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Stechow, L. von

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Author: Stechow, Louise von

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UNRAVELING DNA DAMAGE RESPONSE-SIGNALING NETWORKS THROUGH SYSTEMS APPROACHES



MANUSCRIPT SUBMITTED

Louise von Stechow, Bob van de Water, Erik HJ Danen

SUMMARY

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Genotoxic perturbation holds a central place in cancer formation and ageing, but also is key to cancer therapy by irradiation or chemotherapeutic drugs. Sensing of DNA lesions initiates a highly complex DNA damage response (DDR). This response involves signaling cascades that activate appropriate damage repair pathways, arrest the cell cycle, and ultimately determine cell survival or death. The DDR must be integrated with ongoing signaling and housekeeping processes. With the emergence of high-throughput -omics technologies it has become clear that DNA damage-mediated responses penetrate far deeper than previously appreciated into virtually all cellular signaling pathways. Advances in the last decade have revealed a plethora of early DNA damage-induced changes in posttranslational modifications and subsequent alterations in gene expression profiles, and have provided a glimpse into the assorted rewiring of signal transduction cascades providing biomarkers for chemo- or radiosensitivity. At the same time, genome-wide RNAi screening has provided mechanistic insights in DDR signaling cascades and identified genes involved in mechanisms of cancer resistance to genotoxic therapies. Most recently, distinct -omics datasets have been integrated and sophisticated mathematical models have been applied to the DDR. Here, we review such recent advances that have widened and in some cases, deepened our knowledge of DDR signaling.

The DNA damage signaling response

Recognition of DNA damage elicits a complex signaling response that is aimed at repair or removal of cells carrying irreparable damage. This “DNA damage response” (DDR) involves a fast cascade of posttranslational modifications (PTM) to signaling molecules, transmitting the damage signal throughout the cell and initiating a variety of cellular responses¹. Moreover, DNA damage-induced transcriptional changes provide a slower route for the adjustment of cellular programs after genotoxic perturbations^{2; 3}. Those cellular responses include assembly of DNA repair complexes and halt of cell cycle progression but also terminal alterations, such as removal of damage-carrying cells by apoptosis, senescence or stem cell differentiation^{4; 5} (Fig 1). Finally, unrepaired DNA damage can result in organ toxicity, mutations leading to cancer formation, or depletion of stem cell pools through terminal differentiation or shifts in lineage composition, which have been associated with progeria syndromes and physiological ageing^{4; 5; 6}.

Systems biology approaches to study the DDR

Systems biology constitutes the biological discipline that attempts to describe complex biological phenotypes by investigating the associations of system components and their changes in response to extrinsic or intrinsic perturbations. Taking a holistic approach, systems biology studies of the DDR hold the promise of an unbiased portrayal of the vast amounts of DNA damage-induced cellular changes^{2; 7}. Furthermore, following a

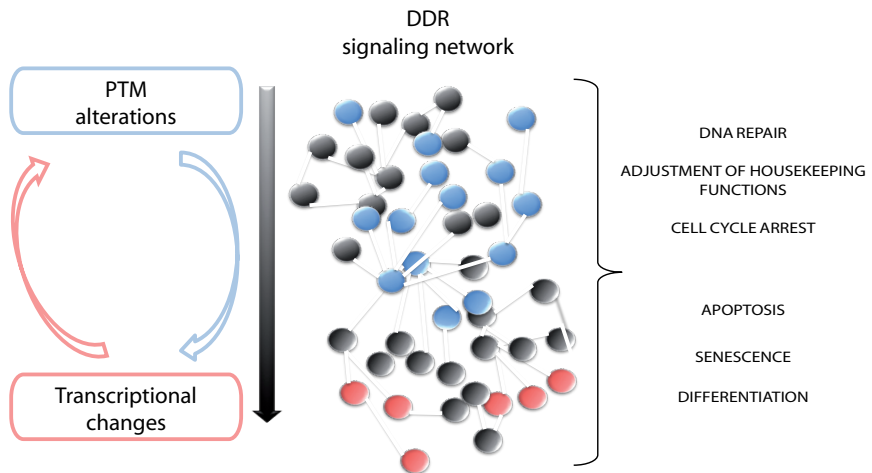


Figure 1. The DNA damage signaling response. Early in the DDR, posttranslational modifications are introduced, which serve to transmit the damage signal and can quickly alter protein-protein interactions. Early signaling events can lead to an arrest of the cell cycle, adjustment of housekeeping functions and recruitment of DNA repair factors. A more slowly occurring transcriptional response to genotoxic stress can determine final outcomes of DNA damage such as induction of apoptosis, senescence or differentiation. Transcription-dependent and independent feedback loops exist for most DDR processes. DNA damage-induced transcriptional and posttranslational alterations represent changes in the DDR signaling network, which can be fast physical interaction changes, but also permanent genetic rewiring.

reductionist approach, smaller scale systems biology studies provide models of DNA damage-induced modulations of cellular behavior, such as oscillatory p53 responses⁸. Both efforts will be essential to gain a comprehensive picture of the complex phenotypes, which comprise the DDR and advance the insight into the processes underlying cancer formation and ageing, and to improve DNA damage-based cancer treatment.

In the last decade high-throughput studies of the cellular responses to various kinds of genotoxic stress have helped to elucidate DDR signaling cascades. Those include monitoring transcriptional, translational, posttranslational and metabolic changes induced by DNA damage; RNAi-based screens to investigate functions of specific DDR signaling molecules; and studies of DNA damage-induced rewiring of signaling networks^{9; 10; 11} (Fig 2). Furthermore, recent studies have begun to integrate different DDR -omics datasets^{12; 13} and provided mathematical models to investigate DNA damage-induced phenotypes^{14; 15}.

Generation and interpretation of large-scale omics datasets

Crucial for systems biology studies is the extraction of phenotype-relevant factors from large-scale datasets. Those can be investigated using data-driven modeling techniques, such as clustering and regression algorithms; often displaying the datasets as heat maps or scatter plots, which show for example principle component analyses^{15; 16; 17}.

Next to these methods, two major approaches have appeared to extract valuable information from large-scale datasets and identify common modules and enriched processes; on the one hand the determination of significantly enriched pathways within large-scale datasets and on the other hand the formation and study of

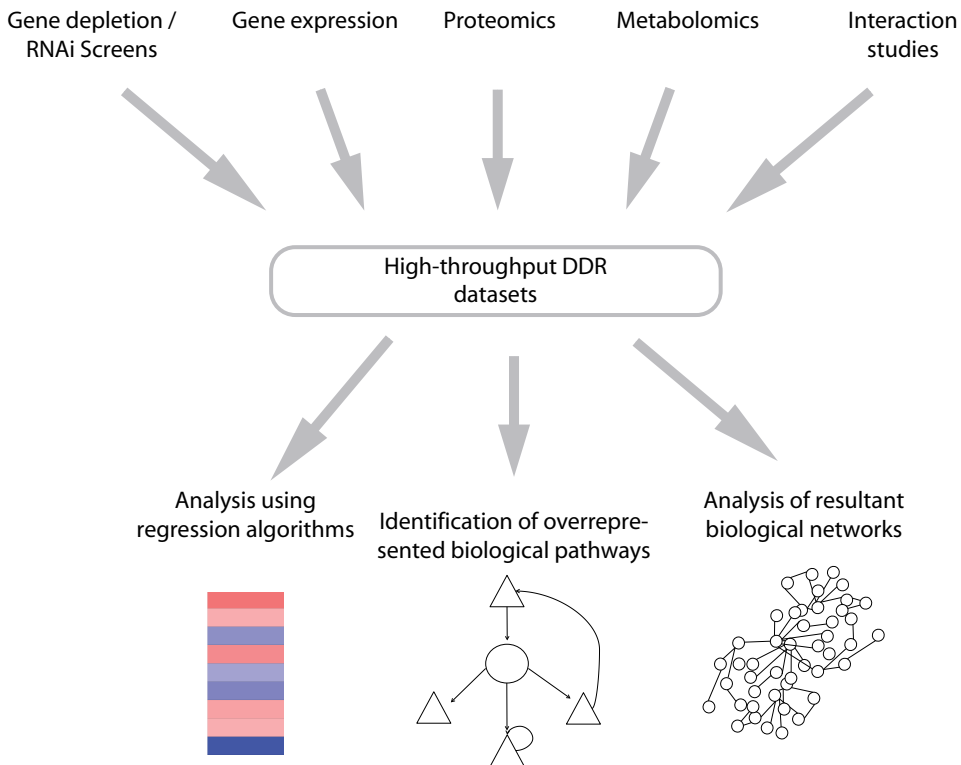


Figure 2. Systems biology approaches using pathway and network analysis. Various sources including proteomics, transcriptomics, metabolomics, functional genomics and interaction studies lead to the accumulation of large-scale datasets monitoring DNA damage-induced cellular responses, which can be analyzed using regression algorithms, biological networks (including regulatory motifs or modules) or the identification of canonical pathways (which allow to uncover regulatory relationships). Those methods can help to build models for the description of biological phenotypes.

biological networks^{16; 18; 19} (Fig 2).

Pathway analysis relies on the identification of commonalties between a group of molecules, which is defined as a biological pathway and a given dataset and has proven an important method to enhance the robustness of cancer biomarkers, extracted from large scale microarray and next generation sequencing datasets^{18; 20; 21}. Pathway analysis can be a useful tool to uncover not immediately obvious regulatory phenotypes from large scale datasets and visualization of expression data as pathway maps can identify regulatory relations, for example between transcription factors and their target genes^{16; 21} (Fig 2).

Network biology is a promising method to decipher complex disease phenotypes, identifying biomarkers and discovering new therapeutic targets^{22; 23; 24}. Biological networks are generally scale-free and depend on so-called hubs, highly interactive nodes, which are vital for network stability. Hub-proteins have been shown to be essential and evolutionary conserved in model organism, such as yeast, but recent studies show that this phenomenon does not necessarily translate into hubs being important disease genes in humans^{19; 23}. However, not only single hubs, but also the organization of different network nodes into modules (describing a dense network

neighborhood with a limited number of nodes) and motifs (describing a small number of nodes which interact in a functional relation) may be significant descriptors of biological phenotypes¹⁶. Genes involved in the same biological processes, are often found in similar network regions and show high connectivity to one another^{25; 26}.

Disadvantages of pathway - and to a lesser extent also of network analyses, lie in their dependency on prior knowledge, which results in a bias towards well-studied biological relationships^{23; 27}. Another challenge is the necessity of generalization, which underlies pathways and networks and often does not take into account organismic, tissue, and cell type specific factors and, in many cases must neglect intracellular organization and timing of signaling events²⁸.

DDR proteomics studies

Early DNA damage-induced signaling events are crucially dependent on the addition of PTMs to signaling molecules and chromatin components²⁹. A special focus has been attributed to the detailed study of the hierarchical phosphorylation and ubiquitination cascade initiated by DNA double strand breaks (DSBs)^{1; 29}. Following recent advances in resolution and speed of Mass Spectrometry-based proteomics³⁰, various studies have been aimed at examining the gross changes in phosphorylation, ubiquitination and acetylation, which are initiated by treatment with γ - or UV-irradiation and different types of genotoxic compounds^{31; 32; 33; 34} (Table 1A, B). These high-throughput studies reveal a complex PTM network, which extends far beyond modification of classical DDR factors. The pioneer phosphoproteomics DDR study performed by Matsuoka et al. indicated that after γ -irradiation damage, the PI3K-related protein kinases ATM and ATR alone lead to phosphorylation of more than 700 proteins, with a total number of 900 phosphorylation events demonstrating the magnitude of the response mediated by those kinases⁹. Peptides phosphorylated in the SQ/TQ ATM/ATR substrate-motif were derived from proteins involved not only in cell cycle inhibition and DNA repair but also in processes not previously linked to the DDR such as RNA splicing⁹ (Fig 3). While subsequent global

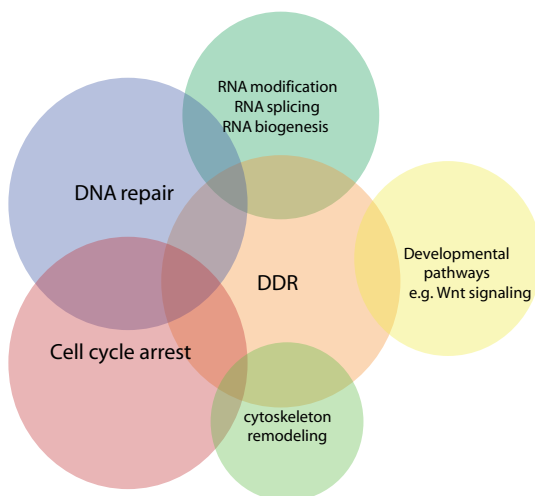


Figure 3. Systems biology studies link novel pathways to the DDR. Recent high-throughput analyses of the DDR reveal a strong enrichment of DNA repair- and cell cycle related pathways but also connect the DDR to a variety of other signaling pathways, including those related to RNA splicing and RNA processing, and to developmental processes. Size of spheres correlates to the frequency with which pathways have been identified in high-throughput DDR studies. Overlapping spheres indicate processes working together to modulated the DDR. For details see tables 1, 2.

phosphoproteomics studies confirmed this crucial role of PI3K-like kinases, studies from our lab and others also identified important contributions of other (downstream) kinases to the DDR, including for example members of the MAP-kinase family^{12; 31; 33; 35}. Two independent studies by Bensimon and Bennetzen analyzed the kinetics of nuclear phosphorylation events after DSB-induction by γ -irradiation or radiometric drugs. Interestingly, both groups emphasized the importance of the early DNA damage-induced phosphorylation response^{31; 35}. Bensimon et al. indicated that this fast ATM, ATR and DNA-PK-dependent phosphorylation response, was associated with nuclear processes such as DNA repair, chromatin binding, and regulation of transcription³¹. Bennetzen et al. showed phosphorylations of DDR-related proteins to occur mainly in the early stages of the DDR phospho-response³⁵. While those studies were restricted to nuclear phospho-events, we recently studied the entire cellular phosphoproteome of cisplatin-treated mouse embryonic stem (ES) cells. Analysis of enriched processes predicted from phosphorylated peptides indicated that besides cell cycle arrest and DNA repair; rearrangements of the cytoskeleton (in pathways mediated by the Rho-GTPases Rac and Cdc42) constitute a major response to DNA damage³³ (Fig 3).

Next to phosphorylation-mediated signaling, ubiquitination of DDR factors has emerged as a central means for signal transmission after different forms of DNA damage, including proteasomal degradation dependent- and independent functions^{32; 36; 37}. Two recent proteomics studies investigated the effect of UV-irradiation on the cellular ubiquitination status (Table 1B)^{32; 37}. Povlsen et al. found a great number of ubiquitination events in factors of the nucleotide excision repair (NER) pathway, which is crucial for the removal of UV-induced DNA lesions. They furthermore identified a decreased ubiquitination of the PCNA interacting molecule PAF15 after UV damage. Alterations of PAF15 ubiquitination could be implicated in PCNA-dependent DNA damage bypass by recruitment of translesion synthesis polymerase η ³². Schwertman et al. identified the factor UVSSA, from a protein complex that increased in ubiquitination after UV damage. UVSSA could be shown to function in transcription-coupled NER (TC NER), by recruiting the deubiquitinase (DUB) USP7, which stabilizes the TC NER master organizer ERCC6³⁷.

Crosstalk between different kinds of PTMs can fine-tune and integrate signaling cascades and interconnect signaling networks which in some cases converge to regulate the function or stability of a single molecule, such as the transcription factor p53³⁸. Crosstalk between phosphorylation routes and other posttranslational modifiers such as methylases or acetyltransferases was highlighted by Bennetzen et al.³⁵ and further elucidated by the multilevel proteomics study by Beli et al.³⁴. Analyzing changes in the phosphoproteome, as well acetylome and proteome, induced by γ -irradiation or Topoisomerase II inhibition, the authors used network analysis to implicate a number of signaling networks and protein complexes involved in ubiquitination and neddylation activities as phosphorylation targets³⁴. Next to attributing a role for the RNA splicing factors PPMG1 and THRAP3 they identified DNA damage-induced phosphorylation of the NF- κ B pathway-member, the DUB CYLD, providing an example for how systems biology can link

individual pathways in a specific cellular context. Overall the study by Beli et al. provides a prospect for the future importance of multi-level proteomics studies of the DDR^{34; 39}.

DDR transcriptomics studies

Various studies in different cell types have described DNA damage-induced global gene expression changes, including both protein coding genes and miRNAs^{3; 40; 41}. In tumor cell lines these studies have characterized the transcriptional response to chemotherapeutic drugs or γ -irradiation with the hope of identifying prognostic and predictive biomarkers. Pivotal examples of how transcriptomics studies link gene expression changes to genotoxic therapy response are shown in table 1C^{40; 42; 43}. However, it proves difficult to identify crucial therapy resistance genes, amongst the plethora of mutations common to cancer genomes. Furthermore, key DDR factors such as p53 and BRCA1 show allelic variation depending on the nature of the mutation or resulting from posttranscriptional mechanisms, such as alternative splicing or differential translation⁴⁴. This indicates that other levels of tumor-specific information will have to be taken into account in addition to transcriptional responses to genotoxic therapy^{28; 45}.

In 2006, Györfy et al. studied gene expression changes in 30 different cancer cell lines, which were treated with 13 anti cancer drugs including DNA damage-inducing (genotoxic) compounds such as cisplatin, doxorubicin, etoposide and mitomycin C and in total identified 76 genes and resistance phenotypes. Most genes were rather specific for a single resistance pattern, while a few genes related to at least six resistance patterns. Those multidrug resistance genes included for example TRIM2 and BIRC2, which had been previously linked to radioresistance⁴². In 2009, Eschrich et al. provided an attempt of integrating sensitivity to γ -irradiation with gene expression profiles in 48 cancer cell lines, using an algorithm that takes into account the cellular background including Ras and p53 status. This led to the formation of a “radiosensitivity network”, which contained 10 hubs, including for example c-Jun, c-ABL and HDAC1. By cluster analysis the authors tested the effect of the different biological variables for the sensitivity and suggested that while Ras-status had a strong effect, p53-status did not⁴³.

Next to studies of DNA damage-induced gene expression changes in cancer cells, different groups have analyzed transcriptomic alterations induced by DNA damage in non-transformed cells, including for example mouse embryonic stem cells⁴⁰. Kruse et al. described an enrichment of p53 target genes, amongst the cisplatin-mediated transcriptomic changes in mouse ES cells, contradicting previous studies which claimed that such cells lack a functional p53 response⁴⁶. Interestingly, the role of p53 in the DNA damage response of ES cells was further refined in a recent Chip-seq study by Li et al., who showed that p53 can repress pluripotency-associated genes, through distal enhancer activity, while differentiation-related genes are activated by p53 in a transactivation-dependent manner⁴⁷.

Studies into the rewiring of physical or genetic interactions in the DDR signaling network

A common feature of biological networks lies in their robustness towards perturbation, which is largely based on the capacity to rewire connection after the loss of central network hubs or edges. Networks can visualize the altered status of interactions representing either physical protein-protein or protein-DNA interactions, or genetic interactions reflecting for example the effect of mutations in different contexts and indicating a rewiring of biological processes⁴⁸ (Table 1D). Cancer cells often display a constant rewiring by replacing commonly silenced signaling routes such as DNA repair pathways and cell cycle checkpoints⁴⁹.

In a study from 2002, Boulton et al. used ⁷⁵ *C.elegans* DDR orthologs, to investigate novel protein-protein interactions using a proteome-wide two-hybrid screen⁵⁰. Relevance of DDR orthologs and their interactors was validated by RNAi in combination with γ -irradiation and subsequent grouping into pheno-clusters that either resembled damage checkpoint- or DNA repair defects. Amongst the novel checkpoint genes, they found the ortholog of human BCL3, which is commonly changed in chronic lymphatic leukemia. Next to identifying a number of novel DNA repair genes, the authors implied the sumo-conjugating enzyme Ubc9 as a *C.elegans* DNA repair and damage checkpoint gene⁵⁰.

Workman et al. studied the physical rewiring of DNA damage-treated yeast cells using a Chip-Chip approach. Promotor binding of 30 DDR-related transcription factors was analyzed in response to treatment with the genotoxic drug methyl methanesulfonate (MMS), revealing vast changes in promoter occupancies after DNA damage induction. The authors validated specific interaction by studying transcriptional changes after MMS treatment, in cells deficient for specific transcription factors. "Deletion buffered" genes, which became unresponsive to MMS after knockdown of specific transcription factors were highly correlated with MMS sensitivity⁵¹.

Two recent studies examined the genetic rewiring of the yeast genome after DNA damage, using "differential epistasis" approaches^{10;52}. Bandyopadhyay et al. found protein complexes stable to perturbation, but connections amongst those complexes changed. Hub-genes were in general found to be more important for MMS sensitivity, with an enrichment of hubs implicated in DNA repair processes. Factors that gained interactions after MMS treatment included the MAP-kinases Stl2 and Bck1 that showed strong interaction with DNA damage checkpoint genes and the centromere binding factor 1 (Cbf1). Other interaction profiles were disrupted by MMS treatment, such as the homologous recombination (HR) factor Rad52 and the histone coding gene HTZ1¹⁰.

Guenole et al. assembled a multiconditional interaction map in response to a panel of genotoxicants⁵². Differential networks varied for the tested agents and possessed the statistical power to identify DNA repair pathways initiated by different agents, but also provided the opportunity to identify novel agent-specific and global DDR factors. Those included the histone acetyltransferase Rtt109, involved in the mutagenic bypass of DNA lesions, but also factors with more global roles, such as the hitherto undescribed

protein Irc21, which was present in a network induced by both MMS and camptothecin and could be linked to damage checkpoints and DNA repair⁵².

Insight in the rewiring of interactions has been proposed to provide biomarkers for cancer progression^{53 54}. This could also be an interesting prospective for analyzing responses to genotoxic therapy in cancer cells as well, as recently shown by Lee et al.¹⁴.

RNAi screens in the context of the DDR

siRNA and shRNA-based screening methods rely on the endogenous process of RNA interference (RNAi), which is characterized by binding of a small RNA molecule to a target mRNA, which induces a reduction in translation of the target transcript, either by the degradation or sequestering of the target mRNA⁵⁵. Various gene family- or whole genome-wide RNAi-based screens have been aimed at shedding light on the DDR, induced by ionizing radiation, genotoxic drugs, DNA repair-targeting drugs such as PARP inhibitors or endogenous DNA damage induction^{11; 56; 57; 58} (Table 2). Interestingly, next to finding crucial new DDR factors these screens have implicated pathways involved in cellular housekeeping functions such as translation, transcription and RNA processing, as well as developmental pathways as crucial regulators of the survival after genotoxic treatment^{12; 59}.

A number of RNAi screens have used the appearance of DNA damage-induced foci as readout, identifying important factors of endogenous DNA repair^{11; 57} and clarifying the hierarchy of the recruitment of DDR mediators and repair factors^{60; 61}. Screens by Paulsen and Adamson and colleagues investigated factors involved in DNA repair and DNA damage prevention, as measured by spontaneous appearance of foci marked by the DSB response factor histone H2AX (γ -H2AX) and the HR factor Rad51, respectively. In a whole genome screen Paulsen et al. identified a novel role of mRNA processing factors for DNA damage prevention. Furthermore they could link an increase in the occurrence of γ -H2AX foci to the neurological disorder Charcot-Marie-Tooth (CMT) syndrome¹¹. Adamson et al. implicated the RNA splicing-associated protein Rbm1 as an important factor for HR-mediated DNA repair⁵⁷.

RNAi screens by Kolas et al. 2007 and Moudry et al. 2012 used 53BP1 foci formation as readout. The Kolas study emphasized the importance of ubiquitination events in the DSB response, by identifying the E3 ubiquitin ligase RNF8⁶⁰, which (similar to the E3 ligase RNF168 that was investigated in a follow-up study⁶²) ubiquitinates histones in response to DNA damage, an event which precedes the recruitment of crucial DSB signaling and repair factors 53BP1 and BRCA1 into DNA damage foci^{60; 62}. Screens by Moudry and colleagues recently provided evidence for roles of the nuclear import factor NUP153 as a crucial determinant of the nuclear recruitment of the DSB response factor 53BP1 itself. Indeed, NUP153-deficient cells showed increased sensitivity to ionizing radiation, which was accompanied by delayed DNA repair⁶¹.

RNAi screens further identified the 9-1-1 and TOPBP1-interacting protein RHINO, which is required for ATR activation⁶³ and the MMS22L-NFKBIL2 complex (identified by two individual groups in 2010), which is involved in the ATR-dependent replication stress

response and the subsequent initiation of HR-mediated repair^{64, 65}. Additionally, shRNA screens targeting cellular DUBs led to the identification of USP1, the DUB that is crucial for removing ubiquitin from FANCD2 in the Fanconi Anemia pathway⁶⁶.

Several RNAi screens have been geared towards DNA damage-induced cell cycle arrest. In a whole genome shRNA screen Hurov et al. identified factors of the Triple T complex as critical modulators of survival in irradiated U2OS cells. Follow-up validation of the factors TTI1 and TTI2 indicated that they are crucially important for checkpoint signaling by regulating the abundance of PI3K-like kinases such as ATM⁵⁶. To globally study the DNA damage-induced G2/M checkpoint network, Kondo and Perrimon performed a genome wide RNAi study in *Drosophila* S2R cells treated with doxorubicin. They identified signaling hubs controlling the G2/M cell cycle arrest, DNA damage repair, DNA replication, chromatin remodeling, and, interestingly, a signaling hub centered on RNA processing⁶⁷ (Fig 3).

Various RNAi-based screening methods have been employed to identify potential new cancer drug targets and synthetic lethal interactions. Synthetic lethality does not necessarily result from genes involved in similar pathways or cellular processes, making an unbiased approach for gene discovery highly relevant⁶⁸. In 2006, Bartz et al. used siRNA screens to identify genes, whose knockdown enhanced the cytotoxic effects of cisplatin, as well as the non-genotoxic drugs gemcitabine and paclitaxel, identifying hits linked to the mechanism of action of the drugs. Knockdown of BRCA1 and BRCA2 was found to specifically sensitize p53-deficient HeLa cells to cisplatin⁶⁹. In an siRNA screen published in 2009 Zhang et al. identified the RRM1 and RRM2 components of the

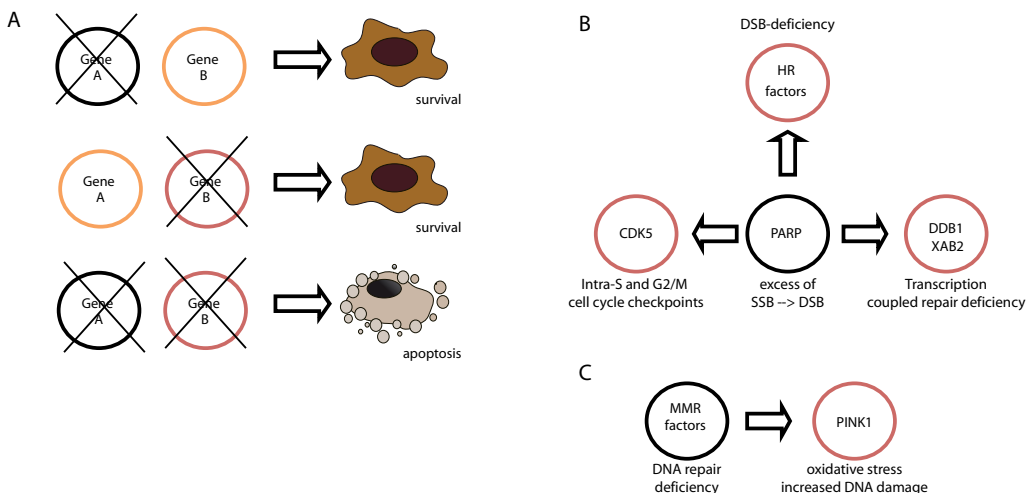


Figure 4. RNAi screens identify novel synthetic lethal interactions. A, synthetic lethality describes the concept, where one gene alone, does not affect cell survival, while loss of a combination of genes induces cell killing. B, synthetic lethality was originally described between PARP and HR factors such as BRCA1 and BRCA2. Recent siRNA screens have identified additional synthetic lethal interactions with PARP, including for example the kinase CDK5 and the transcription coupled-repair factors DDB1, XAB2. C siRNA screens further identified synthetic lethal interactions between proteins of the MMR pathway and the kinase PINK (silencing PINK causes oxidative stress-induced DNA damage, which cannot be repaired by MMR-deficient cells). For details and related references see text.

human ribonucleotide reductase complex, which is required for the formation of dNTPs from NTPs, as targets of the checkpoint kinases and sensitizers to the Topoisomerase inhibitor Camptothecin⁷⁰.

Since the initial description of synthetic lethality between PARP-inhibitors and the BRCA1 and -2 genes and the entry of PARP inhibitors into clinical trials (Fig4A)⁷¹, different screens have aimed to discover novel synthetic lethal interactions with DDR factors. These revealed for example the cyclin-dependent kinase CDK5, which is required for proper execution of intra-S and G2/M checkpoints, and the TCR factors DDB1 and XAB2 as potential targets for sensitization to PARP inhibition^{58; 72} (Fig4B). Next to this HR-dependent synthetic lethality, siRNA screens laid open a synthetic lethal interaction between the PTEN-induced putative kinase1 (PINK1) and proteins of the mismatch repair (MMR) pathway. Knockdown of PINK1 induced oxidative stress selectively in cells deficient for the MMR proteins MSH2, MLH1 and MSH6, leading to enhanced accumulation of DNA damage and loss of cell viability, pointing to a potential selective therapy based on sensitivity of cancer cells with defective MMR for PINK inhibitors⁷³ (Fig 4C).

Mathematical models to decipher the DDR

Different groups have aimed at describing cellular signaling processes using mathematical modeling approaches, which are either based on ordinary differential equations^{74; 75} or other analysis methods, such as regression techniques, Bayesian interference or Boolean networks¹⁵. For the formation of mechanistic computational mathematical models, there is a need for simplification, either by reducing the complexity of the studied phenotype, such as the study of defined circuits and simple relationships, or by reducing the complexity of the studied system e.g. by the use of model organisms such as bacteria or yeast¹⁵.

In 2011, Karschau et al. provided a model for the correlation between DNA repair and cell death in bacteria, by testing the dependency of survival rates on the abundance of repair enzymes, uncovering a counterintuitive relationship between the amount of repair enzymes and survival, which indicates that above a certain threshold the number of repair enzymes is not linked to survival. The authors suggest that the reason for this observation is, that the death rate is not a direct result of the DNA damage but the repair process, which leads to the formation of secondary DSBs⁷⁶.

Following the initial notion that p53 is activated in oscillatory pulses as a result of positive and negative feedback loops⁷⁷ a number of groups aimed to associate the final cellular response to different genotoxic stressors with the mathematical description of dynamic p53 responses. While p53 pulses were damage intensity-dependent for UV damage, which is recognized by the single strand sensor ATR, p53 oscillations were steady and intensity-independent in response to DSB induction by γ -irradiation or NCS^{77; 78; 79}. Two major negative feedback loops regulate p53 pulses: the E3-ubiquitin ligase MDM2 and the phosphatase Wip1, both of which are p53 target genes⁷⁷ (Fig 5A). Differences in the regulations of those feedback loops could be connected to the different

behavior of p53 after UV and IR damage. Wip1 is capable of dephosphorylating and thus deactivating ATM, but ATR, which does not require an activating phosphorylation is unaffected by Wip1. Furthermore, inhibition of MDM2 by ATM occurs faster and via degradation of the MDM2 protein, while ATR-mediated inhibition is slower and does not induce MDM2 degradation⁷⁸ (Fig 5 A).

Different groups used mathematical models to identify modulations of p53 feedback loops that after DSB-inducing damage, lead to sustained p53 activation instead of inducing damage intensity-independent p53 oscillations^{80; 81} (Fig 5B). Purvis et al. modeled the p53 response to identify drug pulses of the MDM2 inhibitor Nutlin that sufficiently switched the oscillatory p53 response to persistent p53 activation, and induction of senescence⁸⁰ (Fig 5B). Modeling of p53 induction feedback loops was further advanced by Choi et al. who used Boolean networks and attractor landscapes to investigate “decision making” in a simplified p53 network, to identify combinations of active molecules, which lead to either cell cycle arrest or apoptosis after DNA damage⁸¹. The model predicted that removal of WIP1 would yield a mixed response, with a less strong bias towards apoptosis induction than induced by removal of the MDM2 feedback loop.

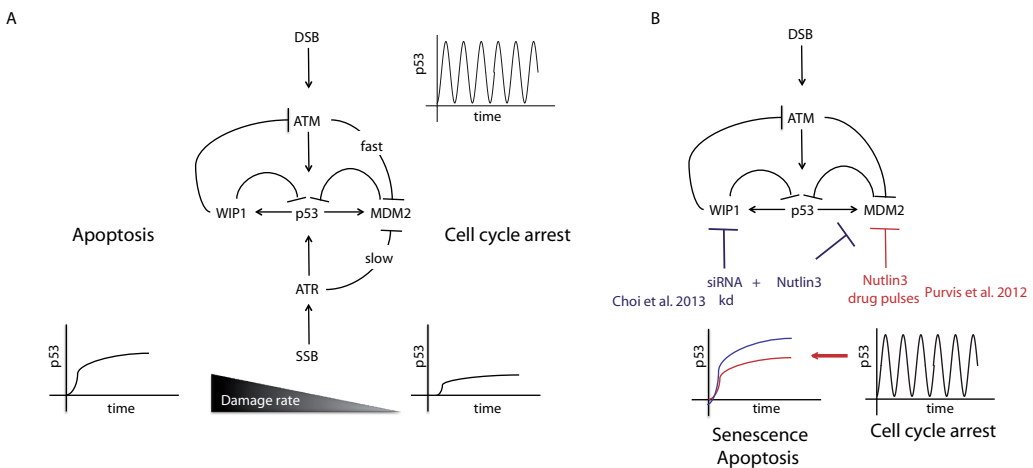


Figure 5. Novel insights into p53 dynamics in the DDR. A, p53 induction in response to different genotoxic stresses shows distinct temporal regulation through distinct feedback loops. DSB-induced, ATM-mediated p53 level oscillations are independent of damage intensity or duration (upper, right). Instead, SSB-induced, ATR-mediated p53 pulses follow the intensity of damage induction and accordingly activate a cellular response (lower left and right). B, Inhibition of negative feedback loops between p53 and its target genes MDM2 by pulsed treatment with the MDM2 inhibitor Nutlin3 triggers a switch from an oscillatory to a sustained pulse p53 response, causing a concurrent switch from cell cycle arrest to senescence or apoptosis. An even more pronounced effect can be obtained by simultaneously silencing Wip1 and MDM2, as shown by Choi et al.. For details and related references see text.

Indeed experimental testing confirmed that knockdown of WIP1 alone did not switch p53 behavior from oscillatory to single pulse induction, while Nutlin3 treatment did. Best results were obtained by simultaneous silencing of both feedback loops, which induced sustained single pulse p53 behavior and apoptosis even in the absence of DNA damage (Fig 5B). Incorporation of large-scale omics datasets into meaningful models remains challenging and it has been suggested that targeted experiments on a medium-throughput scale

might overcome the problems, which are posed by modeling of whole genome data¹⁵. Tentner et al. recently integrated medium-scale time-resolved data, using mathematical modeling. Measuring the damage-induced phosphorylation of DDR marker proteins and survival signaling branches such as ATM and ATR, as well as ERK/MAPK or NF- κ B signaling, the authors used a regression technique, called time-interval-dependent regression to correlate the phosphorylation measurements at each time point to the induction of apoptosis and cell cycle arrest. The model revealed an unexpected role for MAPK/ERK signaling in promoting doxorubicin-induced cell death and cell cycle arrest in different cell types¹³.

The challenge - integration of different DDR omics datasets

Omics integration can enhance sensitivity towards small changes and allow for a higher confidence in linking enriched pathways and signaling networks to biological phenotypes²⁷. This was demonstrated by Cavill et al., who related metabolic and transcriptomic pathway analysis to the sensitivity to platinum-based, genotoxic anticancer drugs, identifying a number of metabolic pathways including the TCA cycle and nucleotide metabolism to be predictors of tumor cell platinum sensitivity⁸². However, integration of different biological datasets beyond conceptual levels remains difficult, largely due to the differences in the primary data, which reflect different kinds of interactions and timeframes¹⁵. We have recently used an integration approach to unravel DDR signaling processes in mouse ES cells treated with cisplatin¹². Integration of siRNA screening data with phosphoproteomics and transcriptomics analyses led to identification of canonical pathways that were significantly enriched in all individual datasets. This allowed the formation of integrated signaling networks based on the “hits” derived from each dataset within those pathways. Besides confirmation of DNA

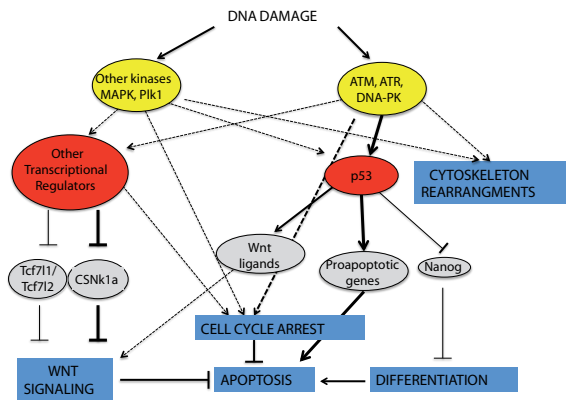


Figure 6. New insights into DDR in pluripotent stem cells. Recent phosphoproteomics studies in ES cells point to activation of a variety of kinases in addition to ATM/ATR/DNA-PK, which directly and indirectly control cellular outcomes, such as cell cycle arrest and cytoskeletal rearrangements. Transcriptomics analyses and RNAi screens have revealed signaling networks involving p53 that lead to induction of pro-apoptotic genes and differentiation through downregulation of Nanog, but also upregulation of Wnt ligands. However, Wnt signaling in response to DNA damage is p53-independent and involves transcriptional downregulation of negative regulators such as Csnk1a1, Tcf711, and Tcf712; which acts as a break on p53-mediated apoptosis. Yellow indicates kinases, red indicates transcriptional regulators, grey indicates target genes and blue indicates cellular outcomes. Dotted lines indicate potential regulatory relationships; continuous lines indicated confirmed regulatory relationships. Weight of lines correlates to relevance of interactions for the regulated outcome. For details and related references see text.

damage- and cell cycle-related processes, this has led to the identification of a novel mode of DNA damage-induced Wnt/ β -catenin signaling that attenuates p53-mediated apoptosis in ES cells¹² (Fig 6).

In an excellent recent study, Lee et al. showed how integration of protein phosphorylation and gene expression data with mathematical modeling can provide deep insight into the rewiring of oncogenic signaling networks in response to combined anticancer treatments. Their systems level time-lapse analysis revealed a time window for co-treatment, in which EGFR inhibition could induce drastic chemosensitization of a subset of triple-negative breast cancer cells¹⁴.

CONCLUDING REMARKS

DNA damage triggers a highly complex cellular response that does not only include DNA repair, cell cycle arrest and cell death, via apoptosis, senescence or other routes, but also has to integrate ongoing cellular housekeeping and signaling functions. Transcriptomics, proteomics and protein- and genetic interaction studies, as well as RNAi screens have opened the door to a much wider view on DNA damage-induced signaling cascades and have revealed interactions between the DDR and other cellular signaling networks. Moreover, integration of the datasets especially in combination with mathematic modeling has begun to reveal some of the dynamic changes in signaling networks that determine the outcome of the DDR for instance in the context of cancer therapy. Still, data production exceeds the possibility of data integration and sophisticated modeling. However, a few pioneer studies have provided a glimpse of future possibilities for systems analysis of DNA damage responses.

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Study	Damage source	Cell system	Main upstream regulators	Regulated processes
Phosphoproteomics studies				
Matsuoka 2007 ¹⁶	γ-irradiation	293T cells	ATM/ATR	DNA replication & repair, cell cycle regulation, RNA modification, nucleotide metabolism
Bensimon 2010 ³²	Neocarzinostatin (NCS)	G361 human melanoma cell line	ATM/ATR/DNAPK CK1, CK2	RNA processing, chromosome organization, DNA repair
Bennetzen 2010 ³⁵	γ-irradiation	GM00130	ATM/ATR/DNAPK CK1, CK2, NEK6, and PLK1	RNA biogenesis, DNA damage, cell cycle
Pines 2011 ³⁴	Cisplatin	Mouse ES cells	ATM/ATR, MAPks, Plk1	DNA repair, cell cycle, Cytoskeleton remodeling, mitosis
Beil 2012 ³⁵	γ-irradiation	U2OS human sarcoma cells	ATM/ATR/DNA-PK	RNA Splicing (Thrap3, PPM1G), NFκB signaling (CYLD)

Table 1A

Study	Damage source	Cell system	Main ubiquitination targets	Regulated processes
Ubiquitinproteomics studies				
Povlsen 2012 ³³	UV-irradiation	U2OS human sarcoma cells	PAF-15	PCNA-dependent DNA damage bypass by recruitment translesion synthesis polymerase η
Schwertmann 2012 ³⁸	UV-irradiation	U2OS human sarcoma cells	UVSSA containing complex	Recruitment of USP7 to TC-NER complexes

Table 1B

Table 1. Proteomics and transcriptomics studies of the DDR. Table 1 lists high-throughput phosphoproteomics (A), ubiquitinproteomics (B), transcriptomics (C) and interaction (D) studies of the DDR, indicating the cell model, genotoxicity source, key identified upstream regulators (kinases/ ubiquitination targets/ transcription factors/rewiring events), and significantly regulated biological processes and pathways.

Study	Damage source	Cell system	Main upstream regulators	Regulated processes
Transcriptomics studies				
Györfi 2006 ⁴³	Cisplatin, doxorubicin, etoposide, mitomycin C	30 cancer cell lines	TRIM2, APOBEC3B, BIRC2, TP52, C10orf38	Multidrug chemoresistance
Eschrich 2009 ⁴⁸	γ-irradiation	48 cancer cell lines	c-Jun, HDAC1, RELA, PKC-beta, SUMO-1, c-Abl, STAT1, AR, CDK1, IRF1	Common radiosensitivity
Kruse 2007 ⁴¹	Cisplatin	Mouse ES cells	p53	DNA damage-induced apoptosis
Table 1C				
Study	Damage source	Cell system	Main rewiring events	Regulated processes
Physical rewiring studies				
Boulton 2002 ⁵¹	γ-irradiation	<i>C. elegans</i>	hBCL3 homologue, exo-3, RFC-4 RAD51 and RAD54 interactors Ubc9	DNA damage checkpoints DNA repair DNA repair & DNA damage checkpoints
Workman 2006 ⁵²	Methyl methanesulfonate	<i>S. cerevisiae</i>	Yap1, Gcn4, Fkh2, Swi4, Sok2, Crt1	Cell cycle regulation, DNA repair, Metabolism, Stress response,
Genetic rewiring studies				
Bandyopadhyay 2010 ¹¹	Methyl methanesulfonate	<i>S. cerevisiae</i>	Slk12, Bck1 Cbf1 RAD52, HTZ1	Cell cycle checkpoint Acetylation response
Guenole 2013 ⁵³	MMS, Camptothecin, zeocin	<i>S. cerevisiae</i>	Rtt109 Neddylation machinery, Irc21	Mutagenic bypass of DNA lesions Cell cycle checkpoints, genomic stability
Table 1D				

Table 1. Proteomics and transcriptomics studies of the DDR. Table 1 lists high-throughput phosphoproteomics (A), ubiquitinproteomics (B), transcriptomics (C) and interaction (D) studies of the DDR, indicating the cell model, genotoxicity source, key identified upstream regulators (kinases/ ubiquitination targets/transcription factors/rewiring events), and significantly regulated biological processes and pathways.

Study	Damage source	Cell system	Readout	Key molecule	Regulated processes
RNAi screens					
Paulsen 2009 ⁷²	Endogenous damage	HeLa	γ -H2AX foci	mRNA processing factors	Charcot-Marie-Tooth (GMT) syndrome
Adamson 2012 ⁸⁸	Endogenous damage	U2OS	Rad51 foci	RNA splicing factor RBMX	HR-mediated DNA repair
Kolas 2007 ⁸¹	γ -irradiation	U2OS human sarcoma cells	53BP1 foci	RNF8	DSB response, histone ubiquitination
Moudry 2012 ⁸²	γ -irradiation	U2OS human sarcoma cells HeLa	53BP1 foci	NUP153	Nuclear import of 53BP1
Nijman 2005 ⁸⁷	Endogenous damage	U2OS human sarcoma cells	FANCD2 immunoblotting	USP1	Deubiquitination of FANCD2
Cotta-Ramusino 2011 ⁸⁴	γ -irradiation	U2OS human sarcoma cells	Cell cycle arrest	RHINO	ATR recruitment
Kondo 2011 ⁸⁸	Doxorubicin	<i>Drosophila</i> S2R cells	G2 arrest	MUS101, MUS312	DSB repair, G2/M cell cycle checkpoint
Hurov 2010 ³⁷	γ -irradiation	U2OS human sarcoma cells	Cell viability	TTI1, TTI2	Intra S, G2/M c cell cycle checkpoint
O'Connell 2010 ⁸⁵	Camptothecin	HeLa	Cell viability	MMS22L-NFKBIL2	Replication stress
Carreras-Puigvert 2013 ¹³	Cisplatin	Mouse ES cells	Cell viability	CSNK1a1	DNA damage-induced Wnt signaling
Bartz 2006 ⁷⁰	Cisplatin	HeLa	Cell viability	BRCA1, BRCA2	Apoptosis in p53-deficient cells
Zhang 2011 ⁷¹	Camptothecin derivatives	MDA-MB-231 breast cancer cells	Cell viability	RRM1, RRM2	Prevention of increased DNA damage accumulation potentially through DNA repair
Lord 2008 ²⁵	PARP inhibition (KU0058948)	CAL51 breast cancer cells	Cell viability	DDI1, XAB2	Transcription coupled DNA repair
Turner 2008 ⁷³	PARP inhibition (KU0058948)	CAL51 breast cancer cells	Cell viability	CDK5, MAPK12, PLK3, PNKP, STK22c, STK36	Intra-S and G2/M cell cycle checkpoints
Martin 2010 ⁷⁴	MMR-deficient cellular background	HEC59, HEC59+Chr2 endometrial cells	Cell viability	PINK1	Accumulation of oxidative DNA damage

Table 2

Table 2. RNAi screens to unravel the DDR. Table 2 lists RNAi-based DDR screens, indicating the cell model, genotoxicity source, key signaling molecules identified, and significantly regulated biological processes and pathways.

