Regulation of DNA damage and immune response pathways by post-translational protein modification

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Introduction

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Insight in the multilevel regulation of NER
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The cellular response to DNA damage

The genetic information that is encoded by DNA harbors all the instructions that are required for proper development and functioning of organisms. Even minor alterations in the genome may disturb normal development and have the potential to drive carcinogenesis, contribute to the progression of ageing-related diseases and underlie hereditary disorders. Conservation of genetic information is thus of upmost importance not only to the life of a single organism, but ultimately also to continuity of species.

The integrity of the vulnerable DNA macromolecule is however continuously threatened by regular endogenous processes that damage the DNA. This damage may result from spontaneous deamination or depurination/depyrimidation or arise from reactions with reactive oxygen species that are produced during normal metabolism[1]. On the other hand, DNA lesions are sometimes created by design, as is exemplified by the programmed induction of DNA double-strand breaks during immunoglobulin differentiation and meiotic chromosomal crossover[2,3]. DNA is furthermore insulted by various exogenous sources, such as ultraviolet (UV) or ionizing (IR) radiation and chemical compounds. If not removed and repaired accurately, replication of DNA damage can result in chromosomal aberrations or mutations that have deleterious effects on cellular functioning and genome stability[4,5].

An essential strategy of cells and organisms to counteract genetic alterations encompasses the continuous surveillance of genomes, hence tracing the occurrence of DNA damage and ensuring its removal. Accordingly, cells react to the presence of DNA lesions by activating the DNA damage response (DDR), which constitutes an elaborate network of signaling cascades that concomitantly coordinate gene expression, chromatin structure adjustments, DNA damage repair and cell cycle progression or, if necessary to preserve genome integrity, accommodate apoptosis[6-8].

DNA damage repair pathways

Evidently, an important part of the DNA damage response is the employment of suitable repair pathways that can repair the large compilation of structurally different DNA lesions, including base damages, bulky lesions, DNA single- and double-strand breaks and DNA crosslinks.

Base excision repair (BER) is the main pathway for removal of damaged DNA bases that cause only minor distortions of the DNA helix, such as 8-oxoguanine and apurinic/apyrimidinic (AP) sites that result from oxidation or depurination/depyrimidation, respectively, as well as single-strand DNA breaks. Repair of these lesions is initiated by cleavage of the damaged DNA by substrate-specific N-glycosylases and processing by AP endonucleases. Restoration of the DNA is next established via short-patch or long-patch BER, which involves re-synthesis of a single nucleotide or replacement of a stretch of nucleotides, respectively, followed by ligation of the remaining nick[9,10].

More bulky DNA damage types are repaired via nucleotide excision repair (NER) – a highly versatile pathway that operates on a broad range of structurally unrelated helix-distorting

lesions. Similarly to BER, NER comprises DNA damage recognition, excision of the lesion, subsequent DNA polymerization to replace the removed stretch of nucleotides, and a ligation reaction[11-13].

The probability of DNA double-strand break formation is highly increased under certain circumstances, which include replication fork stalling or collapse, the presence of multiple adjacent single-strand DNA breaks and exposure to ionizing radiation or chemical compounds. Being active throughout all cell cycle stages, non-homologous end-joining (NHEJ) is the main pathway operating on DNA double-strand breaks in human cells. By rejoining and ligating the broken DNA ends without the use of a homologous DNA sequence, it resolves breaks in a rather straightforward, but often error-prone manner. In contrast, homologous recombination (HR) facilitates error-free repair during the S or G2 phases of the cell cycle. Following resection of the broken ends, a homologous template, generally provided by the sister chromatid, is invaded. This undamaged DNA sequence is then used for DNA re-synthesis, thereby ensuring complete restoration of the original genetic information[14,15].

Covalent crosslinks between the 2 DNA strands (that is interstrand crosslinks, ICLs) block DNA replication and transcription by impeding strand separation. The Fanconi anaemia (FA) pathway is activated by replication fork stalling at ICLs. Following detection of the ICL, incisions on both sides of the lesion lead to breakage of the sister chromatid and unhooking of the ICL and the other sister chromatid. Specialized translesion DNA polymerases are able to synthesize DNA across the ICL, thereby generating a template to repair the DSB in the other chromatid via homologous recombination. NER is responsible for the removal of the remaining ICL adduct. In non-replicating cells, a combination of translesion synthesis and NER is applied to resolve ICLs[16,17].

In addition to the occurrence of damaged DNA, mutations that arise from faulty nucleotide incorporation during DNA replication pose a serious threat to genome stability. Mismatched base pairs that result from DNA damage-induced or spontaneous nucleotide conversion, such as the formation of uracil by deamination of cytosine, are mostly recognized by DNA glycosylases that trigger repair via BER, whereas mismatch repair (MMR) corrects base substitutions that originate from mistakes made by DNA polymerases. Central to this latter pathway is the introduction of nicks at both sides of the mismatched region, which allows processing by exonucleases to remove a stretch of the newly synthesized DNA, including the mismatched DNA bases, and its replacement via DNA synthesis and ligation[18-20].

When encountered during DNA replication, damage that has not accurately been resolved by one of these pathways can initiate post-replication repair (PRR), which comprises different mechanisms to bypass DNA lesions that block progression of the replication machinery. Translesion synthesis (TLS) involves substitution of the replicative DNA polymerase by specialized DNA polymerases that are capable of incorporating bases opposite damaged nucleotides, although at reduced fidelity, for which reason the process is considered to be often error-prone. Conversely, template switching or recombination-dependent events facilitate error-free lesion bypass via pathways that are less well understood[21].

Nucleotide excision repair

The nucleotide excision repair (NER) pathway, its regulation and associated disorders constitute an important area of the research described in this thesis and are therefore discussed in more detail in the following sections.

NER is capable of removing a wide range of bulky lesions that, despite their structural differences, can be recognized and processed owing to their helix-distorting character. Whereas for instance the specialized BER glycosylases directly recognize particular lesions, the proteins involved in the detection of NER substrates act by sensing destabilized base pairing or arrested transcription[22]. In this manner, NER operates on lesions ranging from cisplatin-induced intrastrand crosslinks to bulky adducts caused by polycyclic aromatic hydrocarbons. In human cells, the nucleotide excision repair pathway is the only pathway qualified to remove the covalent linkages between adjacent pyrimidines that are inflicted by sunlight, that is 6-4-photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs). If not accurately and timely removed, these lesions interfere with DNA replication and transcription, perturb cell cycle progression and may promote cancer and accelerated ageing by causing mutations and chromosomal aberrations[11].

NER substrates are detected via one of the NER subpathways, referred to as global genome nucleotide excision repair (GG-NER) and transcription-coupled nucleotide excision repair (TC-NER). GG-NER explores disordered base pairing throughout the whole genome, while the specialized TC-NER pathway removes lesions in active genes that hinder progression of the elongating RNA polymerase. After DNA damage recognition, the NER subpathways converge into a common molecular mechanism that involves DNA unwinding and lesion verification, dual incision and elimination of the excised oligonucleotide, and DNA repair synthesis and ligation[11,13] (Fig. 1).

Importantly, defects in genes that encode proteins involved in NER are associated with a number of disorders (further described in 'Human disorders associated with defects in NER genes'). The nomenclature of many of the core NER proteins, as well as those required for DNA damage recognition via GG-NER or TC-NER, has been derived from the disease that has been linked to mutations in the respective genes. Accordingly, CSA and CSB refer to Cockayne syndrome (CS) complementation groups A and B, respectively, while mutations in the XP proteins XPA through XPG have been found implicated in xeroderma pigmentosum (XP). Defects in UVSSA were shown to cause UV sensitive syndrome (UVsS).

Global genome nucleotide excision repair

By probing the entire genome for disturbed base pairing, the DNA binding protein XPC initiates GG-NER when sensing damage-mediated DNA helix distortion[23-26] (Fig. 1). Until its association with unpaired bases opposite the lesion, stabilization of the XPC monomer is ensured by its incorporation into a heterotrimeric complex additionally comprising RAD23B and CEN2 that serve to prevent XPC ubiquitination and degradation[27-29]. DNA damage detection by XPC is assisted by the UV-DDB dimer, which consists of DDB1 and DDB2 (XPE) and is part of a larger CRL^{DDB2} complex (further discussed in 'Regulation of DNA damage repair by post-translational protein modifications'). Substrate specificity of the UV-DDB

complex resides within the DDB2 protein, which accommodates relatively small lesions in its binding pocket. Damage extrusion by DDB2 exposes the opposing single-stranded DNA and facilitates its subsequent recognition by XPC[11,30-32].

Binding of XPC to a lesion activates lesion verification and subsequent repair by providing a platform for recruitment of the basal transcription factor TFIIH[33,34]. The ATPase and helicase activities of its XPB and XPD subunits, respectively, promote DNA strand separation to create an unwound structure around the lesion, which contributes to damage verification and the assembly of a pre-incision complex that additionally consists of XPA, RPA and XPG[35-38]. The roles of XPA include catalyzing the dissociation of the CAK complex from the core TFIIH complex, corroborating the lesion, and recruiting the XPF-ERCC1 heterodimer[38-41]. RPA, which coats the single-stranded DNA and protects the undamaged strand, assists in damage verification and the positioning of endonucleases XPG and XPF-ERCC1[42,43]. Subsequent dual incision of the DNA is initiated 5′ to the lesion by XPF, after which 3′ incision by XPG results in the release of a fragment of 22 to 30 nucleotides[44]. Following incision by XPF-ERCC1, re-synthesis of the excised DNA is executed by DNA polymerase δ, κ or ε, and DNA ligase 1 or 3 seals the remaining nick to complete the NER process and restore helix integrity[11,44] (Fig. 1).

Transcription-coupled nucleotide excision repair

Stalling of elongating RNA polymerase (RNAPIIo) by DNA lesions in the transcribed strand of active genes initiates fast removal of the road-blocking DNA damage via TC-NER. Accelerated repair as compared to resolution by GG-NER is of prime importance to avoid prolonged transcriptional arrest and consequential cell death[45-48]. Stabilization of the interaction between the stalled RNAPIIo and the SWI/SNF2-like ATPase CSB is considered to be the first step in TC-NER and required for the assembly of a TC-NER-specific complex (Fig. 1). The role of CSB may include remodeling of chromatin and/or the RNAPIIo-DNA interface, which both seem prerequisites for exposure and subsequent repair of the DNA damage. However, these events also rely on the recruitment and activities of other proteins[49-51]. Among these proteins is CSA, which is part of the CRL^{CSA} complex that has common architectural features with the CRL^{DDB2} complex responsible for damage recognition in GG-NER (both further discussed in 'Regulation of DNA damage repair by post-translational modifications')[52]. Its precise role is however yet to be identified. Additionally, a complex comprised of UVSSA and USP7 is recruited specifically to the TC-NER complex, likely to regulate the presence of TC-NER proteins, including CSB, at the site of damage by coordinating their degradations[53-56]. Another protein explicitly involved in TC-NER is the pre-mRNA splicing factor XAB2, which may function as a scaffold by binding XPA[57]. Upon the association of the TC-NER-specific proteins with the stalled RNA polymerase, the pre-incision complex is assembled as described for GG-NER, which involves the recruitment of TFIIH, XPA and RPA, as well as the endonucleases XPG and XPF-ERCC1. DNA damage verification, incision and DNA repair synthesis then continue along the further common NER pathway (Fig. 1). Importantly, accurate DNA damage resolution via TC-NER also includes resumption of transcription. Apart from its role in DNA damage repair, CSB plays a crucial role in the transcriptional restart upon lesion removal via yet to be established mechanisms that may

include chromatin remodeling, reconversion of TFIIH to a transcriptionally active complex and/or the generation of hypophosphorylated RNA polymerase[58-60].

Displacement of the stalled RNA polymerase

Repair of transcription-blocking CPDs seems to be challenged by the presence of the stalled RNAPIIo, which covers around 35 nucleotides that are asymmetrically located around the lesion and especially obstructs the 3' XPG cutting site[61,62]. Several studies have shown that UV irradiation induces degradation of the RNA polymerase in a CSA- and CSB-dependent manner[53,54,63,64]. UV-irradiated cells lacking one of the CS proteins initiate a signaling cascade prompted by the prolonged stalling of RNAPIIo that eventually leads to apoptosis[63,65].

Despite the general believe that the lesion-shielding RNA polymerase needs to be displaced from the site of damage to increase the accessibility by repair factors and to allow repair, its UV-induced degradation might only be a last resort in case the impaired transcription becomes detrimental to the cell[66-68]. A mechanism of backtracking of transcribing RNA polymerase, by sliding backward along the DNA, would enable resumption of transcription after repair and hence seems a more efficient process than transcription termination as a prerequisite for NER. Backtracking has been implicated in many processes, including genome stability maintenance and control of transcription elongation/termination, and additionally has been suggested to have a proofreading function[69,70]. In prokaryotes, it was suggested to be the main mechanism to displace RNA polymerase from the DNA damage during TC-NER and was demonstrated to depend on the helicase UvrD[71,72]. Accordingly, in vitro studies have demonstrated that the absence of UvrD severely inhibits CPD excision by UvrC when the elongating RNA polymerase complex is located at a position that causes shielding of the lesion. Either the addition of UvrD, or the assembly of RNA polymerase at a position upstream of the CPD were shown to restore CPD excision rates. As opposed to backtracking, the DNA translocase Mfd mediates the forward translocation of RNA polymerase and is thought to promote reactivation of transcription when repair is completed[73,74]. Whether TC-NER is driven by backtracking in eukaryotes as well remains to be elucidated. However, this may not be unlikely given the evolutionary conservation of TC-NER and the frequent occurrence of backtracking as a regulatory mechanism at natural transcription pausing sites[69,71].

NER in a chromatin context

Like other DNA-based processes, NER is to a great extent regulated by chromatin status. Especially during GG-NER, highly compacted heterochromatin poses a challenge to repair, which is manifested by the relatively slow removal of bulky lesions, and most likely necessitates carefully modulated and spatiotemporally precise chromatin remodeling events[75]. Such events usually promote transient chromatin decompaction, thereby facilitating access of the repair machinery. While it has been demonstrated that UV-induced DNA damage per se results in histone eviction, local chromatin decondensation is further mediated by ATP-dependent chromatin remodelers that stimulate nucleosome sliding or disassembly[76-78]. In addition, ATPases may drive the exchange of histone variants, such as H2A.Z, which contributes to creating an open chromatin structure around sites of DNA damage[79].

On the other hand, post-translational modification of histones, including ubiquitination, PARylation and acetylation, also modulate the chromatin status to recruit repair proteins and enable DNA damage removal[77,78,80]. GG-NER damage recognition factor DDB2 was shown to stimulate local chromatin unfolding in several manners. Independently of its association with the CRL E3 ubiquitin ligase complex, it was shown to promote chromatin decompaction and/or histone eviction and thereby the assembly of the NER machinery[81]. This notion has been reinforced by studies that demonstrate that DDB2 can promote PARP1-dependent chromatin poly-ADP ribosylation (PARylation, further discussed in 'Regulation of DNA damage repair by post-translational modifications'), which usually leads to a less rigid chromatin environment[82,83]. In addition, this facilitates the recruitment of the chromatin remodeler ALC1 to further restructure UV damage-containing nucleosomes[82]. Furthermore, UV-DDB has been described to promote chromatin remodeling by associating with the INO80 remodeling complex, and by binding to p300 and the STAGA complex that both stimulate repair by mediating chromatin acetylation[84-87]. In addition, DDB2 was shown to recruit the histone acetyl transferase HBO1, which most likely contributes to repair in non-replicating cells, not only by facilitating XPC accumulation at damaged DNA, but also by recruiting the ACF1 and SNF2H chromatin remodelers[88]. As a part of CRL^{DDB2}, DDB2 induces the ubiquitination of all core histones, resulting in nucleosome destabilization, H2A-H2B dimer loss and weakened histone-DNA interaction, which are all hallmarks of accessible chromatin[89-91].

Although transcription-blocking DNA damage is generally encountered in the more relaxed chromatin environment that is required for transcription, repair of these lesions via TC-NER still requires additional chromatin remodeling. For example, a contribution of histone acetylation in human cells was demonstrated by the increased repair of UV-induced lesions via both NER subpathways upon sodium butyrate-induced inhibition of histone deacetylases that mainly affect H4 acetylation[92]. Furthermore, in the mouse CPD removal was delayed upon depletion of HMGN1, which specifically stimulates TC-NER by enhancing H3 acetylation[93-95]. As indicated previously, CSB presumably plays an important role in chromatin remodeling by means of its SWI/SNF2-like ATPase activity and by recruiting other chromatin-modifying complexes.

Although an open chromatin conformation is beneficial at the early stages of NER, upon completion of DNA damage repair the chromatin environment needs to be restored to its original state in order to maintain epigenetic marks and transcriptional status[96]. Several studies have underlined that completion of damage repair itself is not sufficient to restart the transcription machinery and that recovery of transcription requires chromatin reorganization to establish pre-damage levels of gene expression. For example, in addition to the contribution of the histone chaperone proteins CAF1 and ASF1, the restart of stalled RNAPIIo was shown to be promoted by the SPT16 subunit of FACT (facilitates chromatin transcription), which accelerates the damage-induced exchange of H2A and H2B[96-98]. Moreover, roles in transcriptional restart upon DNA damage repair have been identified for the histone chaperone HIRA and the methyltransferase DOT1L, which act by stimulating H3.3 incorporation and H3K79 dimethylation, respectively[99-101]. Thus, next to chromatin relaxation being required for early NER events, specific chromatin remodeling events and histone

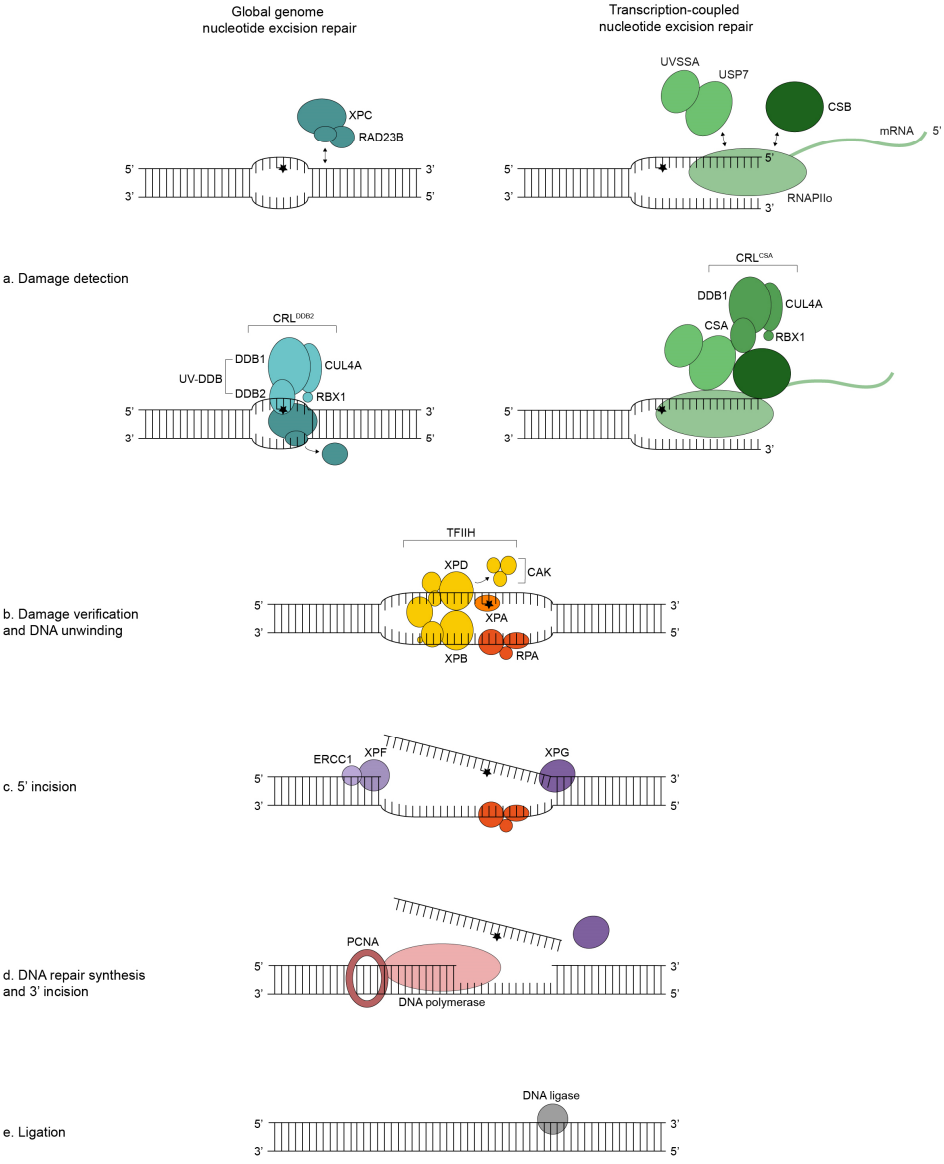

Figure 1. Schematic overview of the nucleotide excision repair pathways
Upon the detection of DNA damage (a) via UV-DDB and XPC (global genome nucleotide excision repair; left panel) or stalling of RNAPII (transcription-coupled nucleotide excision repair; right panel) the subpathways converge into a common molecular mechanism that involves damage verification and DNA unwinding (b), excision of the damaged DNA (c, d), gap filling by DNA synthesis (d) and sealing of the remaining nick (e). A more detailed description of these steps and the involved proteins is given in the main text.

modifications appear to be essential for completion of NER and the continuation of transcription.

Human disorders associated with defects in NER genes

Inherited NER defects are associated with different disorders that display broadly varying symptoms. The (severity of the) clinical outcome is likely explained by a combination of factors, including the subpathway in which the affected protein functions, roles of this protein in other cellular processes and even the specific mutation underlying the manifested disease. Xeroderma pigmentosum (XP) has been linked to defects in seven NER genes (*XPA* through *XPG*) and in a gene encoding DNA polymerase η (XP variant). XP patients are clinically characterized by hypersensitivity to sunlight and predisposition to skin cancer and in a minority of cases (~25%) by severe neurological and developmental problems and ageing. These additional neurodevelopmental abnormalities have been shown to result from mutations in *XPA*, *XPB*, *XPD*, *XPF* or *XPG*[102-104].

Defects in TC-NER genes *CSA* and *CSB* are among others associated with the severe developmental and neurological disorder Cockayne syndrome (CS). Classic CS is characterized by growth failure, premature ageing and progressive neurological degeneration[105,106]. Furthermore, mutations in the CSA and CSB proteins, as well as some of the *XP* genes, have been linked to cerebro-oculo-facio-skeletal (COFS) syndrome – a rare autosomal recessive disease that is classified into the spectrum of CS disorders and outlined by severe developmental delay and facial dysmorphism[105].

Notably, a combined XP/CS phenotype is observed for specific mutations in *XPB*, *XPD*, *XPG* or *XPF*. Other mutations in *XPB* or *XPD* can cause a combination of CS features, though usually not progressively declining, and brittle hair and nails, which is known as trichothiodystrophy (TTD). The explanation for these additional characteristics may be found in the perturbed functioning of TFIIH as a transcription factor that is caused by these particular mutations[102,107].

Most cases of UV sensitive syndrome (UVsS), which is a relatively mild condition that is characterized by photosensitivity without cancer predisposition or neurodevelopmental abnormalities, are ascribed to mutations in the gene encoding the TC-NER protein UVSSA. In addition, UVsS patients with a mutated *XPB* or *XPD* gene have been reported[108]. Remarkably, in a few cases the disorder has also been linked to a defective CS protein, raising the possibility that the TC-NER impairment that is caused by a defect in either UVSSA, CSA or CSB only explains the common hypersensitivity to sunlight[109,110]. Conversely, the neurodevelopmental abnormalities observed for CS patients may arise partly due to defective functioning of CSA or CSB in other cellular processes, in which a role for UVSSA remains to be established[111-113]. Finally, the ability to remove stalled RNAPIIo from sites of DNA damage may contribute to the UVsS phenotype (as discussed in Chapter 2), although no evidence is available yet to support this.

Regulation of DNA damage repair by post-translational protein modifications

Fine-tuning of protein activity is a crucial aspect of all processes that underlie correct cellular functioning and accurate organization and protection of organisms. To a great extent, this is established by a large repertoire of chemical protein alterations, collectively referred to as post-translational modifications (PTMs), which in turn modulate the activity, localization and interactions of already available proteins (Fig. 2). The most common PTMs include the formation of disulfide bonds between cysteine residues, proteolytic cleavage of peptide bonds and the removal or introduction of low-molecular-weight groups. In the DDR, important mechanisms of pathway regulation involve the reversible, covalent addition of functional groups, as has been demonstrated frequently by the induction of PTMs such as ubiquitination, SUMOylation, phosphorylation, PARylation and NEDDylation following DNA damage. Accordingly, these modifications significantly contribute to the spatiotemporal coordination of the different steps that constitute the distinct NER pathways, as well as their interplay with signaling cascades that mediate cell cycle progression and gene expression.

The damage recognition step in GG-NER makes a fine example of the significance and function of several PTMs. Given that UV damage recognition appears to be one of the rate-limiting steps in NER, the intricate interplay between the sensor complexes, that is UV-DDB and XPC-RAD23B-CEN2, is paramount[114]. As a part of the CRL^{DDB2} E3 ubiquitin ligase complex, consisting of DDB2, DDB1, CUL4A/B and RBX1, UV-DDB regulates the retention time of both UV damage recognition complexes[115]. CRL^{DDB2} promotes the ubiquitination of XPC and itself upon UV irradiation[116]. Whilst DDB2 poly-ubiquitination leads to its dissociation from the site of damage, as well as its proteasomal degradation, the atypical poly-ubiquitination of XPC increases its stability at the lesion[116-118]. Recruitment and stabilization of XPC is furthermore controlled by a DDB2-independent interaction with PARP1 and was shown to be enhanced by PARP1's PARylating activity[119]. Notably, the residence time of DDB2 at the damage is regulated by competing post-translational modifications. Poly-APD ribosylation (PARylation) and ubiquitination of DDB2 occur at the same protein region, with the former inhibiting the latter, thereby increasing the half-life of DDB2[82]. The timely removal of DDB2, and later XPC, is controlled by its ubiquitination status and its segregation by VCP/p97, adding an additional regulatory level[120]. Intriguingly, XPC too appears to be tightly regulated by multiple PTMs upon UV irradiation, as it was shown to be ubiquitinated, as well as SUMOylated at several sites[121,122]. These modifications, in contrast to the competitive character of PARylation and ubiquitination of DDB2, appear to behave cooperatively. More specifically, XPC SUMOylation promotes the accumulation of the SUMO-targeted E3 ubiquitin ligase RNF111, which in turn further decorates XPC with non-proteolytic K63-ubiquitin chains[117].

Given that XPC is intrinsically unstable as a monomer, necessitating its association with stabilizing partners RAD23B and CEN2, it is remarkable that RAD23B dissociates upon binding of XPC to damaged DNA[27,81]. Concomitantly, XPC ubiquitination reaches its peak, raising the possibility that, in addition to potential XPC stabilization by PARP1, the non-canonical XPC ubiquitination might initially be read by downstream effectors in a protective

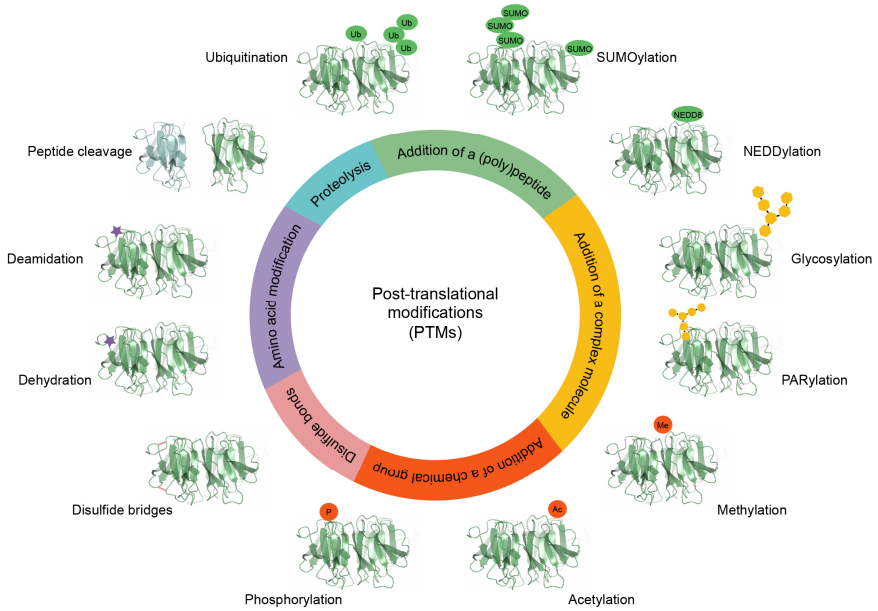

Figure 2. Overview of the most common types of post-translational protein modifications
For each type, examples are shown in a corresponding color.

manner that stabilizes XPC at the damage[117,119]. Following lesion recognition and verification, ubiquitinated XPC is eventually removed by VCP in order to promote the assembly of the downstream repair complex[120].

Similarly to DDB2, the TC-NER factor CSA also assembles into a cullin-RING ubiquitin ligase complex (CRL^{CSA}) consisting of CSA, DDB1, CUL4A/B and RBX1[52]. Although proven essential for TC-NER, the exact roles of CRL^{CSA}, apart from recruiting other repair factors, remain largely elusive. CSA has been suggested to contribute to a last resort mechanism that avoids persistently stalled RNAPIIo by promoting its complete dissociation when NER is compromised (further discussed in 'Displacement of the stalled RNA polymerase'). However, as the ubiquitin ligases NEDD4, elongin A/B/C, Von-Hippel Lindau (VHL) and BRCA1 have also been described to promote ubiquitination and subsequent degradation of stalled RNAPIIo, this raises the possibility that CRL^{CSA}'s catalytic activity is not essential[123-128]. Another potential CRL^{CSA} target is CSB, which likely dissociates after repair of the damage to enable resumption of transcription[129]. Similar to removal of the GG-NER recognition factors, the eviction of CSB is mediated by VCP, which associates with CRL^{CSA} [130]. Additionally, CSA and CSB could be linked via another CRL^{CSA} target that is being recognized by means of a ubiquitin binding domain in CSB. The retention time of CSB might also be regulated by

UVSSA by virtue of the de-ubiquitinating enzyme USP7, which prevents proteasomal degradation of CSB by removing ubiquitin[53]. The association of UVSSA with USP7 additionally seems to prevent degradation of UVSSA itself[131]. Interestingly, UVSSA-USP7 is potentially recruited via CSA as well[55,56]. Although this mechanism requires further study, the diminished survival and recovery of RNA synthesis upon UV irradiation displayed by USP7-depleted cells as compared to wildtype cells, again demonstrate that balanced post-translational modifications are crucial for accurate repair and, in this case, resumption of transcription[53,55].

The activity of both CRL^{DDB2} and CRL^{CSA}, like that of other cullin-RING ligases, is in turn regulated by NEDDylation[132]. Attachment of NEDD8 to the cullin protein is required for activation of their ligase activities, but counteracted by the COP9 signalosome that mediates deNEDDylation by means of its protease subunit CSN5. While association of COP9 keeps both CRL complexes in an inactive state under unperturbed conditions, DNA damage induction causes the release of COP9 and subsequent NEDDylation and activation of CRL^{DDB2} and CRL^{CSA}, although possibly at a different stages of repair[115]. Interestingly, NEDDylation in general coordinates the presence of repair factors in the NER complex (described in Chapter 2), recognizing this modification as an additional layer of regulation.

Next to directly influencing the composition and activity of the repair complex, post-translational modifications also contribute significantly to chromatin remodeling (described at 'NER in a chromatin context'). Interestingly, the activities of the chromatin remodelers themselves are often controlled by PTM's as well. For example, the histone acetyl transferase HBO1 is phosphorylated by ATM/ATR upon DNA damage, which likely contributes to TC-NER in non-replicating cells and is required for its CRL^{DDB2}-mediated ubiquitination and dissociation at later stages[88,133]. Moreover, the ATPase activity of TC-NER factor CSB, which likely contributes to chromatin remodeling, is increased upon its UV-induced dephosphorylation[134].

The above described examples only provide a glimpse of the mechanisms by which PTMs regulate NER, yet give a good impression of how they affect protein stability, retention time and activity. An extra level of complexity is added when multiple post-translational modifications act to complement each other, as for instance is seen for SUMO-targeted ubiquitin ligases, or in contrast serve to establish opposite effects, as was shown for DDB2 ubiquitination and PARylation. Not surprisingly, similar mechanisms are employed throughout all processes of the DDR. Pathway regulation by PTMs is excellently illustrated by modification of PCNA, which influences post-replication repair. During unperturbed replication, yeast PCNA is mainly SUMOylated, enhancing its interaction with the anti-recombinogenic helicase Srs2, which avoids unwanted homologous recombination by disrupting RAD51 filaments[135-138]. In response to replication-stalling damage, PCNA SUMOylation strongly enhances its RAD18-mediated mono-ubiquitination, which can be recognized by TLS polymerases that facilitate (potentially mutagenic) bypass of the lesion[139-141]. Interestingly, extension of the ubiquitin chain by UBC13-MMS2 and RAD5 enables an error-free method that involves template switching[141-143]. The interaction between PCNA and Srs2 is in turn negatively affected by Srs2 SUMOylation, which is increased upon DNA damage induction and in this manner regulates HR-mediated rescue of stalled

replication forks[144,145]. Together these mechanisms greatly affect pathway choice during PRR, exemplifying the importance of PTMs in other aspects of the DNA damage response.
Evidently, PTM-mediated regulation of protein activity contributes not merely to an accurate DDR, yet is essential to control all cellular processes. For example, fine-tuning of protein activity is also broadly applied by the immune system, which induces multiple types of PTMs in response to environmental changes[146].

Other methods of pathway regulation: examples from the immune system

As indicated above, apart from the PTMs of proteins that involve the attachment of small chemical moieties or peptides, several other mechanisms exist to influence protein activities (Fig. 2). Indeed the DDR provides excellent examples of protein regulation by the reversible linkage of functional groups, but the variety of mechanisms that are applied to control pathway activation and execution during the immune response, makes this system a fascinating area of research to gain more insight into other PTMs, such as peptide cleavage.

Zymogen activation by proteolytic cleavage

A fundamental aspect of the immune response is the complement system, which is a network of more than 50 membrane-associated proteins, as well as plasma proteins that act in cascades to mediate a wide range of effector functions contributing to pathogen elimination, such as opsonization, chemotaxis and inflammation[147]. Activation is established through 3 different processes, referred to as the classical, mannose-binding lectin and alternative pathways. Although triggered by different stimuli and initiated by different proteins, they use comparable signal transduction mechanisms that involve sequential peptide cleavages from inactive proteases (zymogens). This activates their proteolytic functions and in turn catalyzes cleavage of the next enzyme (Fig. 3). In this manner the activation of a small number of plasma proteins upon pathogen detection is quickly amplified and enables rapid coating of the pathogen's surface to accelerate its clearance, which is accompanied by a series of inflammatory responses[148]. Evidently, as the activation of only a small number of enzymes can induce a massive response, these processes require tight regulation and inappropriate activation should be avoided. A comparable mechanism is applied by the coagulation system, which encompasses the contact and tissue factor systems and contributes to the innate immune system by increasing vascular permeability and producing agents that assist phagocytic cells. In addition, it serves to induce blood clotting, which is established in a cascade of proteolytic cleavages that eventually lead to the formation of fibrin[149,150]. Likewise, the breakdown of fibrin by the fibrinolytic system involves the conversion of the zymogen plasminogen to the active protease plasmin[151]. Regulation of these processes is of great importance not only to ensure the required blood clot stability, but also to warrant its timely removal without dissolving healthy tissue formations.

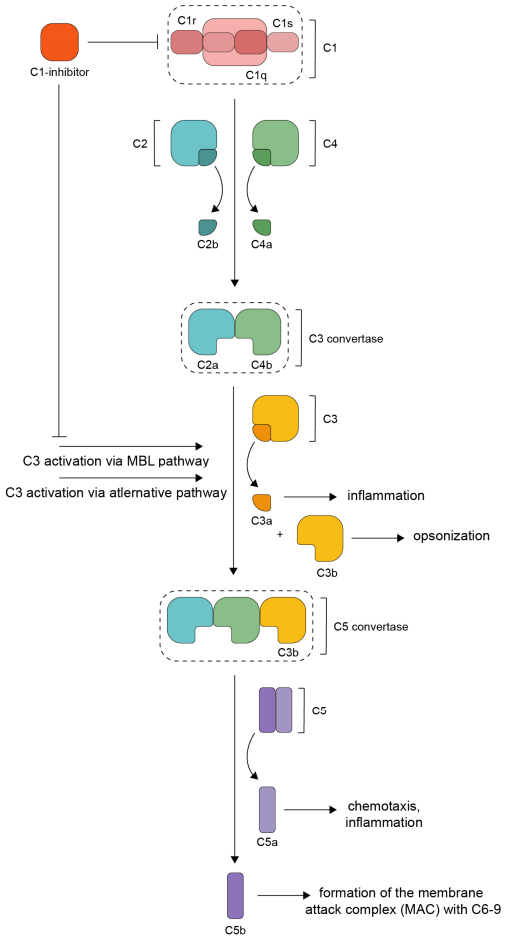

Figure 3. Schematic overview of complement system activation via the classical pathway
Example of a method of pathway regulation and signal amplification that involves sequential proteolytic cleavages. Binding of C1q to an antibody-antigen complex or directly to the pathogen's surface activates C1, which catalyzes cleavage of both C2 and C4 into C2a/b and C4a/b, respectively. C2a and C4b form the C3 convertase that cleaves C3 into C3a/b. While C3a contributes to an inflammatory response, C3b induces opsonization. In addition, C3b together with C2a/C4b forms the C5 convertase that cleaves C5 into C5a/b. C5a stimulates chemotaxis and inflammation, whereas C5b is an essential component of the membrane attack complex (MAC) that is formed on the pathogen's surface to induce its lysis and cell death. The mannose-binding lectin (MBL) and alternative pathways likewise comprise a series of proteolytic cleavages that result in C3 activation. C1-inhibitor avoids inaccurate activation by inhibiting several components of the classical and mannose-binding lectin pathways. A dashed box around a protein complex indicates that it acts as the protease to catalyze the next reaction.

Irreversible protease inactivation by serpins

Essential to the regulation of the above mentioned pathways are the serine protease inhibitors, referred to as serpins, that act as suicide inhibitors on the class of serine proteases of for instance the complement and contact systems[152] (Fig. 3). Well-known examples are antithrombin and α-1-antitrypsin, which both exert their inhibiting actions on multiple proteases. Despite the sometimes poor sequence homology, serpins are characterized by a common structural fold. Fundamental to their inhibitory functions is the presence of the P1-P1' residues, which are presented as a substrate for proteolytic cleavage by the reactive center loop (RCL) that protrudes from the bulk of the protein to be accessible by the protease. Upon cleavage of the P1-P1' bond by the target protease, the insertion of the RCL into the central β-sheet induces a conformational transition, accompanied by the transfer of the protease to the other side of the serpin. The consequential disturbance of the protease's active site makes the hydrolysis of the bond between its serine and the serpin's P1 impossible, resulting in a stable serpin-protease complex that inactivates the protease and is subsequently cleared from circulation and degraded[153,154]. Thus, the actions of serpins provide excellent examples of irreversible protein inactivation by disruption of the enzyme's active site, as opposed to protein activity modulation by post-translational modifications that can be removed by other specialized proteins.

Fascinatingly, the activities of the inhibitory serpins can in turn be regulated by the binding of small molecules, such as polysaccharides. An interesting serpin in this respect is C1-inhibitor, which is a blood plasma protein that is best-known for its anti-inflammatory activity, yet covers a broad range of biological functions. Being the only inhibitor that acts on the first components of the classical and mannose-binding lectin pathways of the complement system, C1-inhibitor plays a crucial role in regulating these cascades by preventing their spontaneous activations. Next to inactivating C1s and C1r (classical complement pathway) and MASP-1 and -2 (mannose-binding lectin pathway), C1-inhibitor downregulates the levels of active kallikrein and factor XII (coagulation/contact system), plasmin and tissue plasminogen activator (fibrinolytic system) and factor XIa and thrombin (coagulation system)[155]. Importantly, deficiency of functional C1-inhibitor underlies hereditary angioedema (HAE), which is characterized by recurrent attacks of potentially life-threatening swelling in various subcutaneous and submucosal tissues due to inadequate activation of the contact system[156,157]. As observed for other serpins, C1-inhibitor activity is enhanced by glycosaminoglycans (GAGs), of which the naturally occurring penta-saccharide heparin and synthetic dextran sulfate have been the most studied in the context of C1-inhibitor potentiation[158-161]. Intriguingly, the effect of GAGs on C1-inhibitor activity appears to be different towards the different target proteases[161]. For example, GAG binding to C1-inhibitor only minimally affects kallikrein inactivation, while it can greatly enhance inhibition of C1s. Gaining more insight into the underlying mechanisms not only offers possibilities for HAE treatment optimization by selective protease inhibition, but also extends our knowledge on protein activity regulation[162].

Although the above described methods of protease (in)activation and modulation of their inhibitors only give a glance at pathway regulation during the immune response, they

elegantly illustrate how every cellular process is tightly controlled by protein fine-tuning and emphasize once more the importance of pathway regulation in the prevention of disease.

Aims and outline

Genetic information has to be protected to ensure correct transmission to the next generation as well as proper functioning on the cellular and organismal level. Upon detection of DNA damage, elaborate response networks are activated that cooperatively protect genome stability by organizing lesion removal and adjusting cell cycle progression. Insight in the regulation of repair and signaling cascades that maintain genome integrity, may improve our understanding of their spatiotemporal coordination and the consequences of inaccurate activation, execution or completion.

The first chapters of this thesis address the regulation of DNA damage response processes, with a focus on the (transcription-coupled) nucleotide excision repair pathway that is crucial for the repair of transcription-blocking DNA lesions such as UV-induced photolesions. As outlined above, defects in NER genes have been associated with multiple disorders, displaying symptoms that vary from mild photosensitivity to severe neurodevelopmental defects. In the last decades, most of the core NER machinery has been described, shifting attention to the molecular mechanisms that either facilitate NER in the context of chromatin or promote the timely and accurate interplay between NER factors and post-translational modifications.

Chapter 2 studies the role of the cullin-RING ubiquitin ligase CRL^{CSA} during TC-NER by inhibiting its NEDDylation-induced activation. It demonstrates the significance of both NEDDylation in general, as well as the presence of CSA for the UV-dependent degradation of RNAPIIo that presumably prevents cell death when NER is compromised. Furthermore, it describes how NEDDylation modulates the interaction between CRL^{CSA} and RNAPIIo.

Whereas this research reveals a potential mechanism to coordinate the presence and activity of CRL^{CSA} at DNA damage, Chapter 3 evaluates how the stability of CSA and assembly of the CRL^{CSA} complex are guaranteed. Mass spectrometry-based approaches to further elucidate the role of CSA uncover the TRiC complex as a stable CSA-interacting factor. Additional functional assays reveal a crucial role for this chaperonin in the stabilization and localization of $(CRL)^{CSA}$.

Chapter 4 describes the discovery and characterization of a new SUMO E3 ligase, Zimp7, that is recruited to laser-inflicted DNA damage. The findings covered by this chapter include a solid interaction with PCNA and convincingly argue for further investigation of the roles of Zimp7 in both the DNA damage response and DNA replication.

In addition to DNA damage that may result from metabolic processes or be caused by exogenous sources, potential infection by for instance bacteria or viruses can pose a serious threat to life. Host defense against invasive pathogens is established via several immune responses, which display distinct methods of protein and pathway regulation as compared to the DDR. Chapter 5 briefly digresses from DNA damage repair, to explain and investigate the potentiation of C1-inhibitor – a protein that modulates multiple immune response

pathways – by the binding of glycosaminoglycans. The herein presented models shed light on how binding of dextran sulfate to C1-inhibitor affects the serpin's activity towards its target proteases to different extents.

Finally, Chapter 6 not only discusses the contribution of the described observations to our understanding of cell-protecting mechanisms and the (clinical) implications of loss of pathway regulation, but also makes recommendations for follow-up studies.

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