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REVIEW

Proteomics insights into DNA damage response and translating this knowledge to clinical strategies

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Genomic instability is a critical driver in the process of cancer formation. At the same time, inducing DNA damage by irradiation or genotoxic compounds constitutes a key therapeutic strategy to kill fast-dividing cancer cells. Sensing of DNA lesions initiates a complex set of signalling pathways, collectively known as the DNA damage response (DDR). Deciphering DDR signalling pathways with high-throughput technologies could provide insights into oncogenic transformation, metastasis formation and therapy responses, and could build a basis for better therapeutic interventions in cancer treatment. Mass spectrometry (MS)-based proteomics emerged as a method of choice for global studies of proteins and their posttranslational modifications (PTMs). MS-based studies of the DDR have aided in delineating DNA damage-induced signalling responses. Those studies identified changes in abundance, interactions and modification of proteins in the context of genotoxic stress. Here we review ground-breaking MS-based proteomics studies, which analysed changes in protein abundance, protein-protein and protein-DNA interactions, phosphorylation, acetylation, ubiquitylation, SUMOylation and Poly(ADP-ribose)ylation (PARylation) in the DDR. Finally, we provide an outlook on how proteomics studies of the DDR could aid clinical developments on multiple levels.

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1 DNA damage response in cancer formation and treatment

Despite the great variety of endogenous and exogenous sources that threaten the integrity of the DNA, our genomes are remarkably stable. This is due to the action of the DNA damage response (DDR). DDR signalling processes comprise the recognition of sites of DNA damage and the recruitment of factors, which transmit and amplify the damage signal, and finally execute the adequate cellular responses [1]. These responses to DNA damage include: chromatin rearrangements to allow access to the damaged DNA, DNA repair, cell cycle arrest, and alignment of cellular housekeeping functions, such as transcription, translation and cellular metabolism [2, 3]. Damage beyond repair can lead to initiation of apoptosis (or other forms of programmed cell death), or senescence. Cells, which survive in the presence of unrepaired damage and re-enter the cell cycle might ultimately become cancerous (Fig. 1) [1]. This is reflected in hereditary cancer syndromes linked to dysfunctional DDR pathways [4] and the enhanced genomic instability in spontaneously arising, non-hereditary types of cancers [5]. Excessive DNA damage has further been associated with accelerated ageing [1, 6].

While silencing of the proper response to DNA damage is seen as an enabling factor of cancer formation [7], on the other hand cancer treatment commonly relies on DNA damage induction by genotoxic drugs or irradiation [8]. In recent years,

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Abbreviations: **ATM**, ataxia-telangiectasia mutated; **ATR**, ATM and RAD3 related; **DDR**, DNA damage response; **DSB**, DNA double strand break; **ICL**, interstrand crosslink; **IR**, γ-irradiation; **MMS**, methyl methanesulfonate; **SUMO**, small ubiquitin-like modifier; **UV**, ultraviolet

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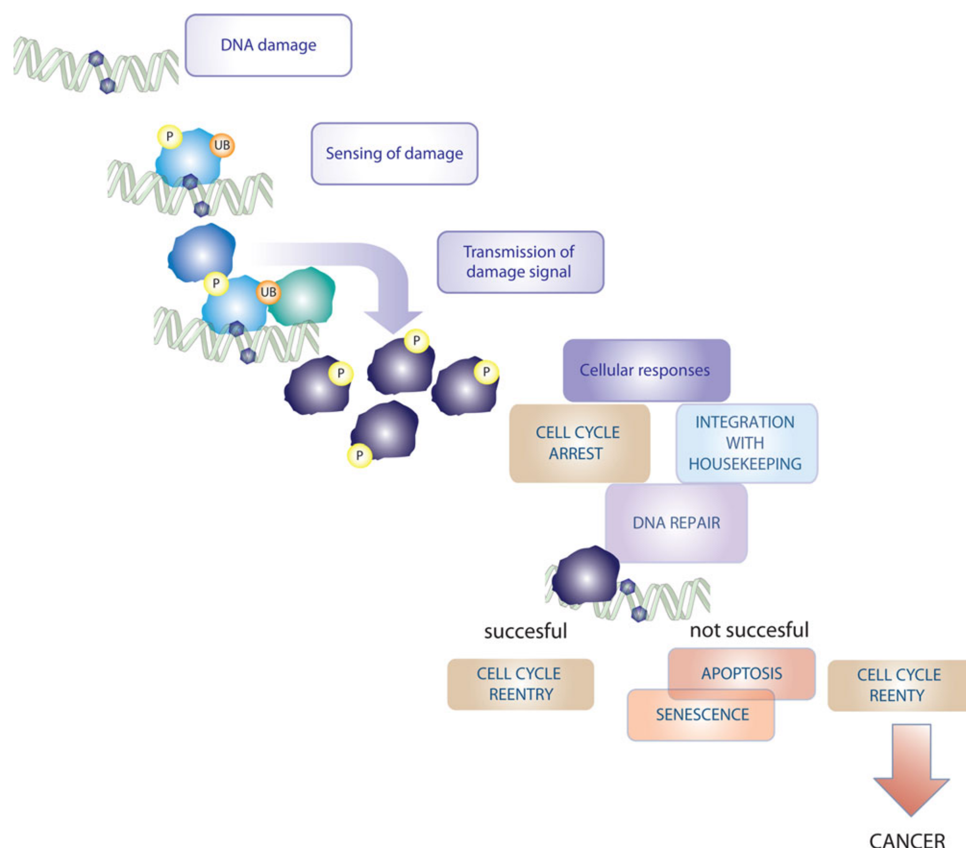

Figure 1. DNA damage signalling response. After sensing of DNA damage by proteins, which are either involved in DNA metabolism, or specifically recruited to aberrant DNA structures, a PTM-based signalling cascade is set into motion. This cascade enhances the nuclear damage signal and leads the damage signal down to effector components, which are involved in DNA repair, cell cycle arrest, and the integration of DNA damage with on-going cellular housekeeping processes. If DNA repair is successful cells can re-enter the cell cycle. If repair is not successful, the initiation of apoptosis or terminal arrest (senescence) can ensue. If cells re-enter the cell cycle in the presence of unrepaired DNA, this can lead to cancer formation.

the potential to specifically exploit DDR defects of tumour cells (e.g. deficiencies in homologous recombination repair) has emerged as a strategy for finding novel drugs and cancer biomarkers [4, 9]. Utilising the concept of synthetic lethality in cancer cells is also emerging as a powerful strategy for anticancer therapy [10, 11].

The DDR comprises a complex signalling network in which proteins and their posttranslational modifications (PTMs) play crucial roles on a multitude of levels.

Proteins involved in DNA metabolism, as well as specialised DNA damage sensor proteins sense various DNA lesions. Often damage sensing proteins are intimately linked with the DNA repair pathways, which repair specific types of lesions [12].

Sensing of aberrant DNA structures generally sets in motion a signalling cascade in which PTMs are added to sensor proteins, chromatin proteins and signalling factors (Fig. 1) [13]. PTM enrichment at sites of damage serves as a recruitment platform for further signalling factors involved in damage sensing, DNA repair, and transmission to downstream effector molecules.

Amongst the earliest activated sensors in the DDR are nuclear protein kinases and E3 ligases, which modify substrate proteins by site-specific phosphorylation and ubiquitylation, respectively [14]. Key upstream modifying enzymes include the PI3-K-related protein kinases ataxia-telangiectasia mutated (ATM) and ATM and RAD3 related (ATR). While ATM reacts to the presence of DNA double strand breaks (DSBs), ATR activity is triggered by RPA-coated single strand breaks [15, 16]. In the response to DSBs also E3 ubiquitin ligases such as RNF8 and RNF168 are crucially important [17].

Enzymes involved in DDR-PTM-cascades, such as kinases and poly(ADP-ribose) (PAR) polymerase (PARP) enzymes have been identified as promising cancer biomarkers and drug targets [4, 18, 19]. The potential to exploit DDR factors for improving the success of cancer therapy makes a better understanding of DNA damage signalling cascades and their apical regulators an important task for researchers today.

A better understanding of the intricate signalling responses evoked by DNA damage requires high-throughput technologies. Mass spectrometry (MS)-based proteomics has emerged as a highly sensitive, high-throughput, technique, which allows snapshots of cellular proteomes at a given cellular state [20, 21]. Shotgun proteomics has tremendous discovery power on multiple levels. The technique allows studying the abundance of proteins [22], their interactions with other proteins or other cellular macromolecules such as DNA [23, 24], and their modification by PTMs [25, 26].

Different groups have attempted MS-based analyses of the responses to different kinds of damage stimuli. Those included studies of PTM changes [27–34], changes in interactions between proteins or between proteins and DNA [35–37], and changes in protein abundance [30–32] (Fig. 2).

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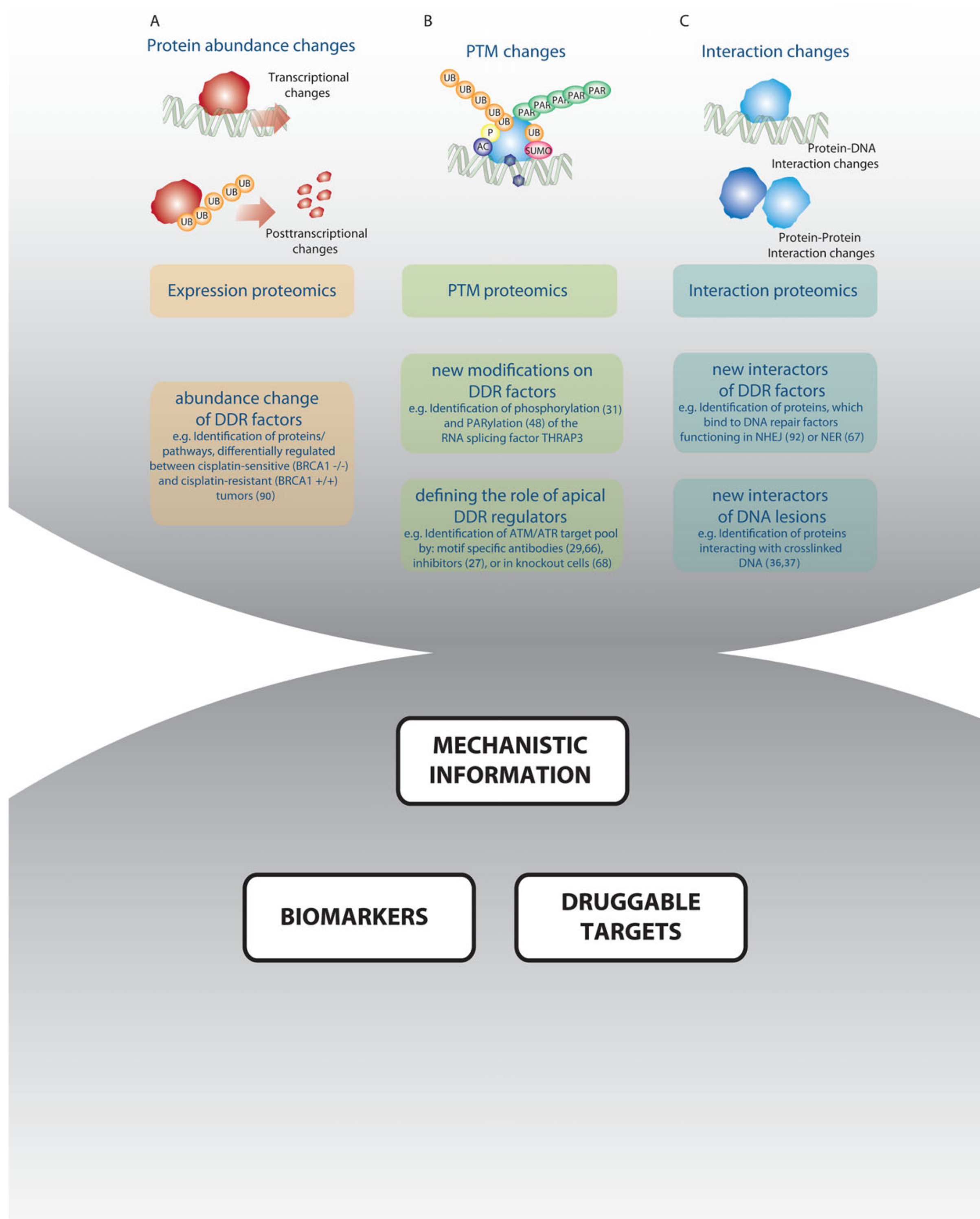

Figure 2. Proteomics techniques to study DNA damage-induced changes in protein interactions, protein abundance and PTM modifications. (A) Expression proteomics can measure changes in protein abundance. Those can result from transcription changing mechanisms or from posttranscriptional mechanisms, which are induced by DNA damage. (B) PTM proteomics can measure PTM changes, which are induced by DNA damage. (C) Interaction proteomics can identify changes in protein-protein and protein-DNA interactions after DNA damage. Those data can help to clarify or corroborate drug mechanisms of action, and lead to identification of drug targets and biomarkers.

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2 Exploring the DDR using MS

In contrast to techniques, which rely on antibodies and thus inherently preclude identification of new proteins and modifications, MS-based proteomics has the power to identify novel players of DDR signalling processes [38].

In shotgun proteomics proteins are generally digested prior to analysis using proteases, in a so-called bottom-up approach. Often, sample complexity is reduced by on- or offline fractionation, or by the enrichment of proteins of peptides prior to MS analysis [39]. Moreover, most workflows include an on-line chromatographic separation step, before peptides are ionised and analysed by MS. Currently, mass spectrometers of the orbitrap type are the most commonly used [39].

The "bottom-up" shotgun proteomics approach entails several limitations. Most shotgun proteomics experiments use trypsin as the exclusive protease, because it generates MS-friendly peptides. This approach, however, neglects the proteome space, to which trypsin is blind [40]. In the future top-down analysis of individual proteins, i.e. direct MS analysis of intact proteins without a protease digestion step [41] and middle-down proteomics using different proteases that generate longer polypeptides than trypsin could increase proteome coverage [42].

Another challenge of proteomics lies in the fact that the target database against which MS spectra are searched is a generic human database. This precludes the possibility to analyse specific mutation profiles of individual cell types, which might be highly relevant when studying cancer genomes that generally undergo massive rearrangements [43]. Proteogenomics approaches, where genomic data from the cell line or tissue sample under investigation are used as reference database, could in the future serve as an elegant means to overcome this problem [44, 45].

The dynamic range of protein abundance within a cell is very large, spanning multiple orders of magnitude. Housekeeping proteins (such as ribosomal proteins), which generally remain steady over a great number of cellular conditions, are often highly abundant. In contrast, levels of signalling-relevant proteins and signalling-relevant PTMs are often low [20]. To capture lowly abundant proteins and PTMs advances in fractionation and enrichment methods can help.

3 Analyzing PTMs in the DDR using MS

In DDR signalling processes, individual PTMs are covalently attached to signalling proteins with different kinetics. Multiple ways exist to dynamically regulate the process of PTM addition. The abundance, localisation and specificity of the enzymes, which add and remove PTMs (e.g. kinases and phosphatases) is regulated upon different stimuli. This regulation depends on transcriptional changes in the expression of these enzymes or alteration in their posttranslational modifications, e.g. by kinase auto-phosphorylation. Moreover, cofactors can either bring enzyme and substrate together or sequester them. Cofactors themselves can also undergo transcriptional and posttranscriptional regulation. In the case of protein-modifiers such as ubiquitin and small ubiquitin-like modifier (SUMO) also the extent of the free pool of modifiers can influence the speed and efficiency of PTM attachment (Fig. 3).

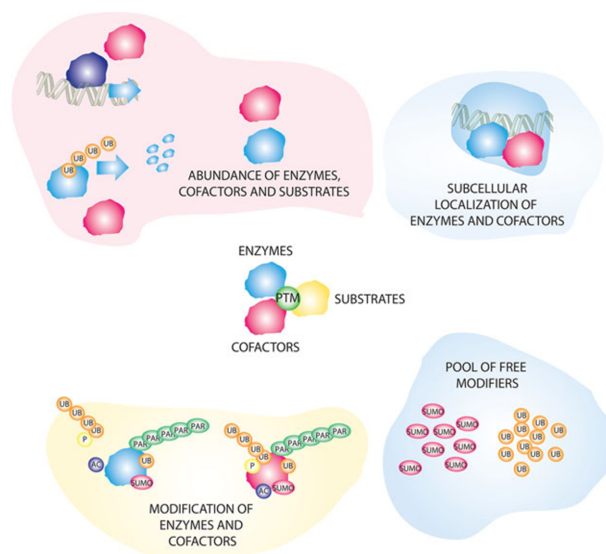

Figure 3. Modes of regulation of PTM responses after DNA damage. Different levels of integration exist for the dynamics and specificity of PTMs. Those include regulation of the (A) abundance (B) localisation (C) modification of enzymes and co-factors. For small protein modifiers regulation can also occur on the level of the pool of free modifiers.

This highly dynamic nature of PTMs can make it difficult to choose the optimal timeframe for the analysis of PTM-responses to DNA damage. Most PTM studies are limited to one or few timepoints, due to the often high requirement of input material in PTM proteomics. It is however, important to consider that the endpoint the researcher chooses will bias the scope of the identified results. While posttranslational responses can be very fast, transcriptional responses might take longer. Moreover, depending on the strength of the damage pulse, choosing a late analysis timepoint might mean that repair of the DNA lesion could already have occurred. Also, the massive contribution of cell cycle changes to the expression and modification of proteins should not be neglected when interpreting the results of DDR studies [46]. It is important to consider the difference between signalling events and cellular responses, which are caused by a halt of cell cycle progression, especially at later timepoints after DNA damage [31].

When analysing PTMs by MS it is important to take into account any special requirements related to sample preparation workflows and MS instrumentation. For highly dynamic PTMs it is important to counteract their reversal by blocking the activity of the responsible enzymes, for example by use of phosphatase inhibitors to preserve phosphorylation, or

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of alkylating chemicals to inhibit deubiquitinating enzymes [20,47]. Also artificial addition of PTMs during sample preparation should be avoided, e.g. by inhibiting the activity of kinases or PARylating enzymes during sample preparation steps by addition of Mg2+ scavengers such as EDTA/EGTA or PARP inhibitors, respectively [20, 48]. Modification usually changes the physiochemical properties of peptides, thus impacting their digestion efficiency and behaviour during chromatography and ionisation [49].

Since modified versions of peptides often exist in substoichiometric quantities, enrichment steps are generally required to analyse those by MS. Enrichment methods can rely on the physicochemical properties or structure/ sequence-specific features of proteins and peptides. Phosphorylated peptides can for example be enriched by using metal ion-based affinity capture (MOAC) or immobilised metal affinity chromatography (IMAC) [50]. Titanium dioxide (TiO_2) metal ion-based affinity capture was used in different studies that provided global snapshots of phosphoproteomes after DNA damage [30–32, 51].

Other enrichment strategies target specific protein sequence, or structural features, using antibodies or binding domains. Antibody-based enrichment is commonly used for enrichment of tyrosine phosphorylated peptides, and has also been successfully applied for enriching ubiquitylated and acetylated peptides in DDR studies [28, 31, 33]. In their landmark study of the phosphorylation response to DNA damage, Matsuoka et al. used antibodies targeting the S/T-Q motif, which is specific for the protein kinases ATM and ATR [29].

Also, binding domains of specific modifications, such as phospho-binding domains can be used to "fish out" their interactors, as shown by Blasius et al. who detected interactors of 14-3-3 proteins in the context of UV treatment [35]. Domains with a high affinity for PAR were further used to enrich PARylated proteins after DNA damage [48].

Nevertheless, for some PTM-types good antibodies are not yet available. Moreover, sample preparation conditions used for IP-based enrichment can interfere with stability of modifications. For enrichment of those proteins, researchers generally rely on the exogenous expression of tagged versions of proteins, as done for studies of SUMOylation responses [52].

While enrichment is still a prerequisite for analysing lowly abundant peptide species, enrichment strategies are generally accompanied by an increase in workload, instrument time and a decrease in reproducibility. Those limit the resolution at the levels of time, cell type and damage-inducer studied.

Most changes in the abundance of proteins or in the occupancy of modified versions of a protein are no on/off situation [53]. Full stoichiometry of PTMs is only reached in rare cases, such as phosphorylation changes during the mitotic phase of the cell cycle [46]. To accurately identify the abundance of peptides, different strategies exist. Those include label-free quantification, Stable isotope labeling with amino acids in cell culture (SILAC)-based quantification and chemical labelling strategies [20]. Most studies of PTM-changes in the DDR used SILAC-based quantitation as method of choice [28, 29, 31, 33].

Another specific challenge posed by PTM proteomics experiments is the downstream computational analysis and bioinformatic interpretation. Multiple modified variants can exist of the same protein, which might have different biological functions. Most DNA damage studies followed the strategy of treating PTM changes similar to changes in protein expression. Using pathway and network analyses DDR PTM studies pointed towards novel signalling routes, implicating RNA metabolism, in particular RNA splicing, in the response to DNA damage [27,29,31,32]. They moreover served to confirm ubiquitylation-mediated regulation of nucleotide excision repair (NER) after ultraviolet light (UV)-exposure [28, 33].

It is, however, important to note that the modification of a signalling molecule does not necessarily correlate with its activity. On the contrary, modification can target a protein for deactivation or even degradation [54]. Moreover, not all modifications are biologically relevant. Indeed, many of them are considered part of the biological noise [55].

Only follow-up studies, using targeted biological experiments can provide final certainty about the relevance of specific PTMs. Targeted validation led to identification of DNA damage-mediated phosphorylation and PARylation of the RNA splicing factor THRAP3 [31, 48], or the ubiquitylation-mediated regulation of RPA [28, 56].

4 Phosphorylation in the DDR

Site-specific protein phosphorylation is the best described PTM functioning in the DDR. Phospho-signalling regulates all stages of the DDR (Fig. 1). Fast phospho-responses lead to the recruitment of DNA repair factors and signalling molecules to damaged DNA [14, 30]. Subsequently, phosphorylation can serve to retain those factors at sites of damage. The phosphorylation of downstream signalling molecules further regulates later cellular responses. Those can "take the long road" by phosphorylating and thus modulating the activity of transcription factors [57, 58]. Among those transcription factors, is the key cellular hub protein p53, termed the *guardian of the genome*. Phosphorylation by DDR kinases can activate p53 by disrupting the regulatory loop between p53 and its negative regulator MDM2 [59–61].

Next to regulating the functions of transcription factors, phosphorylation-mediated signalling can also take a shortcut, by directly regulating downstream effector molecules [58]. Those include for example the CDC25 family of phosphatases, which can remove the highly-conserved inhibitory phosphorylation marks from the N-terminal part of cyclin-dependent kinases (CDKs). Checkpoint kinase-mediated phosphorylation attenuates CDC25 protein stability by priming it for proteasomal degradation. It further induces interaction with 14-3-3, sequestering CDC25 proteins from CDK1. Both mechanisms result in an induction of cell cycle arrest [62, 63].

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Given this highly complex phosphorylation-mediated signalling network, phosphoproteomics can be a vital technique to discover new phosphorylation-mediated phenotypes in the DDR. Those might ultimately be translated into new biomarkers and drug targets [64].

Special interest has been invested into studying the function of the PI3-K-related protein kinases ATM and ATR, which are the principal sensor kinases that are immediately activated after DNA damage. Together with their direct downstream targets Chk1 and Chk2, ATM and ATR regulate a pleiotropic array of processes after DNA damage [16, 51]. Their substrate pool reflects the whole spectrum of the DDR and mediates cell cycle arrest, DNA repair and cell survival [15, 65]. Phosphoproteomics studies have aimed to answer the following questions:

(i) Which targets are comprised in the substrate pool of ATM and ATR?
(ii) Which processes do their targets likely mediate?
(iii) What is the distribution of nuclear and non-nuclear phosphorylation events?
(iv) Which other kinases might be important for the DDR?

The boundaries of discovery within phosphoproteomics analyses of the DDR are defined by choice of enrichment method, MS instrumentation, quantification method, subcellular fractionation, timepoint and damage inducer (Table 1). Indeed, the number of quantified phosphosites within bulk-phosphoproteomics studies increased from earlier studies, which identified around 3000–5000 phosphosites [27, 30] to over 10 000 phosphosites in more recent analyses [31, 32].

Matsuoka et al. identified over 700 ATM/ ATR/ DNA-PK substrates by combining a number of S/T-Q motif antibodies [29]. This study for the first time highlighted the breadth of the ATM/ATR target pool and revealed the intersection of the ATM-mediated DDR with other cellular processes such as PI3K—AKT signalling [29].

Stokes et al. used an S/T-Q antibody-based approach to examine the effect of UV radiation on ATM/ ATR substrate phosphorylation. While they found extensive overlap to the substrates that had been identified by Matsuoka et al. they also found a number of UV-specific substrates. UV radiation leads to a strong activation of ATR kinase. The authors aimed to decipher potentially ATR-specific substrates by testing their phosphorylation in cells from Seckel-syndrome patients, which have very low ATR levels and fail to activate UV-induced ATR-based responses [66].

Subsequent studies analysed nuclear [27, 30] or whole-cell [31, 32] phosphoproteomes in the context of different DNA damage types. Those comprised ɣ-irradiation [30], UV radiation [67], replication stress induced by aphidicolin [68] and stress evoked by various genotoxicants such as cisplatin, neocarzinostatin or etoposide [27, 31, 32].

All studies found an enrichment of the ATM/ATR-substrate motif [S/T-Q] among DNA damage-induced phosphorylation sites [27, 30–32, 67, 68]. Bennetzen et al., who performed a time-resolved analysis, found ATM-dependent phosphorylation sites amongst the early responders, in line with ATM mediating fast responses to DNA damage [30]. While Bensimon et al. found only 10% of the identified phosphosites carrying an S/T-Q motif; addition of the ATM inhibitor KU55933 counteracted 60% of DNA damage-modulated phosphosites [27]. Similarly Mazouzi et al. found an enrichment of the S/T-Q motif for around 50% of phosphosites induced by 4 h of aphidicolin treatment, over 70% of which were mediated by ATM. Their findings highlighted the role of ATM-signalling in early replication stress. At the later replication stress timepoint, 24 h, the number of ATM-regulated sites decreased to around 50% [68].

Different studies suggested ATM-dependent and independent activation of the NFκ-B signalling pathway [51]. Interestingly, Choi et al. performed MS-based analysis of ATM-dependent protein composition of different cellular compartments. They found that the chromatin association of ANXA1, a protein that has been linked to NFκ-B signalling, depended on ATM activity [69]. Furthermore, also Beli et al. found DNA damage-induced phospho-regulation of members of the NFκ-B pathway [31].

Studies, which analyzed whole-cell phosphoproteome changes after DNA damage, allowed deciphering the different dynamics and biology of nuclear and non-nuclear phosphorylation events [31, 32]. Beli et al. found that DNA damage-induced phosphorylation events were enriched in the nuclear compartment, which was particularly true for S/T-Q phosphosites [31]. While nuclear phosphorylation was mainly related to DNA metabolic processes, cytoplasmic events were enriched for proteins involved in cell cycle regulation [31]. Pines et al. found processes related to cytoskeleton rearrangements changed after DNA damage in embryonic stem cells [32].

Despite the clear overrepresentation of S/T-Q motif-containing peptides after different types of DNA damage, phosphoproteomics studies of the DDR suggested the modulation of the activity of other kinases. Proline-directed phosphorylation, which is common for both cell cycle kinases and stress kinase family members, was found enriched among peptides, whose phosphorylation decreased after DNA damage [27, 31]. This might be due to the activation of phosphatases or decreased activity of kinases.

A number of kinases were phospho-targets themselves, including cytoplasmic kinases involved in cytoskeleton rearrangements [32]. Interestingly, phosphosites on p38, BUB1 and OXSR1 conformed to S/T-Q motifs [31]. Nevertheless, it is important to stress that phosphorylation of a protein is not the same as its activation.

Taken together, phosphoproteomics studies of the DDR indicated that next to the clearly vital and wide-ranging effect of ATM and ATR, other kinases might be important in the DDR [51]. Kinases, ordinarily involved in other cellular signalling events, such as stress kinases or cell cycle kinases, can be drawn into DDR signalling processes. Phosphorylation of those kinases on S/T-Q motifs suggests extensive crosstalk

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Table 1. Proteomics studies of the DDR

Study	Cell line	DNA damage-inducer	Timing	Enrichment	Number of sites/ proteins	Major affected pathways/ factors
Matsuoka 2007 [29]	293T cells	γ—irradiation	1 h after IR	S/T-Q motif specific AB	905 phosphosites on 700 proteins induced after IR	Identifying the scope of ATM/ ATR targets in response to IR. Connecting ATM/ ATR signalling to other pathways such as PI3K/ AKT
Stokes et al. [66]	M059K glioblastoma cells, GM18366 Seckel syndrome cells and GM00200-matched control cells	UV 50 mJ/cm^2	2 h after UV	S/T-Q motif specific AB	570 sites phosphorylated in UV-damaged cells	Identifying the scope of ATM/ ATR targets in response to UV. Analyzing ATR-specific responses in Seckel syndrome cells
Bensimon 2010 [27]	G361 human melanoma cell line	Neocarzinostatin (NCS)	10, 30, 120, and 360 min	TiO_2	2871 phosphosites on 1099 proteins	Deciphering the ATM-dependent nuclear phosphoproteome. Identification of an ATM-dependent phosphosite on ATM, which is required for ATM chromatin retention.
Bennetzen 2010 [30]	GM00130	γ—irradiation	5 timepoints: 0 min, 5 min, 20 min, 1h, 8 h	ERLIC and TiO_2	5204 phosphosites, 594 regulated	Temporal, nuclear phosphoproteome analysis. S/T-Q phosphosites are among the early responders
Pines 2011 [32]	Mouse ES cells	Cisplatin	4 h	SCX and TiO_2	11 034 unique phosphopeptides 3395 proteins	Global phosphoproteome. proteome and transcriptome analysis. Differential regulation of processes related to cytoskeleton rearrangements.
Beli 2012 [31]	U2OS human sarcoma cells	γ—irradiation etoposide	1 h 24 h	TiO_2 for phospho IP for acetylation	11 500 phosphosites 1800 acetyl sites	Global phosphoproteome proteome and acetylome analysis. Linking of RNA-splicing related factors to DNA-damage induced phosphorylation responses.
Povlsen 2012 [33]	U2OS human sarcoma cells	UV-irradiation	1 h after UV	Di-Gly AB SCX	6700 UB sites	Proteome-wide analysis of ubiquitylation changes after UV. Identification of PAF15 mono-ubiquitylation.
Elia et al. 2015 [28,56]	HeLa	UV (40J/m2) IR (10Gy)	1 h after UV or IR	Di-Gly AB for UB FACET-IP For AC	33 500 UB sites 16 740 acetyl sites	Combination of global ubiquitin and acetyl proteomics. Global increase in K6- and K33-linked polyubiquitination. Cullin-RING ligases mediate 10% of DNA damage-induced ubiquitination events.
Hendriks et al. 2015 [80]	HeLa and U2OS	Methyl methane-sulfonate (MMS)	90 min	FLAG-SUMO-2 (HeLa) His10-SUMO-2-IRES-GFP (U2OS)	755 SUMO-2 sites, 362 regulated after MMS	SUMOylation of chromatin modifiers, transcription factors, DNA repair factors, and nuclear body components.
Xiao et al. 2015 [52]	U2OS	Hydroxy Urea	2 h, 24 h	His10-SUMO-2 pulldown	566 SUMO target proteins	SUMO network including replication factors, transcriptional regulators, DNA damage response factors
Jungmichel et al. 2013 [48]	U2OS cells	H_2O_2, MMs, UV, IR	1 h for genotoxic stresses, 10 min for H_2O_2	Af1521 domain pulldown	165 proteins, which significantly increase in PARylation	DNA repair factors and proteins involved in RNA metabolism targets for PARylation after (genotoxic) stress. PARylation affects the nuclear relocalisation of THRAP3.

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Table 1. Continued

Study	Cell line	DNA damage-inducer	Timing	Enrichment	Number of sites/ proteins	Major affected pathways/ factors
Warmoes et al. 2013 [90]	Murine BRCA1-/-, p53-/- tumors; CDH1-/-, p53-/- tumors	cisplatin	24 h	Gel-based proteomics	167 differentially expressed proteins in BRCA1-/-, p53-/- tumors 98 differentially expressed proteins in CDH1-/-, p53-/- tumors	DNA repair, DNA metabolism, and chromosome segregation enriched in in BRCA1-/-, p53-/- tumors. Fatty acid metabolism in CDH1-/-, p53-/- tumors. Identification of FASN, as a cispltin sensitizer
Mazouzi et al. [68]	ATM+/+/ ATM -/- MEFs ATMIN +/+, ATMIN -/- MEFs	1 μM aphidicolin	4 h 24 h	Fe(III)-NTA-based phosphoenrichment	13 801 phosphosites on 4094 proteins	Deciphering the ATM- and ATMIN- dependent phosphoproteome and transcriptome in response to replication stress. Identification of ATMIN-dependent phosphorylation of CRMP2.
Boeing et al. [67]	HEK293	UV 30 J/cm^2	3 h after UV	Di-Gly AB For ubiquitylated peptides	10 000 UB sites, 900 regulated by UV 635 UV-regulated phosphosites	Multi-omics study of the UV response. Identifies a function for the melanoma-associated kinase STK19 in the DDR.

with the “classical” DDR kinases [51]. This complex phosphorylation network is likely destined to function as a cellular buffer, ensuring the faithful execution of the DDR, even if one of the players is missing. For instance, p53-deficient cells have been shown to rely on a p38-MAPK/MK2 signalling module for checkpoint activation [70, 71]. Indeed, only a few factors within the intricate phospho-signalling network are vital for cellular survival, including the kinase ATR, as well as its downstream target Chk1 [15, 61].

4.1 Ubiquitylation and SUMOylation in the response to DNA damage

Ubiquitin, a small 76 amino acid long protein highly conserved in eukaryotes, is covalently attached onto target lysines involving a three-enzyme process [47]. The ubiquitin machinery classically includes an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase [72]. The large number of E3 ubiquitin ligases confers specificity within the ubiquitin system [73]. Removal of ubiquitin requires deubiquitinase (DUB) enzymes [74].

Importantly, ubiquitin can be added to a number of lysines of ubiquitin itself, resulting in a variety of ubiquitin chains, including K6, K11, K48 and K63 linkage [75], whereas, K11 and K48 ubiquitin chains generally target proteins for proteasomal degradation, other types of ubiquitin chains and mono-ubiquitylation events can alter protein features.

Next to ubiquitin a number of other small ubiquitin-like protein modifiers exist. Amongst them, SUMO is probably the best studied in the DDR [52, 76, 77]. SUMO, similar to ubiquitin, is added in a 3-step process. A major difference to the ubiquitin system lies in the fact that the number of SUMOylases is much smaller than that of kinases and ubiquitinases, comprising only one E2 and a limited number of E3 ligases [78].

Both substrate degradation and change of protein properties induced by the ubiquitin- and SUMO-systems are highly relevant for DDR signalling processes [47, 73] (Fig. 2) [78]. Ubiquitylation regulates the signalling response downstream of DNA double strand breaks, as well as DNA repair pathways such as NER or Fanconi Anemia [78]. SUMOylation also regulates various nuclear processes, including transcription and cell cycle regulation. A number of DNA repair factors can be found SUMOylated after DNA damage [77].

However, the induction of degradation of protein substrates by the ubiquitin proteasome systems can pose a challenge in the interpretation of ubiquitin proteomics results [28, 33]. The decrease in an ubiquitylated peptide species can result either from its deubiquitylation or from its degradation [33]. To identify cases in which protein degradation is responsible for decrease in ubiquitylated peptides, a comparison to the un-modified proteome is a possibility. Alternatively, including proteasome inhibitors can enrich for species, which are targeted for degradation by ubiquitin after DNA damage. Those include for example the cell cycle regulators CDC25A and CDC25B [28]. However, proteasome inhibition massively boosts the overall abundance of ubiquitylated proteins and leads to a depletion of the pool of free ubiquitin. Thus, extra care has to be taken in the interpretation of proteomics data from such treatments.

Different enrichment strategies were employed to analyse the ubiquitin-response to UV radiation or γ-irradiation (IR) treatment, as well as SUMOylation responses to replication stress and methyl methanesulfonate (MMS) [28, 33, 34, 52, 67, 77]. Schwertmann et al. used the FK2-ubiquitin

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antibody to enrich ubiquitylated proteins after UV treatment [34]. The studies by Povlsen et al., Elia et al. and Boeing et al. utilised antibodies that recognise the glycine dipeptide (di-Gly), which results from tryptic cleavage of ubiquitylated peptides [28, 33, 67]. While the use of di-Gly antibodies allows bulk enrichment of great numbers of ubiquitin sites, and permits studying endogenous proteins, it entails certain limitations. Not only ubiquitin, but also the ubiquitin-like modifiers NEDD8 or ISG15 leave a di-Gly remnant on the acceptor lysine. The modifying molecule cannot be distinguished using this strategy [47]. Nevertheless, the amount of ISG15 or NEDD8-modified proteins was found to be negligible in comparison to ubiquitylated proteins [79]. While di-Gly antibodies cannot identify the ubiquitin chain linked to an individual peptide, the bulk changes in different types of linkages can be quantified. Interestingly, Elia et al. found a strong increase in K6- and a less pronounced increase in K33-linked ubiquitin chains after UV but not IR exposure [28]. K6-chains have been related to BRCA1, a key DDR protein in vitro [75].

Xiao et al. and Hendriks et al. used expression of epitope-tagged, exogenous SUMO molecules [52, 80]. Since SUMO modifications are quickly removed by deSUMOylases during sample preparation, and to date no suitable deSUMOylase inhibitor exists, expression of tagged SUMO versions provides a viable alternative, allowing lysis under harsh conditions [80].

Upstream regulators of phosphorylation responses can be explored by identification of linear sequence motifs, which can help inferring upstream kinases (or kinase families). Also inhibitor studies can aid identifying kinases, which are responsible for phosphorylation responses [27, 30, 31]. In contrast, linear target motifs are much less prevalent for the ubiquitin system and good inhibitors for E3 ligases are rare [81]. Elia et al. made a first attempt to identify upstream enzymes, which might be relevant for bulk ubiquitin changes after DNA damage, by combining the Cullin-Ring (CRL) ligase inhibitor MLN4924 with DNA damage induction. They found that CRLs mediated around 10% of UV-induced ubiquitylation events [28].

Similar to phosphorylation, also ubiquitylation and SUMOylation were found to be enriched in the nuclear compartment after DNA damage [28, 33, 80]. Moreover, ubiquitylation and SUMOylation events were particularly enriched on proteins, which are involved in the repair of DNA lesion caused by the damage inducers employed. Those included for example protein ubiquitylation of factors involved in the NER pathway, which is crucial for the repair of UV lesions [28,33,34], or SUMOylation of factors involved in the response to replication stress, which is caused by Hydroxyurea treatment [52]. Moreover, processes related to the mitotic spindle, were found changed after UV and IR, with many of those proteins decreased in ubiquitylation [28].

Identification of the specific sites of SUMOylation has posed a considerable challenge to researchers. Hendriks et al. [80]. combined SILAC-based quantification of SUMOylated proteins with label free site-identification. They were able to map 755 SUMO-2 sites, of which 362 were regulated after MMS treatment. Interestingly, next to identifying proteins involved in DDR processes, Hendriks et al. found that MMS also induced SUMOylation of chromatin proteins. SUMOylation targets were moreover found to functionally interact, further establishing the concept of SUMO group modifications.

4.2 Other PTMs: acetylation and PARylation

Next to ubiquitylation and phosphorylation, a number of other modifications are emerging to be relevant in the DDR. Prevalent examples are two types of PTMs, acetylation and PARylation, which are of high clinical relevance. Inhibitors of PARP enzymes and histone deacetylases (HDACs) have entered clinical trials or clinical use in cancer treatment [19,82].

Lysine acetylation impacts gene expression by modifying chromatin interacting proteins, including histone tails and non-histone proteins. HDACs have been implicated as radiation sensitizers, and acetylation has been shown to regulate a number of DNA damage-relevant processes [48, 83]. Amongst the prominent DDR targets of acetylation is the transcription factor p53. Acetylation enhances p53 protein stability and transcriptional activity. The latter is further aided by acetylation-dependent chromatin relaxation in p53 target genes [84].

A number of proteomics investigations set out to analyse acetylation responses to DNA damage [28, 31, 85]. DNA damage-induced acetylation changes were enriched in the nuclear compartment [28, 31, 85]. However, both acetylation and deacetylation changes were found to be significantly less pronounced than changes in (de)ubiquitylation and (de)phosphorylation at 1 h after IR and UV damage [28,31]. Interestingly, Bennetzen et al. found an early wave of deacetylation 5 min after IR [85].

In a recent large-scale proteomics screen, Elia et al. improved the enrichment protocol for acetylation sites, by combining it with deep SCX fractionation. This yielded an unprecedented depth in acetylation site identification, which equaled the range of ubiquitin- and phosphoproteomics studies [28]. A number of known DDR factors, such as DNAPK and PARP1, showed dynamic acetylation after DNA damage [28].

Another PTM, whose relevance for the DDR is increasingly appreciated is PARylation. PARylation is a reversible posttranslational modification that is excessively added to proteins and other biomolecules after DNA damage. Inhibitors of PARP enzymes are in clinical studies for cancer treatment and have been shown to be especially successful in the context of HR-deficiency [9]. The PARP inhibitor Olaparib was recently approved by the FDA for treatment of cancer patients [19].

Despite the fact that PARylation cascades are highly relevant for the DDR, high-throughput studies of PARylation were hampered by the difficulty of PAR enrichment. In 2013,

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Jungmichel et al. used an Af1521 domain, which showed strong ADP-Ribose-binding features, to fish out PARylated proteins from cellular lysates, which had been exposed to different DNA damage stimuli [48]. Using as SILAC approach the authors compared the wild-type PARP binding domain to a mutant version, which lacked affinity to PARylated proteins. Similar to other studied PTMs, PARylation events after DNA damage were enriched in the nucleus. The authors confirmed a large number of DNA repair factors as PARylation targets after DNA damage. Moreover, similar to studies of the phosphorylation machinery, the authors also found proteins involved in RNA metabolism to be PARylated in the context of (genotoxic) stress. Those included the RNA splicing factor THRAP3, whose localisation had been shown to also depend on phosphorylation in an earlier study [31].

5 Studying PTM crosstalk by MS

PTM crosstalk is a key means for signal integration in the DDR. Enzymes that function in specific PTM pathways are targets for modification by other PTMs, including for example the phosphorylation of ubiquitinases [30, 31]. While crosstalk on the pathway level suggests reciprocal regulation of different PTM classes, functional validation is required to draw final conclusions about PTM-based regulation of enzymes.

Next to regulating enzymatic activity of other PTM-modifiers, different PTMs can also converge on the same protein, sometimes even on the same amino acid residue. The addition of multiple PTMs has great regulatory potential, including the modulation of positive and negative interactions [54]. The combinatorial logic of different PTMs is large, considering the different types of PTMs, as well as their potentially different functional outcomes. This PTM integration is vital in regulating DDR signalling hubs, such as the DNA clamp loader PCNA or the transcription factor p53 [78, 86].

Many different types of modifications target lysines. Among those are acetylation, methylation, ubiquitylation and SUMOylation. This phenomenon presents the idea of specific lysine residues in signalling proteins to function as cellular modification hubs that integrate different PTM pathways. Indeed, different studies suggested the potential for reciprocal regulation between ubiquitylation and acetylation [79,85]. The potential crosstalk between the acetylation and ubiquitylation system in response to DNA damage was tackled by Elia et al. on a global level. However, they only discovered a small proportion of reciprocal modification (increased ubiquitylation and decreased acetylation or vice versa) on the same lysine residue [28].

It is important to note that the peptide-centric approach severely limits the ability of MS to analyse PTM crosstalk. PTMs, which are further apart than the typical length of a tryptic peptide cannot be analysed. Moreover, different PTMs might require different enrichment strategies and cannot be properly analysed within the same sample. Finally, determination of site occupancy would be required to truly assess crosstalk between PTMs on a global scale.

5.1 Changes in protein abundance after DNA damage

DNA damage can regulate protein expression on multiple levels. Bulkier DNA lesions can directly block transcription [87]. Moreover, signalling downstream of DNA damage can lead to changes in gene expression and posttranscriptional mechanisms can change the stability of RNA transcripts or proteins [88]. Due to those posttranscriptional mechanisms, gene expression levels are not always well correlated with protein abundance. Indeed, the correlation between gene transcripts and proteins seems to be highly context-dependent [89]. In line with this observation, Pines et al. found little direct correlation between regulation on RNA and protein level, when comparing the cisplatin response in embryonic stem cells. They confirmed this lack of correlation using targeted biology experiments [32].

Different studies analysed the regulation of protein abundance after DNA damage. Overall, changes in protein levels were much less pronounced than PTMs changes [31]. Proteins, which did change after DNA damage were enriched for target genes of the transcription factor p53, which is regulating pleiotropic responses after different types of DNA damage [31, 32, 86].

In 2013, Warmoes et al. searched for protein biomarkers in cisplatin-sensitive, BRCA1- and p53-deficient and cisplatin-resistant BRCA1-proficient, p53-deficient mouse tumors. They analysed proteome changes 24 h after cisplatin treatment, a timepoint were DNA damage induction in tumor cells was evident, yet no excessive amounts of apoptosis did occur. Enriching cisplatin-regulated protein networks for functional information they found categories associated with "M-phase" and "chromosome segregation" and "DNA metabolic process/deoxyribonucleotide metabolic process" enriched in BRCA1-deficient tumors. They further discovered enrichment of proteins involved in fatty acid metabolism in cisplatin resistant tumors. Indeed, knockdown of one of the identified fatty acid metabolism factors, FASN, could sensitize resistant tumor cells to cisplatin treatment [90].

An interesting mechanism of transcriptional repression after DNA damage was proposed by Hendriks et al., who suggested that SUMOylation of chromatin modifiers could lead to transcriptional repression after DNA damage [77, 80]. They found SUMOylation of various chromatin proteins. Those included the transcriptional co-activators P300 and CBP, SUMOylation of which had been previously reported to suppress transcription. They moreover discovered SUMOylation-mediated recruitment of the histone demethylase JARID1C, which led to demethylation of the transcriptionally-activating histone marks H3K4me2 and H3K4me3. Those and other SUMO-mediated changes in chromatin modifiers might act in concert to repress transcription after MMS treatment [80].

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5.2 Changes in protein-protein interaction and protein-DNA interaction induced by DNA damage

Signalling in the DDR requires recognition of the presence of damaged DNA. The initial recognition of a DNA lesion induces a great number of rearrangements in the nuclear architecture. Those include the recruitment of DNA damage-specific proteins to the DNA, alterations in chromatin e.g. to make the damage accessible, or form docking platforms for repair factors, and changes in proteins involved in DNA metabolism, which are already present at the DNA [14, 91]. Altogether those rearrangements result from changes in protein-protein and protein-DNA interactions, which can be measured by MS-based interaction proteomics (Fig. 2).

A number of studies analysed the proteins binding to a single DDR factor or a whole group of proteins, functioning in the same DDR pathway. Xing et al. used MS to analyse proteins binding to 19 factors functioning in the DSB repair pathway NHEJ [92], whereas Boeing et al. analysed the interactome of the NER factors CSB and RNAPII in the context of UV stress [67].

Next to studying the interactions of a single protein or a group of proteins another strategy lies in the pull-down of a specific protein-binding domain. Phospho-binding domains were found to be crucial for early signalling processes in the DDR. Those include BRCT, FHA and 14-3-3 domains, to name a few [93]. In 2014, Blasius et al. analysed the 14-3-3 interactome in the context of UV radiation and caffeine-mediated PI3K-kinase family inhibition [35]. 14-3-3 proteins are highly conserved phospho-binders, which regulate a number of cellular functions, such as cell cycle halt by binding to Chk and CDC25 proteins [58]. Next to known damage-induced binders, such as Chk1, the authors found proteins and protein complexes involved in RNA metabolism. Those included the nuclear exosome component Rbm7 [35].

Two elegant studies recently combined novel MS-technology with sophisticated follow-up experiments to decipher the recruitment of proteins to cross-linked DNA [36, 37]. Interstrand crosslinks (ICLs) are extremely toxic lesions, which affect both transcription and replication by hindering the crucial separation of the DNA strands. ICLs are formed by exposure to chemotherapeutic drugs such as cisplatin or mitomycin C and their repair involves a complex mixture of repair pathways, including the Fanconi Anemia pathway [94].

Raeschle et al. developed a technique they termed chromatin mass spectrometry (CHROMASS) to decipher protein recruitment during ICL repair. They used cross-linked and undamaged sperm chromatin, which underwent replication in Xenopus extracts, and analysed protein binding at different timepoints. The authors found DNA repair factors strongly enriched in the damaged chromatin, compared with the undamaged one. This enrichment depended on DNA replication, as the recruitment of those factors was inhibited by replication inhibitor geminin [37].

Liang et al. created a DNA structure that contained a single, well-defined ICL. They incubated this DNA structure with nuclear extracts of HeLa cells, which had been exposed to mitomycin C. Using MS, they identified the protein UHFR1, which was enriched at ICL-DNA compared with the control [36]. UHFR1 was recruited to chromatin after treatment with crosslinking agents, and was required for recruitment of the Fanconi Anemia pathway component FANCD2 [36]. Those two initial analyses open up possibilities for the study of other DNA lesions, by pulling down damaged DNA and the binding components for other types of DNA lesions.

5.3 Outlook: clinical relevance of proteomics studies of the DDR

Proteomics can be relevant for multiple steps of drug discovery processes, including the identification of novel drug targets, highlighting drug mechanisms of action and biomarker discovery [95] (Fig. 2). In the future, proteomics might also become relevant as a diagnostic tool.

5.4 Proteomics as a tool to discover new drug targets, biomarkers and drug mechanisms of action in model systems

Most proteomics studies of the DDR to date focused on the description of global responses to a single or limited number of DNA damage inducers in model systems. Those studies could identify known and novel signalling routes and highlight their key players. Those are especially valuable for providing a better understanding of drug mechanisms of action, but can also help identifying potential new drug targets and biomarkers.

In the future, powerful proteomics technologies can be a valuable source for network medicine approaches, which base biomarkers and drug targets on a network of events (protein signature), rather than a single marker or target [96]. Pioneering studies, such as mid-level resolution phosphorylation analyses by the Yaffe lab, could predict sensitivity to DNA damage-inducing drugs in breast cancer cells [97]. Initial efforts have explored the predictive power of large-scale phosphoproteomics datasets in the study of signalling pathways in model organisms and drug sensitivity in cancer cells [98, 99]. Nevertheless, predictive modelling generally requires a high-resolving power of time-points, high reproducibility and high coverage, in order not to miss crucial data points. Proteomics analyses are now on a good way to attain the speed, sensitivity and reproducibility that will allow designing studies with high numbers of timepoints, replicates and different DNA damage-inducers.

5.5 Diagnostic clinical application of proteomics

To take the next step into the clinic, proteomics will have to master the challenges posed by mass spectrometric analyses

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of tissues [53]. This is relevant for biomarker discovery from tissue samples, and for proteomics becoming a true diagnostic tool.

Next to the aforementioned limitations of MS-based proteomics analysis, specific challenges for tissue proteomics relate to tissue availability. Those are particularly critical for analysis of prognostic patient biopsy samples. Sample amounts are especially relevant for PTM studies, whereas for expression proteomics nowadays minute amounts of sample might suffice.

Next to the availability of samples also their quality is important. It is not always feasible to gain fresh samples. Formalin-fixed, paraffin-embedded (FFPE) samples pose a viable alternative for both proteomics and PTM proteomics [100]. However, it is worth noting that ischemia-induced artifacts can occur during the preparation of tissue samples. Those can drastically change patterns of dynamic PTMs such as phosphorylation [101, 102].

Another challenge lies in the inherent heterogeneity of tissue samples. On the quest for biomarkers, this can pose a distinct challenge to the researcher. Heterogeneity between samples requires an increase in the number of replicates in order to identify confident biomarkers [53]. Heterogeneity amongst tissue cell types or amongst different tumor cell populations can potentially be addressed by tissue microdissection prior to MS analysis [103]. Targeted MS approaches could further serve as an alternative to biomarker discovery from patient samples [104].

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