benzo rings only (such as BP and DBP) must be composed of at least four rings and arranged in a fashion that it contain a bay- or fjord-region. There are exceptions to this rule, but in general, there seems to be a positive correlation between the number of benzo rings and carcinogenicity [Boström *et al*., 2002]. The International Agency for Research on Cancer (IARC) have classified carcinogenic PAHs (Table 1) as carcinogenic to humans (group 1), probably carcinogenic to humans (group 2A), possibly carcinogenic to humans (group 2B) or not classifiable (group 3).

**Table 1.** IARC classification of PAHs used in this thesis

| PAH compound | Number of rings | IARC group* |
|---|---|---|
| Acenaphthene | 3 | 3 |
| Acenaphthylene | 3 | not evaluated |
| Anthracene | 3 | 3 |
| Benz[*a*]anthracene | 4 | 2B |
| Benzo[*c*]chrysene | 5 | not evaluated |
| Benzo[*g*]chrysene | 5 | 3 |
| Benzo[*b*]fluoranthene | 5 | 2B |
| Benzo[*k*]fluoranthene | 5 | 2B |
| Benzo[*ghi*]perylene | 6 | 3 |
| Benzo[*c*]phenanthrene | 4 | 2B |
| Benzo[*a*]pyrene | 5 | 1 |
| Chrysene | 4 | 2B |
| Dibenz[*a,h*]anthracene | 5 | 2A |
| Dibenzo[*a,l*]pyrene | 6 | 2A |
| Fluoranthene | 4 | 3 |
| Fluorene | 3 | 3 |
| Indeno[1,2,3-*cd*]pyrene | 6 | 2B |
| Phenanthrene | 3 | 3 |
| Pyrene | 4 | 3 |
| Naphthalene | 2 | 2B |

* IARC Monograph vol. 32, 82 and 92 (in preparation).

Benzo[*a*]pyrene is often used as an indicator for PAHs in mixtures and the cancer potency of PAHs are often compared to, and ranked relative to BP. Toxic equivalency factors (TEFs) is a system where compounds have been given a relative number compared to a reference compound. TEFs can be used for large groups of compounds with a common mechanism of action, when there are limited data except for one reference compound. The TEF value for a substance is based on; 1) that a reasonably well-characterized reference compound exists, 2) the toxic effects are qualitatively similar for all members of the class, 3) TEFs for different toxic end points are similar, and 4) the toxic effects of different compounds in a mixture are additive. For PAHs, the reference compound is BP, and its TEF is set to one. There exist different TEF values for individual compounds, depending on what studies the authors based their evaluation on [Boström *et al*., 2002].

# DNA DAMAGE CELL SIGNALING

Maintaing genomic stability and regulating cell survival and death in response to DNA damage is essential for the organism. In this thesis, some proteins involved in these processes have been studied after exposure to polycyclic aromatic hydrocarbons and their reactive intermediates.

## THE PI3K-LIKE PROTEIN KINASE FAMILY

The phosphatidylinositol 3-kinase like protein kinases (PIKKs) respond to various stresses by phosphorylating key proteins in the corresponding response pathway [Shiloh, 2003]. In mammals, four members of the PIKK family are known to be involved in the DNA-damage response; ataxia-telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), the DNA-dependent protein kinase (DNA-PK), and ATM related kinase/suppressor of morphogenesis in genitalia-1 (ATX/SMG1).

## P53

The tumor-suppressor protein p53 is a transcription factor with a central role in the regulation of cell cycle, DNA repair, senescence and apoptosis (Figure 4). In response to environmental or intracellular stresses, p53 acts as a node for incoming signals, which it transduces mainly by its function as a transcription factor [Meek, 2004]. *In vitro* studies have shown that several thousand genes are directly regulated by p53, but if this many genes are affected in real life is unclear [Soussi and Wiman, 2007].
In unstressed cells, the levels of the p53 protein are low and have very little effect on cell fate [Oren, 2003]. A key regulator of p53 is Mdm2, and Mdm2 is described in more detail below. Upon stress, the interaction between Mdm2 and p53 can be abolished, resulting in p53 stabilization and transcription of p53 responsive genes [Oren, 2003].

The human p53 protein consists of 393 amino acids and is organized into four functional domains. These domains are; the N-terminal domain containing the transcriptional function of p53, the DNA binding domain in the core of the protein, the tetramerization domain, and the regulatory domain in the C-terminal of the protein containing the nuclear localization and export signals. Several proteins involved in the transcription machinery can bind to the transcription activation domain of p53 and thereby activate transcription of target genes. Negative regulators of p53, *e.g.* Mdm2, may also bind to the transcriptional activation domain, and the binding of proteins to this region of p53 can be modified by posttranslational modifications, such as phosphorylations [Römer *et al*., 2006].

Posttranslational phosphorylations of p53 affect its stabilization. So far, seventeen phosphorylation sites are identified [Bode and Dong, 2004; Römer *et al*., 2006]. Serine 15 is a functionally important recidue within the p53 N-terminal region [Fiscella *et al*., 1993]. In response to DNA damage, p53 can be phosphorylated on Ser15 and this phosphorylation impairs the ability of Mdm2 to inhibit p53, and promotes accumulation and functional activation of p53 [Shieh *et al*., 1997]. The kinase ATM

may phosphorylate p53 Ser15 [Banin *et al*., 1998], as well as the ATR kinase [Tibbetts *et al*., 1999] and the DNA-PK [Shieh *et al*., 1997] in response to DNA damage.
Another important phosphorylation site of p53 is serine 46. Phosphorylation of p53 Ser46 has no affect on Mdm2 interaction, but is associated with apoptosis [Mayo *et al*., 2005]. This is dependent on the concentration of the damaging agent, where in this case [Mayo *et al*., 2005], a high dose of etoposide resulted in a switch of gene promoter targeting leading to apoptosis.
Other posttranslational modifications of p53 include acetylation, ubiquitination and sumoylation [Römer *et al*., 2006].

From the discovery of p53 in 1979 to the late 80's, p53 was thought to be a tumor oncogene [Kastan and Berkovich, 2007]. The finding of p53 mutations and p53 gene loss in tumors, as well as the fact that overexpression of p53 suppresses cell growth and transformation, resulted in the reclassification of p53 as a tumor suppressor gene.
The p53 protein does not function correctly in most human cancers [Vogelstein *et al*., 2000]. Mutations in p53 are found in approximately 50% of all cancers [Soussi and Wiman, 2007], and in the other cases, it is either inactivated indirectly through binding of viral proteins or through alterations in other proteins that interact with p53 [Vogelstein *et al*., 2000].

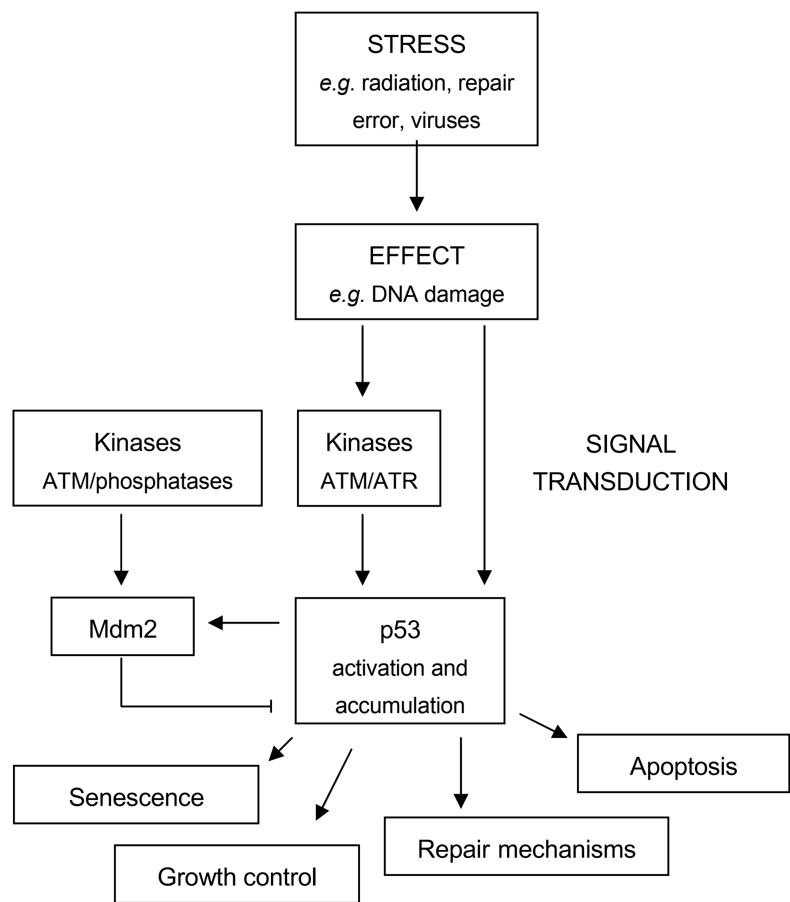


**Figure 4.** Schematic view of the p53 pathway. Different types of stress can result in activation of kinases, which may transduce the signal to p53 and thereby activate it. The activation of p53 can result in different outcome depending on the input signal. (Adopted from [Römer *et al*., 2006])

## MDM2

The murine double minute 2 gene (*mdm2*), was first identified as one of three genes (*mdm1, mdm2* and *mdm3*) that were overexpressed in a spontaneously transformed mouse BALB/c cell line [Iwakuma and Lozano, 2003]. Further studies showed that, when cells overexpressing either of the genes were subcutaneously injected into nude mice, only the *mdm2* encoding product could induce tumorigenicity [Fakharzadeh *et al*., 1991]. It was also found that *mdm2* is an evolutionary well-conserved gene that provides a fundamental cellular function.

The full-length protein of Mdm2 consists of 491 amino acids and contains functional domains, such as a p53 binding site, nuclear localization sequence, nuclear export sequence, acidic domain, zinc finger, RING finger, and nucleolar localization sequence [Meek and Knippschild, 2003]. Mdm2 has an E3 ubiquitin ligase function that is dependent on the RING finger domain. The ubiquitination of proteins is a complex process involving E1, E2 and E3 proteins. The E1 enzyme binds ubiquitin and activate it for further processing, the E2 conjugating enzyme recieves the activated ubiquitin and transfer it to the E3 enzyme, which covalently binds the ubiquitin to the substrate and targets the substrate for degradation. The Mdm2 E3 ligase can ubiquitinate p53 on lysine residues and is thereby a negative regulator of p53 [Iwakuma and Lozano, 2003].

Mdm2 may be phosphorylated in response to different stimuli, and these posttranslational modifications affect its function [Meek and Knippschild, 2003]. In response to genotoxic stress, phosphorylation of Mdm2 and p53 can be induced, which prevent their interaction and result in p53 stabilization [Oren, 2003]. The DNA-damage responsive kinase ATM may phosphorylate Mdm2 at Ser395 [Maya *et al*., 2001]. This phosphorylation plays an important role in the DNA-damage induced stabilization of p53 [Khosravi *et al*., 1999; Maya *et al*., 2001]. The Mdm2 Ser395 phosphorylation is located within the 2A10 epitope (consisting of aa 255-266 and 389-400) and is also associated with a decreased binding of the monoclonal 2A10 antibody, a binding that can be restored by treatment with alkaline phosphatase [Balass *et al*., 2002; Maya and Oren, 2000]. Studies of benzo[*a*]pyrene and dibenzo[*a,l*]pyrene showed that Mdm2 2A10 phosphorylation can be used as a sensitive marker for genotoxicity [Pääjärvi *et al*., 2008; Malmlöf *et al*., 2008].

Many studies have shown that Akt mediates the phosphorylation of Mdm2 at Ser166 and Ser186 [Mayo and Donner, 2001; Zhou *et al*., 2001; Gottlieb *et al*., 2002]. In contrast to Mdm2 2A10 phosphorylation, the Ser166 phosphorylation is associated with increased turnover of p53, inhibition of p53-mediated trans-activation and protection against p53-mediated cell death [Meek and Knippschild, 2003].

The Mdm2 oncoprotein promotes cell survival and cell cycle progression by inhibiting the p53 tumor suppressor protein. The *Mdm2* gene can be up-regulated in tumors by gene amplification, increased transcription or enhanced translation [Momand *et al*., 1998]. In the tumors tested, the overall *Mdm2* amplification frequency was 7% with the highest frequency in soft tissue tumors (20%) [Momand *et al*., 1998]. It was also found that *Mdm2* and *p53* mutations generally do not occur in the same tumor.

Overexpression of the Mdm2 protein is predictive of high grade, aggressive, metastatic malignancies refractory to chemotherapy [Mayo *et al*., 2005].

## P53/MDM2 REGULATION

Mdm2 and p53 are involved in an autoregulatory loop (Figure 5). The *Mdm2* gene has a p53 responsive element so that wild-type p53 induce transcription of *Mdm2*. The Mdm2 protein can in turn bind to p53 and decrease its transcription factor activity. In this way, the p53 protein regulates the *Mdm2* gene transcription, and the Mdm2 protein regulates the p53 activity, creating an autoregulatory feedback loop [Wu *et al*., 1993]. Studies of Mdm2-null created mice showed that loss of Mdm2 is early embryonic lethal [Jones *et al*., 1995; Montes de Oca Luna *et al*., 1995]. However, if the mice were also p53-null, the lethality was reversed, indicating that the mice lacking Mdm2 died because of unregulated p53 activity.

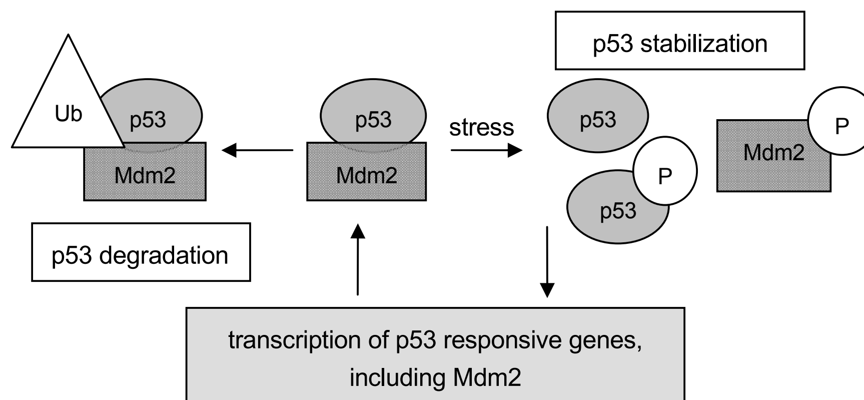


**Figure 5.** The Mdm2-p53 autoregulatory loop. Under normal conditions, Mdm2 control the p53 level by ubiquitinating p53 and addressing it to degradation. Upon stress, the interaction is lost and p53 can function as a transcription factor, which also increase the level of Mdm2, which limits the active period of p53.

## HISTONE H2AX

DNA in eukaryotes is packed into nucleosomes, which forms the basic building unit of chromatin. In turn, each nucleosome consists of 147 base-pairs of DNA wrapped around a core of eight histone proteins, two of each H2A, H2B, H3 and H4 histones, linked with H1. H2AX is a member of the histone H2A family and in a normal human fibroblast, H2AX composes about 10% of the H2A compartment, and a cell contains about 6 million H2AX molecules [Takahashi and Ohnishi, 2005]. H2AX has a specific role in the maintenance of genomic stability. This was first observed by Rogakou *et al*., when they found that DNA DSBs induced by ionizing radiation (IR) rapidly results in a specific phosphorylation on Ser139 in the C-terminal of H2AX [Rogakou *et al*., 1998]. The phosphorylated form of H2AX is denoted γH2AX or H2AXγ.

The amount of γH2AX per DSB correspond to a great region, estimated to about 2 megabases of chromatin [Rogakou *et al*., 1998] and the development of a specific

γH2AX antibody confirmed this phosphorylation pattern and it is referred to as nuclear γ-foci [Rogakou *et al*., 1999]. Many repair factors has been shown to co-localizing to these foci, *e.g.* the tumor supressor protein Brca1 and the MRN complex (Mre11, Rad51 and Nbs1) that is involved in repair [Paull *et al*., 2000]. A role for γH2AX has been implied at sites of DNA damage, cell cycle checkpoints, regulated gene recombination events and tumor suppression. H2AX phosphorylation may be induced by a variety of origins, such as external damage, replication fork collision, apoptosis and dysfunctional telomeres [Fernandez-Capetillo *et al*., 2004], and is mediated by ATM, ATR or DNA-PK [Downey and Durocher, 2006].
The H2AX phosphorylation in response to ATM/ATR is involved in homologous recombination repair (HR), while DNA-PK phosphorylation of H2AX is involved in the recognition of DNA lesions by non-homologous end joining (NHEJ), suggesting that H2AX has a central role in both repair pathways. The γH2AX may also have a role in the global chromatin relaxation, which could have an effect on GGR in NER [Escargueli *et al*., 2008].

## OTHER SIGNALING PROTEINS

A network of signaling pathways, checkpoint pathways, mediates the arrest or delay of cell cycle progression that provides time to repair or prevent cell proliferation if the damage is to severe (Figure 6). This includes sensor proteins that monitor the genome for abnormalities and help generate signals, which are amplified and propagated by mediators and signal transducers. The mediators and signal transducers, in turn, affect downstream checkpoint effectors that connect the checkpoint with the core cell cycle machinery. In spite of this general hierarchical arrangement, the cell cycle checkpoint mechanisms rarely operate in simple and linear pathway. It is often difficult to discriminate a strict biochemical order, and the checkpoint proteins often involve self-reinforcing autocatalytic loops [Lukas *et al*., 2004].

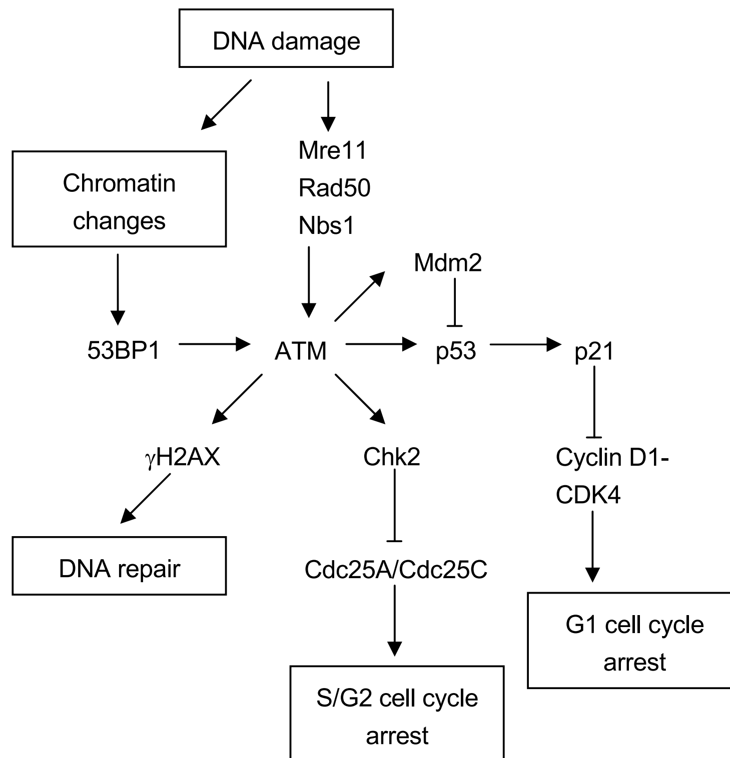


**Figure 6.** Schematic overview of the DNA damage proteins investigated in this thesis. (Adopted from [Lukas *et al*., 2004; Zgheib *et al*., 2005])

***Chk2***

The checkpoint kinase 2 (Chk2), is an effector protein that becomes activated in response to DNA damage, mainly by ATM although crosstalk with other kinases exists, and its activation involves dimerization and autophosphorylation. Chk2 is a mobile protein and may thus rapidly spread a local DNA damage signal to other soluble and mobile proteins, such as Cdc25A, leading to cell cycle arrest in the S/G2 phase. Mice deficient in Chk2 are viable, fertile and do not show a tumor-prone phenotype except when exposed to carcinogens and possibly late in life. However, in some studies, increased resistance to ionizing radiation was observed, and defects in p53 function and checkpoint responses, as well as in apoptosis [Bartek and Lukas, 2003].

***p21***

The p21 protein is considered one of the most important and potent effector molecules of p53, and p53 directly activates p21 expression by binding to its promoter. Thus, in cancers with inactivated p53, the p21 levels are decreased. The kinase inhibitor p21 can bind to cyclin D1-CDK4 and thereby arrest the cell cycle progression in G1 [Abukhdeir and Park, 2008].

***Cyclin D1***

Cyclin D1-CDK4 is an important target for growth factor signals in promoting G1 cell cycle progression. This complex phosphorylates the retinoblastoma protein, Rb, and relieves the Rb mediated inhibition of E2F transcription factors. The accumulation of

cyclin D1 is regulated throughout the cell cycle at multiple levels, including transcription, posttranslational modifications and cellular localization, and overexpression of cyclin D1 has been observed in a number of human cancers [Gladden and Diehl, 2005].

### ***53BP1***

The p53 binding protein, 53BP1, rapidly localizes to sites of DNA damage. The precise position of 53BP1 in the DNA damage signaling is being debated. It has been shown that 53BP1 localizes to sites of DNA damage normally in cells with inactive ATM, suggesting that 53BP1 is an upstream sensor [Mochan *et al*., 2004]. However, other investigations have shown that 53BP1 is downstream of ATM [Minter-Dykhouse *et al*., 2008]. The 53BP1 protein has a binding sequence for γH2AX, and in mouse cells lacking γH2AX, 53BP1 is recruited to the site of damage but is not retained there [Mochan *et al*., 2004].

# AIM OF THE THESIS

In general, the aim was to better understand the cellular effects of oxidative stress- and PAH-induced DNA damage. This is essential since damage to the DNA could initiate carcinogenesis.

In paper I, the aim was to study the difference in susceptibility to oxidative stress in DNA and RNA. This was investigated since RNA oxidation, and the implication of it, is much less studied. In more recent years, RNA oxidation has been found to be important in a number of diseases, hence there is a need for further investigation.

The aim of paper II was to find out more about the differences in carcinogenic potency between two categories of PAHs, the bay-region BPDE and the fjord-region DBPDE. More specifically, we investigated the effect these DEs had on a protein involved in the recognition of DNA damage in an attempt to elucidate why DBPDE-adducts are not removed as efficiently as BPDE-adducts.

In the third study, paper III, the aim was to investigate if the findings in paper II together with the findings in [Malmlöf *et al*., 2008; Pääjärvi *et al*., 2008] could be generalized to include several PAHs of the bay- or fjord-region category.

The aim of the last study, paper IV, was to investigate the effect complex mixtures of PAHs had on DNA damage signaling. The mixtures were derived from contaminated soils. The aim of this study was also to evaluate if BP, which is often used as an indicator substance for complex PAH mixtures, really is a good indicator. We also investigated if the TEF-value system could be used to predict the effects we found on the DNA damage signaling proteins.

# RESULTS AND DISCUSSION

In all studies, human carcinoma cell lines were used as models.

The A549 cells are a human lung carcinoma cell line. The A549 cells express, among other CYPs, CYP1A1 and CYP1B1, and they may be up-regulated in response to PAHs. The cells also have phase II activity of glutathione transferase and glucuronyl transferase [Castell *et al*., 2005].

The HepG2 cells are a human-derived hepatoma cell line. It retains the activities of various phase I and phase II enzymes important in the activation and detoxification of procarcinogens, such as PAHs. The HepG2 cells have CYP1A enzymes that are necessary for PAH activation. The cells also express EH, which together with the CYP enzymes enables the formation of the ultimate carcinogenic metabolites of PAHs. The HepG2 cells also have active phase II enzymes, such as glutathione transferases and sulfo transferases [Knasmüller *et al*., 1998].

## PAPER I

The "oxygen paradox" states that oxygen is dangerous to the very life-form for which it has become an essential component of the energy production [Davies, 2000]. We live in an oxygen rich environment and require oxygen for our existence, and yet as byproducts of our energy production, reactive oxygen species (ROS) are formed [Wilson III *et al*., 2003]. Exposure to lifestyle or environmental agents, such as alcohol, cigarette smoke, high fat diet, pesticides, car exhaust, UV light and IR may also result in ROS production [Wilson III *et al*., 2003]. These reactive byproducts can potentially attack most cellular components (*e.g.* DNA, RNA and proteins) and if the damage is not properly repaired, it could for instance promote cancer.
Of the four DNA bases, guanine is the most easily oxidized due to its lower oxidation potential [Kawanishi *et al*., 2001]. Through the Fenton reaction, hydrogen peroxide ($H_2O_2$) can form a hydroxyl radical ($^{\bullet}OH$), which in turn can interact with guanine and form 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) [Kawanishi *et al*., 2001]. The formation of 8-oxodGuo can cause misreplication of DNA that may lead to G-C $\rightarrow$ T-A transversions, and carcinogenesis. Luckily, we have a battery of defense mechanisms that protect us against oxidative damages. The non-enzymatic defense mechanisms include *e.g.* glutathione, ascorbic acid (vitamin C) and $\alpha$-tocopherol (vitamin E), and the enzymatic defense includes superoxide dismutase, catalase and glutathione peroxidase [Valko *et al*., 2006]. If an oxidative lesion does occur on DNA, we also have repair systems that may correct it, if it is found. The base excision repair (BER) can take care of oxidative DNA damage with the assistance of the human 8-oxoguanine glycosylase 1 (hOGG1) enzyme [Evans and Cook, 2004].
Most of the research on oxidative damages has focused on DNA, as a target for oxidation, a substrate for repair and as a source of mutations with attendant pathological consequences, while RNA is less studied. Compared to DNA, oxidative damages to mRNA may have more limited impact on cellular functions due to the

presence of multiple copies of mRNA, its degradation and inheridity [Evans and Cook, 2004]. However, implications of oxidative damages to RNA and DNA have been shown to be associated with Parkinson's disease [Zhang *et al*., 1999] and Alzheimer disease [Nunomura *et al*, 2001]. Specific repair enzymes for RNA has been observed, one such mechanism that limit the availability of oxidatively modified RNA to the translational processes is the Y-box-binding protein 1 (YB-1). The YB-1 can bind to 8-oxoGuo containig mRNA and remove it from the translational material and thereby minimise aberrant protein production [Hayakawa *et al*., 2002].

In this study, we investigated the differences in oxidative susceptibility between DNA and RNA. The human lung cancer cell line A549 was exposed to $^{18}$O-labeled hydrogen peroxide ([$^{18}$O]-$H_2O_2$) and the total DNA and RNA was extracted in parallel. Using high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-MS/MS), the $^{18}$O-labeled 8-oxo-7,8-dihydroguanosine ([$^{18}$O]-8-oxoGuo) and $^{18}$O-labeled 8-oxo-7,8-dihydro-2'-deoxyguanosine ([$^{18}$O]-8-oxodGuo) were analyzed. We found that exposure to [$^{18}$O]-$H_2O_2$ resulted in a dose-response formation of both [$^{18}$O]-8-oxoGuo and [$^{18}$O]-8-oxodGuo, where it was approximately 14-25 times more common in RNA than in DNA. Analysis of the kinetics of formation and removal of the oxidized adducts showed that the half-lives for adducts in RNA and DNA was approximately 12 h and 20 h, respectively.

### PAPER II

DE-DNA adducts from bay- and fjord-region PAHs have different carcinogenic potency. This variation is suggested to be related to the structural features of their DNA adducts and how these adducts, or their associated conformational changes in DNA, are recognized by surveillance systems, and removed by NER before replication [Geacintov *et al*., 1997; Dip and Naegeli, 2005; Buterin *et al*., 2000]. The (-)-*anti*-DBPDE is found to be more refractory to NER than the (+)-*anti*-BPDE [Dreij *et al*., 2005].
Understanding the factors underlying the considerable difference in tumorigenic potency between bay- and fjord-region PAHs is of great interest. In an attempt to find out more about how these two DEs affect cells, we studied the phosphorylation of histone H2AX. Phosphorylation of histone H2AX promotes an open chromatin structure at the site of a DNA DBS and thereby facilitates the repair [Paull *et al*., 2000]. It also helps with the recruitment of other factors involved in the repair.

We investigated the effect of (-)-*anti*-DBPDE and (+)-*anti*-BPDE on γH2AX in human A549 cells. If and to what extent bulky and stable DNA adducts from these DEs induce γH2AX has not been studied, nor has the influence on the response of their differences in adduct structural and topological features. As a positive control in the Comet assay and of the cells ability to evoke and signal the DNA damage responses mediated by γH2AX, we used hydrogen peroxide ($H_2O_2$).

First we performed Comet assay to assess any formation of DNA strand breaks in response to the different treatments. The A549 cells were either exposed to 1.0 or 5.0 μM (+)-*anti*-BPDE, 0.1 or 0.5 μM (-)-*anti*-DBPDE for 3 or 6 h, exposed to 100 μM $H_2O_2$ for 1 h, or mock treated with DMSO for 3 h. We found that 1 h treatment with 100 μM $H_2O_2$ readily induced DNA strand breaks. However, the results of both (+)-*anti*-BPDE and (-)-*anti*-DBPDE did not differ from control cells exposed to DMSO. This indicated that (+)-*anti*-BPDE and (-)-*anti*-DBPDE did not induce any DNA strand breaks in the cell system used. This is in line with previous studies showing that these DEs are chemically stable and do not spontaneously decompose into strand breaks in DNA [Pruess-Schwarts *et al*., 1988; Melendez-Colon *et al*., 1997].

Immunocytochemical detection of γH2AX after treatment with 100 μM $H_2O_2$ for 1 h revealed formation of γH2AX in a great majority of the cells. Exposure to 1.0 μM (+)-*anti*-BPDE or 0.1 μM (-)-*anti*-DBPDE also gave rise to γH2AX, although not all cells were affected and with differences in the response between the two DEs. When studied over time, (+)-*anti*-BPDE induced a transient γH2AX, with approximately 35% of the cells exhibiting γH2AX after 1 h followed by a decline and lower levels at 3 and 6 h. On the other hand, exposure to (-)-*anti*-DBPDE resulted in a constantly increasing γH2AX up to 6 h, where approximately 35% of the cells exhibited γH2AX. Similar results were obtained with Western blotting, but with this technique, we found the highest levels of γH2AX at 3 h for both DEs. This was followed by a more drastic decrease in γH2AX for (+)-*anti*-BPDE than for (-)-*anti*-DBPDE, which remained at almost the same γH2AX level after 6 h. The difference between the two methods most likely reflects that immunocytochemistry is based on single cell responses and is more sensitive, while Western blot is based on pooled cells. Overall, these results correlates

with the previous finding that (+)-*anti*-BPDE adducts are more easily removed by NER than (-)-*anti*-DBPDE adducts [Dreij *et al*., 2005]. Western blot analysis also showed a concentration dependent γH2AX response after exposure to the DEs.
When the γH2AX response was monitored for up to 24 h with immunocytochemistry, no further reduction in the number of γH2AX positive cells was found after exposure to (+)-*anti*-BPDE compared to 6 h. However, 24 h after exposure to the (-)-*anti*-DBPDE, the level of γH2AX positive cells had increased to approximately 75%. This time point was associated with dead cells floating in the medium after (-)-*anti*-DBPDE exposure, consistent with the finding of high acute toxicity of DBPDE in addition to its high mutagenic and carcinogenic potency [Luch *et al*., 1994]. There is also the possibility that at later time points, some γH2AX foci could reflect cells undergoing programmed cell death [Rogakou *et al*., 2000], or that the γH2AX is present within cryptogenic γ-foci observed in senescent cells and suggested to mark non-repairable DNA lesions [Sedelnikova *et al*., 2004].

In this study, we demonstrated that removable DNA adducts derived from BPDE and the more persistent adducts from DBPDE cause H2AX phosphorylation. The results indicate that the structural features of the adducts, and the associated DNA conformational change, dictate the kinetics and magnitude with which γ-foci are assembled in response to the genotoxic insult. We suggest that the increased mutagenic potential of DBPDE compared to BPDE correlates with the relative slow, or even absent, dephosphorylation and/or removal of γH2AX resulting in persistent and most likely repair insufficient γ-foci in response to DBPDE.

## PAPER III

This study is a continuation of paper II. Here we investigated the effect on H2AX, Mdm2 and p53 phosphorylation in response to DEs from two bay-region PAHs and three fjord-region PAHs to find out if the previous findings [Malmlöf *et* al., 2008; Mattsson *et al*., 2008; Pääjärvi *et al*., 2008] could be extended to bay- and fjord-region DEs in general. The bay-region DEs used here were derived from chrysene (CDE) and dibenz[*a,h*]anthracene (DBADE), and the fjord-region DEs were derived from benzo[*c*]chrysene (B[*c*]CDE), benzo[*g*]chrysene (B[*g*]CDE) and benzo[*c*]phenanthrene (B[*c*]PhDE).

The A549 cells were exposed to 0.001-1 μM of the DEs for 1-24 h. To study the different signaling proteins with Western blot, the cells were lysed in a lysis buffer containing protease inhibitors and the proteins subjected to SDS-Page gel electrophoresis. We found that after 3 h exposure to 0.001-1 μM of the DEs, higher levels of Mdm2 2A10 phosphorylation was observed in response to the fjord-region DEs, with an indication of concentration dependence for B[*c*]PhDE and B[*c*]CDE. The Mdm2 2A10 phosphorylation was weaker for the bay-region DEs. High concentration of fjord-region DEs induced p53 stabilization, but the bay-region DEs did not. The p53 Ser15 phosphorylation correlated with the p53 stabilization and was only apparent at the high concentration of fjord-region DEs. The p53 Ser46 phosphorylation, which is associated with apoptosis, showed similar response as the p53 Ser15 phosphorylation, indicating the toxicity of the concentration. In addition, the phosphorylation of H2AX was also only detected in response to the higher concentration of fjord-region DEs, correlating with p53 Ser15 phosphorylation.

The high concentration of the fjord-region DEs had a sustained effect on Mdm2 2A10 phosphorylation, p53 stabilization and p53 Ser15 phosphorylation as monitored 24 h after treatment. The persisting response on these DNA damage signaling proteins indicated the severity and persistence of the induced damage.

An immunocytochemical analysis of the γH2AX response at 1, 3 and 6 h after treatment with 1.0 μM of the DEs revealed formation of γH2AX. The fjord-region DEs induced a stronger and more persisting response compared to the bay-region DEs. The fjord-region B[*c*]CDE evoked a weak γH2AX response at 1 h, followed by and increase in both the number of cells affected and the intensity, which sustained at 6 h. The γH2AX response after DBADE treatment was less intense, affected less number of cells and had decreased at 6 h.

In conclusion, fjord-region DEs affect Mdm2, p53 and H2AX more than bay-region DEs, where Mdm2 2A10 phosphorylation was the most sensitive marker. The results correlate with the fjord-region DEs being more powerful mutagens than bay-region DEs.

## PAPER IV

PAHs in contaminated soil are an environmental problem that imposes a risk to humans, and risk levels are often difficult to estimate due to the complexity of the contaminants present. Those types of evaluations are often based on one or a few indicator substances, or TEF-scales. In this study, we compared the effect on cell signaling of contaminated soil collected at six different industrial settings in Sweden, all containing complex mixtures of PAHs. Using Western blotting and immunocytochemistry, we investigated the impact these soil extracts had on DNA damage signaling in HepG2 cells.

Since BP often is used as an indicator substance for complex PAH mixtures, one strategy was to expose the cells to the soil fractions calculated to contain a certain level of BP. The results were then compared between different samples, and to the exposure of the equal concentration of pure BP. The other strategy was to expose the cells to a certain amount of soil. In general, we found that the BP content of a soil extract could not explain the impact the soil had on the different proteins studied.

We found that very little soil extract was needed to induce Mdm2 2A10 phosphorylation in response to both the PAH containing fraction and the oxy-PAH containing fraction. As little as 0.0065 mg soil/ml medium of the PAH fraction from the Hässleholm sample, corresponding to a BP content of 0.001 μM, induced a very strong response. This is in line with the previous studies, indicating how sensitive the Mdm2 2A10 phosphorylation is [Malmlöf *et al*., 2008; Pääjärvi *et al*., 2008]. Mdm2 2A10 phosphorylation is involved in p53 stabilization, however, we could not detect any p53 stabilization under the conditions evaluated here. This probably reflects that Mdm2 2A10 phosphorylation is a more sensitive marker and that higher concentrations of the soil fractions might be needed to induce p53 stabilization. The decrease in Mdm2 levels in response to high exposure, or in response to very contaminated fractions, could reflect a transcriptional down-regulation of Mdm2 as previously shown with BPDE [Pääjärvi *et al*., 2004].

The PAH and oxy-PAH containing soil fractions gave rise to persistent γH2AX phosphorylation still apparent after 24 or 48 h. Exposing the cells to 1 μM BP resulted in a transient and comparable weak γH2AX that was reversed after 24 h. When the BP concentration was increased to 10 μM, it seemed to induce persistent damage as the phosphorylation sustained at 24 h. The oxy-PAH containing soil fraction evoked γH2AX already at 0.01 mg soil/ml medium, indicating this fraction's high DNA damaging potency. We also found sustained nuclear foci of 53BP1 at 48 h, correlating with the observed γH2AX. Together, this suggests persistent DNA damage.

Phosphorylation of Mdm2 at Ser166 is suggested to be a possible marker for tumor promotive activity by attenuating the p53 response [Pääjärvi *et al*., 2005]. We found that the PAH containing soil fractions induced Mdm2 Ser166 phosphorylation and that it correlated with Erk phosphorylation. Erk has been shown to mediate the Mdm2 Ser166 phosphorylation in HepG2 cells [Malmlöf *et al*., 2007]. BP alone did not induce Mdm2 Ser166 phosphorylation.

Effects on cell cycle are another factor that potentially could influence tumor promotion. In general, increasing concentrations of the PAH containing soil fractions resulted in a decrease in cyclin D1 and increase in p21, indicating a stop in the $G_1$-phase of the cell cycle. The effects of BP alone were the opposite.
The effects on cell proliferation, as measured with the MTT assay, showed a reduced cell proliferation for all soil fractions except for Boden, which has the lowest PAH content.

This study demonstrates that neither the BP concentration nor the available TEF-scales are sufficient to predict the risk of complex PAH mixtures, at least not for predicting effects on DNA damage signaling. Further, our investigation demonstrates that established approaches to evaluate carcinogenic potentials of PAH mixtures in contaminated soil are insufficient and are in need of new biotests. This study also point out the need to understand how different PAHs interact. We suggest that the endpoints studied here may be useful for evaluating the carcinogenic potency of PAHs in contaminated soil.

## FUTURE PERSPECTIVES

To further elucidate the differences between bay- and fjord-region DEs, the assembly of NER factors at the site of the DNA adducts could be studied. It would be interesting to find out where in the sequence of NER factor assembly the phosphorylated H2AX is located. Cell lines deficient in different NER factors are available, and it would be interesting to study if the deficiencies affect the H2AX phosphorylation, or if H2AX is upstream of NER recognition factors.

In this context, it could also be informative to study the assembly of other damage signaling proteins to find out if the foci around a BPDE adduct, which is more easily removed, differ from the foci assembled around a DBPDE adduct, which result in persistent γH2AX. Studies on which kinase(s) is involved in the H2AX phosphorylation in response to the PAH DEs could also provide useful information.

The investigation of complex mixtures of PAHs in contaminated soil could be continued in different ways. For instance, it would be interesting to find out if the PAH fractions contain heavier PAHs, such as DBP, which is a very potent carcinogen. Many analytic methods have trouble to separate this type of compounds.

One could also make mixtures of selected PAHs to try to elucidate if there is a combination of PAHs that could represent the observed effects. If such a mixture exists, those PAHs could be used as indicators to complement BP, which often is used as an indicator by itself in an insufficient manner.

To be able to investigate the effect environmental contamination of PAHs have on humans, it would be interesting to study the effect on these signaling pathways in cells that easily could be obtained from *e.g.* a blood sample.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Den första studien i den här avhandlingen handlar om oxidativa skador på arvsmassan, DNA. Fria syreradikaler kan ge skador på DNA och om dessa skador inte repareras kan de leda till förändringar som i sin tur kan resultera i cancer. Hur mycket fria syreradikaler man utsätts för påverkas av livsstilsval, som t.ex. alkohol konsumtion, cigarrettrökning och fettinnehållet i dieten, men även miljöfaktorer, som t.ex. bilavgaser, UV-ljus och strålning. Fria syre radikaler bildas också som biprodukter när våra celler omvandlar födan till energi. Vårt DNA är uppbyggt upp av fyra baser; adenin, guanin, cytosin och tymin, och av dessa baser är guanin känsligast för oxidation. När guanin får en oxidativ skada kan det leda till att det blir ett fel vid kopieringen av DNA och att fel bas sätts in. Som tur är har vår kropp flera olika sätt att skydda sig mot dessa fria radikaler. Vitamin C och vitamin E är exempel på ämnen som kan ge skydd, och vi har enzymer som katalyserar nedbrytningen av fria radikaler. Skulle en oxidativ skada på DNA uppstå, så har vi även reparationsvägar för att ta bort skadan, men om skadan av någon anledning slinker igenom detta försvar, kan det resultera i att det bildas nya celler som inte innehåller rätt information och detta kan leda till utveckling av cancer.

När det gäller oxidativa skador är inte RNA lika väl studerat. I den första studien använde vi oss av en human cancer cellinje och visade att RNA var känsligare för oxidativstress än DNA. Vi såg också att de oxidativa skadorna på RNA försvann nästan dubbelt så fort som motsvarande skador på DNA, vilket speglar den högre omsättningen av RNA än DNA. (En cancer cellinje innebär att man isolerat celler från en tumör och att man sedan odlar dem i flaskor där man tillsätter ett medium som gör att cellerna kan växa och föröka sig).

Polycykliska aromatiska kolväten (PAH efter engelskans "Polycyclic Aromatic Hydrocarbon") finns i vår omgivning och bildas vid ofullständig förbränning av organiskt material, som t.ex. kol och olja. Till de största källorna hör utsläpp från industrin, uppvärmning av våra bostäder, men även bilavgaser m.m. bidrar. Våra livsstilsval påverkar också hur mycket PAHer vi utsätts för, t.ex. cigarrettrökning ökar ens exponering. PAHer är carcinogena föreningar, vilket betyder att de ger en ökad risk för att utveckla cancer. PAHer är fettlösliga och relativt inaktiva ämnen och för att vår kropp ska kunna bli av med dem behöver de göras vattenlösliga så de kan försvinna ut via urinen. När det handlar om hur vi utsätts för främmande ämnen brukar man prata om exponering, upptag, aktivering och utsöndring. Exponering kan ske vi hud, intag av föda eller inandning. För PAHer är födan vår största exponeringskälla, om man inte röker. Bladgrönsaker kan till exempel innehålla PAHer genom att de deponerats på ytan via små luftburna partiklar. En annan födokälla som innehåller PAHer är stekt eller grillat kött eller fisk. PAHer behöver som sagt göras mer vattenlösliga för att vår kropp ska kunna utsöndra dem. Detta sker med hjälp av ett enzymsystem som kopplar på andra molekyler som ändrar vattenlösligheten för PAHn. Men under denna process bildas så kallade reaktiva intermediärer som är mycket reaktiva och som kan sätta sig som en klump på DNA, en DNA addukt. Dessa skador på DNA kan göra att det sker en mutation, dvs att det blir ett permanent fel, som kan leda till utvecklingen av cancer.

I den här avhandlingen har några PAHer studerats och de carcinogena metaboliterna av dem. För att försöka ta reda på mer om hur dessa ämnen kan ge upphov till cancer har vi undersökt hur de påverkar vissa signalsystem i våra celler. Dessa signaler utgörs av hur nivåerna av olika proteiner förändras. Vi har tittat på p53 som är ett så kallat tumör-suppressor protein. Det innebär att p53 hjälper till att skydda oss från att celler som innehåller fel i sitt DNA ska få dela sig och bilda nya celler. Det gör p53 genom att stoppa celldelningen och se till att felet blir reparerat eller se till att cellen dör. Men p53 är inte ensam om detta ansvar, utan samarbetar med många andra proteiner. Här nämns t.ex. Mdm2 som är ett oncoprotein. Ett oncoprotein har egenskaper som innebär att det kan ge tillväxtfördelar, vilket kan leda till cancer. Mdm2 och p53 är i normala fall under väldig hård kontroll, bl.a. genom att de reglerar varandra. När p53 är aktiv bidrar det till ökad mängd Mdm2 som i sin tur håller nere p53 nivån, detta kallas en självreglerad återkoppling.

Ett annat protein som undersökts i våra studier är histonen H2AX. En histon är ett protein som hjälper till att förpacka DNA så att de ca 2 meter DNA som finns i stort sett i varje cell i människan kan få plats. Vid en skada på DNA kan H2AX förändras vilket underlättar för reparationssystem att hitta skadan. Vi har även studerat hur PAHer påverkar andra signaleringsprotein som är viktiga för att stoppa celler som innehåller skador så att de inte delar sig och kan leda till utveckling av cancer.

I de studierna vi gjort om PAHer och deras reaktiva intermediärer har vi sett att de PAHer som är mer carcinogena har en annan påverkan på de olika signalproteinerna än de som är mindre carcinogena. Vi har visat att komplexa PAH-blandningar som finns i förorenad jord har tydliga effekter på cellernas signalvägar för skada. Vi har också sett att det inte går att förutspå dessa effekter med de metoder som traditionellt använts.

# ACKNOWLEDGEMENTS

The studies presented in this thesis were carried out at the Division of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institutet.
It would like to thank all my nice and talented co-workers and my dear family and friends, who have supported me, and in different ways contributed to this thesis.

I would like to express my warmest thanks to my main supervisor Ulla Stenius, who took me on at the very end when it was the most needed. Your enthusiasm and deadlines were much welcome to help me push myself to finish.

I would also like to express my gratitude to my co-supervisor Bengt Jernström, who was my main supervisor for most of my PhD time. Thank you for always having your door open, and for providing a good combination of knowledge together with Ulla.

My second co-supervisor, Ian Cotgreave. You introduced me to "life" at IMM by letting me do my masters degree project in your group. Although you soon left IMM, I am thankful that you gave me the opportunity to start my research education.

My co-authors
Edyta Bajak, who got me started in the lab and who taught me methods and so on. It was a nice collaboration and we finally published.
Tim Hofer, Jean-Luc Ravanat and Carine Badouard, thank you for the collaboration.
Maria Malmlöf, thank you for being so easy and nice to collaborate with.
Albrect Seidel, for providing us with diol epoxides.
Staffan Lundstedt, for supplying us with soil extracts and for your help with the manuscript.

People in the Biochemical Toxicology group
Katarina, for being a great friend and for always having time to listen and give advice. You are such a strong and lovely person! I will come visit often and interrupt your work ☺. Erika, for being so nice and a perfect roommate, and for sharing some of the stress of being close to finishing. Astrid, for being a great roommate, for having as hard as me to keep quiet and for spreading energy. Jingwen, you're also a nice roommate. Johan Å, for bringing some enzyme kinetics into our lives. Vanina, for being the nicest postdoc. Ilona, it is great to have you back. Rebecca, Oras, Marjolein, Ralf, Julia, Bengt F, Roland, Johan H, Monica, you're all a part of making our division so great.

Colleagues at Plan 3
Anna, Emma, Lina, Daniel, Sabina, Maria H, Kina, IngaLill, AnnaLena, Annika H, Helen, and all the rest, you all help to make the nice plan 3 atmosphere.

Former people at Plan 3
Therese, IMM is not the same without you, and Louise, I'm looking forward to the next dinner with the two of you and lots of great food. Kristian & Linda, thanks for showing

me around NY. Salomon, Sara, Carolina, Ylva, Rebecca L, Annika S, Gudrun, it was nice to have you as colleagues.

Vänner från Universitetet
Lotta, du är en fantastisk vän. Du finns du alltid där och du känner mig så bra. Vi hade otroligt kul under molbi-tiden! Tack för alla middagar, kräftskivor på ditt landställe, nyår i fjällen mm. Olle, André och Benita, ni var också en del av molbi-livet och ni såg till att det aldrig blev tråkigt. Vi hade de bästa ”Floydiga” middagarna! Linus & Malin, ni är så gulliga, tack för allt trevligt vi har!

Tjejerna i Skåne
Tack Helena, Anna, Maria med familjer för att ni alltid har varit bra vänner och för att ni alltid finns där, även om vi inte ses lika ofta längre. Vi har känt varandra sen barnsben och ni betyder mycket. Jag ser fram emot nästa fika!

Min underbara lilla stora familj
Mamma & Anders, Pappa & Jane, min bror Henrik & Elna, tack för att ni alltid ställer upp och alltid finns där, ni betyder väldigt mycket ♥. Mina bonussyskon Fredrik, Johanna, Therese, Johan och Anna med familjer, det är alltid trevligt med lite bonus i livet! Farmor och Morfar, ni är alltid så snälla och måna om mig.

Tack Anki & Anders och Ulrika för allt ni hjälper Johan och mig med och för resorna till Guadeloupe och San Francisco.

Sist men inte minst, min Johan. Tack för att du får mig att må bra och vara lycklig. You are my lobster! PussPuss ♥ You!

Tack alla!

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