Proteomics Reveals Global Regulation of Protein SUMOylation by ATM and ATR Kinases during Replication Stress

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Graphical Abstract

Highlights

- We examine global SUMO and phospho-response interplay in replication stress
- TOPBP1 is highly SUMOylated and phosphorylated by DNA inter-strand crosslinking
- ATR inhibition leads to hyper-SUMOylation of members of the ATR activation complex
- ATR and ATM regulate global SUMOylation upon replication stress and fork breakage

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In Brief
Munk et al. use mass spectrometry-based proteomics to analyze the interplay between SUMOylation and phosphorylation in replication stress. They analyze changes in the SUMO and phosphoproteome after MMC and hydroxyurea treatments and find that the DNA damage response kinases ATR and ATM globally regulate SUMOylation upon replication stress and fork breakage.

Data and Software Availability
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Proteomics Reveals Global Regulation of Protein SUMOylation by ATM and ATR Kinases during Replication Stress


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SUMMARY

The mechanisms that protect eukaryotic DNA during the cumbersome task of replication depend on the precise coordination of several post-translational modification (PTM)-based signaling networks. Phosphorylation is a well-known regulator of the replication stress response, and recently an essential role for SUMOs (small ubiquitin-like modifiers) has also been established. Here, we investigate the global interplay between phosphorylation and SUMOylation in response to replication stress. Using SUMO and phosphoproteomic technologies, we identify thousands of regulated modification sites. We find co-regulation of central DNA damage and replication stress responders, of which the ATR-activating factor TOPBP1 is the most highly regulated. Using pharmacological inhibition of the DNA damage response kinases ATR and ATM, we find that these factors regulate global protein SUMOylation in the protein networks that protect DNA upon replication stress and fork breakage, pointing to integration between phosphorylation and SUMOylation in the cellular systems that protect DNA integrity.

INTRODUCTION

DNA replication is a tremendously challenging, time-consuming, and vital task for eukaryotic organisms. The maintenance of genomic integrity during this process is challenged by endogenous and exogenous factors that cause replication forks to slow and stall, and, in extreme cases, this leads to DNA breakage (Halazonetis et al., 2008). Cells are equipped with a complex DNA damage response (DDR) consisting of protein networks that enable them to cope with replication stress (RS), and a malfunction in these systems can result in genomic instability and oncogenesis (Jackson and Bartek, 2009). These protective signaling pathways require the precise spatial and temporal coordination of DDR components, which is achieved by dynamic and specific post-translational modifications (PTMs) (Polo and Jackson, 2011). In particular, protein phosphorylation is a well-established driver of the RS response, with the ATR (ataxia telangiectasia and Rad3-related protein) kinase functioning as the key initiator and orchestrator (López-Contreras and Fernandez-Capetillo, 2010; Shiloh, 2001). Depletion of this central kinase leads to replication fork breakage and genomic instability, instigating a phosphorylation response mounted by the ATM (ataxia telangiectasia mutated) kinase, which mediates repair and checkpoint activation upon double-strand breaks (DSBs) (Murga et al., 2009; Smith et al., 2010). ATM and ATR belong to the same atypical serine/threonine kinase family (the phosphatidylinositol 3 kinase-related kinases [PIKK]-related kinases) with similar substrate sequence specificity (Kim et al., 2009), but they have unique triggers. Although ATR responds to the accumulation of single-stranded DNA (ssDNA) and regulates replication, ATM is the key mediator of the cellular response to DSBs. DNA-dependent protein kinase (DNA-PK) is the third member of this kinase family; however, its functions are confined to local repair processes (Meek et al., 2008).

Phosphorylation, however, must act in concert with other PTMs, such as ubiquitylation, to elicit efficient responses to genotoxic insults (Ulrich and Walden, 2010). The functions of PTMs in the DNA damage and RS responses have therefore been subject of intense investigations, individually (Beli et al., 2012; Bennetzen et al., 2009; Danielsen et al., 2011; Jungmichel et al., 2013) and in concert (Gibbs-Seymour et al., 2015; González-Prieto et al., 2015; Hunter, 2007). More recently, studies have revealed the significance of protein SUMOylation in the DDR, and deregulation of the small ubiquitin like modifier (SUMO) system has been shown to confer genomic instability (Bergink and Jentsch, 2009; Bursomanno et al., 2015; Jackson and Durocher, 2013; Xiao et al., 2015). Using various RS-inducing agents, these studies have shown that the SUMOylation status of a number of proteins is modulated when DNA replication is perturbed (García-Rodríguez et al., 2016). Furthermore, it has been demonstrated that phosphorylation and SUMOylation
intersect at various levels (Gareau and Lima, 2010). A phosphorylation-dependent SUMO modification (PSDM) motif has been suggested to prime SUMOylation (Hietakangas et al., 2006) by enhancing the binding of the SUMO E2 enzyme UBC9 (Mohideen et al., 2009), and phosphorylation was also found to regulate the function of SUMO-interacting motifs (SIMs) (Stehmeier and Muller, 2009). However, a potential global coordination of the SUMOylation response and the well-known phosphorylation response to RS remains unexplored.

Quantitative mass spectrometry (MS)-based proteomics and developments in enrichment methodologies have seen tremendous developments in recent years (Hendriks and Vertegaal, 2016). State-of-the-art MS technologies allow the identification of thousands of SUMOylation sites (Hendriks et al., 2017; Lamoliatte et al., 2014, 2017; Schimmel et al., 2014; Tammalsalu et al., 2014) and tens of thousands of phosphorylation sites from cellular systems (Francavilla et al., 2017; Mertins et al., 2016; Olsen et al., 2010). In this study, we utilized complementary proteomics strategies to identify the interplay between the global SUMOylation and phosphorylation responses to replication stressors. We identified regulation of thousands of phosphorylation sites and hundreds of SUMOylation sites in response to treatment with the DNA inter-strand crosslinking (ICL) agent mitomycin C (MMC) and hydroxyurea (HU), with a number of proteins co-regulated by both PTMs. Our investigations revealed that the well-established apical responders to RS and RS-induced DSBs, ATR and ATM, both modulate protein SUMOylation at various stages of the RS response. Our findings not only identify an intersection between phosphorylation and SUMOylation in the RS response but also reveal further levels of signaling regulation in this response by the two most prominent kinases of the DNA damage and RS responses.

RESULTS

Global SUMOylation Changes upon MMC Treatment

To investigate the interplay between the SUMOylation and phosphorylation responses to RS, we treated U-2-OS osteosarcoma cells with MMC (Figure 1A). MMC, a widely used chemotherapeutic agent in the treatment of various cancers, induces ICLs, thereby impeding normal replication fork progression and causing RS. To study the effects of MMC during DNA replication, cells were synchronized at the G1/S checkpoint by 24 hr of thymidine blocking and were thereafter released into S phase with or without MMC for 8 hr (Figure 1B; Figure S1A). After an 8 hr release into MMC, western blotting confirmed increased phosphorylation of checkpoint kinases, CHK1 at S435 and CHK2 at T68, as well as increased levels of phosphorylation of S140 on histone H2AX (γH2AX) (Figure S1B). These phosphorylation sites are known targets of ATR and ATM, indicating that our experimental conditions generate RS (ATR activation) and DSBs (ATM activation).

For MS-based global analysis of SUMOylation, we used two previously described SUMO enrichment approaches to quantify changes in protein SUMOylation and SUMO acceptor sites (Hendriks et al., 2014; Schimmel et al., 2014) on a global scale (Figure 1B). SUMOylated proteins were identified and quantified by immunoprecipitation (IP) of SUMO2-conjugated proteins from U-2-OS cells stably expressing FLAG-SUMO2-Q87R (Figure 1C; Figure S1C). The Q87R mutation allows for identification of SUMO sites after tryptic digestion because of the resulting remnant (Schimmel et al., 2014). To confidently distinguish SUMOylated from non-SUMOylated proteins, control IPs were also performed from the parental U-2-OS cell line because non-SUMOylated proteins would be underrepresented in these compared with FLAG-SUMO2-Q87R-expressing cells (Figure 1B). Complementarily, we mapped SUMOylation acceptor sites by enrichment of SUMOylated peptides from His10-tagged SUMO2-K0-Q87R-expressing U-2-OS cells (Figure 1B; Xiao et al., 2015). Tryptic peptides from all enriched samples were analyzed by nano-scale liquid chromatography-tandem MS (LC-MS/MS) on a Q-Exactive high-frequency (HF) instrument (Kelstrup et al., 2014). We used stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) for accurate MS-based quantification, and differentially labeled SILAC cells showed comparable cell cycle distributions upon synchronization (Figure S1A). The SUMO2 expression levels in the two stable cell lines were 3- to 4-fold higher than in the parental cells, as observed by MS full scans from proteome measurements and by western blotting (Figures S1D and S1E).

All raw LC-MS/MS files were processed and analyzed together using the MaxQuant software suite (http://www.coxdocs.org/doku.php?id=maxquant:start) with a 1% false discovery rate at the peptide, site, and protein levels (Cox and Mann, 2008). From this analysis, we confidently identified 3,453 proteins (Table S1). Ratios from proteome measurements of these conditions revealed that the protein abundances in the MMC-treated FLAG-SUMO2-Q87R cells were largely unchanged compared with the equivalently treated parental cells. We therefore reasoned that we could determine the proteins significantly SUMOylated in FLAG-SUMO2-Q87R cells using ratio cutoffs of 2 SDs from the mean (95th percentile) of this ratio distribution (Figure S1F). This analysis resulted in a cutoff of 1.7-fold change, by which 702 proteins were deemed to be SUMOylated (Figure 1D; Table S1). Using the same strategy for the MMC-treated and untreated FLAG-SUMO2-Q87R cells, a resulting ratio cutoff of 1.5 resulted in 187 proteins having significantly increased SUMOylation upon treatment with MMC (Figure 1D; Figure S1G; Table S1). Additionally, we mapped 311 unique SUMO acceptor sites (Figure 1E). Sequence motif analysis of these showed a strong preference for a glutamate two residues downstream of the modified lysine (Figure 1E), conforming to the previously described SUMOylation consensus motif (WKXE) (Sampson et al., 2001). By separately analyzing SUMOylated peptides with or without this motif, we found that, indeed, the known SUMO consensus motif is predominant, with the inverted SUMO motif the secondmost overrepresented (Figure 1E).

To determine the cellular compartments and biological processes in which the SUMOylated proteins are involved, we performed a gene ontology (GO) enrichment analysis. In agreement with previous studies, we found that the majority of SUMOylation occurs on nuclear proteins that are involved in transcription (Figure S1H; Flotho and Melchior, 2013). Further, among the proteins with MMC-regulated SUMOylation, we identified 24 transcription factors for which 24 target genes were...
found to be co-regulated by at least two of these. Interestingly, these target genes were highly enriched in proteins involved in apoptosis and cancer development (Table S1). GO analysis of the 187 proteins with increased SUMOylation after MMC treatment also revealed this trend, and, furthermore, these proteins are involved in histone ubiquitylation and DNA repair (Figure 1F).

Many of the identified proteins known to function in DNA repair clustered together in a functional network based on search tool for recurring instances of neighboring genes (STRING) database analysis (Szklarczyk et al., 2015). Fanconi anemia factors, BRCA1 (breast cancer type 1 susceptibility protein), and TOPBP1 (DNA topoisomerase 2-binding protein 1) were among the regulated SUMOylated proteins after MMC treatment (Figure 1G). These proteins are well-known to play important roles in response to ICL-induced RS and DNA damage. The regulation of SUMOylation levels on these proteins upon MMC treatment indicates that this modification may modulate their function in this response.

Global Phosphorylation Changes upon MMC Treatment
To study the potential interplay between the SUMOylation and phosphorylation responses to MMC, we used a streamlined quantitative phosphoproteomics workflow (Batth et al., 2014) to enrich phospho-peptides from FLAG-SUMO2-Q87R U-2OS cells synchronized and treated with MMC in the same manner as for SUMOylation mapping. Tryptic digests of whole-cell lysates were separated by offline high-pH reverse-phase fractionation, and phospho-peptides were enriched with TiO2 beads prior to LC-MS/MS (Figure S2A). We quantified 20,900 high-confidence phosphorylated sites, of which 650 were induced (SILAC ratio above 1.5) after 8 hr of MMC treatment (Figure 2A; Table S2). Proteins with induced phosphorylation were primarily nuclear and involved in DNA repair, as determined by GO analysis, similar to our findings for SUMOylated proteins that were induced by MMC treatment (Figure 2B; Figure S2B).

We performed sequence motif analysis of the 650 upregulated phosphorylation sites to identify protein kinases that were activated in the response to MMC treatment. A strong overrepresentation of glutamine (Q) at the position directly C-terminal to the phosphorylation sites (P+1) indicated activation of the ATM and ATR kinases, both of which are known to preferentially phosphorylate substrates on serine/threonine residues that are followed by a glutamine (S/T-Q) (Figure 2C). Indeed, we found that 170 (26%) of the phosphorylation sites upregulated by MMC treatment confer to the S/T-Q motif. Moreover, MS spectra show a clear induction of ATM and ATR target phosphorylation sites on ATM itself and CHK1, respectively (Figure 2D). Conversely, phosphorylation sites on proteins from other signaling pathways, as exemplified by ERK1, remained largely unperturbed (Figure 2D). Functional network analysis of proteins with increased phosphorylation reveals two highly interconnected clusters of phosphoproteins involved in the DDR, DNA replication, and cell cycle (Figure 2E). A number of these proteins were also found to have increased SUMOylation, indicating that phosphorylation and SUMOylation are modulating proteins in the same pathways in the RS response to MMC treatment.

Central DDR Proteins Are Highly Phosphorylated and SUMOylated in Response to MMC
To elaborate on this hypothesis and uncover a potential interplay between the SUMOylation and phosphorylation responses to MMC, we integrated our large-scale proteomics datasets of the two modifications. First we evaluated the datasets for potential biases arising from the MS strategies used for enrichment and detection of proteins with these modifications. The distribution of the relative protein copy numbers (intensity-based absolute quantification [iBAQ] values) from the proteome, the phosphorylated proteins, and the SUMOylated proteins in these datasets revealed that all three groups of proteins had similar distribution patterns with no apparent abundance biases (Figure S3A). We then assessed the overlap between the datasets and found that 540 proteins harbored at least one SUMOylation and phosphorylation event (Figure 3A). This comprises two-thirds of the SUMOylated proteins we identified, corresponding to the proportion of the total proteome that is reported to be phosphorylated at any given time (Olsen et al., 2010). Although only 17 of these proteins were found to have upregulation of both modifications upon MMC treatment, this subset included UIMC1 (BRCA1-A complex subunit RAP80), BRCA1, BARD1 (BRCA1-associated RING domain protein 1), and TOPBP1, which are proteins with well-established key functions in the DDR (Figures 3A and 3B; Table S3). We therefore find that quantitative analysis of proteins co-regulated by both PTMs is a powerful means to determine and prioritize key players in cellular signaling networks.

To elaborate on the mechanism of regulation of these two PTMs in RS, we further investigated the roles of most prominent DDR- and RS-activated kinases, ATR and ATM, in modulating RS-induced SUMOylation (Smith et al., 2010). These kinases are well-known initiators and key modulators of the global phosphorylation and ubiquitylation responses to DNA damage and RS (Shiloh, 2001). Indeed, ATR is activated upon 8 hr of
MMC treatment after thymidine release, as observed by increased phosphorylation of its direct target CHK1 on S345, which can be further attenuated with an ATR inhibitor (ATRi) (Figure S3B). Interestingly, TOPBP1, an important co-activator of ATR, was the highest co-modified protein upon MMC treatment (Figure 3B). By SUMO enrichment from both FLAG-SUMO2-Q87R and His10-tagged SUMO2-K0-Q87R cells, we were able to confirm that, indeed, TOPBP1 SUMOylation is increased over time with MMC treatment (Figure 3C). Because the His10-based pull-down procedures involved lysis and enrichment under harsh denaturing conditions, these findings confidently demonstrate that TOPBP1 is indeed differentially SUMOylated by RS and that the observed changes are not due to TOPBP1 interactions with other SUMO-regulated target proteins. Interestingly, TOPBP1 SUMOylation was further induced upon co-treatment of MMC with ATRi, also at earlier time points (Figure 3D). Although TOPBP1 SUMOylation is increased upon treatment with MMC or ATRi only, the combination of the two is required for massive hyper-SUMOylation (Figure 3D). ATM is also activated under these conditions, as indicated by increased CHK2 and H2A.X phosphorylation (Figure 3D; Figure S3B), and, interestingly, hyper-SUMOylation of TOPBP1 upon MMC and ATRi co-treatment was significantly reduced by ATM inhibition (Figure 3D). Thus, in contrast to

**Figure 2. Phosphoproteomics Analysis of MMC-Treated cells**

(A) Overview of the number of phosphorylated peptides and proteins from phosphoproteomics analyses of cells treated as shown in Figure S2A.

(B) GOCC and GOBP analysis of proteins with regulated phosphorylation sites after MMC treatment using InnateDB.

(C) Motif enrichment analysis of 360 MMC-dependent phosphorylation sites, done with IceLogo.

(D) Full MS spectra of phosphorylated peptides from ATM, CHK1, and ERK1.

(E) Two highly interconnected molecular complex detection (MCODE) clusters from functional network analyses of all proteins with regulated phosphorylation sites. MCODE was set to determine clusters with the “Haircut” approach, a minimum node score cutoff of 0.2; K-core was set to 2, and max depth to 100. See also Figure S2 and Table S2.
well-known phospho-induced SUMOylation, it appears that modulation of phosphorylation networks can also reduce SUMOylation in this context, expanding the repertoire of phospho-SUMO crosstalk.

These observations are in accordance with the induction of DNA DSBs and ATM activation that arises upon RS in combination with checkpoint inhibition (Toledo et al., 2013; Figure 4A). To validate our observation that central DDR kinases modulate hyper-SUMOylation of TOPBP1 upon MMC treatment and determine whether such regulation occurs on other proteins, we performed an additional label-free quantitative proteomics screen. Here we analyzed enriched SUMOylated proteins from MMC-treated cells in combination with the ATM inhibitor (ATMi) and ATRi (Figure 4B; Figure S4A; Table S4). We confirmed that TOPBP1 is hyper-SUMOylated by co-treatment with MMC and ATRi and that this was attenuated upon addition of ATMi (Figures 4C and 4D). Remarkably, ATR itself and its constitutive interactor ATR interacting protein (ATRIP), which localizes ATR to TOPBP1 for activation, both displayed the same hyper-SUMOylation pattern as TOPBP1 (Figures 4C and 4D). Although SUMOylation of ATRIP and ATR has previously been reported in response to UV and HU treatments (Wu et al., 2014), we found that hyper-SUMOylation of ATR, ATRIP, TOPBP1, and XRCC6 (X-ray repair cross-complementing protein 6) arises upon RS in combination with checkpoint inhibition. Importantly, STRING-based functional network analysis of SUMOylation targets significantly regulated upon MMC treatment with and without ATRi and ATMi reveals that these consist of core ATR-activating proteins and DDR responders, showing remarkable orchestration of this functional group (Figure S4B; Jentsch and Psakhye, 2013).

Together, these proteomics experiments suggest that regulation of phosphorylation and SUMOylation occurs within overlapping networks of RS responders and that these may be subjected to common control by the same apical DDR kinases.

![Figure 3. Integrated Analyses of SUMOylation and Phosphorylation Datasets](image-url)

(A) Overlap of all identified and regulated SUMOylation and phosphorylation substrates.

(B) Functional network analysis of the 17 proteins with regulated phosphorylation and SUMOylation.

(C) Validation of TOPBP1- and BRCA1-regulated SUMOylation in FLAG-SUMO2 U-2-OS cells blocked for 24 hr with thymidine and then treated for 8 hr with or without MMC. FLAG-IP, FLAG-based immunoprecipitation; His-PD, His-based pull-down.

(D) Western blot analysis of TOPBP1 SUMOylation upon treatment for 8 hr with MMC with and without ATR inhibitor (ATRi, ATR-45) and ATMi (ATMi, KU55933). See also Figure S3 and Table S3.
Atm and Atr Modulate a Global Sumoylation Response to Rs

We next sought to determine whether modulation of protein sumoylation by ATM and ATR was a general mechanism under other conditions of RS. Using HU, an inhibitor of dinucleotide triphosphate (dNTP) synthesis that causes DNA replication fork stalling, we could reproduce the pattern of topBP1 sumoylation observed for MMC with and without ATRi and ATMi co-treatment (Figure 5A). TopBP1 sumoylation was increased upon 3 hr of HU treatment, further massively enhanced by co-treatment with ATRi, and then attenuated by addition of ATMi (Figure 5A). However, after 30 min HU and ATRi treatment, only a modest increase of TopBP1 sumoylation was detected. This pattern is in accordance with replication forks breaking after longer treatment with replication stressors and checkpoint inhibition, thereby also inducing ATM signaling (Figure 5A; Figure S5A). Furthermore, treatment with high-dose ionizing radiation (IR), which also induces DSBs and ATM activation, did not induce TopBP1 sumoylation, indicating that this regulation is specific to RS-associated DNA breaks (Figure 5A). We further validated this pattern of TopBP1 sumoylation using two different pharmacological inhibitors for ATM and ATR and with one CHK1 inhibitor (CHK1i) (Figure S5A). Analogous to ATR, inhibition of CHK1, a
Figure 5. Deep Proteomics Analysis of Phosphorylation and SUMOylation in RS and Replication Fork Breakage

(A) Western blot analysis of TOPBP1 SUMOylation and markers of ATR and ATM activity upon treatment with HU with and without ATRi (ATR-45) and/or ATMi (KU55933).
prominent substrate and mediator of ATR checkpoint signaling, results in replication fork breakage and ATM activation (Figure S5A). Interestingly, TOPBP1 was also hyper-SUMOylated upon CHK1i and HU co-treatment (Figure S5A). Collectively, these observations indicate that modulation of the SUMOylation response to RS by these central DDR kinases could be a general regulatory mechanism and not only specific to MMC treatment.

To elaborate on the magnitude of this mechanism, we performed a large-scale proteomics experiment to analyze SUMOylation and phosphorylation site regulation under these conditions. Specifically, we enriched SUMOylated and phosphorylated peptides from cells treated with HU in combination with and without CHK1i and ATMi for analysis by LC-MS/MS (Figures S5B and SSC). CHK1i was used rather than ATRi to permit initiation of the RS response by ATR. Four biological replicates were performed, and each sample was analyzed twice by MS for label-free quantification (Figure S5D). We identified 3,465 SUMOylated peptides corresponding to 1,590 SUMOylation acceptor sites, of which 2,450 peptides were quantified at least three times under at least one of the three treatment conditions (Figure 5B; Table S5). Using ANOVA significance testing to compare the dynamics of the modifications between treatments, 1,375 SUMOylated peptides, corresponding to 816 SUMO acceptor sites, were deemed to be regulated under at least one condition (Figure 5B). Similarly, 3,373 high-confidence phosphorylation sites were found to be modulated, and 127 proteins harbored changes of both PTMs (Figures 5B and 5C). To determine whether there was interdependency between SUMOylation and phosphorylation in our dataset (for example, with the PDSM motif; Hietakangas et al., 2006), we analyzed our raw MS data to identify co-occurring phosphorylation sites on the enriched SUMO peptides. We identified 127 phosphorylation sites in the SUMO-enriched dataset, of which 26 were on SUMOylated peptides (Table S5). Although the overlap is modest, 64% of these phosphorylation sites harbored a proline in the residue directly C-terminal to the phosphorylated serine/threonine residue, conforming to part of the PDSM motif (ΨKxExxSP) (Table S5).

We further analyzed our dataset to determine the degree of control the DDR kinases exert on protein SUMOylation in response to RS. It is evident from the number of significantly perturbed SUMOylation acceptor sites that regulation of this modification by ATM and ATR is a global mechanism in the response to RS because more than 50% of the quantified sites were significantly regulated (Figure 5B). We performed unsupervised hierarchical clustering of the regulated phosphorylation sites and SUMOylated peptides to determine the dynamics of this regulation (Figure 5D; Figure S5E). For both modifications, we identified a cluster that showed the same dependency on CHK1 and ATM as observed for TOPBP1 by western blotting (Figure 5D). In this cluster, protein SUMOylation and phosphorylation sites increased upon co-treatment of HU with CHK1i compared with HU alone and was attenuated upon further addition of ATMi (Figure 5D; Figure S5E). Interestingly, GO analysis revealed that these clusters were enriched in proteins involved in DNA replication and recombination (Figure 5D; Figure S5E). Among the SUMO-regulated proteins in this cluster were key regulators of DNA replication and homologous recombination, such as TOP2A (DNA topoisomerase 2-alpha), BLM (Bloom syndrome protein), and BRCA1 as well as its constitutive interactor BARD1 (Figure 5D). Moreover, the dynamics of the modifications in these specific clusters are in accordance with the expected and observed phosphorylation profiles of targets of ATR and ATM (Figures 5A and 5D; Figures S5A and S5E). Additionally, a cluster of proteins with significantly increased SUMOylation upon HU and CHK1i co-treatment, but unchanged by addition of ATMi was enriched in proteins involved in DDR and DNA repair (Figure 5D). This included UIMC1, RBBP4 (CtIP), and, interestingly, also topoisomerase I-binding arginine-serine-rich protein (TOPORS), a dual ubiquitin/SUMO E3 ligase that is known to play a role in the DDR (Lin et al., 2005; Marshall et al., 2010). Noteworthy, a substantial fraction of SUMOylation sites were modulated inversely, being unaffected or only slightly modulated by CHK1 inhibition but increasing dramatically upon co-inhibition of ATM (Figure 5D). This further indicates that ATM is a central regulator of protein SUMOylation in the DDR and, possibly, more specifically in protein deSUMOylation. This subset of SUMO-regulated proteins was enriched for housekeeping biological processes such as RNA metabolism, transcription, and chromatin remodeling (Figure 5D). Our findings demonstrate that SUMOylation is regulated globally in response to RS by the chief DDR kinases ATM and ATR.

**DISCUSSION**

Context-specific and dynamic post-translational protein modifications are well-established regulators of the signaling pathways that protect eukaryotic DNA integrity during the tremendous task of replication. Advancements in speed, resolution, and sensitivity of MS-based technologies have revolutionized the study of global PTM biology (Olsen and Mann, 2013). With this rise in global PTM data, it has become evident that efficient cellular responses, such as those that safeguard genomic integrity, require the precise and timely coordination of several
PTMs and the different enzymes that regulate them (Papouli et al., 2005). Integrated analysis of PTMs is therefore pertinent for our understanding of the molecular mechanisms that respond to DNA damage and RS. Using state-of-the-art proteomics methodologies, we mapped nearly 1,400 regulated SUMOylation acceptor sites and 3,300 regulated phosphorylation sites in response to the chemotherapeutic agents MMC and HU. Our study reveals that SUMOylation is regulated by the most dominant, apical DDR kinases ATR and ATM, which are known to initiate and coordinate the phosphorylation responses to RS and replication fork breakage.

In accordance with previous studies, we found that RS elicits increased SUMOylation of the core ATR-activating proteins, including TOPBP1 and ATRIP. Interestingly, previous studies have shown that the SUMOylation of ATR and its constitutive interactor ATRIP is necessary for efficient ATR-dependent checkpoint signaling (Wu and Zou, 2016; Wu et al., 2014). Further, here we showed that TOPBP1, a key co-activator of ATR, undergoes increased SUMOylation in response to MMC-induced RS. This indicates that SUMOylation of this factor, in addition to that of ATR and ATRIP, may be important for ATR-dependent checkpoint signaling. However, further biochemical and molecular biological analyses are required to confirm the precise role of TOPBP1 SUMOylation in ATR activation. In addition, our data suggest that SUMOylation is a common and relevant modification of a number of proteins involved in ATR activation in response to RS.

We aimed to uncover the interplay between phosphorylation and SUMOylation of protein networks in the RS response. Using an integrated proteomics approach, we found that protein SUMOylation was widely modulated by the main regulatory kinases that mediate the phosphorylation response. Parallel proteomics analysis of changes in these two PTMs revealed co-regulation of a number of central RS and DDR responders, including BRCA1, BARD1, and TOPBP1. BRCA1 SUMOylation and phosphorylation have individually been found to play a key role in the function of this protein because SUMOylation has been shown to increases its ubiquitin ligase activity (Morris et al., 2009). It will be interesting in future analyses to determine whether there is co-dependency or cross-regulation of these modifications in the proteins that harbor both phosphorylation and SUMOylation and, in particular, the relevance of this for the functions of central DDR proteins.

Our approach, to study co-regulated SUMO- and phosphorylated proteins, proved to be successful to identify key co-modified targets. TOPBP1 was the most highly co-regulated protein in our dataset upon 8 hr of MMC treatment, and we found that TOPBP1 SUMOylation was heavily modulated by ATR inhibition during RS and by ATM upon replication fork breakage. We found this particularly interesting because these central DDR kinases (particularly ATM) are well known to orchestrate various PTM-based networks upon threats to the DNA (Smith et al., 2010). However, the effect of the apical DDR kinases on global protein SUMOylation in response to DNA damage and replication stress has not yet been shown. We determined that such regulation by kinases not only applies to TOPBP1 but, further, to over 800 nearly 1,400 SUMOylation acceptor sites in response to HU-induced RS, demonstrating global regulation of SUMOylation by these kinases in the maintenance of genome stability. Interestingly, we observed decreased SUMOylation of a large subset of proteins upon ATM inhibition under conditions that induce replication fork breakage. This suggests that ATM may be important for global deSUMOylation to maintain and control physiological levels of protein SUMOylation.

In our bioinformatics analysis of proteins with increased SUMOylation upon treatment with MMC and HU, we found clusters of co-regulated proteins that are known to function together in the RS response. In addition to the ATR activation proteins, BRCA1 and BARD1, we also found Fanconi anemia proteins and DSB response proteins, like MDC1, NBN, and CtIP. This is particularly interesting in light of the recent idea that SUMO functions as a molecular glue to mediate protein complex formation under specific cellular states and that this modification takes form of a “SUMO spray” (Jentsch and Psakhie, 2013). A consequence of this hypothesis is that SUMOylation should occur on functionally related proteins to promote cooperation and interaction in protein networks, and this is precisely what we observed in our dataset. Interestingly, we found that proteins co-modified by SUMOylation and phosphorylation generally have many regulated sites in response to RS. This poses a challenge for functional studies because site-directed mutagenesis of specific SUMOylation acceptor sites has been shown to result in little effect on overall protein SUMOylation or function (Jentsch and Psakhie, 2013).

Here, we present an integrated analysis of global protein phosphorylation and SUMOylation in RS responses and the largest resource to date of regulated SUMOylation targets under these conditions. We propose that increased SUMOylation occurs on specific and relevant factors in response to distinct DNA lesions, as illustrated by the SUMOylation dynamics upon RS and RS-induced DSBs. Our data suggest that these SUMOylation responses are orchestrated by the apical kinases ATR and ATM in parallel with or as part of their phosphorylation signaling. These findings, and further investigations of the co-regulation of these two modifications, are currently of great interest because the induction of RS-provoked DSBs is increasingly used in chemotherapy to induce cancer cell killing (Li and Heyer, 2008). In light of the essential role of SUMO in the maintenance of genomic integrity (Bergink and Jentsch, 2009; Jackson and Durocher, 2013), the increasing interest in this system as a druggable target (Kessler et al., 2012) will require the understanding of how its perturbation affects global signaling networks.

EXPERIMENTAL PROCEDURES

Further details and an outline of the resources used in this work can be found in the Supplemental Experimental Procedures.

Cell Culture

Human U-2-OS osteosarcoma cells were cultured in complete DMEM. For SILAC-based experiments, cells were SILAC-labeled as reported previously (Hekmat et al., 2013; Olsen et al., 2006). For further details regarding cell culture, synchronization, and drug treatments, see the Supplemental Experimental Procedures.

Stable Cell Line Generation

To generate stable cell lines for SUMO enrichment, U-2-OS cells were infected with a lentivirus encoding either FLAG-tagged SUMO-2 (FLAG-SUMO2) or
His10-SUMO-2-KO-Q87R (His10-S2-K0), as described previously (Hendriks et al., 2014; Schimmel et al., 2014). Further details are provided in the Supplemental Experimental Procedures.

SUMO Target Protein Enrichment
Enrichment of SUMOylated proteins was performed as described previously (Schimmel et al., 2014). Briefly, cell were harvested in lysis buffer and sonicated prior to enrichment of SUMOylated protein using monoclonal ANTI-FLAG M2 beads (Sigma) for 90 min at 4°C with rotation. Following washes, the bound proteins were eluted using 1 mM FLAG-M2 epitope peptide and, thereafter, filtered through an Amicon Ultra 10 kDa nominal molecular weight limit (10k MWL) spin filter (Millipore). The resulting proteins were processed by in-gel digestion for LC-MS/MS analysis. For details regarding enrichment of SUMO target proteins and in-gel digestion, see the Supplemental Experimental Procedures.

SUMO Peptide Enrichment
SUMOylated peptides were enriched as described previously by Hendriks et al. (2014). Briefly, thirty 15-cm plates of U-2-OS cells per condition were harvested in PBS, lysed in 6M guanidine-HCl lysis buffer, and sonicated. SUMOylated proteins were enriched from equal amounts of protein for each condition by nickel-nitrilotriacetic acid (Ni-NTA) agarose beads overnight at 4°C. Proteins were eluted using 500 mM imidazole twice. The eluted proteins were filtered and concentrated in spin filters digested with endolysylpeptidase (LysC). SUMOylated peptides were subsequently enriched with Ni-NTA agarose beads at 4°C for 5 hr and eluted using 500 mM imidazole. The enriched peptides were filtered and concentrated prior to digestion with trypsin and analysis by LC-MS/MS. For detailed SUMO peptide enrichment procedures, see the Supplemental Experimental Procedures.

MS Analysis
Peptide mixtures were analyzed using the EASY-nLC system (Proxeon, Odense, Denmark) connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described previously (Kelstrup et al., 2012). Details are provided in the Supplemental Experimental Procedures.

Raw Data Processing
Raw data were analyzed using MaxQuant v1.4.1. and v1.5.11 against the protein quantification. Nat. Biotechnol. 26, 1367–1372.

Bioinformatics Analysis
All functional network analysis were done using the STRING database (Szklarczyk et al., 2015) and further processed with Cytoscape (www.cytoscape.org). Hierarchical clustering and ANOVA t test were performed using Perseus. For ANOVA, the false discovery rate (FDR) threshold was set to 0.05. Sequence motif analysis was performed using IceLogo (Colaert et al., 2009). Details are provided in the Supplemental Experimental Procedures.

DATA AND SOFTWARE AVAILABILITY
The accession number for the raw mass spectrometric data reported in this study is ProteomeXchange Consortium: PXD006361.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.09.059.

AUTHOR CONTRIBUTIONS
J.O.S. performed the experiments described in Figures 1, 2, 3, and 4. Z.X. performed the experiments described in Figure 5. T.S.B. provided help and performed part of the dataset presented in Figure 2. G.F. validated the experiments presented in Figure S5. L.v.S. and A.J.L.-C. supervised J.O.S. and S.M. and gave critical input on the manuscript. A.C.O.V. supervised Z.X. and J.V.O. supervised J.O.S. and S.M. generated and analyzed the data shown in the remaining figures. S.M., A.C.O.V., and J.V.O. conceived the study, designed the experiments, critically evaluated the results, and wrote the manuscript.

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