Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response

Graphical Abstract

Highlights

- Proteomic discovery of DNA damage-regulated ubiquitination and acetylation sites
- FACET-IP approach improves acetylation site detection
- Atypical K6- and K33-polyubiquitin linkages increase in response to UV radiation
- SCF-Cyclin F mediates EXO1 ubiquitination in response to UV radiation

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In Brief

Elia et al. have globally profiled ubiquitination, acetylation, and phosphorylation in the DNA damage response (DDR). Their valuable datasets of 33,500 ubiquitination and 16,740 acetylation sites implicate K6- and K33-linked polyubiquitination in the DDR, demonstrate that CRLs mediate 10% of DDR ubiquitination events, and reveal that EXO1 is an SCF-Cyclin F substrate.

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Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response

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SUMMARY

Execution of the DNA damage response (DDR) relies upon a dynamic array of protein modifications. Using quantitative proteomics, we have globally profiled ubiquitination, acetylation, and phosphorylation in response to UV and ionizing radiation. To improve acetylation site profiling, we developed the strategy FACET-IP. Our datasets of 33,500 ubiquitination and 16,740 acetylation sites provide valuable insight into DDR remodeling of the proteome. We find that K6- and K33-linked polyubiquitination undergo bulk increases in response to DNA damage, raising the possibility that these linkages are largely dedicated to DDR function. We also show that Cullin-RING ligases mediate 10% of DNA damage-induced ubiquitination events and that EXO1 is an SCF-Cyclin F substrate in the response to UV radiation. Our extensive datasets uncover additional regulated sites on known DDR players such as PCNA and identify previously unknown DDR targets such as CENPs, underscoring the broad impact of the DDR on cellular physiology.

INTRODUCTION

In response to genotoxic stress, cells evoke a complex signaling network known as the DNA damage response (DDR) (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). The DDR serves to protect genomic integrity by coordinating pathways involved in DNA repair, cell-cycle arrest, gene transcription, apoptosis, and senescence. Execution of the DDR relies upon a dynamic array of protein modifications, with phosphorylation playing a central role. Using quantitative proteomics to identify over 700 substrates of the pivotal DDR kinases ATM and ATR, we previously demonstrated the extensive breadth of phosphorylation-dependent signaling in the DDR (Matsuoka et al., 2007). Since this initial study, the repertoire of known DDR-regulated phosphorylation events has expanded significantly to more than 1,300 proteins (Beli et al., 2012; Bennetzen et al., 2010; Bensimon et al., 2010; Stokes et al., 2007).

In addition to phosphorylation, it is now evident that the DDR depends upon other protein modifications such as ubiquitination (Bekker-Jensen and Mailand, 2011; Jackson and Durocher, 2013; Messick and Greenberg, 2009). Ubiquitin-dependent signaling regulates post-replication repair, the Fanconi Anemia (FA) pathway, the double-strand break (DSB) response, nucleotide excision repair (NER), and cell-cycle arrest. Ubiquitination in these pathways can function to recruit interacting proteins, to allosterically alter protein function, or to target proteins for proteasomal degradation. These roles are often associated with different polyubiquitin chain linkages. For example, K63 linkages serve as a scaffold for signaling complex assembly during template switching and the DSB response, whereas K48 linkages lead to proteasomal degradation. Notably, the atypical K6 linkage has also been associated with the DDR, as the BRCA1/BARD1 ubiquitin ligase can assemble K6 linkages in vitro and in overexpression systems. However, whether endogenous K6 linkages increase after DNA damage, and to what extent, is currently unknown. This unresolved issue is critical to determining whether K6 ubiquitination occurs physiologically in the DDR (Kulathu and Komander, 2012).

In addition to ubiquitination, other lysine-linked protein modifications, such as acetylation, participate in the DDR. Acetylation regulates p53 activation (Brooks and Gu, 2011), DNA damage kinase signaling (Altmeyer et al., 2013; Floyd et al., 2013; Kaidi and Jackson, 2013; Sun et al., 2007), homologous recombination (Kaidi et al., 2010; Tang et al., 2013), nucleotide excision repair (Fan and Luo, 2010), non-homologous end-joining (Miller et al., 2010), and base excision repair (Hasan et al., 2002). Comparison of ubiquitination and acetylation proteomic datasets has demonstrated that many sites of lysine ubiquitination and acetylation overlap, leading to speculation that in certain conditions, reciprocal regulation of acetylation and ubiquitination may occur at common sites (Bennetzen et al., 2013; Kim et al., 2011; Wagner et al., 2011). However, no proteomic studies have surveyed both DDR ubiquitination and acetylation together nor sequenced acetylation sites deeply enough in cell lines in order to make meaningful conclusions about reciprocal regulation.
Figure 1. Quantitative Profiling of Ubiquitination in Response to UV and Ionizing Radiation

(A) Schematic overview of approach to profile ubiquitination regulated by UV or IR. Three biological replicates were performed for each stimulus and three sequential IPs for each replicate, giving a total of nine IPs per stimulus.

(B and C) Experimental reproducibility indicated by Pearson correlation coefficients for log₂(L/H) ratios for three biological UV replicates (B) and for three biological IR replicates (C).

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To directly compare the relative prevalence of ubiquitination, acetylation, and phosphorylation in the DDR, we have quantitatively profiled all three modifications in response to both UV and ionizing radiation. For ubiquitination, we employed a proteomic strategy that enriches for ubiquitin-derived di-Glycine remnants on trypsinized peptides (Emanuele et al., 2011; Kim et al., 2011; Poulsen et al., 2012; Udeshi et al., 2013; Wagner et al., 2011). To functionally characterize DDR-regulated sites, we utilized secondary perturbations in 8 proteomic screens, producing a dataset that includes more than 33,500 ubiquitination sites. To detect acetylation sites on a similar scale, we developed an approach (FACET-IP) that allowed us to identify over 10,800 acetylation sites in a single experiment. These integrated datasets provide valuable insight into how the DDR remodels the proteome. We demonstrate that SCF-Cyclin F ubiquititates EXO1 in response to UV radiation and that K6- and K33-linked polyubiquitination undergo bulk increases in response to DNA damage.

RESULTS

Systematic Profiling of Ubiquitination in Response to UV and Ionizing Radiation

To globally examine ubiquitination in the cellular responses to UV and ionizing radiation, we performed ubiquitin remnant profiling and metabolic labeling in cell culture. Heavy isotope-labeled cells were left untreated, while light cells were stimulated with either UV or IR. Lysates were combined, digested with trypsin, and peptides containing ubiquitin-derived di-Glycine remnants were enriched with an antibody against di-Glycine. Three biological replicates were performed for each stimulus, as well as 3 sequential immunoprecipitations (IPs) for each replicate, giving a total of 9 IPs per stimulus (Figure 1A, Table S1). We attained strong reproducibility in quantitation between replicates for both the UV (Figure 1B) and IR screens (Figure 1C). In further support of the quality of this data, we identified proteins known to be ubiquitinated in response to UV and/or IR treatment, including FANCD2, FANC1, PCNA, and XPC (Figure 1D). Moreover, the levels of ubiquitination after UV versus IR stimulation were consistent with immunoblot results (Figure 1D): IR induced less FANCD2 ubiquitination and failed to stimulate PCNA ubiquitination, as previously reported (Ulrich, 2009). Notably, UV radiation regulated more sites than IR, as reflected in the histograms in Figure 1E. This regulated enrichment is consistent with UV-stimulating ubiquitination pathways not activated by IR, such as those coordinated by the stress-activated kinases p38 and JNK in response to non-genotoxic stimuli (Srinivas et al., 2005; Tsuchiya et al., 2010; Villumsen et al., 2013).

Importance of Proteasome Inhibition in Profiling DNA Damage-Induced Ubiquitination

In response to DNA damage, ubiquitination can lead to the proteasomal degradation of substrates such as CDC25A, CDC25B, SETD8, and EXO1. We failed to detect increased ubiquitination of these proteins upon UV or IR stimulation, likely because of their rapid degradation. We therefore repeated UV and IR screens in the presence of the proteasome inhibitor MG132 (Figure 2A, Table S2). We again observed a strong correlation in quantitation between replicates for both the UV (Figure 2B) and IR screens (Figure 2C). Pre-treatment with MG132 markedly increased the detection of diGly peptides from proteins whose ubiquitination is known to cause proteasomal degradation (Figure 2D). This allowed us to identify the UV-induced ubiquitination of numerous known degraded proteins including CDC25A, CDC25B, SETD8, EXO1, CDC6, and DDB2 (Figure 2E). In the absence of MG132, importantly, many ubiquitinated peptides within these proteins actually decreased upon UV stimulation (Figure 2F). Reversal of these decreases with MG132 (Figure 2F) demonstrated they were due to protein degradation rather than deubiquitination. These results highlight the importance of proteasome inhibition in comprehensive investigation of ubiquitination dynamics. Equally important, however, is ubiquitin profiling in the absence of MG132, as proteasome inhibition can cause ubiquitin pool depletion and thereby diminish the inducibility of non-degradative ubiquitination events. We observed this phenomenon for PCNA and XPC (Figure 2G), whose UV-induced ubiquitination is known to be non-proteolytic (Poulsen et al., 2013; Sugasawa et al., 2005; Ulrich, 2009).

Ubiquitin Profiling in Fractionated Nuclear Extracts Expands the DDR Ubiquitinome

In addition to ubiquitin profiling in whole-cell lysates, we performed diGly IPs from nuclear extracts. For these experiments, we fractionated nuclear lysates with strong cation exchange chromatography (SCX) prior to diGly enrichment (Figure S4A, Table S3). IPs were performed from ten fractions for each UV or IR stimulus. We employed this fractionation procedure in order to allow comparison with acetylation and phosphorylation enrichment strategies described below. Combining sites from all of our DNA damage ubiquitin remnant experiments, representing 18 biological replicates and 68 diGly IPs, we identified 33,504 sites and quantified 26,009 sites (Figure S1A). We identified a total of 2,197 sites whose abundance increased greater than 2-fold (log2(L/H) ratio ≥ 1) in response to UV and 741 sites that were upregulated greater than 2-fold by IR.

Pathways and Sites Regulated by DDR Ubiquitination

We performed gene set enrichment analysis on proteins whose ubiquitination either increased or decreased greater than 2-fold following UV or IR radiation. In response to UV, we enriched for multiple DNA repair pathways, including NER and the FA pathway, as well as DNA damage-induced checkpoint pathways (Figure 3A). Interestingly, we also enriched for functions related to the mitotic spindle, including centrosome amplification, chromosome alignment, chromatid cohesion, and chromosome

(D) Log2(L/H) ratios for diGly sites on proteins known to be ubiquitinated in response to UV and/or IR. For both FANC2 and PCNA, the reduced or absent levels of IR-induced ubiquitination, relative to UV-induced ubiquitination, are consistent between proteomic and immunoblot results.

(E) Histograms depicting log2(L/H) ratios for all quantified diGly sites in the three UV replicates (left) and three IR replicates (right).

(F) Log2(L/H) ratios for all quantified sites of PCNA ubiquitination found in both the UV and IR datasets. Error bars in all plots represent the SEM of unique MS1 quantifications for the indicated site. See also Figure S1.
segregation (Figures 3A and 3C). Notably, we found that 10 centromere proteins (CENPs) were deubiquitinated in response to UV or ionizing radiation (Figure 3B). This regulation may be related to crosstalk between the DDR and the spindle checkpoint, which was also enriched in our screens (Figures 3A and 3C), or may alternatively involve CENP proteins participating in non-centromere-related DDR functions. Consistent with this idea, the proteins CENP-S and CENP-X are known components of the FA core complex and are necessary for MMC resistance (Singh et al., 2010; Yan et al., 2010), while the proteins CENP-A, -N, -T, -U have been reported to localize to sites of laser microirradiation (Zeitlin et al., 2009). At the other end of the spindle, we identified UV-regulated proteins associated with the centrosome (Figure 3C), including the centriolar satellite proteins AZI1, PCM1, and MIB1, whose UV-induced deubiquitination was recently reported (Villumsen et al., 2013). We also identified proteins involved in spindle microtubule assembly, including 6 kinesin motor proteins and 4 of 8 subunits from the HAUS complex (Figure 3C), whose depletion results in destabilization of kinetochore microtubules (Lawo et al., 2009).

In addition to identifying previously uncharacterized DDR targets, we uncovered additional ubiquitination sites on known DDR players. Ubiquitination of PCNA on K164 is a well-known DDR event that leads to the recruitment of translesion polymerases or the translocase ZRANB3 (Ciccia et al., 2012). We identified UV-induced ubiquitination of PCNA on not only K164, but also the additional site K117 (Figure 1F). Prior reports have demonstrated that ubiquitination of K107 in S. cerevisiae PCNA occurs in response to the failure of Okazaki fragment ligation arising from deletion of DNA ligase I and that this ubiquitination is necessary for RAD53 phosphorylation (Das-Bradoo et al., 2010b). This finding has led to the proposal that different types of...
of DNA damage (nicks in nascent strands versus replication-blocking lesions in template strands) may elicit ubiquitination on different sites in *S. cerevisiae* PCNA (Das-Bradoo et al., 2010a). Our results suggest that similar regulation may occur in human cells. Notably, depletion of DNA ligase-I in human cells causes PCNA ubiquitination on an unknown site (Das-Bradoo et al., 2010b).

**Systematic Profiling of Acetylation in Response to UV and Ionizing Radiation**

Proteomic studies have demonstrated that many sites of lysine ubiquitination and acetylation overlap, suggesting that reciprocal regulation of acetylation and ubiquitination may occur at common sites (Bennetzen et al., 2013; Kim et al., 2011; Wagner et al., 2011). Importantly, no prior study has sequenced acetylation in cell lines deeply enough to make meaningful conclusions about coregulation. To examine such coregulation in the DDR, we sought to identify acetylation sites at a scale similar to that possible for ubiquitination and thus developed the approach FACET-IP (Fractionated ACETylation IP). Peptides from nuclear lysates were separated by strong cation exchange chromatography (SCX) into ten fractions, and acetylated peptides were enriched with a pan-acetyl antibody from each fraction (Figure 4A). SCX improved the number of acetylation sites identified in a single acetylation IP. Whereas a single IP from 20 mg of total cellular peptides yielded 1,006 sites (Figure 4B, Table S5), IP from a single SCX fraction containing only 1–2 mg of peptides produced up to 2,556 sites (Figure 4B). Over ten SCX fractions,
Figure 4. FACET-IP Identifies Acetylation Sites Regulated by UV or Ionizing Radiation
(A) Schematic for proteomic identification of acetylation sites by FACET-IP. 
(B) Number of acetylation sites (blue) and acetylated proteins (red) detected by FACET-IP among 10 SCX fractions in the UV screen (range 745–2,556 sites, 523–1,220 proteins). Elution of fractions 1–5 occurred using a KCl gradient to 91 mM and fractions 6–10 using isocratic 350 mM KCl. Dashed lines represent the number of acetylated sites (1,006 sites, blue) and proteins (554 proteins, red) identified in a single IP from 20 mg of nuclear lysates.

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this method generated over 10,800 sites per 20 mg of total cellular peptides (Figure S2A). Including pilot fractionation studies using whole-cell lysates, we have identified a total of 16,740 acetylation sites (Table S3), which is the largest collection of sites in a single study to date (Choudhary et al., 2014).

To examine UV- and IR-regulated acetylation, we utilized SILAC in conjunction with FACET-IP (Figure 4A). We identified 12,456 sites and quantitated 10,192 sites for the UV and IR experiments together (Figure 4C). This dataset is nearly 5 times larger than that for a previous study of etoposide (10 μM 24 hr) and IR (10 Gy 1 hr) treatment and 20 times larger when considering acetylation events that occur within 1 hr and thus are less prone to cell-cycle or transcriptional effects (Figure S2B) (Bell et al., 2012). The success of our technique is demonstrated by our ability to detect UV and IR-induced p53 acetylation on its known site K382 (Figure 4D). We identified 219 sites whose acetylation increased at least 2-fold in response to UV radiation and 107 in response to IR (Figure 4C, Table S5). Increased acetylation occurred on multiple proteins with known roles in the DDR, including RPA1, RPA3, ERCC8, ERCC4, and APEX1 (Figure S3A). We also observed de-acetylation of known DDR factors, including RAD50, XRCC1, BLM, CLSPN, EXO1, USP7, and MSH2. These latter proteins may be targets of histone deacetylase inhibitors, possibly explaining how this class of drugs causes DNA damage sensitization, the mechanism for which is currently unclear.

Lastly, we determined the frequency of ubiquitination and acetylation co-regulation at common sites following UV radiation. Among 2,276 quantitated sites that were both ubiquitinated and acetylated, 237 underwent a ≥2-fold increase in ubiquitination, and only 5 of these sites experienced a reciprocal ≥2-fold decrease in acetylation (Figure 4E). Similarly, among 180 sites undergoing ≥2-fold deubiquitination, only 5 experienced an increase in acetylation (Figure S3B). These results suggest that reciprocal regulation of ubiquitination and acetylation is not a frequent event in the DNA damage response. It is possible that different cellular populations are ubiquitinated versus acetylated on the same sites.

Comparative Analysis of Ubiquitination, Acetylation, and Phosphorylation in the DNA Damage Response

Given a meaningful number of acetylation sites regulated by DNA damage, we set out to quantify the relative prevalence of ubiquitination, acetylation, and phosphorylation regulation in the DNA damage response. To allow for an accurate comparison among the three modifications, we quantitated ubiquitination and phosphorylation using the same approach we utilized for acetylation: SCX fractionation of nuclear lysates. Nuclear peptides were fractionated by SCX, and ubiquitinated peptides were then enriched with diGly antibody and phosphopeptides with IMAC (Figure S4A, Tables S3 and S6). We found that the percentage of sites that increased at least 2-fold in response to UV radiation was similar for phosphorylation (8.4%) and ubiquitination (8.5%) and was over 3 times greater than that for acetylation (Figure 4F). When looking at removal of modifications, we also found that UV-induced deubiquitination and dephosphorylation were more prevalent than deacetylation (Figure S4B). These results suggest that regulation of ubiquitination is as common as phosphorylation in the DNA damage response and that both modifications are more common than acetylation.

We next compared DNA damage-induced ubiquitination, acetylation, and phosphorylation on specifically DNA repair proteins (belonging to the Gene Ontology category GO: 0006281). To depict a heatmap summary of inducible sites on DDR proteins, we included 2-fold-induced sites from this work and from all prior proteomic studies of phosphorylation and acetylation. We found fewer instances of inducible acetylation than inducible ubiquitination or phosphorylation on proteins within the DNA repair category (Figure 4G). Moreover, the number of inducible sites on these proteins was greater for ubiquitination and phosphorylation than for acetylation (Figure 4G). Notably, DDR components were among the most heavily acetylated proteins in the proteome, with DNAPK and PARP1 falling among the top 12 proteins with the most acetylation sites (Figure S3C). Despite the presence of many acetylation sites on DDR proteins, our results collectively suggest that acetylation regulation by DNA damage is significantly less common than for ubiquitination and phosphorylation, consistent with previous findings that compared acetylation and phosphorylation (Bell et al., 2012).

K6- and K33-Linked Polyubiquitination Occur in Response to UV Radiation

Lysine linkages between ubiquitin monomers within polyubiquitin chains are associated with different physiological roles. K48- and K11-linked polyubiquitination lead to proteasomal degradation, whereas K63-linked polyubiquitination serves as a scaffold for signaling complex assembly. Increases in both K48 and K63 linkages occur on specific proteins in response to IR and UV radiation. K48 linkage is involved in the degradation of cell-cycle proteins such as CDC25A and CDT1, while K63-linked polyubiquitination occurs on the scaffolds H2A and PCNA to recruit proteases that promote DNA repair.

Our ubiquitin profiling efforts revealed no changes in bulk K48 or K63 linkages upon IR or UV radiation. These findings are expected given the plethora of biological processes that utilize K48 and K63 linkages outside of DNA damage signaling. Much to our surprise, however, we did detect bulk increases in K6 (3.6-fold), and to a lesser extent K33 (1.8-fold), linkages in...
response to UV but not ionizing radiation (Figure 5A). Importantly, we observed increases in K6 and K33 linkages in all 7 biological UV replicates and in none of 6 IR replicates (Figures 5B and 5C), supporting the strong reproducibility of our data. For the K6 linkage, these replicates represent a total of 76 unique peptide measurements for the UV data and 68 measurements for the IR data. Remarkably, increases in K6 linkage occurred for all 76 unique peptides in the UV data and none of the 68 peptides in the IR data (Figure 5B). Similarly, increases in K33 linkage occurred in all of 27 unique peptide measurements for the UV data and none of 21 unique peptide measurements for the IR data (Figure 5C). These results demonstrate that endogenous K6 and K33 linkages increase in response to DNA damage, the implications for which are discussed below.

**Cullin-RING Ligases Are Responsible for 10% of UV-Induced Ubiquitination Events**

Determining E3 ligases upstream of ubiquitination events is important for a systems analysis of DNA damage-induced ubiquitination. While few pharmacologic inhibitors of ubiquitin ligases are available for this purpose, Cullin-RING ligase (CRL) activity can be probed with the drug MLN4924. MLN4924 inhibits CRL neddylation, which is necessary for the activation of all CRL ligases. To identify UV-induced ubiquitination events mediated by CRLs, we performed ubiquitin remnant profiling with UV radiation and MLN4924. Both heavy and light cells were treated with UV radiation and with MG132, while only light cells were treated with MLN4924 (Figure 6A, Table S4). Di-Glycine IPs were performed in triplicate and yielded strong correlation between replicates (Figure 6B). We identified 206 sites on 116 proteins whose ubiquitination increased at least 2-fold in response to UV radiation and decreased at least 2-fold in response to MLN4924 treatment (Figure 6C). These data suggest that approximately 10% of UV-induced ubiquitination events are mediated by CRLs (Figure 6C). Our approach successfully identified many proteins known to be ubiquitinated by CRLs in response to UV radiation, including CDC25A, SETD8, XPC, DDB2, and CDC25B (Figure 6D), demonstrating the validity of our approach.

**SCF-Cyclin F Mediates EXO1 Ubiquitination in Response to UV Damage**

In addition to known CRL substrates, we also identified previously uncharacterized targets of CRLs in the response to UV radiation. For example, the nuclease EXO1 is known to be ubiquitinated and degraded by the proteasome in response to HU-induced replication fork stalling (El-Shemerly et al., 2005). Our proteomic analyses detected EXO1 ubiquitination in response to UV radiation (Figure 2E, Table S2). Consistent with UV-induced ubiquitination mediating proteasomal degradation, we did not detect EXO1 ubiquitination proteomically in the absence of proteasome inhibition (Figure 2D, Table S1), and MG132 inhibited EXO1 degradation detected by immunoblot (Figure S5A). Also consistent with immunoblot results (Figure S5B), our proteomic data indicated that EXO1 ubiquitination was stimulated by UV but not IR (Figure S5B, Table S2). Importantly, the E3 ligase for EXO1 has not been previously identified. Our proteomic data indicated that UV-induced ubiquitination of EXO1 at K796 was inhibited by MLN4924 (Figure 6D). To validate this finding, we demonstrated by immunoblot that EXO1 degradation was maximal in late S/G2 phase of the cell cycle and that this degradation was inhibited by MLN4924 (Figure 6D). To determine which of five Cullins in the CRL family mediate EXO1 ubiquitination, we expressed dominant-negative mutants of each Cullin and found that CUL1 was responsible for EXO1 degradation (Figure 6F). These findings establish EXO1 as an SCF substrate in the DDR.
To determine the F-box protein responsible for EXO1 ubiquitination, we sought to identify candidates interacting with EXO1, preferably in a DNA damage-induced fashion. We therefore treated 293T cells with 4NQO (in the presence of MG132) and screened for proteins interacting with HA-tagged EXO1 by mass spectrometry. We found that the F-box protein Cyclin F (CCNF) associated with EXO1 in 4NQO-stimulated, but not non-damaged, cells (Figure 7A). Importantly, immunoblotting of HA-EXO1 immunoprecipitates with a Cyclin F antibody confirmed the interaction (Figure 7B). MG132 treatment was necessary to detect binding, consistent with degradation of EXO1 occurring upon Cyclin F association (Figure 7B). MG132 additionally increased the levels of Cyclin F, whose own stability is known to be regulated by the proteasome (D’Angiolella et al., 2013). Importantly, stimulation with 4NQO enhanced interaction of the proteins beyond that with MG132 alone (Figure 7B). These results demonstrate that binding of Cyclin F to EXO1 is induced by DNA damage.

We next investigated whether Cyclin F functionally regulates EXO1 stability. We found that depletion of multiple other F-box proteins (b-TCRP, FBXW7, SKP2, and FBXO18) failed to prevent 4NQO-induced EXO1 degradation (Figures 7C and S6A), while depletion of Cyclin F strongly inhibited its degradation (Figure 7C). We confirmed this result using four individual siRNAs, all of which efficiently depleted Cyclin F and suppressed EXO1 degradation (Figures 7D and 7E). Notably, the degree of Cyclin F depletion correlated with inhibition of EXO1 degradation, as shown for an additional siRNA that was less effective in blocking protein degradation rather than deubiquitination (Povlsen et al., 2012), emphasizing the importance of discriminating between these causes of diGly site reduction. However, ubiquitin profiling should not be performed exclusively in the presence of proteasome inhibition, as it can cause ubiquitin pool depletion and impair the detection of non-degradative ubiquitination events (Figure 2G). Combining results from complementary UV screens performed with MG132 or MLN4924 led to the discovery that EXO1 is a CRL substrate. Integrating orthogonal screens allows for functional site characterization and represents a strength of our datasets.

**FACET-IP Improves Global Acetylation Profiling in the DDR**

In addition to ubiquitination, we profiled acetylation and phosphorylation in response to UV and IR. We found that DDR-regulated ubiquitination is as prevalent as phosphorylation and significantly more common than acetylation. To identify acetylation sites on a scale similar to that for ubiquitination, we developed the approach FACET-IP, which allowed identification of over 10,800 sites in a single experiment. Our improvement in acetylation site yield does not result simply from performing additional IPs per biological replicate. We found that a single IP from 20 mg of total cellular peptides generated 1,006 sites. This number was surpassed for 7 of 10 IPs performed from single SCX fractions (range 1,240–2,556 sites) despite the presence of only 1–2 mg peptides in each fraction (Figure 4B). SCX fractionation prior to acetylpeptide enrichment likely prevents overwhelming of the antibody by highly abundant acetylpeptides such as those arising from histones. Separating these peptides from the bulk population likely enhances access of the antibody to less-abundant peptides.

**Ubiquitin Regulation of Mitotic Spindle Proteins**

We detected DDR-regulated ubiquitination sites on kinetochore and other mitotic spindle proteins (Figure 3C). Regulation of kinetochore-microtubule attachment and chromosome alignment by DDR factors has been previously reported (Rozier et al., 2013). Our data suggest that ubiquitin signaling may mediate DDR regulation of the kinetochore. We found that 10 centromeric proteins (CENPs) were deubiquitinated in response to UV and/or IR. This regulation may represent the DDR interfacing with the spindle assembly checkpoint or alternatively the involvement of CENPs in non-centromere-related DDR functions that require CENP localization to DNA damage sites as described (Singh et al., 2010; Yan et al., 2010; Zeitlin et al., 2009). An additional possibility is that CENP ubiquitination regulates chromosome positional stability (Soutoglou et al., 2007) or enhanced chromosome mobility (Dimitrova et al., 2008) that has been observed in different DNA damage settings. Yet another possibility is that ubiquitination of CENPs may regulate recombination between repetitive DNA sequences found at centromeres. Future studies to elucidate the function of CENP deubiquitination are likely to yield important insights into the DDR.

**SYSTEMATIC ORTHOGONAL PROFILING OF UBIQUITINATION IN THE DDR**

We have globally profiled ubiquitination in response to UV and ionizing radiation, utilizing secondary perturbations in 8 orthogonal screens (Figure S1) and producing a dataset of over 33,000 ubiquitination sites. Our work highlights the importance of ubiquitin profiling in the presence of proteasome inhibition, which facilitates detection of proteolytic ubiquitination events. In the absence of proteasome inhibition, we would have failed to detect UV-induced EXO1 ubiquitination (Figure 2D). Profiling in the presence of proteasome inhibition also helps to distinguish inducible deubiquitination from protein degradation (Figure 2F). In a prior proteomic study, UV-induced decrease in diGly sites on the PCNA-binding protein PAF15 was found to result from protein degradation rather than deubiquitination (Povlsen et al., 2012), emphasizing the importance of discriminating between these causes of diGly site reduction. However, ubiquitin profiling should not be performed exclusively in the presence of proteasome inhibition, as it can cause ubiquitin pool depletion and impair the detection of non-degradative ubiquitination events (Figure 2G).
Figure 6. Screen for CRL-Mediated Ubiquitination Events in the DDR Identifies EXO1 as an SCF Substrate

(A) Diagram of approach to identify UV- or IR-induced ubiquitination events mediated by CRLs. Light cells were treated with 10 μM MLN4924 and 5 μM MG132 for 30 min, while heavy cells were treated with 5 μM MG132 for 30 min. Both heavy and light plates were then irradiated with 40 J/m2 UV or 10 Gy IR and harvested 1 hr later.

(B) UV

L = MLN4924 + UV + MG132
H = UV + MG132

R

Rep1

Rep2

Rep3

R 0.89

Rep2 Log2(L/H)

Rep1 Log2(L/H)

IR

L = MLN4924 + IR + MG132
H = IR + MG132

R 0.81

Rep1 Log2(L/H)

Rep2 Log2(L/H)

(C) UV induced sites (2197)  Proteins with UV-induced sites (1153)

MLN4924 regulated

206 1991

116 1037

(D) Graph showing Log2(L/H) for different conditions.

(E) Hrs 0 2 4 6 8 10 12 AS

4NQO - + - + - + - + - + - +

EXO1

Vinculin

S G2/M G1

6 hrs after G1/S release

MLN4924 - - + +

4NQO - + - +

EXO1

Vinculin

(F) dnCullin

4NQO - + - + - + - + - + - +

EXO1

Vinculin

1 2 3 4 5 EV

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K6- and K33-Linked Polyubiquitination in Response to UV Radiation

K6-linked polyubiquitination has been associated with the DDR through the ubiquitin ligase BRCA1. Prior studies have shown that BRCA1 can assemble K6-linked polyubiquitin chains in vitro and in cells when overexpressed (see references in Kulathu and Komander, 2012; Christensen et al., 2007). However, these studies did not examine whether DNA damage can actually induce K6 polyubiquitination, nor did they assay for endogenous K6 linkages. Here, we demonstrate that UV, but not IR, increases endogenous K6-linked polyubiquitination. BRCA1 localizes to UV-induced foci, functions in post-replication repair, and stabilizes stalled replication forks (Pathania et al., 2011; Schlacher et al., 2012). It therefore has known roles in the response to replication stress and represents a candidate for regulating UV-induced K6 ubiquitination. Involvement of BRCA1’s ubiquitin ligase in this response would provide a new function for the ligase, which is dispensable for homologous recombination repair of DSBs (Reid et al., 2008).

In addition to K6-linked polyubiquitination, we discovered a smaller UV-induced increase in K33 ubiquitination. Little is known about K33 ubiquitination, and it has not been previously linked to the DDR. For both K6 and K33 linkages, it is significant that they underwent bulk increases in response to UV radiation. It is surprising that ubiquitin linkages of any type would undergo UV-induced aggregate changes detected by mass spectrometry, as this requires that the linkage is produced in such high quantity that it overwhelms levels resulting from all other concomitant cellular processes. For example, it is well established that K63- and K48-linked ubiquitination are induced by DNA damage, yet we observed little or no bulk increases in these linkages upon UV or ionizing radiation, likely because of their significant involvement in other processes. Thus, there exists a strong and unique relationship between UV radiation and polyubiquitination involving K6 and K33 linkages that raises the question as to whether these linkages are largely dedicated to the DNA damage response.

EXO1 Is an SCF-Cyclin F Substrate in the Response to Replication Stress

EXO1 is a member of the RAD2 family of structure-specific nucleases with 5’-3’ processive exonuclease activity and 5’-3’ flap endonuclease activity. It functions in multiple DNA metabolic processes, including mismatch repair, DSB resection during homologous recombination, and Okazaki fragment processing (Tran et al., 2004). We demonstrate that EXO1 is ubiquitinated and degraded by the proteasome in response to UV radiation and 4NQO, both of which cause replication stress. Prior reports have shown that EXO1 deletion suppresses replication fork instability caused by RAD53 deletion mutants in S. cerevisiae (Segurado and Diffley, 2008). Furthermore, EXO1 mediates the resection and degeneration of stalled replication bubbles in RAD53 mutant cells (Cotta-Ramusino et al., 2005). EXO1 is present at replication forks due to its roles in mismatch repair and lagging strand synthesis. The purpose of its degradation during replication stress may therefore be to prevent unwanted resection of stalled replication forks. Notably, 4NQO-induced EXO1 destabilization also occurs during G2, where its degradation may serve to prevent excessive DSB resection during homologous recombination. Unchecked nucleases can threaten genomic stability, so limiting their activity through negative feedback circuitry is important for avoiding unintended toxicity (Ciccia and Elledge, 2010). Importantly, we show that EXO1 ubiquitination is mediated by SCF-Cyclin F. Increase in basal EXO1 upon Cyclin F depletion (Figures 7C–7E) is reminiscent of CDC25A elevation upon β-TCP depletion (Figure S6A) and may result from basal suppression of EXO1 levels by endogenous DNA damage or may indicate an additional role for SCF-Cyclin F ubiquitination of EXO1 in the absence of damage. Although Cyclin F was the founding member of the F-box protein family (Bai et al., 1996), relatively few of its substrates have been identified to date (D’Angiolella et al., 2013), and EXO1 now expands this important list.

Our findings on SCF-Cyclin F-mediated EXO1 ubiquitination and K6- and K33-linked polyubiquitination demonstrate the utility of our proteomic datasets, which are likely to yield additional valuable insights into DDR pathways.

EXPERIMENTAL PROCEDURES

SILAC Sample Preparation

HeLa cells were cultured in lysine- and arginine-free DMEM containing 10% dialyzed FBS. Light media was supplemented with 50 mg/ml L-lysine and 40 mg/ml L-arginine, while heavy was supplemented with 50 mg/ml L-lysine- U-13C6-15N2 and 40 mg/ml L-arginine-U-13C6-15N4 (Cambridge Isotope Labs). For all SILAC screens (except those described in Figure 6), heavy cells were not damaged, while light cells were treated with 40 J/m² UV or 10 Gy IR and then harvested 1 hr later. For the MG132 screens in Figure 2, both light and heavy cells were treated with 5 μM MG132 for 30 min prior to UV or IR treatment of the light cells. For the MLN4924 screens in Figure 6, light cells were treated with 10 μM MLN4924 and 5 μM MG132 for 30 min, while heavy cells were treated with 5 μM MG132 for 2 hr followed by 1 μg/ml 4NQO for 2 hr. Propidium iodide-stained cells from each time point were analyzed by flow cytometry, and lysates were blotted for EXO1. Bottom: HeLa cells were released from a double-thymidine arrest and harvested 6 hr later. At 4 hr prior to harvest, they were treated with 5 μM MLN4924 for 2 hr followed by 1 μg/ml 4NQO with 5 μM MLN4924 for 2 hr.

(B) Correlation coefficients for log2(L/H) ratios for three biological replicates identifying CRL substrates in the presence of UV and for two biological replicates in the presence of IR.

(C) Overlap of sites and proteins ubiquitinated greater than 2-fold in response to UV (from UV, UV-MG, and UV-SCX datasets) with sites whose ubiquitination is suppressed more than 2-fold by MLN4924 (from UV-MLN dataset).

(D) Identification of proteins whose ubiquitination is stimulated by UV and inhibited by MLN4924. Blue bars represent log2(L/H) ratios of diGly sites from UV-MLN dataset (Figure 2A), while red bars represent ratios from UV-MLN dataset (Figure 6A). Error bars represent the SEM of unique MS1 quantifications. EXO1 represents a previously uncharacterized CRL substrate.

(E) Top: HeLa cells were released from a double-thymidine block and harvested at 2 hr intervals. At 2 hr prior to each point of harvest, cells were treated with 1 μg/ml 4NQO for 2 hr. Propidium iodide-stained cells from each time point were analyzed by flow cytometry, and lysates were blotted for EXO1. Bottom: HeLa cells were released from a double-thymidine arrest and harvested 6 hr later. At 4 hr prior to harvest, they were treated with 5 μM MLN4924 for 2 hr followed by 1 μg/ml 4NQO with 5 μM MLN4924 for 2 hr.

(F) HeLa cells infected with lentiviruses expressing dominant-negative Cullins (with C-terminal truncations that prevent E2 binding) or empty vector (EV) were harvested 2 hr after 1 μg/ml 4NQO treatment and blotted for EXO1. See also Figure S5.
Figure 7. SCF-Cyclin F Mediates EXO1 Ubiquitination in Response to UV Damage

(A) HA-tagged EXO1 was immunoprecipitated from 293T cells treated with 5 μM MG132 for 30 min followed by 1 μg/ml 4NQO for 2 hr. EXO1 complexes were eluted with HA peptide and interacting proteins identified by mass spectrometry.

(B) 293T cells expressing HA-tagged EXO1 were treated with 5 μM MG132 for 30 min followed by 1 μg/ml 4NQO for 2 hr, and HA immunoprecipitates were blotted for Cyclin F. Asterisk represents a non-specific cross-reacting band.

Legend continued on next page.
were treated with 5 μM MG132 for 30 min. Both light and heavy cells were then irradiated with 40 J/m² UV or 10 Gy IR and harvested 1 hr later. For all screens, cells were harvested, washed with PBS, and lysed in denaturing buffer consisting of 8 M urea, 20 mM HEPES (pH 7.5), 1 mM β-mercaptoethanol, 2.5 mM sodium pyrophosphate, and 1 mM Na3VO4 with sonication. Heavy and light lysates were combined in a 1:1 ratio, and proteins (20 mg) were reduced with 4 mM DTT, alkylated with 5.5 mM chloroacetamide, and digested with trypsin overnight at room temperature. The solution was acidified with trifluoroacetic acid (TFA) and clarified by centrifugation. The supernatant was then desalted on a Sep-Pak C18 column (Waters), and peptides were lyophilized.

Enrichment of DiGly, Acetylated, and Phosphorylated Peptides
For diGly and acetylation proteomics, lyophilized peptides were dissolved in IP buffer (50 mM MOPS [pH 7.2], 10 mM sodium phosphate, 50 mM NaCl) and then enriched by overnight incubation with protein A agarose beads conjugated to a monoclonal diGly antibody (Cell Signaling Technology) or polyclonal acetyl-lysine antibody (ImmuneChem). Beads were washed with IP buffer followed by water, and enriched diGly-modified or acetylated peptides were eluted with 0.15% TFA. For diGly screens in Figures 1, 2, and 6, three sequential immunoprecipitations were performed. Eluates were desalted by stage tip chromatography and lyophilized before analysis by LC-MS/MS.

For phosphorylation proteomics, one-half of each lyophilized SCX peptide fraction was dissolved in IMAC buffer (250 mM acetic acid, 30% acetonitrile) and then enriched by 90 min incubation with precharged IMAC resin (PhosSelect iron affinity gel; Sigma-Aldrich). Beads were washed with IMAC buffer and bound peptides eluted with 50 mM Tris, 300 mM NH4OH (pH 10). The remainder of each lyophilized SCX fraction was dissolved in TiO2 buffer (2 M dihydroxybenzoic acid, 50% acetonitrile, 0.1% TFA) and enriched by 90 min incubation with Tiatop TiO2 beads (GL Sciences). Beads were washed with TiO2 buffer followed by 50% acetonitrile, 0.1% TFA, and bound peptides were eluted as for IMAC. Eluted peptides from both IMAC and TiO2 enrichment were desalted by stage tip chromatography and lyophilized before MS analysis.

Mass Spectrometry
Lyophilized peptides enriched by diGly IP, FACT-IP, or IMAC/TiO2 were dissolved in 5% acetonitrile/5% formic acid. Using a Famos autosampler (LC Packings), they were loaded onto a reversed phase microcapillary column (100 μm I.D.) packed first with 5 mm of Magic C4 resin (5 μm, 100 Å; Michrom Bioreources) followed by 20 cm of Magic C18AQ resin (3 μm, 200 Å; The Nest Group). Ubiquitin-derived diGly and acetylated peptides were separated using a gradient of 5%–27% acetonitrile in 0.125% formic acid over 180 min and detected in a LTQ-Orbitrap Velos Pro mass spectrometer (ThermoFisher). IMAC/TiO2-enriched phosphorylated peptides were separated using a 95 min gradient and detected in an LTQ-Orbitrap XL mass spectrometer (ThermoFisher).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.05.006.

ACKNOWLEDGMENTS
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REFERENCES


Supplemental Figures

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Figure S1. Number of ubiquitinated sites and proteins identified and quantified in diGly proteomic screens. Related to Figures 1, 2, 6, and S4.

(A-B) Total number of diGly modified sites (A) and proteins (B) identified and quantified in UV and IR diGly proteomic screens. False discovery rates (FDRs) are indicated.
Figure S2. Identification of acetylation sites by FACET-IP. Related to Figure 4.

(A) Number of acetylation sites identified from 20 mg of total cellular peptides after a single acetylation IP (red bar) or after FACET-IP (blue bar, data from UV screen); see Table S5.

(B) Overlap of all quantified acetylation sites from both our UV and IR screens with all quantified acetylation sites from a prior study (see Table S2 of Beli et al., 2012) that examined acetylation in response to 10 Gy IR (with 1 hour recovery) or to 24 hour of 10 μM etoposide.
Figure S3. DDR regulated acetylation sites. Related to Figure 4.

(A) Interaction network of DNA repair proteins whose acetylation either increases (green) or decreases (red) in response to UV radiation. Network generated by Ingenuity. Heat map represents Log₂(L/H) ratios. For proteins with multiple regulated sites, the mean Log₂(L/H) ratio was calculated. Arrows indicate the direction of interactions, and lines without arrowheads indicate binding.

(B) Heat map representing Log₂(L/H) ratios for ubiquitination (upper) and acetylation (lower) among 180 quantified sites that were both acetylated and ubiquitinated and that underwent a more than 2-fold decrease in ubiquitination in response to UV radiation.

(C) Distribution of acetylation sites identified per protein. The top 12 proteins with the most acetylation sites are listed in the inset.
Figure S4. Quantitative comparison of phosphorylation, ubiquitination, and acetylation in response to ultraviolet and ionizing radiation. Related to Figure 4.

(A) Diagram for enrichment of ubiquitination sites (by diGly IP) and phosphorylation sites (by IMAC/TiO₂) from HeLa nuclear extracts fractionated by SCX. The same protocol was used for the identification of UV and IR regulated acetylation sites in Figure 4A.

(B) Percentage of quantified ubiquitination, acetylation, and phosphorylation sites that decrease in response to UV radiation. All values are derived from proteomic screens (anti-diGly, anti-acK, or IMAC/TiO₂) involving SCX fractionation of nuclear lysates.
Figure S5. EXO1 is degraded by the proteasome in response to UV but not ionizing radiation consistent with proteomic results. Related to Figure 6.

(A) HeLa cells were treated with 1 µg/mL 4NQO and harvested 2 hours later. Where indicated, they were also pre-treated with 5 µM MG132 for 30 min prior to 4NQO addition.

(B) EXO1 ubiquitination occurs in response to UV but not ionizing radiation. Blue bar represents Log2(L/H) ratio of the diGly site from the UV-MG132 screen (Figure 2A) while red bar represents ratio from the IR-MG132 screen (Figure 2A). For the EXO1 immunoblot, HeLa cells were treated with 30 J/m² UV, 1 µg/mL 4NQO, or 10 Gy IR and harvested one hour later.
**Figure S6. Identification of the F-box protein responsible for EXO1 ubiquitination.** Related to Figure 7.

(A) HeLa cells transfected with siFF or a siRNA pool against the F-box proteins β-TRCP1 and β-TRCP2 were treated with 1 μg/mL 4NQO for 2 hours and lysates blotted with the indicated antibodies.

(B) HeLa cells transfected with siFF or individual siRNAs against CCNF were treated with 1 μg/mL 4NQO for 2 hours and lysates blotted for EXO1. EXO1 band intensities, measured by Image J, were normalized to tubulin, and ratios from 4NQO to UNT (untreated) lanes calculated. CCNF mRNA depletion was measured by RT-qPCR.

(C) HeLa cells transfected with siFF or siCCNF-1 were released from a double thymidine arrest and harvested 3 hours later. At 2 hours prior to harvest, they were treated with 1 μg/mL 4NQO for 2 hours.
Supplemental Tables

Table S1: DiGly sites identified and quantified from three UV replicates and three IR replicates, as described in Figure 1.

Table S2: DiGly sites identified and quantified from three UV and two IR replicates performed in the presence of MG132, as described in Figure 2.

Table S3: DiGly sites identified and quantified from UV- or IR-treated nuclear lysates fractionated by SCX prior to enrichment, as described in Figure S4.

Table S4: DiGly sites identified and quantified from three UV-MLN4924 replicates and two IR-MLN4924 replicates, as described in Figure 6.

Table S5: Acetylation sites identified and quantified from UV- or IR-treated nuclear lysates using FACET-IP, as described in Figure 4.

Table S6: Phosphorylation sites identified and quantified from UV- or IR-treated nuclear lysates fractionated by SCX prior to IMAC or TiO₂ enrichment, as described in Figure S4.
Supplemental Experimental Procedures

Cell culture, antibodies, siRNAs

HeLa and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. siRNAs were reverse transfected into HeLa cells at 20 nM using Lipofectamine RNAiMAX reagent (Invitrogen), and cells were harvested 2 days later. The following antibodies were used: Cyclin F (Santa Cruz sc-952), CDC25A (Abcam ab2357), CDC25B (Cell Signaling 9525), EXO1 (Bethyl A302-639A), FANCD2 (Epitomics 2986-1), HA (Roche 12013819001), PCNA (Abcam ab18197), β-Tubulin (Cell Signaling 2128), and Vinculin (Sigma V9131). The following individual siRNAs were purchased from Dharmacon: siCCNF-1 (CCAGUUGUGUGUCUGCAUUA), siCCNF-2 (GCACCCGGUUUAUCAGUUA), siCCNF-3 (UAGCCUACCUCUACAAUGA), siCCNF-4 (GCACCCGGUUUAUCAGUUA), siCCNF-5 (GACAAGCGCUAUGGAGAAA), siFF (CGUACGCGGAUAACUUCGAAU). The following siRNA SMART pools were purchased from Dharmacon: CCNF (M-003215-02), β-TRCP-1 (M-003463-01), β-TRCP-2 (M-003490-01), FBXO18 (M-017404-00), SKP2 (M-003324-04), FBXW7 (M-004264-02).

SCX fractionation of nuclear peptides

For the proteomic comparison of DDR-regulated acetylation (FACET-IP in Figure 4), ubiquitination (Figure S4A), and phosphorylation (Figure S4A), HeLa cells were lysed in 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, and 0.1% Triton-X. Nuclei were isolated by centrifugation at 1300g and lysed in denaturing buffer, as described in the procedure section on SILAC sample preparation. Prior to acetyl-, diGly-, or phospho-enrichment, purified peptides were fractionated by strong cation exchange (SCX) chromatography as previously described (Villen and Gygi, 2008). Lyophilized peptides (20 mg) were resuspended in SCX Buffer A (7 mM KH₂PO₄, pH 2.65, 30% acetonitrile) and loaded onto a 9.4 mm x 200 mm column packed with polysulfoethyl aspartamide (5 μm particle size; 200 angstrom pores; PolyLC). They were then separated using a 35 minute gradient of 0% to 26% Buffer B (7 mM KH₂PO₄, pH 2.65, 30% acetonitrile, 350mMKCl) followed by isocratic elution.
with Buffer B. Ten or twelve fractions were collected and lyophilized. They were then dissolved in 0.1% trifluoroacetic acid, desalted on a SepPak C18 column (Waters), and lyophilized again.

**Peptide identification and quantification**

MS/MS spectra were searched using version 28 of the Sequest algorithm against a database containing all human proteins (IPI version 3.6) in both forward and reversed orientations. Since lysine residues in the diGly and acetylation screens may contain two variable modifications (isotopic label and either diGly remnant or acetylation), each of these MS experiments underwent two searches, the first of which designated the lysine mass as its natural abundance value, while the second increased its mass by 8.014199 to account for heavy labeling. The light- and heavy-labeled peptides from these two searches were then combined using customized scripts. Variable modifications included heavy labeling of arginine (10.008269), oxidation of methionine (15.994946), and either diGly modification of lysine (114.042927), acetylation of lysine (42.010564), or phosphorylation of serine, threonine, and tyrosine (79.966330), while static modifications included carboxyamidomethylation of cysteine (57.021464). The precursor mass tolerance was set to 25 ppm, and three missed cleavages were allowed.

The target-decoy method was used to estimate false discovery rates (Elias and Gygi, 2007), and linear discriminant analysis (LDA) was employed to filter ubiquitinated peptides to an initial 1% peptide-level FDR. Subsequently, peptides were assembled into proteins and further filtered to a protein-level FDR of < 1%, as described (Huttlin et al., 2010). Final FDRs are provided in Figure S1. Localization of diGly, acetylation, and phosphorylation sites was performed using a modified version of the A-score algorithm (Beausoleil et al., 2006) as previously reported (Kim et al., 2011). Sites scoring above 13 (p < 0.05) were considered localized, and peptides with sites failing to meet this threshold were discarded. Peptides with sequences allowing only one possible modification site are unequivocally localized and were assigned scores of 1000. Due to the inability of trypsin to cleave immediately after ubiquitinated or acetylated lysines, we did not allow di-Gly modification or acetylation to occur on C-terminal lysine residues.

Peptide quantification was performed using extracted ion chromatograms as described (Kim et
To ensure reliable quantification, we required that the signal-to-noise (S/N) values for heavy and light species each be greater than 5.0. If this criterion was not met, the peptide was still included if one of the two S/N values was above 10.0. Peptides without quantification values in Tables S1-6 failed to meet one of these criteria. Light-to-heavy ratios for quantified peptides underwent Log2 transformation. To quantify sites of diGly modification, acetylation, or phosphorylation, matching peptides were grouped together and the median Log2 value among these peptides determined. The standard deviation was also calculated as an indication of variability. Peptides containing more than one diGly, acetylation, or phosphorylation site were not used for site quantitation. Log2 values for modification sites were normalized based on the median Log2 values of non-modified peptides where necessary. Sites with a more than two-fold increase or decrease in L/H ratio were considered to be regulated.

**Bioinformatic Analysis**

Ingenuity Pathway Analysis (IPA) was used to determine canonical pathways and functions enriched among proteins with ≥ 2-fold increase in ubiquitination in response to UV radiation in the UV, UV-MG132, or UV-SCX datasets (Figure 3A). Significance was determined using the right-tailed Fisher’s exact test. IPA was also utilized to generate the interaction network of DNA repair proteins (belonging to the Gene Ontology category GO:0006281) whose acetylation is ≥ 2-fold increased or decreased in response to UV (Figure S3A). To generate the heat map summary in Figure 4G of inducible ubiquitination, acetylation, and phosphorylation sites on DNA repair proteins (in GO:0006281), we included two-fold induced sites from this work and from all prior proteomic studies of phosphorylation and acetylation (Beli et al., 2012; Bennetzen et al., 2010; Bennetzen et al., 2013; Bensimon et al., 2010; Matsuoka et al., 2007; Stokes et al., 2007). The heat map was produced using the heatmap.2 tool of the statistical environment R, with color variation representing the number of upregulated sites on each protein.

**Cell-cycle synchronization**

HeLa cells were synchronized at G1/S by treatment with 2.5 mM thymidine for 20 hours, followed by release into fresh DMEM for 8 hours and subsequent treatment with 2.5 mM
thymidine for 16 hours. Cells were washed and then released into DMEM for the indicated times. Harvested cells were fixed in 70% ethanol and resuspended in 20 μg/mL propidium iodide and 100 μg/mL RNAse. Flow cytometry was performed on a BD-LSRII Flow Cytometer (Becton Dickinson). Data was collected using BD FACS Diva software (Becton Dickinson) and cell cycle analysis was performed using FloJo Software.

**Identification of EXO1-interacting proteins**

293T cells expressing HA-tagged EXO1 were treated with 5 μM MG132 for 30 minutes followed by 1 μg/mL 4NQO for 2 hours and then lysed in low-salt lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM NaF, 0.5% NP40, pH 7.5) containing 2 mM N-Ethylmaleimide, protease inhibitor tablet (Roche), and phosphatase inhibitor cocktails I and II (Calbiochem). Lysates were clarified by centrifugation at 14,000g for 10 minutes. The insoluble pellet was then sonicated in low-salt lysis buffer and clarified again by centrifugation at 14,000g. Supernatants from both centrifugation steps were combined and immunoprecipitated with monoclonal anti-HA agarose (Sigma) for 2 hours at 4°C. Beads with bound complexes were washed 4 times in low-salt lysis buffer and eluted with 500 μg/mL HA peptide (Sigma). EXO1-interacting proteins were TCA precipitated, digested with trypsin, desalted using Stage tips, and analyzed by LC-MS/MS.

**RT-qPCR analysis**

RNA was isolated from cells using the RNAeasy Plus kit (Qiagen) and reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen #18080-044) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was performed on an Applied Biosystems 7500 Fast PCR machine using Platinum Cybergreen Super Mix with Rox dye (Invitrogen #11733-046) and primer pairs for CCNF (left, 5’-GGGAACCTGAAGCTCTTTGA; right, 5’-GACAGGCTTTAGGATAGGG) and ACTB (left, 5’-GCTACGAGCTGCCTGACG; right, 5’-GGCTGGAAGAGTGCCTCA). CCNF mRNA level was normalized to ACTB mRNA.
Supplemental References


