Aus der Institut für Pharmazie und Biochemie – Therapeutische
Lebenswissenschaften der Johannes Gutenberg-Universität Mainz

Consequences of DNA damage for gene transcription: direct effects of modified nucleobases and the role of base excision repair

Folgen von DNA Schäden für die Gentranskription: direkte Auswirkungen modifizierter Nukleobasen und der Einfluss der Basenexzisionsreparatur

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1) Kitsera, N., A. Khobta, and B. Epe (2007). "Destabilized green fluorescent protein detects rapid removal of transcription blocks after genotoxic exposure." *Biotechniques* **43**(2): 222-227. *(Journal article, experimental, IF=2.7)*

2) Khobta, A., N. Kitsera, B. Speckmann, and B. Epe (2009). "8-Oxoguanine DNA glycosylase (Ogg1) causes a transcriptional inactivation of damaged DNA in the absence of functional Cockayne syndrome B (Csb) protein." *DNA Repair (Amst)* **8**(3): 309-317. *(Journal article, experimental, IF=4.1)*

3) Khobta, A., S. Anderhub, N. Kitsera, and B. Epe (2010). "Gene silencing induced by oxidative DNA base damage: association with local decrease of histone H4 acetylation in the promoter region." *Nucleic Acids Res* **38**(13): 4285-4295. *(Journal article, experimental, IF=8.0)*

4) Khobta, A., T. Lingg, I. Schulz, D. Warken, N. Kitsera, and B. Epe (2010). "Mouse CSB protein is important for gene expression in the presence of a single-strand break in the non-transcribed DNA strand." *DNA Repair (Amst)* **9**(9): 985-993. *(Journal article, experimental, IF=4.1)*

5) Kitsera, N., D. Stathis, B. Lühnsdorf, H. Müller, T. Carell, B. Epe, and A. Khobta (2011). "8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1." *Nucleic Acids Res* **39**(14): 5926-5934. *(Journal article, experimental, IF=8.0)*

6) Luhnsdorf, B., N. Kitsera, D. Warken, T. Lingg, B. Epe, and A. Khobta (2012). "Generation of reporter plasmids containing defined base modifications in the DNA strand of choice." *Anal Biochem* **425**(1): 47-53. *(Journal article, experimental, IF=3.0)*

7) Khobta, A. and B. Epe (2012). "Interactions between DNA damage, repair, and transcription." *Mutat Res* **736**(1-2): 5-14. *(Journal article, review, IF=2.9)*

2 SUMMARY

The presence of damaged nucleobases in DNA can negatively influence transcription of genes. One of the mechanisms by which DNA damage interferes with reading of genetic information is a direct blockage of the elongating RNA polymerase complexes – an effect well described for bulky adducts induced by several chemical substances and UV-irradiation. However, other mechanisms must exist as well because many of the endogenously occurring non-bulky DNA base modifications have transcription-inhibitory properties in cells, whilst not constituting a roadblock for RNA polymerases under cell free conditions. The inhibition of transcription by non-blocking DNA damage was investigated in this work by employing the reporter gene-based assays. Comparison between various types of DNA damage (UV-induced pyrimidine photoproducts, oxidative purine modifications induced by photosensitisation, defined synthetic modified bases such as 8-oxoguanine and uracil, and sequence-specific single-strand breaks) showed that distinct mechanisms of inhibition of transcription can be engaged, and that DNA repair can influence transcription of the affected genes in several different ways.

Quantitative expression analyses of reporter genes damaged either by the exposure of cells to UV or delivered into cells by transient transfection supported the earlier evidence that transcription arrest at the damage sites is the major mechanism for the inhibition of transcription by this kind of DNA lesions and that recovery of transcription requires a functional nucleotide excision repair gene *Csb* (*ERCC6*) in mouse cells. In contrast, oxidised purines generated by photosensitisation do not cause transcriptional blockage by a direct mechanism, but rather lead to transcriptional repression of the damaged gene which is associated with altered histone acetylation in the promoter region. The whole chain of events leading to transcriptional silencing in response to DNA damage remains to be uncovered. Yet, the data presented here identify repair-induced single-strand breaks – which arise from excision of damaged bases by the DNA repair glycosylases or endonucleases – as a putative initiatory factor in this process. Such an indirect mechanism was supported by requirement of the 8-oxoguanine DNA glycosylase (OGG1) for the inhibition of transcription by synthetic 8-oxodG incorporated into a reporter gene and by the delays observed for the inhibition of transcription caused by structurally unrelated base modifications (8-oxoguanine

and uracil). It is thereby hypothesized that excision of the modified bases could be a general mechanism for inhibition of transcription by DNA damage which is processed by the base excision repair (BER) pathway. Further gene expression analyses of plasmids containing single-strand breaks or abasic sites in the transcribed sequences revealed strong transcription inhibitory potentials of these lesions, in agreement with the presumption that BER intermediates are largely responsible for the observed effects. Experiments with synthetic base modifications positioned within the defined DNA sequences showed that inhibition of transcription did not require the localisation of the lesion in the transcribed DNA strand; therefore the damage sensing mechanism has to be different from the direct encounters of transcribing RNA polymerase complexes with DNA damage.

Altogether, this work provides new evidence that processing of various DNA base modifications by BER can perturb transcription of damaged genes by triggering a gene silencing mechanism. As gene expression can be influenced even by a single DNA damage event, this mechanism could have relevance for the endogenous DNA damage induced in cells under normal physiological conditions, with a possible link to gene silencing in general.

3 SYNOPSIS OF THE CUMULATIVE HABILITATION THESIS

3.1 Introduction

3.1.1 Effects of DNA damage on the expression of genetic information

Alterations of normal DNA structure (chemical modifications of the sugar-phosphate backbone as well as misincorporation, modification or loss of nucleobases) are unavoidable under normal physiological conditions because of the continuous exposure of DNA in cells to endogenous and environmental DNA damaging agents, various enzymatic activities and spontaneous decay (Friedberg, 2003; Lindahl, 1993). Immediate consequences of DNA damage are loss of the information stored in the damaged DNA strand as well as possible impacts on copying of genetic information by DNA polymerases and its reading by RNA polymerases. Persisting DNA damage in growing cells can cause mutations during replication which can lead in subsequent cell generations to malignant transformation and tumour development. On the other hand, accumulation of DNA damage in non-dividing cells is regarded as a plausible cause of senescence and cell death, thus contributing to physiological ageing (de Boer et al, 2002; Hyun et al, 2008; Kamileri et al, 2012; Kenyon & Gerson, 2007).

Failure to execute efficient transcription in the presence of DNA damage is also believed to contribute to pathological ageing, being a major pathogenic mechanism in Cockayne syndrome (CS) and cerebro-oculo-facio-skeletal (COFS) syndrome – human genetic diseases which are characterised by segmental progeria, neurodegeneration, and growth retardation (Cleaver et al, 2009). Affected individuals are deficient in a specialised DNA repair pathway termed transcription-coupled nucleotide excision repair (TC-NER) which is specialised in accelerated removal of modified nucleobases preferentially from transcribed DNA strand of actively expressed genes because of mutations in genes encoding for the CSA or CSB proteins. Skin fibroblasts from transgenic mice carrying mutations in *Csa* and *Csb* genes have similar DNA repair defects and are also characterised by impaired recovery of transcription after exposure to DNA damaging agents, altogether indicating that impaired transcription is most probably caused by massive stalling of the elongating RNA polymerase complexes caused by DNA damage in transcribed genes (van der Horst et al, 2002; van der

Horst et al, 1997). According to current estimates of the ENCODE project, as much as 75% of human genome is transcribed, of which up to 57% is transcribed in single cell lineages (Djebali et al, 2012). Considering the widespread occurrence of transcription in the genome, blockage of transcriprional elongation by DNA damage could have a huge biological impact even at physiologically low levels of DNA damage.

In addition to transcriptional stalling at damaged nucleobases in transcribed DNA, several other mechanisms by which DNA damage can interact with transcription are described in the literature. Depending on the chemical structure and size of DNA lesion, a fraction of DNA damage can be bypassed by transcribing RNA polymerases occasionally resulting in altered ribonucleotide sequence in a process termed RNA mutagenesis or "transcriptional mutagenesis" (Doetsch, 2002). Transcriptional mutagenesis can cause phenotypic changes in cells of various organisms from bacteria to mammals (Bregeon et al, 2003; Saxowsky et al, 2008; Viswanathan et al, 1999) and thereby is proposed to have a broad physiological importance. Besides the damage in transcribed gene regions, DNA base modifications in regulatory gene elements can affect expression of genes as well, for instance by changing binding affinities of transcription factors to their target nucleotide sequences (Hailer-Morrison et al, 2003; Marietta et al, 2002). However, coding gene regions and the regulatory elements constitute together not more than a few per cent of genomes of higher organisms (Consortium, 2011). Consequently, the chance of these genome elements to be affected by DNA damage is relatively low and the physiological significance of such DNA damage for transcription under real-life conditions is expected to be much smaller than of damage in the entire transcribed DNA.

3.1.2 Effects of DNA damage on transcription in cell-free systems

Damaged nucleobases and sites of base loss usually do not cause major distortions of the helical structure of DNA. As long as DNA damage is not associated with interruption of template DNA strand, damaged DNA can be transcribed by RNA polymerases at normal rate until the damage site is encountered. Further fate of the transcription elongation complex is determined to a large extent by size and stereochemistry of the DNA adduct, but apparently also depends on structural and catalytic properties of the RNA polymerase enzyme and possibly on adjacent nucleobases in DNA (Scicchitano et al, 2004). Stalling of the elongating transcription complex at the damage site can be caused by inability of the active site of the RNA polymerase to accommodate the modified base, hindered incorporation of the ribonucleotide opposing to the modified base, or inability of the RNA polymerase to translocate (for instance, if incorporation of the ribonucleotide causes a misalignment of the transcript end). The extent of a kinetic impediment during each of these steps determine the manifestation of the transcription block as either a permanent arrest or transient stalling of the elongation complex.

The largest part of experimental data on interaction of DNA damage with elongating RNA polymerases comes from transcription systems reconstituted in vitro (either with purified proteins of with cell extracts). Most of base modifications investigated up to date could be bypassed with varying degrees of efficiency by RNA polymerases from various sources (reviewed in references (Scicchitano et al, 2004; Tornaletti, 2005)). Several DNA base modifications have been reported to cause transcriptional mutagenesis (Clauson et al, 2010; Kuraoka et al, 2003; Viswanathan et al, 1999), and it is therefore expected that transcriptional bypass of DNA damage in general can be associated with substantial error rates. However, the structural basis for inaccurate bypass of various DNA base modifications remains to be explored yet. On the other hand, a few base modifications have been reported to cause a virtually complete block of elongation in the reconstituted transcription systems, manifested as accumulation of transcripts interrupted in correspondence to the damaged nucleotide whereas virtually no full-length transcripts could be produced. The best described DNA base modifications causing premature transcript termination as a result of a permanent elongation block are cyclobutane dimers of T (Donahue et al, 1994; Tornaletti et al, 1997), as well as cisplatin induced GG and GNG covalent adducts (Larsen et al, 2004;

Tornaletti et al, 2003) and acetylaminofluorene monoadducts of G (Donahue et al, 1996), all of which represent large structural modifications of DNA. In all cases, localisation of the damage in the template DNA strand is a prerequisite for the inhibition of transcription, indicating that truncation of the transcripts in proximity to the damage sites is caused by direct collisions of elongating RNA polymerases with DNA damage.

Investigations of transcriptional effects of structurally smaller base modifications, such as products of DNA oxidation or hydrolysis, resulted in rather heterogeneous results, reporting either incomplete block or no effect at all (Scicchitano et al, 2004; Tornaletti, 2005). One possible explanation for these inconsistencies is that protein preparations used for reactions could be contaminated with minor amounts of active DNA glycosylases which would excise the damage, generating abasic sites and/or DNA strand breaks, both of which are potentially transcription blocking (Kathe et al, 2004; Tornaletti et al, 2006). On the other hand, essential cellular co-factors of efficient transcription could be missing in some reconstituted transcription reactions. As an example, no RNA polymerase II transcription block could be observed at a common oxidative nucleobase modification 8-oxoG in reactions performed with nuclear extracts of HeLa cells (Kathe et al, 2004), although some pausing or blockage of transcription at the damage site was reported by several groups in reactions reconstituted with purified proteins (Kuraoka et al, 2003; Larsen et al, 2004; Tornaletti et al, 2004). However, supplementation of transcription reactions with accessory factors (elongin, CSB, TFIIS) could reconstitute normal transcription thus indicating that 8-oxoG essentially does not block elongation (Charlet-Berguerand et al, 2006; Kuraoka et al, 2007).

3.1.3 Inhibition of global and gene-specific transcription by DNA damage in cells

One approach for the investigation of the effects of DNA damage on transcription in cells is measuring of global or gene-specific transcription following the exposure to a damaging agent. Such experimental setup is well suited for assessing the regulation of transcription in response to stress conditions. However, it has to be kept in mind that such transcriptional stress responses are not necessarily triggered by DNA damage because, as a rule, damaging chemicals or physical agents are promiscuous in their molecular targets. An important exception is UV-irradiation, which damages DNA with a rather high selectivity. Consequently, also the effects of UV on transcription in cells are largely mediated by damage to DNA. It is long known that DNA damage induced by UV causes an overall decrease of RNA synthesis in cells (Mayne & Lehmann, 1982). Based on the property of UV-induced DNA adducts (primarily, cyclobutane pyrimidine dimers) to interrupt transcription upon being directly encountered by tracking RNA polymerase complexes (Brueckner et al, 2007; Donahue et al, 1994), this global decrease of transcription has been attributed to stalling of the elongating RNA polymerase at the bulky DNA modifications (Tornaletti et al, 2003). In accordance with this model, recovery of RNA synthesis after UV irradiation in turn requires an efficient repair of UV-induced DNA lesions (cyclobutane pyrimidine dimers and 6-4 photoproducts) (Hanawalt, 1994). These considerations allow indirect detection of both DNA damage (as a decline of global transcription) and repair (as recovery of RNA synthesis in cells following the damage). Thanks to the property of UV-induced DNA damage to block transcription, recovery of global RNA synthesis (RRS) in UV-irradiated sells is widely used as a diagnostic marker for human diseases which are caused by inadequate nucleotide excision repair (NER). The defective RRS is a characteristic phenotype caused by mutations in genes associated with Cockayne syndrome and several genes associated with xeroderma pigmentosum (XP), but not by mutations in the XPC gene which is only required for repair of untranscribed DNA regions.

In addition to global decline of transcription, DNA damage by UV induces numerous gene-specific transcriptional changes which are caused by activation of cellular DNA damage response signalling (Lagerwerf et al, 2011). The activation of stress response pathways can be even more pronounced, depending on the nature of a DNA damaging agent. So transcriptional responses of cells to ionising radiation, many cancer drugs, oxidants,

alkylants, etc., can be to a major extent determined by stress-induced factors. As a result, significant changes in transcriptions can already occur at relatively low DNA damage loads, thus making the direct effects of DNA damage on transcription hard to detect. Another difficulty is that genotoxic agents, apart of a few exceptions, have multiple molecular targets in cell, of which DNA in not the most vulnerable. Therefore, it is often not possible under experimental conditions to generate desired levels of DNA damage without causing cytotoxicity by irreversible damage of various cellular compartments. For these reasons, direct effects of such types of DNA damage on transcription (either global or gene-specific) are explored to a much smaller degree than the effects of UV.

Of particular interest is investigation of potential transcription-modulatory activities of endogenously generated DNA base modifications, because some of these DNA lesions apparently account for the pathological symptoms of Cockayne syndrome, a human disease associated with an impaired recovery of RNA synthesis after DNA damage. However, for obvious reasons, knowledge about the effects of such base modifications on transcription can hardly be obtained by extrapolation of the effects observed in experimental systems where the damage is inflicted from the outside of cells. Therefore, some alternative experimental approaches are required in order to adequately investigate such effects. One of the available experimental strategies is host cell reactivation assay (HCR) based on the use of an expression vector containing DNA damage of a defined nature in a reporter gene which is delivered by transfection or viral infection into undamaged host cells (Athas et al, 1991; Carreau et al, 1995; Slebos & Taylor, 2001). Damage in the transcribed sequence of a reporter gene can cause interruption of transcription. Recovery from the transcription block (gene reactivation) is only possible in cells that repair the damage (**Figure 1**). For DNA damage with proven transcription blocking potential, recovery of expression of damaged reporter genes is proportional to the host cell repair capacity (Ganesan et al, 1999). For instance, in a study of Spivak and Hanawalt the decrease of expression of a reporter β-galactosidase gene was proportional to the UV dose applied to the plasmid DNA, and this decrease was more pronounced in CS-B and $UV^{S}S$ cells (both lacking a functional CSB gene) than in host cells derived from healthy individuals (Spivak & Hanawalt, 2006).

As a method to study direct effects of DNA damage on gene transcription, HCR provides several benefits over damaging of cells. Most important advantages of the HCR

assay are the avoidance of cellular stress responses and better options to manage the amounts and chemical nature of DNA damage in a vector DNA compared to the whole genome. HCR is no less sensitive that global transcription assays in detection of transcription blocking potential of bulky DNA UV-photoproducts (Henning et al, 1995) or adducts induced by chemicals and drugs (Dabholkar et al, 1990; Dean et al, 1988; Stevnsner et al, 1995). It is also promising as an assay for interactions of transcription machineries with other kinds of DNA damage, such as oxidative damage.

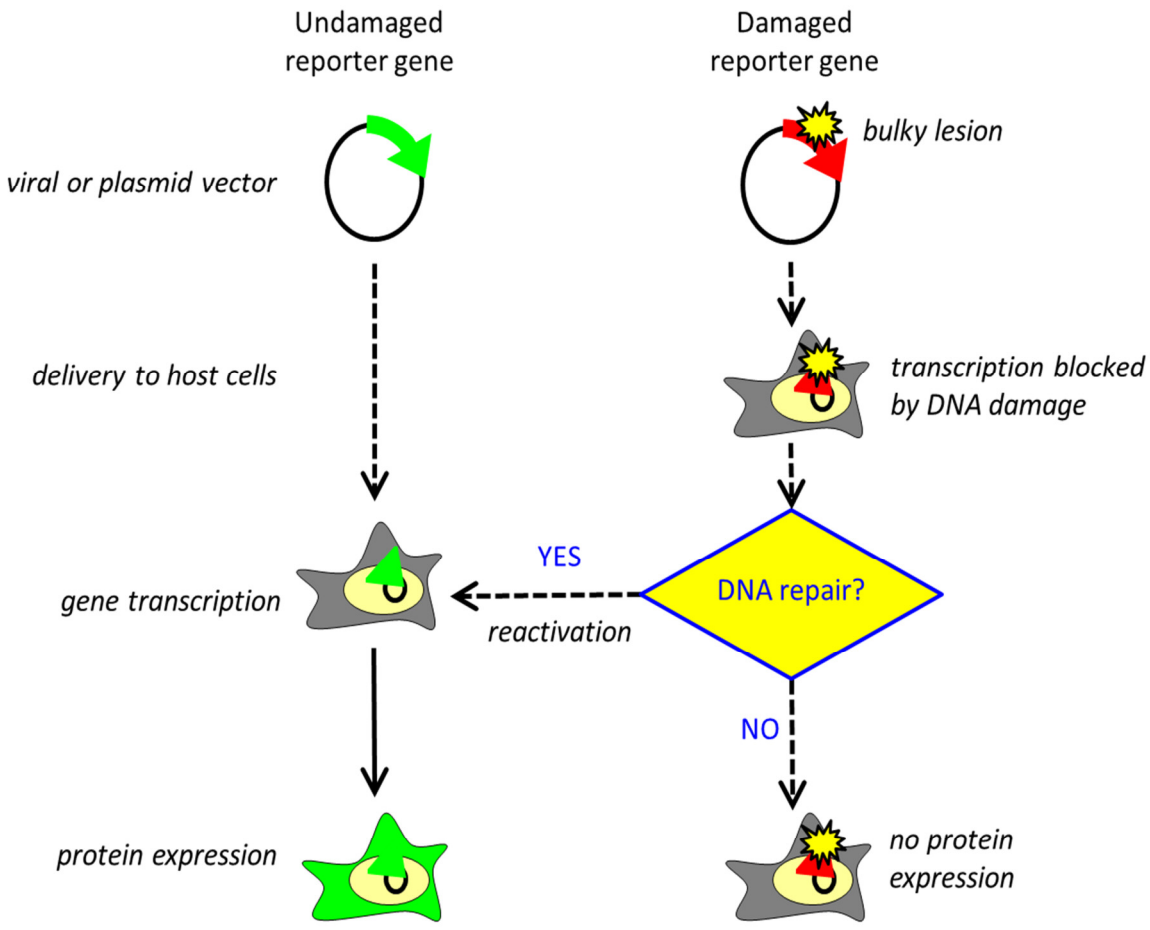

Figure 1 | Principle of the host cell reactivation assay (HCR).

3.1.4 Inhibition of gene expression by non-bulky DNA base modifications: the curious case of 8-oxoG

8-Oxo-7,8-dihydroguanine (8-oxoG) is one of the most common base modifications endogenously generated in cells (Ohno et al, 2006; Pflaum et al, 1997). It arises from the damage of DNA by reactive oxidative species such as superoxide anions, hydroxyl radicals and hydrogen peroxide. The structure, chemical reactions of DNA leading to formation of 8-oxoG, as well as the mechanisms of its mutagenicity are well studied and described elsewhere (Cadet et al, 1999; Hsu et al, 2004; Maga et al, 2007; Shibutani et al, 1991; Steenken, 1997). Base excision repair pathway provides a universal mechanism of removal of 8-oxoG over all domains of life. In eukaryotes initiation of repair relies on the OGG family of DNA glycosylases with OGG1 being the only excision activity detectable in mammalian cell extracts (Klungland & Lindahl, 1997; Osterod et al, 2001; Radicella et al, 1997; Rosenquist et al, 1997). Thus, it appears unlikely that other glycosylase could significantly contribute to BER of this DNA base modification. Despite this, some residual repair is clearly present in OGG1-null cell lines (Osterod et al, 2002), what requires a backup repair pathway for 8-oxoG. It is still disputed whether the nucleotide excision repair pathway (NER) could overtake this function in the absence of OGG1 (Berquist & Wilson, 2012; D'Errico et al, 2008).

Because of evidence that active transcription could accelerate repair of 8-oxoG (Le Page et al, 2000a; Le Page et al, 2000b), the NER subpathway denoted as transcription-coupled repair (TC-NER) has been assumed to be a plausible repair mechanism of 8-oxoG in transcribed DNA. The interactions of 8-oxoG with elongating RNA polymerases have been intensely investigated thereafter, resulting in surprising and sometimes contradictory reports. Even though 8-oxoG does not block elongation by RNA polymerases in reconstituted transcription systems, evidence has been provided that this lesion might have an inhibitory effect on transcription in mammalian cells. HCR studies of a plasmid vector (Spivak & Hanawalt, 2006) and of a recombinant adenovirus (Leach & Rainbow, 2011) demonstrated that expression of the reporter genes was decreased following the DNA damage by methylene blue photosensitisation. The magnitude of the observed effects was particularly strong in CS cell lines which lack TC-NER; it has been therefore assumed that transcription was inhibited by encounters of transcription complexes with unrepaired 8-oxoG. However, this thesis could not be definitively confirmed, because a single 8-oxoG placed in the

transcribed strand of a plasmid-borne luciferase gene failed to display any significant transcription block upon transfection of OGG1-null mouse host cells even when OGG1 deficiency was combined with functional deletion of the *Csb* gene (Larsen et al, 2004), while the presence of a strong block has been subsequently claimed in the same cell model by another group (Pastoriza-Gallego et al, 2007). The conflicting host cell reactivation results of the 8-oxoG containing vectors call for re-evaluation of the transcription-blocking potential of this nucleobase modification.

Although the observations of decreased expression of vectors carrying 8-oxoG were sometimes interpreted as the result of blockage of elongating RNA polymerases at the damage site, there is a difficulty in reconciling such a view with the apparent absence of transcription blockage under cell free conditions (Charlet-Berguerand et al, 2006; Kathe et al, 2004; Kuraoka et al, 2007; Larsen et al, 2004; Tornaletti et al, 2004) and with the available structural data (Damsma & Cramer, 2009). One possible explanation could be that under the HCR experimental setup there is always a delay of at least several hours between vector delivery to host cells and the measurement of the reporter gene expression. Depending on DNA repair activities of the host cells, a substantial fraction of DNA damage can be repaired or, at least, excised during this time. If very efficient, repair would reduce the chances of the damage to be encountered by transcribing RNA polymerases. Moreover, given that several catalytic steps are required for the accomplishment of BER (**Figure 2**), it is overall possible that transcription-blocking structural intermediates (abasic site or a single-stranded break) could be encountered during transcription. Even a subtle quantitative disbalance between single BER components could uncouple the sequential steps of DNA damage processing and potentially alter the probability of such encounters. Consequently, in order to clarify whether certain type of DNA damage can directly inhibit transcription, it would be necessary to perform gene expression analyses in cells whose DNA repair systems fail to detect this particular kind of DNA damage. Such DNA repair-deficient cell lines are available from the knockout mice. They were used, among others, in the present study as host cells for analyses of expression of exogenous damaged DNA.

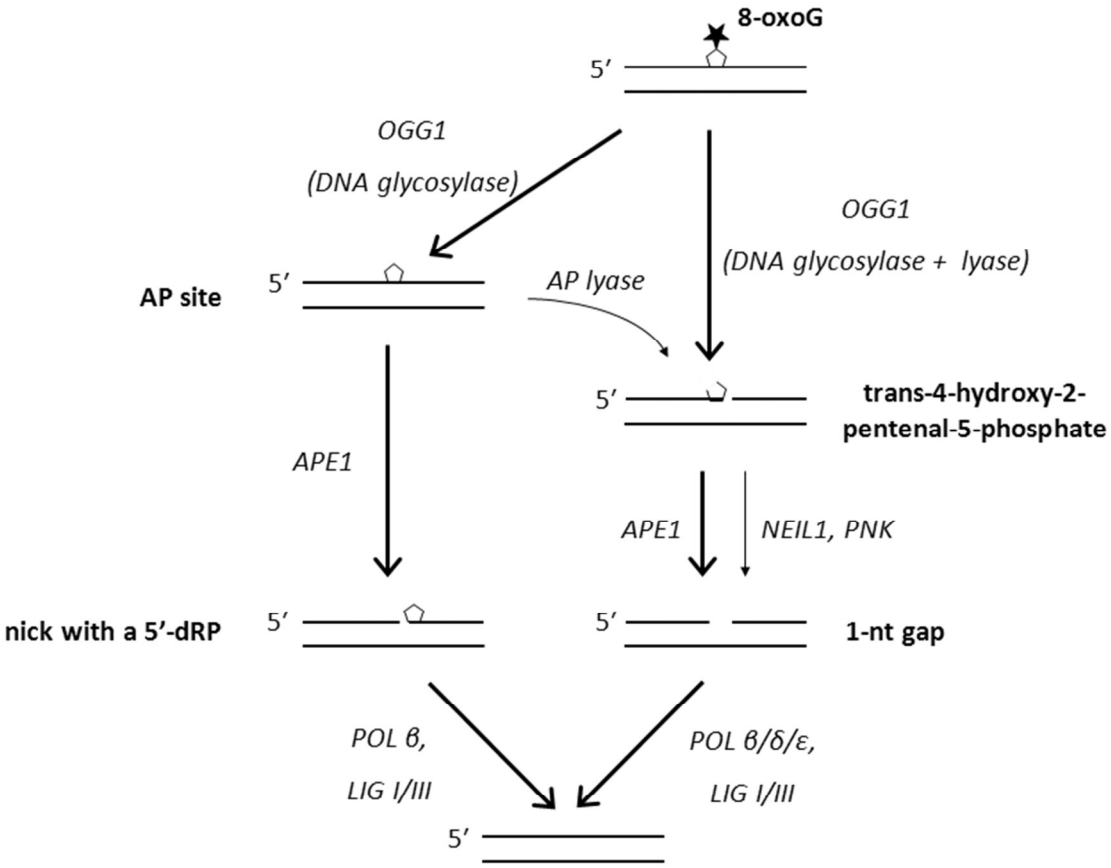

Figure 2 | Scheme of base excision repair of 8-oxoG, as reconstituted with a minimal set of purified repair proteins (Frosina et al, 1996; Pascucci et al, 2002; Sobol et al, 2000). Separable catalytic activities of OGG1 as a DNA glycosylase and a DNA lyase enable several possible scenarios for the subsequent repair steps which comprise structurally distinct intermediates. Excision of the damaged base generates an apurinic site (AP site), 3′-phosphate bond of which can optionally undergo further hydrolysis by the β-lyase activity of OGG1, resulting in a single-strand break (SSB) with the rest of the sugar attached to the 3′ DNA end in the form of an unsaturated aldehyde. It is subsequently hydrolysed by the AP site endonuclease APE1 to generate a 1-nt gap with a 3′-OH. Alternatively, APE1 can directly excise the AP site, generating a 3′-OH and a deoxyribose phosphate (dRP) attached to the 5′ DNA end. Both SSB structures generated by alternative processing of 8-oxoG are recognised by the DNA polymerase β (pol β), which incorporates G into the 1-nucleotide gap created by APE1. Removal of the 5′-dRP residue usually occurs concomitantly with the DNA synthesis step, thanks to the pronounced intrinsic lyase activity of pol β. DNA ends produced by pol β are ligated by DNA ligase I or DNA ligase III/XRCC1 complex. Bolder arrows show the predominant enzymatic activities.

3.1.5 Aims and objectives of the present work

The research presented in this habilitation thesis was directed towards the understanding of the mechanisms underlying the inhibition of transcription by DNA damage. Because the knowledge on the influences of DNA base modifications and strand breaks on transcription has been largely based on results obtained in cell-free systems, the experimental work of this thesis was primarily focused on the effects of several structurally distinct types of DNA damage on expression of the affected genes in cells. First of all, the inhibition of expression of an individual gene had to be measured following the exposure of cells to an exogenous DNA-specific damaging agent (such as UV irradiation) which generates transcription-blocking photoproducts. This task was accomplished by construction of a cell line stably expressing a reporter protein with a very rapid turnover, which is described in **Publication 1** (Kitsera et al, 2007). Next, the effects of oxidative DNA damage in an exogenous plasmid-borne gene on the gene expression had to be characterised by host cell reactivation assay and compared with the effects of UV-induced DNA damage. This was achieved by employment of a truly quantitative flow cytometry-based procedure for the gene expression analyses following the transfections of cell lines deficient in diverse repair genes, as described in **Publication 2** (Khobta et al, 2009). As a surprisingly strong inhibitory effect of oxidative DNA damage on transcription has been found in these experiments, further research was focused on the understanding of the precise mechanism of inhibition of transcription in the absence of a direct RNA polymerase blockage. The specific aims included identification of peculiarities of the chromatin structure caused by the oxidative base damage in **Publication 3** (Khobta et al, 2010a) and characterisation of the effects of repair intermediates, such as single-strand breaks, on transcription in **Publication 4** (Khobta et al, 2010b). Moreover, single DNA base modifications of defined chemical structure had to be inserted into the transcribed or the non-transcribed DNA strands and the effects of these synthetic nucleobase modifications had to be investigated depending on their localisation and on the DNA repair competence of the host cell line. This was accomplished in **Publications 5** and **6** (Kitsera et al, 2011; Luhnsdorf et al, 2012).

3.2 Overview of own research work

3.2.1 Stably replicating episomal vector for analyses of gene expression in the presence of DNA damage

Publication 1 (journal article, experimental): Kitsera, N., Khobta, A.[1], and Epe, B. (2007) Destabilized green fluorescent protein detects rapid removal of transcription blocks after genotoxic exposure. ***Biotechniques***, 43, 222-227

Authors' contributions: A. Khobta designed the experiments, performed preliminary experimentation, and wrote the manuscript; N. Kitsera performed the experiments; B. Epe advised on research planning and contributed to writing the manuscript.

Transcriptional responses of cells exposed to DNA damage induced by exogenous agents (such as UV-induced pyrimidine photoproducts) are well studied on the scale of global RNA synthesis, but not so much on the level of single genes. I chose a reporter transgene encoding for the enhanced green fluorescent protein (EGFP) as a model for investigation of the effects of DNA damage on transcription of a single gene, because the EGFP expression levels can be directly measured in individual cells by fluorescence detecting techniques (Cormack et al, 1996; Soboleski et al, 2005). However, detection of short-lasting perturbations of transcription appeared problematic because the average repair times of DNA base modifications are usually much shorter than the protein lifetime of conventional EGFP in cells (about 26 hours). To achieve a more rapid protein turnover, we modified the untranslated region (UTR) of the EGFP gene (**Figure 3**) by attaching a DNA sequence coding for a proline-glutamate-serine-threonine-rich (PEST) sequence motif and several AU-rich elements (AREs), which provide powerful degradation signals for the protein and mRNA, respectively (Kitsera et al, 2007)[2]. After trying several vector backbones for the EGFP expression construct, we chose an episomally replicating non-viral vector pMARs (Jenke et al, 2004) because it showed remarkably steady levels of gene expression in stably transfected host cells (Kitsera, 2011). The newly-designed EGFP-ODC fusion protein showed half-life of <40 minutes in stably transfected HeLa cells (Kitsera et al, 2007).

[1] Corresponding author

[2] This publication

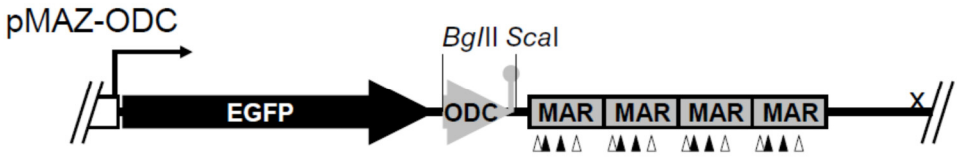

Figure 3 | Fragment of the pMAZ-ODC episomal vector carrying a gene for the newly-constructed short-leaved EGFP-ODC fusion protein. The canonical EGFP coding sequence is fused to a fragment of the exon 10 of the mouse ornithine decarboxylase gene (ODC) containing a well characterised PEST motif. Matrix attachment regions (MARs) essential for the episomal maintenance of the vector were provided within the original pMARs backbone. Black and white arrowheads show two kinds of putative AREs in the 3'-UTR of the gene. Reproduced in a reduced form from Kitsera et al. (2007) *Biotechniques* 43: 222-227.

Stationary levels of the short-lived EGFP-ODC protein in stably transfected cells were sensitive to perturbations of the gene transcription cased either by chemical inhibitors or by DNA damage induced by exposure of cultivated monolayers to 254 nm UV-C (Kitsera et al, 2007). The inhibitory effect of UV-induced DNA damage on the gene transcription was dose-dependent (**Figure 4**): the fractions of cells losing the EGFP fluorescence thereby showed a clear correlation with the probability of damage generation in the transcribed region of the gene. Extrapolation of quantitative data from the literature reporting the frequencies of generation of the pyrimidine photoproducts by UV to the doses applied in our experiments showed good agreement with the notion that the decrease of the EGFP expression was caused by stalling of transcribing RNA polymerase II at DNA damage sites, assuming that only DNA adducts located in the transcribed DNA strand would block elongation (Kitsera, 2011; Kitsera et al, 2007). Another indication for the proposed mode of action is a gradual recovery of the EGFP expression (**Figure 5**), which started several hours after the exposure to UV, being thus consistent in the time scale with the reported repair kinetics for the UV-induced DNA damage (Ljungman, 1999; Spivak et al, 2002).

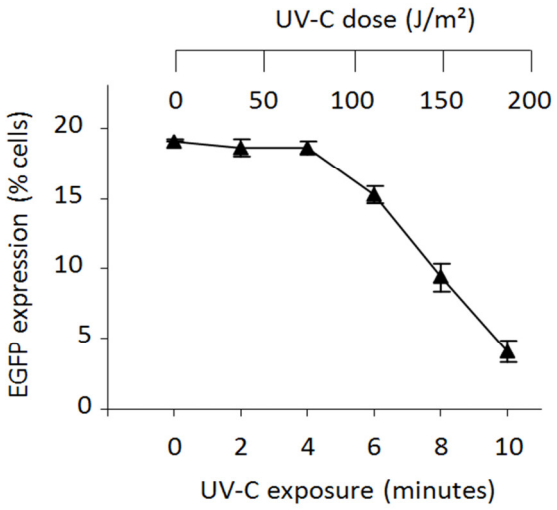

Figure 4 | Percentages of EGFP-positive cells remaining 150 minutes after exposure to a range of UV-C doses. Modified from Kitsera et al. (2007) *Biotechniques* 43: 222-227.

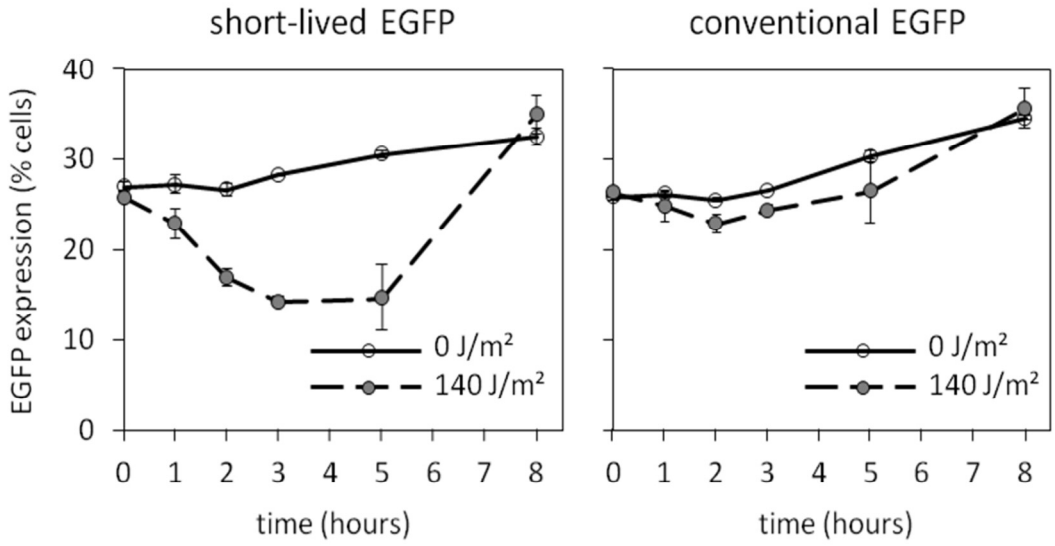

Figure 5 | Inhibition of EGFP expression in cells stably transfected with episomal vectors encoding either for the short-lived EGFP-ODC fusion protein (left) of for a conventional EGFP (right) at various times following the irradiation with UV-C (140 J/m²). Experimental details are provided in Kitsera et al. (2007) *Biotechniques* 43: 222-227.

The decrease of EGFP expression following the UV irradiation was readily observable in cells carrying a gene for the short-lived EGFP-ODC fusion protein, but not in cells expressing an equally-sized conventional EGFP gene encoded by an analogous episomal vector (**Figure 5**), thus showing that of two proteins the short-leaved EGFP is better suited for detection of transient perturbations of transcription. This property characterises our destabilised EGFP as an excellent reporter for investigation of dynamics of downregulation of gene transcription. After our publication, the vectors (and in some cases cell lines) were dispatched upon requests to more than 20 labs. The cell line stably expressing the short-lived EGFP was adopted by Miltenyi Biotech GmbH for testing the efficiencies of transcriptional gene silencing by siRNAs (Dr. Thomas D. Rockel, personal communication). I hope and anticipate that this model will find further applications for investigation of cellular mechanisms of toxicity of chemical substances and physical agents which specifically target DNA.

3.2.2 Host cell reactivation of the expression vector containing oxidative base damage generated by photosensitisation: the roles of the 8-oxoguanine DNA glycosylase (OGG1) and of the Cockayne syndrome B (CSB) proteins

Publication 2 (journal article, experimental): Khobta, A.[1], Kitsera, N., Speckmann, B., Epe, B. (2009) 8-Oxoguanine DNA glycosylase (Ogg1) causes a transcriptional inactivation of damaged DNA in the absence of functional Cockayne syndrome B (Csb) protein. ***DNA Repair***, 8, 309-317

Authors' contributions: A. Khobta conceived and optimised the modified host cell reactivation assay, designed the experiments, performed most of the experiments, wrote the manuscript; N. Kitsera performed time-course host cell reactivation experiments in repair-deficient MEFs; B. Speckmann performed preliminary experiments; B. Epe supervised the work, participated in planning the research, and took part in writing of the manuscript.

Several studies of transcriptional responses to oxidants provided indications for a decreased global transcription in the exposed cells (Heine et al, 2008; Kyng et al, 2003). Generation of oxidative DNA damage has been proposed as a plausible factor responsible for inhibition of transcription by these agents, based on the assumption that tracking of the elongating RNA polymerase II complexes would be substantially inhibited by such DNA damage. However, no strong effect of oxidative DNA base modifications on the elongation rate by RNA polymerases isolated from various sources could be subsequently demonstrated in reconstituted transcription systems containing various chemically defined base modifications in the template DNA (Charlet-Berguerand et al, 2006; Kathe et al, 2004; Kuraoka et al, 2007; Larsen et al, 2004; Tornaletti et al, 2004). To resolve this apparent contradiction, the efficiency of transcription of templates containing oxidative DNA base modifications had to be measured in cells not subjected to irradiation or exogenous oxidative stress in order to avoid unspecific stress responses. This could be achieved by transfection of damaged vector DNA containing a suitable reporter gene into non-damaged cells in an approach known as "host cell reactivation" (HCR) – a strategy which was successfully applied in the past for identification of DNA repair defects in the actively transcribed genes, leading to cloning of the human CSA (ERCC6) gene (Henning et al,

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1995). The principle of the method is that any persistent transcription-blocking damage can be detected as a decrease of the reporter gene expression. Consequently, the inhibition of transcription is typically more severe in repair-deficient cells.

I designed an improved HCR protocol and optimised it with the participation of co-workers Bodo Speckmann and Nataliya Kitsera to achieve a truly quantitative readout of expression of the reporter EGFP gene under conditions of transient cell transfections (Khobta et al, 2010a; Khobta et al, 2009[1]). Our method exploits a pair of expression vectors encoding for fluorescent proteins with different emission spectra (EGFP and DsRed-Monomer) whose expression can be measured independently in transfected mammalian host cells by fluorescence-activated techniques, such as flow cytometry. This provides an advantage of selecting of individual transfected cells by detection of the expression of a fluorescent protein which is used as a tracer (DsRed-Monomer is generally preferable for this purpose because of its weaker fluorescent signal compared to that of EGFP). We showed that amount of expression of another gene (EGFP) in these pre-selected cells is directly proportional to the number of gene copies (**Figure 6**), making the system potentially well suited for quantitative detection of transcription-blocking DNA damage.

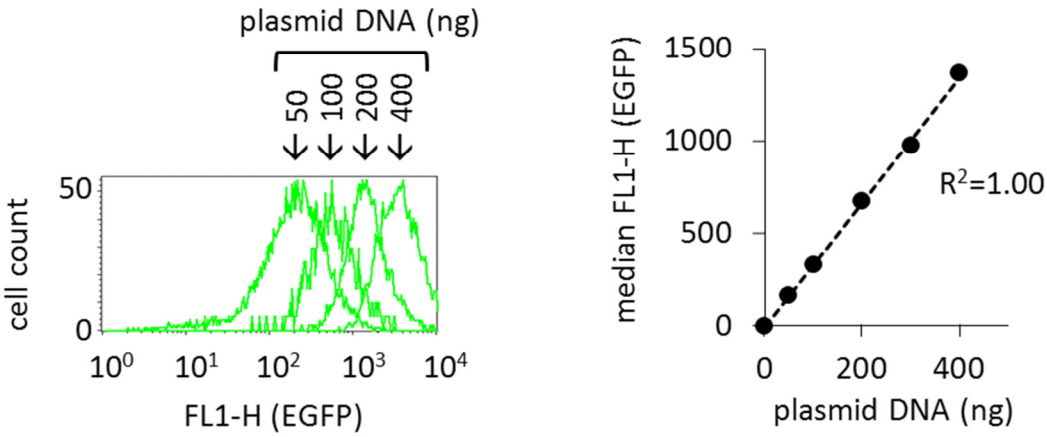

Figure 6 | Quantitative analyses of the EGFP fluorescence in the transfected HeLa cells by flow cytometry and a linear dependence of the EGFP expression on the amounts of the pEGFP-mODC-ZA plasmid. Transfected cells were gated based on the expression of the tracer plasmid encoding for DsRed-Monomer, amounts of which were kept constant (400 ng) in all transfections.

[1] This publication

To verify that expression of the plasmid-borne EGFP transgene is sensitive to the transcription blocking DNA damage, small amounts of such damage – a mixture of cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine photoproducts (6-4PP) – were generated by UV-B irradiation in an EGFP-encoding pZA plasmid. The numbers of CPD (which are the predominant DNA modifications induced by UV-B) were quantified by digestion with endonuclease V of bacteriophage T4, and plasmids containing on average 3 or 9 CPD (corresponding respectively to 0.5 and 1.5 CPD per transcribed strand of the EGFP gene) were chosen for transfection into mammalian cells and transcription analyses. Host cell reactivation assays were performed in the homozygous *Csb*$^{mut/mut}$ mouse embryonic fibroblasts (MEFs), because these cells are deficient in CPD removal from transcribed DNA strand of endogenous genes (van der Horst et al, 1997). Remarkably, already 0.5 CPD per transcribed strand of the EGFP gene caused an about two-fold decrease of the average EGFP fluorescence per transfected cell in *Csb*$^{mut/mut}$ MEFs (Khobta et al, 2009), thus reflecting a high extent of transcription blockage in the presence of CPD and confirming a high sensitivity of the assay. This effect was enhanced with the increase of the DNA damage load to 1.5 CPD per transcribed strand of the EGFP gene (9 CPD per plasmid molecule) resulting in only 10% residual EGFP fluorescence, compared to the undamaged control. The same amounts of DNA damage caused a much milder decrease of the gene expression in the repair-proficient *Csb*$^{wt/wt}$ cell line. In the latter case, initial decrease of the gene expression was followed by a gradual recovery (**Figure 7**), being thus consistent with removal of the damage by DNA repair. No recovery of gene expression was observed in repair deficient *Csb*$^{mut/mut}$ MEFs monitored for 48 hours.

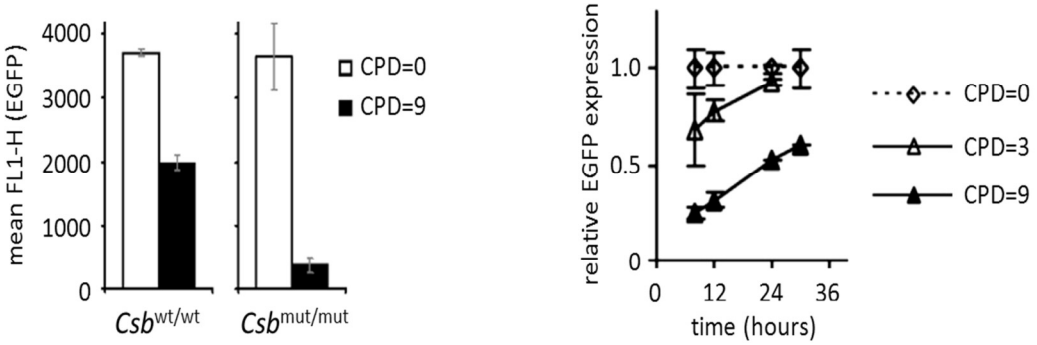

Figure 7 | Expression analyses of plasmids containing the indicated numbers of cyclobutane pyrimidine dimers (CPD) induced by UV. Quantification of the EGFP fluorescence in *Csb*$^{wt/wt}$ and *Csb*$^{mut/mut}$ MEFs by flow cytometry 24 hours post transfection (left) and time course of the EGFP expression in wild-type MEFs (right).

Next, the effects of oxidative DNA base damage on the reporter gene transcription were investigated in wild-type, *Ogg1*$^{-/-}$, *Csb*$^{mut/mut}$ and *Csb*$^{mut/mut}$*Ogg1*$^{-/-}$ cells. This combination of cell lines provides a useful model system for studying the effects of the oxidative nucleobase damage on transcription in the absence of repair, because base excision repair of 8-oxoG is absent or, at least, very inefficient in mouse embryonic fibroblast (MEF) cell lines with a homozygously disrupted *Ogg1* gene (Osterod et al, 2001; Saxowsky et al, 2008). The plasmids were damaged prior to transfection by irradiation with a halogen lamp in the presence of methylene blue as a photosensitiser to generate on average 1 or 3 oxidised guanines per plasmid molecule, as quantified by treatments with the *E. coli* formamidopyrimidine DNA glycosylase (Fpg). Because the induction levels of oxidised pyrimidines, abasic sites, and single strand breaks were many folds lower (Khobta et al, 2009), these DNA lesions could not interfere to a significant extent with the gene transcription. If the oxidative base modifications induced by the photosensitiser would cause blockage of transcription of the damaged DNA, this was expected to cause in the OGG1-null cell lines a stronger decrease of EGFP expression than in the repair proficient OGG1 wild-type (wt) cells. Nevertheless, transfection of the same amounts of the same preparations of the damaged plasmids into different MEF cell lines showed that this was not exactly the case. The effect of damage on EGFP expression was observed in all MEF cell lines and had the strongest magnitude in *Csb*$^{mut/mut}$ MEFs whose both *Ogg1* alleles were wild-type. Combined deficiency in both OGG1 and CSB proteins did not result in additive effects, but rather caused in a significantly improved expression of the damaged plasmids (2-fold reduction of gene expression against the 5-fold in the *Csb*$^{mut/mut}$*Ogg1*$^{wt/wt}$ MEFs in the presence of the same amounts of the DNA damage). This result was further confirmed by quantitative analyses of mRNA transcripts by reverse transcription and real-time PCR (Khobta et al, 2009). Remarkably, we found no indication for a time-dependent recovery of expression of the oxidatively damaged gene in any of the MEF cell lines tested, including the fully repair proficient *Csb*$^{wt/wt}$*Ogg1*$^{wt/wt}$ cells (Khobta et al, 2009). These results show that prolonged inhibition of expression of the oxidatively damaged DNA cannot be regarded as a bona fide marker of the repair deficiency of the host cells, what is strikingly different from the results obtained with the UV-damaged expression vectors (**Figure 7**), and thus undermine the traditional way of interpretation of host cell reactivation data. The reasons

for the persistent inhibition of gene transcription by oxidative nucleobase damage were further investigated in course of the research described in subsequent sections, where the effects of several structurally defined base modifications and single-strand breaks were addressed more specifically.

Altogether the results provided evidence for a pronounced negative effect of oxidative damage induced by photosensitisation on gene expression. The inhibition of transcription reported here was probably caused by 8-oxoG, which was the predominant base modifications under our experimental conditions. In the presence of a functional *Csb* gene, no major effect of the *Ogg1* gene status on expression of the damaged plasmid gene was found. A plausible explanation of this result could be that the presence of CSB protein enables transcription of the damaged gene, regardless of the functional states of base excision repair. The results thereby demonstrate that the *Csb* gene product has an essential function for transcription in the presence of the oxidative base damage, while OGG1 can be potentially harmful for expression of oxidatively damaged DNA if the CSB protein is absent.

3.2.3 Gene silencing as a mechanism of transcriptional inhibition by oxidative DNA base damage induced by photosensitisation

Publication 3 (journal article, experimental): Khobta, A.[1], Anderhub, S., Kitsera, N., Epe, B. (2010) Gene silencing induced by oxidative DNA base damage: association with local decrease of histone H4 acetylation in the promoter region. ***Nucleic Acids Res***., 38, 4285-4295

Authors' contributions: A. Khobta designed, performed and supervised the experiments, wrote the manuscript; S. Anderhub and N. Kitsera took part in experiments jointly with A. Khobta or performed them under his supervision; B. Epe took part in planning the experiments and writing the manuscript.

The nature of the inhibitory effect of oxidative damage induced in plasmid DNA by photosensitisation on transcription was further investigated in repair-proficient host cells. Each of the two vectors – pEGFP-mODC-ZA (encoding for EGFP) and pDsRed (encoding for DsRed-Monomer) – was damaged by exposure to a range of light doses in the presence of methylene blue and transfected to HeLa cells, which are easy to transfect and are proficient in synthesis of large amounts of the fluorescent proteins within short times after transfection. Both vectors demonstrated a damage-dependent decrease of expression of the reporter genes upon transfection into HeLa cells. The magnitude of the inhibition of transcription was clearly dependent on the light doses, no matter which of the two vectors had been damaged (Khobta et al, 2010a)[2].

The influence of oxidative DNA damage (oxidised guanines) on plasmid expression were next analysed at various times after delivery into the host cells. Only small effects of the photosensitisation-induced DNA damage on the reporter gene expression were detected at early times after transfection (up to 8 hours). However, a strong decrease of the protein expression, which was specific for a plasmid that has been exposed to light in the presence of methylene blue as a photosensitiser was developing at later times even if a few randomly distributed oxidised guanines were present in the plasmid DNA (Khobta et al, 2010a). This finding suggests that the effect of oxidative DNA base modifications on gene expression

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[2] This publication

must be different from the direct blockage of elongating RNA polymerases at the damage sites, otherwise the inhibitory effect of DNA damage on gene transcription would be immediately observable. The latter was clearly not the case, because fluorescence emitted by the protein expressed from the damaged plasmid was visible along with fluorescence of the reference protein in all cells analysed 8 hours after co-transfection, but vanishing from a large fraction of cells at later time points (**Figure 8**).

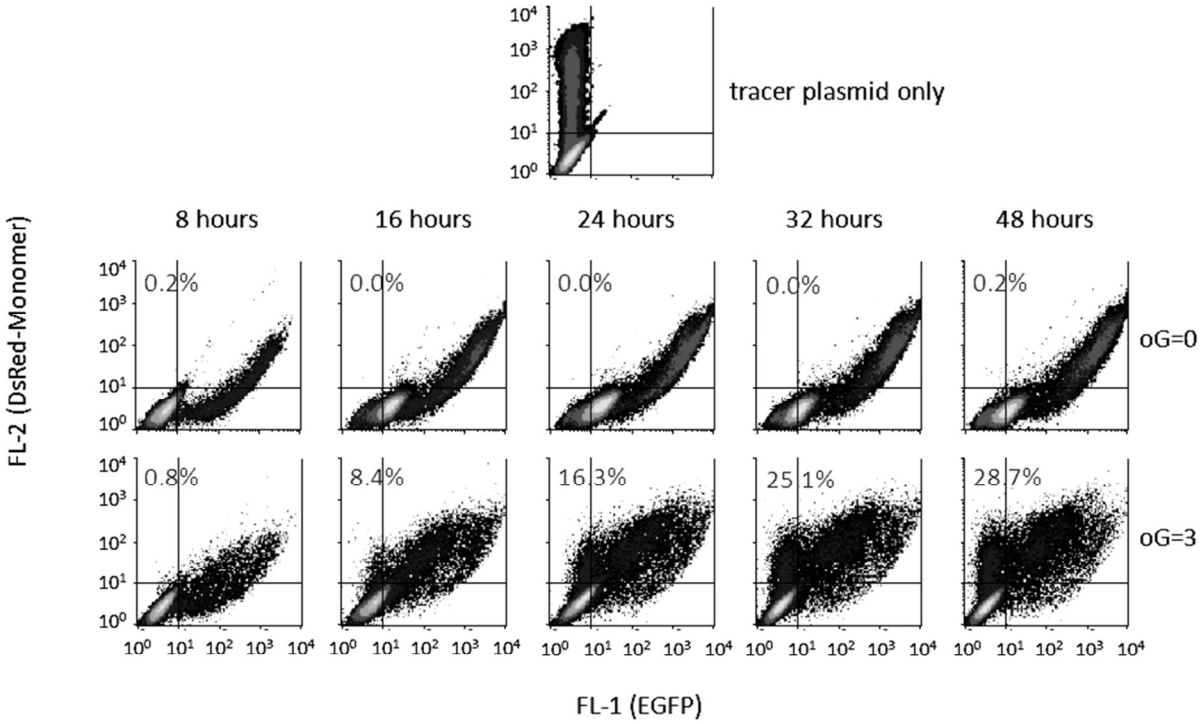

Figure 8 | Time-course of expression of the EGFP-encoding plasmid damaged by incubation with methylene blue as a photosensitiser in HeLa cells (analysed by flow cytometry). Dark-incubated plasmid contained no Fpg-sensitive products of guanine oxidation (oG=0). Light-damaged plasmid contained on average 3 oxidised guanines per DNA molecule (oG=3). Transfected cells are localised in upper two quadrants (UL+UR) of scatter-plots. Overlayed numbers show fractions of transfected cells which have no EGFP expression, calculated as UL/(UL+UR)×100%. Modified from Khobta et al. (2010) *Nucleic Acids Res* 38: 4285–4295.

The observed decrease of expression of the plasmid DNA containing the photosensitisation-induced oxidative base damage could be either the consequence of inefficient delivery of the damaged plasmid to the host cells or its degradation after transfection, or transcriptional downregulation of the plasmid-borne genes in the host cells. Quantitative analyses of plasmid DNA recovered after prolonged (24 hours) incubations in the host cells demonstrated that delivery or intracellular degradation of damaged vector DNA are of a minor relevance. On the other hand, the expression of the plasmid-borne gene copies persisting in the cells is decreased several fold in consequence of the DNA damage, as measured by the transcript to DNA copy number ratios (Khobta et al, 2010a). As the transcription rate of the co-transfected undamaged reference plasmid did not decrease in these cells, the decreased rate of transcription of the reporter gene situated in the plasmid exposed to the DNA damaging agent has to be explained by a selective gene silencing mechanism acting in cis.

Epigenetic marks present in the vector DNA residing in the transfected cells were investigated aiming on the identification of the gene silencing mechanism operating in the damaged DNA. No CpG methylation was found by bisulphite sequencing of several plasmid clones analysed 24 hours after transfection (unpublished results), thus indicating that de novo cytosine methylation was not a primary mechanism of transcriptional silencing. Instead, a chromatin mediated gene silencing was assumed to be a more likely candidate mechanism, because it has been demonstrated that folding of the transgene into chromatin takes place within hours after transfection. Moreover, both transcription activatory and repressive histone marks were found in the EGFP transgene by chromatin immunoprecipitation 24 hours after transfections (Khobta et al, 2010a).

Quantitative analyses of acetyl-histone H3, acetyl-histone H4, methyl-histone H3 (H3K9Me2 and H3K27Me3) and histone H1 in the gene promoter, the protein coding region and the 3' untranslated region (UTR) of the EGFP gene could detect alterations of the chromatin structure, which were specific for the damaged vector. Namely, the amount of histone H4 acetylation was decreased in the promoter region in comparison to the corresponding undamaged control. This decrease was mild, but reproducibly observable in multiple experiments. In addition, the repressive chromatin marks (histone H3 K9Me2 and K27Me3) and the linker histone H1 showed some re-distribution along the gene body,

demonstrating a shift towards the promoter region, albeit without a significant overall increase in the damaged gene over the undamaged one (Khobta et al, 2010a). Altogether, the chromatin immunoprecipitation data demonstrated an association of transcriptional silencing of the oxidatively damaged transgene with covalent modifications of histone proteins in the gene promoter region.

We further tested whether transcriptional silencing of the oxidatively damaged gene could be prevented by a histone deacetylase inhibitor trichostatin A (TSA) or by an inhibitor of DNA methylation 5-aza-2′-deoxycytidine. A complete recovery of gene expression to the levels of non-damaged vector could not be achieved, however the levels of gene expression significantly increased in TSA-treated cells in a dose dependent manner (Khobta et al, 2010a). By contrast, no change of expression of the damaged plasmid was observed in cells continuously incubated with 5-aza-2′-deoxycytidine. These results thus strongly suggest that the mechanism of transcriptional silencing is mediated, at least in part, by the induction of histone deacetylation.

The finding that persistent transcriptional silencing can be induced by oxidative DNA damage is of potential great biological importance, because high amounts of such damage (several hundreds per cell per day) are generated in all cells of the organism under normal physiological conditions. If a similar gene silencing mechanism operates in chromosomal genes, it might be capable of significantly perturbing in a stochastic manner the expression of individual genes in individual cells.

3.2.4 Inactivation of the reporter gene transcription by single-strand breaks in different DNA strands and the role of CSB protein

Publication 4 (journal article, experimental): Khobta, A.[1], Lingg, T., Schulz, I., Warken, D., Kitsera, N., Epe, B. (2010) Mouse CSB protein is important for gene expression in the presence of a single-strand break in the non-transcribed DNA strand. ***DNA Repair***, 9, 985-993

Authors' contributions: A. Khobta designed, supervised and partly performed the experiments, wrote the manuscript; T. Lingg performed most of the experiments under the supervision of A. Khobta; I. Schulz performed the analyses of repair kinetics; D. Warken performed preliminary experiments under the supervision of or jointly with A. Khobta; N. Kitsera performed some of the transfections and all experimentation with cells of human origin; B. Epe participated in design of the experiments and writing of the manuscript.

Repair of frequently occurring types of DNA base damage requires excision of the modified bases by the specific DNA glysosylases and hydrolysis of the phosphodiester bond 5' from the damage site by the apurinic/apirimidinic site endonuclease (APE1). Transient single-strand breaks (SSBs) are thus unavoidably generated concomitantly with every repair event. This makes the repair-induced SSBs a most frequent type of the endogenously generated DNA damage in cells. It is reasonable to assume that in genes transcribed at high rates these SSBs can be encountered by elongating RNA polymerase complexes before the repair is accomplished. The plasmid-based host cell reactivation assay is well suited for investigation of the effects of such encounters on transcription of an individual gene insofar as defined amounts of SSBs can be easily generated extracellularly in plasmid DNA.

Previously, while performing host cell reactivation experiments with photosensitiser-damaged DNA, we noticed that SSBs (induced in circular DNA containing oxidised purine bases by treatment with a bacterial DNA glycosylase Fpg and thus resembling the physiological base excision repair intermediates) cause a strong inhibition of transcription upon transfection into mammalian host cells (Khobta et al, 2009). Since it was interesting to investigate whether the magnitude of inhibition of transcription depends on the SSB position with respect to the

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damaged gene, I sought for a possibility of generation of a unique SSB into the EGFP transgene in a DNA strand- and position-specific manner. I found out that the protein-coding DNA sequence contained two sites for the strand-specific nicking endonucleases Nt.Bpu10I and Nb.Bpu10I. A silent AG→TC mutation was then introduced in the codon 209 of the EGFP gene. This mutation eliminated one of the Bpu10I sites, while the remaining site could now be used for induction of strand-specific nicks in the plasmid DNA either in the transcribed strand of the EGFP gene ("bottom strand") by Nb.Bpu10I or in the non-transcribed strand ("top strand") by Nt.Bpu10I, as shown in **Figure 9**. It was subsequently confirmed that Nt.Bpu10I and Nb.Bpu10I generate nicks of identical chemical structure (3'-hydroxylated and 5'-phosphorylated) which are fully ligatable (Khobta et al, 2010b)[1].

The results of host cell reactivation of plasmid DNA transfected to HeLa cells showed that a single nick in either of the DNA strands was sufficient to cause a strong decrease in the EGFP gene expression (as judged from quantification of the EGFP-specific fluorescence in transfected cells) without notably influencing the number of DNA copies delivered to the host cells (as measured by real-time quantitative PCR). Interestingly, the nick in the transcribed strand of the EGFP gene caused a smaller (less than two-fold) decrease in the gene expression than the nick in the complementary DNA strand (more than a three-fold-decrease).

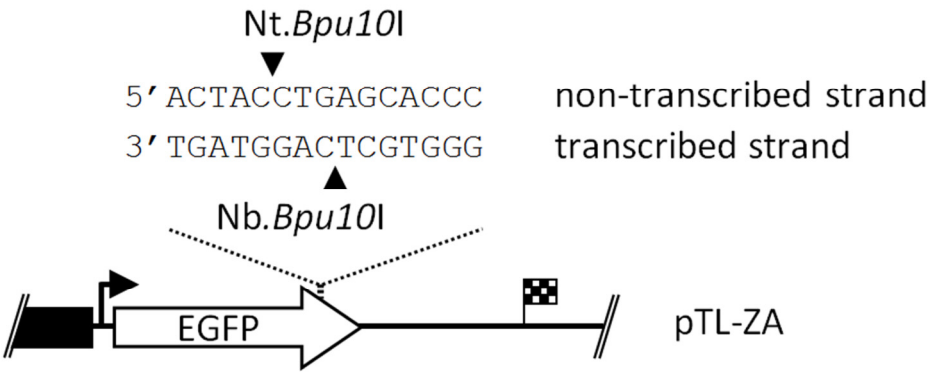

Figure 9 | DNA sequence and location of the unique Bpu10I site (5'-CCTNAGC-3') within the EGFP gene in the pTL-ZA plasmid. Arrowheads above and below the sequence indicate positions of the nicks introduced by Nt.Bpu10I (the top, non-transcribed DNA strand) and Nb.Bpu10I (the bottom, transcribed strand). Open arrow indicates the protein-coding sequence. The broken arrow and the flag mark transcription start and termination sites, respectively.

[1] This publication

The functional CSB protein is known to be important for recovery of transcription by RNA polymerase II (RNAPII) after UV-induced DNA damage (Ganesan et al, 1999; Selby & Sancar, 1997), however the requirements of CSB for the RNAPII transcription in the presence of SSBs had not been explored so far. To investigate this, we compared expression of the plasmids containing an enzymatically induced nick in either the transcribed or the non-transcribed DNA strand of the EGFP gene in MEFs obtained from the wild-type mice and from the CSB-null mice homozygous for the disrupted *Csb* allele ($Csb^{mut/mut}$). Inhibition of the transgene expression by unique SSBs in wild-type cells quantitatively resembled the effects previously observed in HeLa cell line, once again supporting a starker effect of the damage in the non-transcribed strand. The inhibitory effects of SSBs on the EGFP expression were strongly enhanced when the host MEFs were CSB-null, especially in case when the enzymatically-induced SSB was present in the non-transcribed DNA strand (Khobta et al, 2010b).

Since transcription failure in correspondence to DNA damage is believed to be responsible for the clinical features of Cockayne syndrome (CS) in humans, the expression of the nicked plasmid substrates was also assessed in immortalised human cell lines derived from a CS-B patient or from healthy individuals. However, no major differences between the CS-B cell line (CS1ANps3g2) and two control cell lines (MRC-5 VA1 and WI-38 VA13) could be observed in the expression levels of the nicked plasmid substrates (Khobta et al, 2010b). The observation of discrepant transcriptional responses to the same kind of DNA damage between the human CS cells and the mouse CSB-null cells indicates the limited usefulness of one or both of these models for understanding of the molecular defect of transcription in CS cells. It is unlikely that deficient transcription of nicked DNA in CSB-null MEFs is caused by an uncontrolled genetic factor because very similar effects were reported in two cell lines independently derived from different animals (Khobta et al, 2010b). Thus, the absence of a significant difference in responses to SSBs between the normal and CS-B human cells indicates that the transcriptional phenotype caused by CSB deficiency is different between the human and mouse models of disease. These differences can be either the result of transformation of human cells by the SV40 large T-antigen or reflect some inherent interspecies differences in transcriptional responses to DNA damage.

In summary, the results show that the inhibitory effects of the nicks in DNA on transcription are present in apparently all cell types and that the nicks situated in coding (non-transcribed) DNA strand are generally more harmful. In mouse cells, the absence of a functional *Csb* gene exacerbates the transcriptional defect in the presence of a single nick in the template DNA. Together with the demonstration of a negative role of the OGG1 DNA glycosylase for the expression of the methylene blue damaged DNA in a previous study (Khobta et al, 2009), the data suggest that the repair-induced SSBs could potentially be responsible for the inhibition of transcription by oxidative base damage and that CSB must be important for prevention of the inhibition of transcription by these repair intermediates. Another important finding is that RNAPII transcription in CSB-deficient MEFs is particularly sensitive to nicks situated in the non-transcribed DNA strand, thus revising the common belief that CSB protein is only relevant for processing of DNA damage situated in the transcribed strand and indicating that the role of CSB in transcription of damaged DNA is uncoupled from function of this protein in transcription-coupled repair of damage situated preferentially in the transcribed DNA strand.

3.2.5 Inhibition of gene expression by single 8-oxoG specifically positioned within the transcribed region of a reporter gene

Publication 5 (journal article, experimental): Kitsera, N., Stathis, D., Lühnsdorf, B., Müller, H., Carell, T., Epe, B., Khobta, A.[1] (2010) 8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1. ***Nucleic Acids Res.***, 39, 5926-5934

Authors' contributions: N. Kitsera optimised the methodology, co-designed and performed most of the experiments; B. Lühnsdorf performed the host cell reactivation analyses of uracil and AP-site; D. Stathis, H. Müller and T. Carell provided the oligonucleotides containing single 8-oxoG; B. Epe participated in writing and editing of the manuscript. A. Khobta authored the project, designed and supervised the experiments, wrote the manuscript.

8-OxoG is the most frequent base modification generated by oxidation of DNA in the presence of methylene blue as a photosensitiser. It was therefore presumed that inhibition of transcription of the methylene blue-damaged reporter plasmids in the host cell reactivation assays (Khobta et al, 2010a; Khobta et al, 2009) was most likely caused by 8-oxoG itself or by its excision products in the repair-proficient host cells. Still, to formally proof the potential of 8-oxoG to inhibit transcription and to understand the mechanism of this inhibition, it was necessary to investigate expression of a gene in which 8-oxoG would be present in a defined position in the transcribed DNA strand and in the absence of any collaterally occurring DNA damage. Such plasmid molecules have been previously constructed by isolation of single-stranded form of plasmid DNA from the M13 filamentous phage, annealing of a synthetic oligonucleotide containing a single 8-oxoG to the circular DNA, and re-synthesis of the complementary DNA strand in vitro (Bregeon & Doetsch, 2004). However, we found out that plasmid substrates obtained by this method are not well suited for the quantitative host cell reactivation assays for a number of reasons, most important of which are low yield of the covalently closed plasmid DNA and contamination with chemicals, as discussed by my collaborator Nataliya Kitsera in her doctoral thesis (Kitsera, 2011). Instead, we proposed an alternative method (described in the next chapter) for an efficient

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incorporation of synthetic oligonucleotides containing 8-oxoG or other modified bases into the plasmid-borne EGFP reporter gene. The synthetic oligonucleotides were incorporated on places of the native single stranded 18-mers excised from the plasmid DNA in a DNA strand-specific manner by the nicking endonucleases Nt.Bpu10I and Nb.Bpu10I. The plasmids containing single 8-oxoG bases in the opposing strands of the EGFP gene and the corresponding plasmids harbouring the unmodified synthetic oligonucleotides were thus obtained (**Figure 10**).

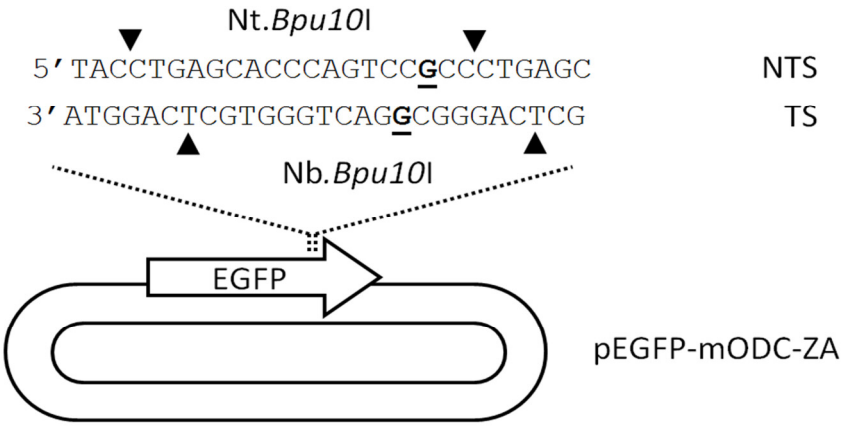

Figure 10 | A fragment of DNA sequence of the EGFP gene, containing the sites nicked by the Nt.Bpu10I and Nb.Bpu10I strand-specific endonucleases (arrowheads) in the non-transcribed (NTS) and the transcribed (TS) DNA strands. The excised single-stranded 18-mers were subsequently exchanged for synthetic oligonucleotides containing either G or 8-oxoG in the positions underlined.

Plasmid substrates containing single synthetic 8-oxoG in the transcribed and the non-transcribed DNA strands of the EGFP gene were constructed, and the effects of 8-oxoG on EGFP expression were measured in HeLa cells. The plasmid containing synthetic 8-oxoG in the non-transcribed DNA strand demonstrated an about twofold decrease of EGFP fluorescence (compared to the control plasmid), while parallel transfections of plasmid DNA with 8-oxoG positioned in the transcribed strand of the EGFP gene detected no influence of the base modification on the expression of the reporter protein, compared to the control plasmid (**Figure 11**). This result was confirmed by the quantitative analyses of the RNA transcripts by real time quantitative PCR, whereas the amount of the gene copies delivered to and residing in the host cells did not differ between the plasmids containing G and single 8-oxoG. Moreover,

analyses of the integrity of the RNA transcripts detected in cells provided no evidence for truncations of the EGFP mRNA in the presence of 8-oxoG (Kitsera et al, 2011)[1]. The absence of the effect 8-oxoG situated in the transcribed DNA strand on the gene transcription together with the inhibition of transcription by a single 8-oxoG situated in the non-transcribed DNA strand indicate that the mode of interaction of this base modification with transcription is clearly distinct from direct blockage of the elongating RNA polymerase II.

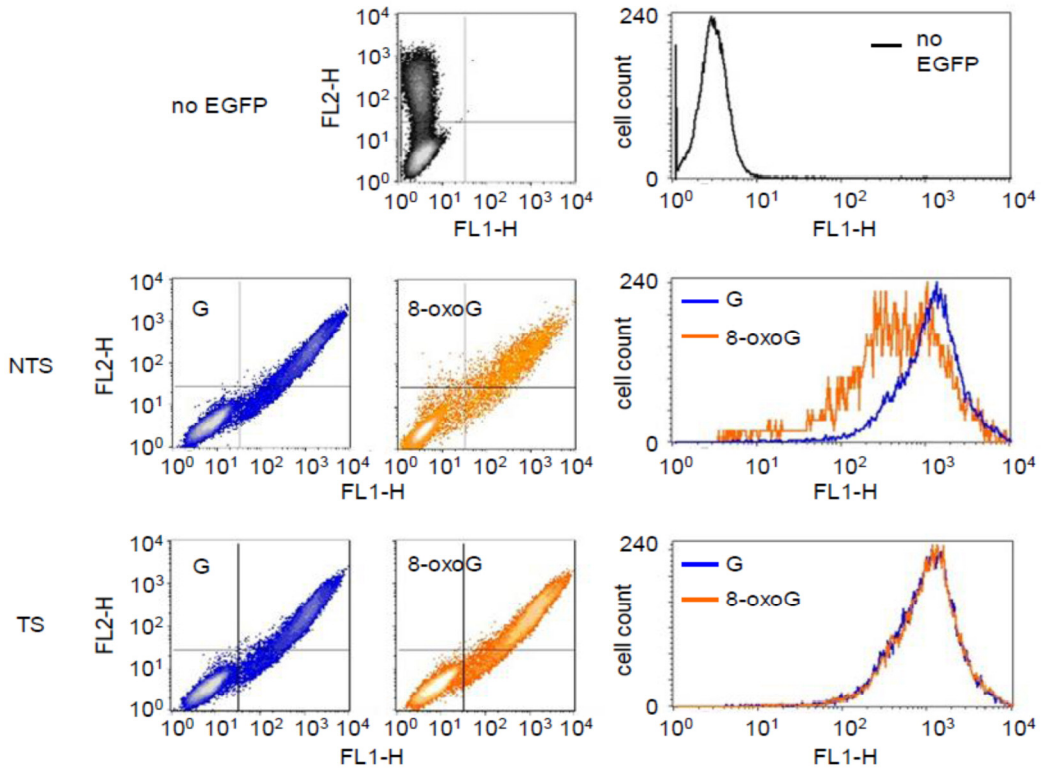

Figure 11 | Expression of DsRed-Monomer and EGFP in HeLa cells 24 hours after co-transfection with equal amounts of the corresponding plasmids. Flow cytometry of cells transfected with the EGFP-encoding plasmid constructs containing guanine (G) and 8-oxo-7,8-dihydroguanine (8-oxoG) either in the transcribed (TS) or the non-transcribed (NTS) DNA strands. The overlaid fluorescence distribution plots (right panels) specifically show cells marked by the expression of the tracer DsRed-Monomer protein (a subpopulation localised in the upper two quadrants of the dot density plots). Reproduced in a reduced form from Kitsera et al. (2011) *Nucleic Acids Res* 39: 5926-5934.

The indirect nature of the inhibition of transcription by single 8-oxoG was further confirmed by time-course of expression of the plasmids containing single synthetic 8-oxoG

[1] This publication

(Kitsera et al, 2011). If the decrease of the EGFP gene expression was a consequence of a direct effect of 8-oxoG on transcriptional elongation, DNA repair would lead to reactivation of the damaged gene; i.e., the inhibitory effect on transcription should decrease in time, as it has been previously reported for the UV-damaged plasmids (Khobta et al, 2009). Contrary to this expectation, the data show that 8-oxoG had no effect on the EGFP expression up to 8 hours after transfections. Inhibition of transcription by 8-oxoG in the non-transcribed strand of the gene appeared and proceeded with a continually increasing magnitude between 8 and 48 hours post-transfection. A similar time-course was documented for the plasmid containing single 8-oxoG in the transcribed DNA strand, but with a much smaller magnitude of the inhibitory effect on the reporter gene expression (Kitsera et al, 2011).

Time-course of expression of plasmids containing single 8-oxoG in the reporter EGFP gene showed that the inhibition of gene transcription can only occur after some latency time of incubation, thus indicating that the damaged DNA possibly undergoes secondary modification in the host cells. To investigate the effects of unprocessed 8-oxoG on the gene expression, further transfection experiments were performed in immortalized mouse embryonic fibroblast (MEF) cell lines deficient in base excision repair of 8-oxoG (OGG1-null), transcription recovery after DNA damage (CSB-null) or both (OGG1- and CSB-null). The results demonstrated that single 8-oxoG leads to a significant decrease of the gene expression in the *Ogg1*$^{wt/wt}$ but not in the repair deficient OGG1-null (*Ogg1*$^{-/-}$) MEFs, suggesting that the decrease of transcription in the OGG1-proficient cells is caused by the repair-induced DNA single-strand breaks (SSBs), rather than by the base modification itself. In the *Csb*$^{-/-}$ genetic background the inhibition of the gene expression by single 8-oxoG was even stronger than in the wild-type MEFs, but was again completely abolished in the absence of OGG1, once again demonstrating the absence of the transcription blockage by this DNA base modification and also demonstrating that functional OGG1 protein is needed for the inhibition of transcription by 8-oxoG (Kitsera et al, 2011). As in human cells, the inhibition of gene expression did not require the presence of 8-oxoG in the transcribed DNA strand. On the contrary, somewhat stronger inhibition of the EGFP expression was observed if 8-oxoG was present in the non-transcribed strand of the gene.

The results of host cell reactivation of plasmids containing single 8-oxoG in cell lines with different statuses of the OGG1 gene infer the possibility that other base modifications

might be converted in a similar manner into transcription-blocking SSBs by the cellular repair mechanisms. This was tested by construction of plasmids containing a non-blocking base uracil and the analyses of their expression in various host cell lines. Transcription of the plasmids containing single uracil within the EGFP gene was strongly decreased in the CSB-null cell lines, but not in the CSB-proficient MEFs, thus suggesting that CSB prevents either formation of harmful repair intermediates or their interaction with transcription apparatus (Kitsera et al, 2011).

The magnitude of the inhibition of gene transcription by single uracil was not apparently influenced by position of the modified base within the transcribed or the non-transcribed DNA strand (Kitsera et al, 2011), thus indicating that the signal for cessation of transcription most likely did not originate from the stalled transcription complexes. If the repair intermediates (an abasic site or an SSB) in the opposing DNA strands are equally harmful, then an explanation is needed for the observed quantitative differences between the negative effects of single 8-oxoG in different DNA strands. There is a possibility that repair of the two tested plasmid substrates may differ qualitatively or quantitatively. For instance, different repair efficiencies (or mechanisms) between the transcribed and non-transcribed DNA strands of the gene or modulation of excision efficiency of 8-oxoG by neighbouring DNA bases could be responsible. These mechanisms are currently under investigation.

3.2.6 A method for strand- and position-specific incorporation of single base modifications into plasmid DNA

Publication 6 (journal article, experimental): Lühnsdorf, B., Kitsera N., Warken, D., Lingg, T., Epe B., Khobta, A.[1], Generation of reporter plasmids containing defined base modifications in the DNA strand of choice. ***Anal. Biochem.,*** doi:10.1016/j.ab.2012.03.001

Authors' contributions: B. Lühnsdorf, N. Kitsera and D. Warken performed the experiments under the supervision of A. Khobta; T. Lingg performed preliminary experiments; B. Epe participated in writing and editing of the manuscript; A. Khobta designed and supervised all the experiments, wrote the manuscript.

Plasmid substrates containing a single DNA base modification can be efficiently produced by annealing and ligation of a modified synthetic oligonucleotide with a circular DNA containing the matching single-stranded gap (Donahue et al, 1996). Such gapped DNA circles can be conveniently generated by treatments of a plasmid DNA with sequence-specific nicking endonucleases (Wang & Hays, 2001), which have been engineered as single subunit mutants of heterodimeric restriction enzymes. This class of enzymes already found multiple applications (Zheleznaya et al, 2009), promising new tools for strand-specific modification of DNA molecules. Unfortunately generation of pairs of the mutants with catalytic selectivities directed to opposing DNA strands remained technically challenging. This is why only a few couples of such enzymes have been cloned and purified thus far (Chan et al, 2011). Luckily, the protein-coding sequence of the EGFP gene in the pEGFP-mODC-ZA plasmid was found out to contain two Bpu10I sites tandemly located with an interval of 18 nucleotides, which can be nicked by the mutant enzymes Nt.Bpu10I (cutting the coding DNA strand) and Nb.Bpu10I (cutting the transcribed strand). Digestion of the vector DNA with one of these nicking endonucleases generates two nicks in a chosen DNA strand of the EGFP gene, thus allowing an exchange of the excised 18-mer for a matching synthetic oligonucleotide, containing a chemically defined base modification in a position of choice (Luhnsdorf et al, 2012)[2].

[1] Corresponding author

[2] This publication

The straightforward strand exchange approach proved very efficient for construction of plasmids containing single uracil or 8-oxoG – the nucleobase modifications which are involved in conventional Watson-Crick base pairing within the DNA helix. On the other hand, DNA base modifications which do not form correct base pairing or cause structural distortions of DNA helix could not efficiently compete with the native excised oligonucleotides (Luhnsdorf et al, 2012). To facilitate the incorporation of such modified bases into vector DNA, the excised strand fragments could be depleted in a preliminary step by melting and re-annealing in the presence of the correspondent complementary oligonucleotides. The excess of single stranded oligonucleotides and low molecular weight duplexes could be subsequently easily removed by ultrafiltration. As a result, circular vector DNA with a single-stranded gap suitable for annealing and ligation of modified synthetic oligonucleotides was obtained. A summary of the experimental flowchart for targeted incorporation of paired and unpaired DNA base modifications into vector DNA is shown (**Figure 12**).

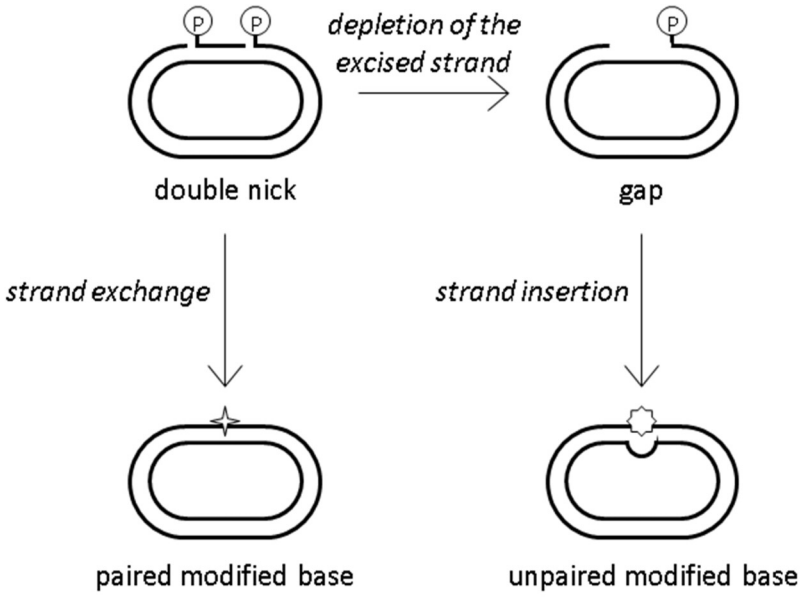

Figure 12 | Flowchart for incorporation of synthetic oligonucleotides containing modified bases into double-nicked DNA. DNA strand-specific double nicks were generated in the EGFP coding sequence by the nicking endonucleases Nt.Bpu10I and Nb.Bpu10I, as described in the text. Exchange of only one DNA strand is shown for clarity. Adapted with minor adjustments from Lühnsdorf et al. (2012) *Anal Biochem* 425: 47-53.

As the first proof of the method, DNA base modifications involved in the Watson-Crick-type base pairing (dU paired with dA and 8-oxo-dG paired with C) were incorporated into the opposing DNA strands of the EGFP-encoding pEGFP-mODC-ZA vector by a direct strand exchange procedure. Next, the oligonucleotides with imperfect base pairing because of the contained base modifications (dU opposed to dG and deoxythymine glycol opposed to dA) were successfully incorporated by insertion into the gapped plasmid substrates. In all cases, high yields of covalently closed vector DNA were obtained and the presence of single nucleobase modifications verified, as documented by gel-electrophoresis of DNA following the excision with the specific DNA glycosylases (**Figure 13**).

In summary, we conclude that the proposed method permits the DNA strand-specific incorporation of virtually any DNA base modification into the protein-coding sequence of the plasmid-borne EGFP gene. The obtained hybrid vector constructs harbouring modified synthetic oligonucleotides have been successfully applied for analyses of the effects of single 8-oxoG nucleobases on the gene expression in mammalian host cells (Kitsera et al, 2011). Investigation of the effects of other base modifications on gene transcription and of repair kinetics of various DNA base modifications are underway.

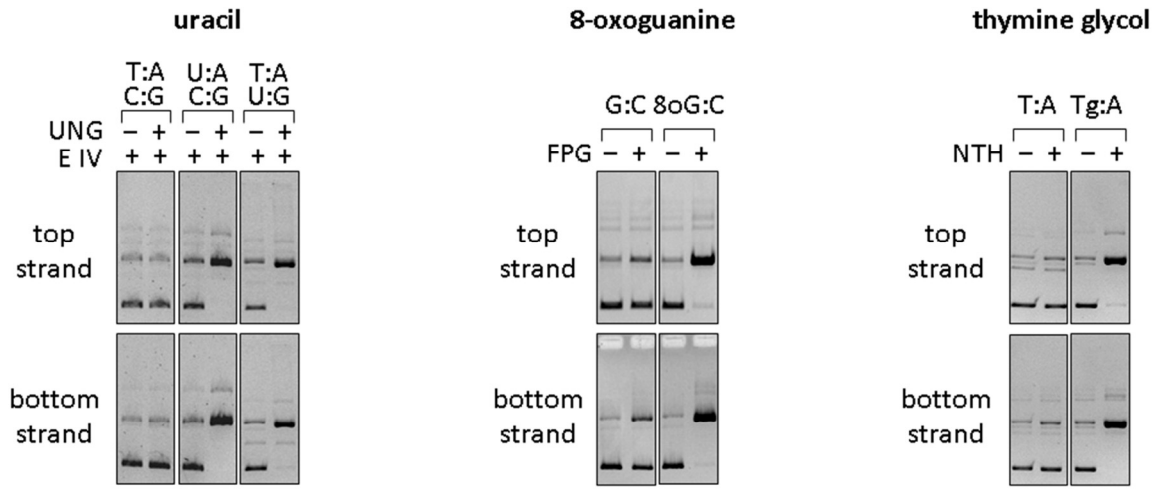

Figure 13 | Analyses of covalently closed vector DNA containing single uracil, 8-oxoguanine or thymine glycol in the DNA strand of choice of the EGFP gene. The presence of the modified DNA bases was assessed with specific DNA repair enzymes: uracil-DNA glycosylase (UNG), endonuclease IV (E IV), formamidopyrimidine-DNA glycosylase (FPG), and endonuclease III (NTH). Adapted from Lühnsdorf et al. (2012) *Anal Biochem* 425: 47-53.

3.2.7 Achievements and outlook

Publication 7 (journal article, review): Khobta, A. and Epe, B. (2012). Interactions between DNA damage, repair, and transcription. ***Mutation Res.,*** 736, 5-14.

Authors' contributions: Jointly written by the co-authors with a major involvement of A. Khobta.

Transcriptional responses of cells to DNA damaging agents can be generalised or gene-specific, depending on the nature and the load of DNA damage. The signalling cascades are activated in cells by potentially lethal DNA damage (such as double strand breaks, interstrand crosslinks) or when the damage is massive. This results in coordinated activation and repression of the specific sets of genes (e.g. those relevant for cell cycle, DNA repair, apoptosis, etc.), constituting an element of the general "DNA damage response" (DDR). Much less is known about transcriptional response to physiologically low levels of non-cytotoxic DNA damage that do not induce DDR. It is reasonable to assume that such damage can perturb expression of the affected genes in cis. Because of the stochastic nature of DNA damage, these effects are not measurable in populations of cells. Still, it is plausible that expression of vital genes is affected in individual cells and it remains to be established to which extent this can contribute to disease and ageing in organisms.

Local DNA damage can interfere with reading of genetic information either directly or indirectly. Some of the mechanisms, whose relevance is supported by experimental evidence, are summarised in **Figure 14**. So, stalling of RNA polymerase II (Pol II) at bulky DNA base adducts (cyclobutane pyrimidine dimers and some others) causes cessation of transcription that requires a specialised transcription-coupled nucleotide excision repair (TCR) pathway to be reversed (Mellon et al, 1987), (Laine & Egly, 2006). It is postulated (mostly based on results from cell-free models) that only damage situated in the transcribed DNA strand can efficiently inhibit transcription (**Figure 14**). The second mechanism is erroneous bypass of DNA damage by Pol II which causes transcriptional mutagenesis and can lead to accumulation of mutant proteins and phenotypic transitions in cells (Bregeon et al, 2003; Saxowsky et al, 2008; Viswanathan et al, 1999). In addition, multiple reports testify that various DNA base modifications can alter the binding of transcription factors to their specific DNA sequence

motifs thereby modulating the transcriptional activity (reviewed in (Khobta & Epe, 2012)[1]). However, none of these mechanisms could explain the inhibition of transcription by structurally small, non helix-distorting and non elongation-blocking nucleobase modifications, such as 8-oxoguanine, uracil, thymine glycol, etc. Data presented in this work demonstrate for the first time that excision by the DNA glycosylase OGG1 is necessary for inhibition of transcription by 8-oxoguanine (Kitsera et al, 2011) and together with yet unpublished data provide evidence in support for such mechanism of transcriptional inhibition by other DNA base modifications as well.

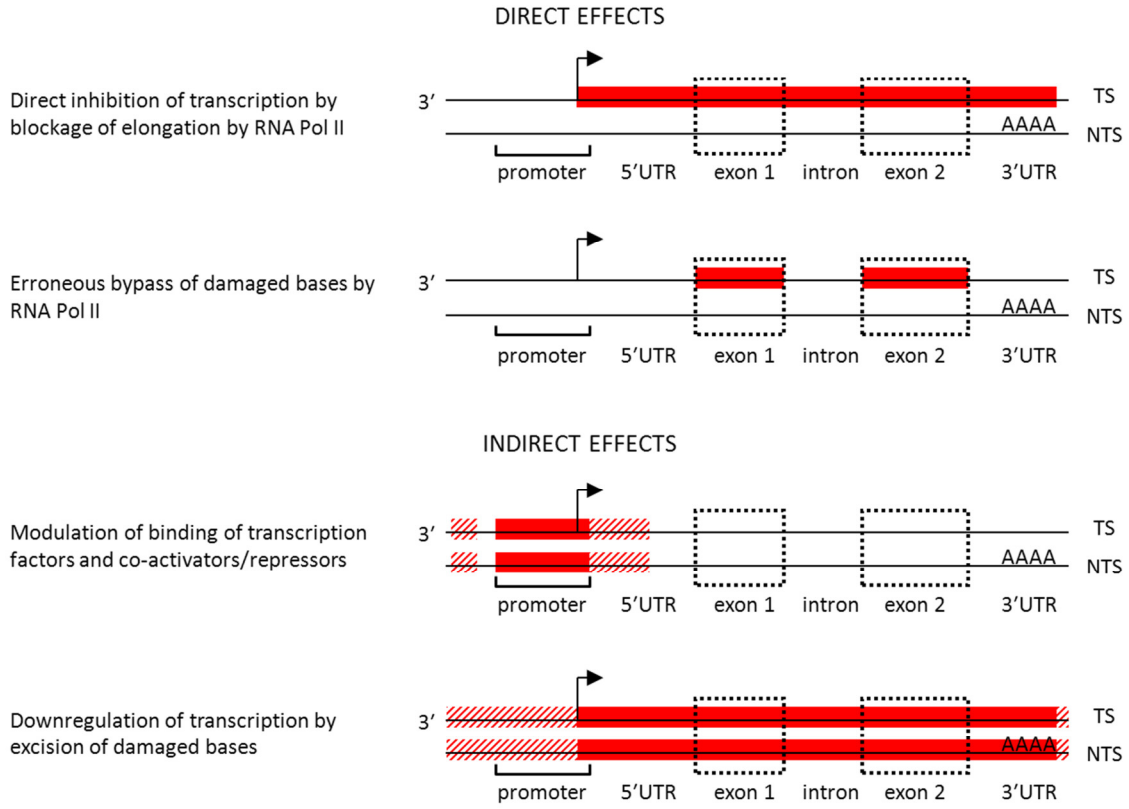

Figure 14 | A simplified view of mechanisms by which DNA base modifications can interfere with transcription, depending on the chemical structure and position within the gene. Gene elements relevant for inhibition of transcription by various types of DNA damage are highlighted with red colour. Hatched filling is used wherever the inhibitory effects on transcription are expected from the current state of knowledge, but have not yet been experimentally verified. The transcribed and the non-transcribed DNA strands (TS and NTS, respectively) are shown separately. Broken arrow shows transcription start site. Modified from Khobta et al. (2012) *Mutation Res* 736: 5-14.

[1] This publication

It is important to underline that the inhibition of transcription by 8-oxoguanine, uracil, and thymine glycol does not require that the base modifications are localised in the transcribed DNA strand (Kitsera et al, 2011), (A. Khobta and B. Lühnsdorf, unpublished results). Moreover, the very strong inhibition of transcription by low density of oxidative base damage generated by photosensitisation (Khobta et al, 2010a; Khobta et al, 2009) suggested that transcription could even be inhibited by damage generated outside of the gene (**Figure 14**). At least for 8-oxoguanine, this has been recently confirmed with a single synthetic 8-oxoguanine positioned upstream from the promoter region (A. Khobta and J. Allgayer, unpublished results). This finding explains why inhibition of gene transcription by oxidative base modifications is stronger in magnitude that the effect of equal numbers of transcription blocking cyclobutane pyrimidine dimers induced by UV (Khobta et al, 2009; Spivak & Hanawalt, 2006).

The results discussed above (Khobta et al, 2009; Kitsera et al, 2011) identify the recognition of 8-oxoguanine by OGG1 as an initiatory event in the mechanism of downregulation of gene transcription by this oxidative base modification, while the downstream scenario is not yet clarified. One possibility is that the entire gene silencing mechanism is triggered by encounters of transcribing RNA polymerase II with the repair-induced single-strand breaks. Such idea would be consistent with a very strong inhibitory effect of enzymatically generated nicks on gene transcription documented in our study (Khobta et al, 2010b). Alternatively, the entire gene silencing mechanism could be initiated either by some interaction partners of OGG1 or by proteins binding to the repair-induced single-strand breaks. The latter possibilities are favoured by our observation of a significant inhibitory effect on transcription by single 8-oxodG localised outside of the transcribed gene sequence (A. Khobta and J. Allgayer, unpublished results). Identifications of proteins which mediate the mechanism of transcriptional repression will be a major issue for future research. In this context, it would be important to further investigate the role of CSB protein in transcription of damaged DNA, since our data show that CSB improves transcription of genes containing 8-oxoG, uracil (Kitsera et al, 2011) and single-strand breaks (Khobta et al, 2010b).

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5 APPENDED REPRINTS