A Proteome-wide Analysis of Kinase-Substrate Network in the DNA Damage Response

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The DNA damage checkpoint, consisting of an evolutionarily conserved protein kinase cascade, controls the DNA damage response in eukaryotes. Knowledge of the in vivo substrates of the checkpoint kinases is essential toward understanding their functions. Here we used quantitative mass spectrometry to identify 53 new and 34 previously known targets of Mec1/Tel1, Rad53, and Dun1 in Saccharomyces cerevisiae. Analysis of replication protein A (RPA)-associated proteins reveals extensive physical interactions between RPA-associated proteins and Mec1/Tel1-specific substrates. Among them, multiple subunits of the chromatin remodeling complexes including ISW1, ISW2, INO80, SWR1, RSC, and SWI/SNF are identified and they undergo DNA damage-induced phosphorylation by Mec1 and Tel1. Taken together, this study greatly expands the existing knowledge of the targets of DNA damage checkpoint kinases and provides insights into the role of RPA-associated chromatin in mediating Mec1 and Tel1 substrate phosphorylation in vivo.

Cells are highly responsive to their environment, especially DNA damaging agents. Damaged DNA in cells is rapidly sensed and turned into signals by the DNA damage checkpoint to control many processes, including cell cycle progression, DNA replication and repair, and gene transcription (1). The DNA damage checkpoint consists of several evolutionarily conserved protein kinases (2, 3). Understanding the function of the DNA damage checkpoint requires knowledge of their in vivo substrates. Although the regulation of DNA damage checkpoint kinases has been studied extensively, the knowledge of their in vivo substrates is limited. This can be attributed to the lack of suitable technology to detect low abundant phosphorylation in cells. With the use of stable isotope labeling, the advancement of high mass resolution mass spectrometry (MS), the recent development of analytical and computational tools by many laboratories (4–9), changes in low abundant and regulatory phosphorylation in cells are increasingly detected. Combined with the use of genetics, in vivo kinase substrates have been identified using a quantitative mass spectrometry approach (10).

In the yeast Saccharomyces cerevisiae, Mec1 and Tel1, homologs of the mammalian ATR and ATM kinase, respectively, function at the top of the signal transduction cascade in the DNA damage checkpoint (1–3). Mec1 is primarily responsible for the activation of downstream checkpoint kinases including Rad53 (11, 12), whereas Tel1 has a more prominent role in regulating telomere length (13). Interestingly, deletion of both MEC1 and TEL1 leads to a synergistic increase in gross chromosomal rearrangements, indicating their redundant role in genome maintenance (14, 15). Mec1 is recruited to the site of DNA damage via replication protein A (RPA) that coats single-stranded DNA. Tel1 on the other hand is recruited by the Mre11-Rad50-Xrs2 complex, which recognizes DNA double-stranded breaks (DSBs) (16–19). Importantly, DSBs in cells undergo 5' to 3' resection to generate 3' single-stranded DNA overhangs, which recruit RPA and Mec1. Several proteins including Mre11-Rad50-Xrs2 complex, Sae2, Dna2, Exo1, and Sgs1 participate in the DNA DSB resection process (20, 21). Many of them have been shown to act as substrates of Mec1 and Tel1 (22–25). Moreover, a number of Mec1 and Tel1 substrates have been identified, including histone H2A, Rtt107, Slx4, Cdc13, Ies4, Rad9, Mrc1, and Rad53 (26–32), likely more remain to be identified (10).

Among the most studied Mec1 and Tel1 substrates is the Rad53 kinase, a homolog of the mammalian Chk2 kinase (11, 12, 33). Rad53 helps to maintain stalled DNA replication forks (34, 35), which may be mediated by its substrates Exo1, Dbf4, and possibly others (36, 37). The Dun1 kinase is phosphorylated and activated directly by Rad53 (38–40). Following its activation, Dun1 phosphorylates Sml1, a ribonucleotide reductase (RNR) inhibitor, leading to Sml1 degradation and increased dNTP levels in cells (41, 42). Both Rad53 and Dun1 appear to control the phosphorylation of Rfx1/Crt1 (43), which regulates the transcriptional induction of RNR genes. Acting in parallel to Rad53, the Chk1 kinase also acts downstream of Mec1 and Tel1 (33), although it appears to have a more limited role compared with Rad53 in S. cerevisiae.

Despite extensive studies, current knowledge of the substrates of DNA damage checkpoint kinases in yeast appears to be far from complete. Our previous study identified a number of new substrates of Mec1, Tel1, and Rad53 in yeast, yet several known substrates of these kinases were not identified (10). Fur-
thermore, recent MS analyses of ATM and ATR substrates in mammalian cells have identified several hundred potential substrates (44, 45). To obtain a more in-depth and comprehensive knowledge of the substrates of DNA damage checkpoint kinases in yeast, we applied a recently developed multidimensional chromatography technology (8), stable isotope labeling via amino acids in culture (SILAC) (5) and high-resolution MS to determine substrates of Mec1/Tel1, Rad53, and Dun1 in cells following DNA damage treatment by the DNA alkylating agent methyl methanesulfonate (MMS).

**EXPERIMENTAL PROCEDURES**

**Yeast Strains Used and SILAC Growth Conditions**—Standard yeast cell culture and genetic methods were used. A list of the yeast strains used is shown in Supplemental Table S7.

**Experimental Method for Large-scale Quantitative MS Screen**—SILAC medium consisting of CSM-Lys-Arg with 2% glucose is supplemented with 30 mg/liter of lysine (either isotopically light or heavy), 20 mg/liter of arginine (either isotopically light or heavy), and 80 mg/ml of proline. Heavy isotope containing l-arginineHCl (1^15N, 98%; 1^13C, 98%) and l-lysineHCl (1^15N, 98%; 1^13C, 98%) were purchased from Sigma. For DNA damage treatment, WT and kinase-null cells were grown in the SILAC medium to an A600 nm of 0.3 and then treated with 0.05% MMS for 2 h. Cells were then harvested and lysed using the glass bead beating method (8). For each large-scale MS screen, equal amounts of proteins extracted from WT and each kinase-null cell were combined. 5 mg of the combined protein extracts were used to purify phosphopeptides using an immobilized metal affinity column as previously described (8, 10). Fractionation of phosphopeptides using a hydrophilic interaction column was described previously with the following two modifications (8). First, purified phosphopeptides were dried and then resuspended in 60% acetonitrile in water. The sample was then loaded into a hydrophilic interaction column. Second, 0.04% trifluoroacetic acid was used in Buffer C instead of 1% phosphoric acid. After elution, each hydrophilic interaction column fraction was dried under vacuum, resuspended in 0.1% trifluoroacetic acid, and loaded via a Dionex autosampler to the RP-LC and Orbitrap LTQ mass spectrometer. From each large-scale MS screen, 24 hydrophilic interaction column fractions were analyzed by LC-MS/MS. Each MS screen was repeated twice to ensure that similar results were obtained.

**Analyzing Phosphorylation of Purified Protein Complexes by LC-MS/MS**—Tandem affinity purification (TAP)-tagged cells were grown in 0.5 liters of SILAC medium containing either light or heavy isotopically labeled lysine/arginine until an A600 nm of 0.3, then one (labeled by heavy Lys/Arg) was treated with 0.05% MMS for 2 h. Cells were then harvested and lysed using a glass bead beating method (8). Typically 50 mg of total protein extract was incubated with 25 μl of IgG beads (Amerham Biosciences) for 4 h in the cold room. Then the IgG beads were washed 3 times by 1 ml of TBS buffer. A Tev protease solution consisting of 0.5 μg of Tev protease in 200 μl of TBS buffer with 1 mM dithiothreitol was added and the sample incubated overnight in a cold room. Next, 5–10% of the Tev-eluted sample was analyzed by silver staining. The remaining samples were combined, mixed with 50 μl of 8 M urea with 0.1 M Tris, pH 8.0, and then processed for MS analysis as previously described (8). Initially, 10% of the peptides were analyzed directly by MS. The remaining 90% of the sample was further purified using an immobilized metal affinity column and then analyzed by MS as described previously (8).

**Data Analysis Method for Large-scale MS Screens**—Raw MS/MS spectra were searched by SEQUEST on a Sorcerer system using a composite yeast protein data base, consisting of both the normal yeast protein sequences and their reversed protein sequences as a decoy to estimate the false discovery rate in the search results (6). The following parameters were used in the database search: semi-tryptic requirement; a mass accuracy of 10 ppm for the precursor ions; differential modification of 8.0142 daltons for lysine and 10.00827 daltons for arginine; differential modification of 79.966331 daltons for phosphorylation of serine, threonine, and tyrosine; and a static mass modification of 57.02145 daltons for alkylation cysteine residues. XPRESS was used to quantify all the identified peptides using a mass tolerance of 10 ppm (10). The search results were then filtered using the following criteria: a probability score over 0.8; Sprank scores of less than 5; mass tolerance of no more than 10 ppm; a requirement for valid XPRESS ratios, phosphorylated peptides, and the identification of the either heavy lysine- or arginine-labeled peptides. The use of these criteria typically yields over 10,000 phosphopeptides from all three large-scale MS screening experiments with an ~0.02% false discovery rate based on the number of hits in the decoy data base (6).

For any phosphopeptide to be considered as dependent on Mec1/Tel1, Rad53, or Dun1, a threshold 4-fold more abundant in WT than the corresponding kinase-null mutant was used. Furthermore, to be considered as a Dun1-specific target, the phosphopeptide of interest must be dependent on Dun1, Rad53, and Mec1/Tel1. To be considered as a Rad53-specific target, the phosphopeptide must be Rad53 dependent, Mec1/Tel1 dependent, but not Dun1 dependent. To be considered as a direct Mec1/Tel1 target, each phosphopeptide of interest must be Mec1/Tel1 dependent but not Rad53 and Dun1 dependent, and contain a phosphorylated SQ/TQ site. The use of these requirements led to the classification of specific targets of Mec1/Tel1, Rad53, and Dun1 for further manual analysis. For manual examination, the MS/MS spectra were examined to identify ions with the loss of phosphate, which is characteristic of Ser/Thr-phosphorylated peptides, and to confirm that most of the major ions were properly assigned and the assignment of the phosphate group to the specific site was correct. For quantification using XPRESS (10), a Gaussian-like elution profile was confirmed for the heavy isotope-labeled peptide with at least a signal/noise ratio over 5. Most of the quantification errors were due to a failure to properly locate the elution profile of a peptide of lower intensities. Thus, manual inspection was used to eliminate such cases. Because a stringent set of criteria was used (see above), manual inspection confirmed most of the identified and quantified phosphopeptides. Moreover, most of the kinase-specific phosphopeptides were found only in WT cells and not the corresponding kinase-null cells, thus revealing a highly stringent kinase dependence of kinase-specific phosphopeptides (see details in Supplemental Tables S1–S3).
RESULTS

Identifying Substrates of DNA Damage Checkpoint Kinases by Large-scale Quantitative MS Screens—Three independent large-scale MS screens were performed to identify specific targets of Mec1/Tel1, Rad53, and Dun1 (see Fig. 1A for experimental strategy). Using results from the Mec1/Tel1 screen as an example, the ratios of the integrated intensity of heavy over light isotopically labeled phosphopeptides are plotted (see Fig. 1B). As shown in Fig. 1B, the median of the relative abundance ratios is close to 1, and most phosphopeptides show a relative abundance ratio within 4-fold. Compared with the mec1Δ tel1Δ cells, significantly more phosphopeptides are 4-fold more abundant in WT cells than those that are 4-fold less abundant in WT cells. Therefore, the deletion of MEC1 and TEL1 causes a preferential loss of some phosphorylation. Based on this observation, any phosphopeptide that is 4-fold more abundant in WT than in mec1Δ tel1Δ cells is considered Mec1/Tel1-dependent. Similar results are also found for the Rad53 and Dun1 screens (not shown), thus a 4-fold cutoff was used to determine kinase-dependent phosphopeptides from the Mec1/Tel1, Rad53, and Dun1 screens, whose overlaps are analyzed using Venn diagrams (see text for further details).

Over 14,000 unique phosphopeptides from 2186 proteins are found in the Mec1/Tel1 screen. Using a 4-fold ratio as a cutoff, 238 Mec1/Tel1-dependent phosphopeptides are found. Using the same criterion, 177 and 77 phosphopeptides are found to be Rad53-dependent and Dun1-dependent, respectively. Because Mec1 and Tel1 control the activity of Rad53, which in turn controls the activity of Dun1, we next examined the overlap among the kinase-dependent phosphopeptides. 17 phosphopeptides are dependent on Mec1/Tel1, Rad53, and Dun1; thus they are considered as candidates for being Dun1-specific targets. 42 phosphopeptides are dependent on both Mec1/Tel1 and Rad53, yet independent of Dun1; thus they are considered as candidate Rad53 targets. Finally, 169 phosphopeptides are Mec1/Tel1 dependent and independent of both Rad53 and Dun1. Among them, 87 phosphopeptides con-
tained the expected consensus phosphorylation site, SQ/TQ, of Mec1 and Tel1 (46). Therefore they are considered to be Mec1/Tel1-specific. These kinase-specific phosphopeptides were subjected to further manual examination (see “Experimental Procedures” for details), leading to identification of 75, 33, and 13 phosphopeptides specific to Mec1/Tel1, Rad53, and Dun1, respectively (see Fig. 1C). They are derived from 58, 24, and 12 potential substrates of Mec1/Tel1, Rad53, and Dun1, respectively (see Table 1 and supplemental Tables S1–S3). Due to the large scope of these large-scale MS screens, some phosphopeptides might be found in one MS screen but not the others. Here we chose to consider only those phosphopeptides that satisfy the criteria above, which may lead to the loss of some potential kinase targets.

Functional Classification and Physical Interactions of the Kinase-specific Targets—Among the 87 kinase-specific targets, 15 of them are known targets from previous studies (see supplemental Table S4), 19 of them were also identified as targets in our prior study (10), and 53 targets are identified here for the first time (see Fig. 2A). Thus this study greatly expands the existing knowledge of the substrates of Mec1/Tel1, Rad53, and Dun1. Moreover, most known substrates of these kinases are found here, which not only validates our approach, but also illustrates the scope and depth of the present study. However, we cannot exclude the possibility that variations in cell cycle stages and/or prolonged MMS treatment might alter protein abundance of some of the targets. For example, DNA damage-induced expression of Rnr3 and Rad54 are known (47, 48), which may contribute to their elevated phosphorylation in WT cells. Nevertheless, in most other cases, either there is no previous report of DNA damage-induced protein abundance changes, or our own studies show that their protein abundances are not affected by MMS treatment (see below). Furthermore, most WT and kinase-null cells are expected to be in the S phase following MMS treatment. Possible variations of cell cycle stages between asynchronous WT and kinase-null cells are likely insufficient to account for the complete loss of phosphorylation on most kinase-specific targets in the corresponding kinase-null mutants (see supplemental Tables S1–S3). Therefore, most of these findings here are likely direct kinase substrates, although further studies are needed to establish each newly identified substrate as direct and functional.

Functional classification shows that most Mec1 and Tel1 specific targets function in nuclear processes, including mRNA transcription, DNA damage checkpoint, DNA replication and repair, chromatin remodeling, and cell cycle control (see Table 1 and Fig. 2B). Among them are 10 previously known substrates, including Hta1, Rad9, Mrc1, Rfa1, Rfa2, Rtt107, Srx4, Ies4, Rad53, and Mec1. Eleven proteins were also previously identified (10), including Cbf1, Dpb4, Msh6, Sp7, Cbf5, Prp19, Ysh1, Hsp12, Hpr1, Sum1, and Asg1. It should be noted that the method used previously differs greatly from the one used here, which may explain the differences in the findings (10). In the end, 37 new candidate substrates of Mec1 and Tel1 are identified here. Among Rad53-specific targets, Dun1, Mrc1, Nup1, Nup60, Dbf4, Rx1, and Rph1 are known from previous studies. In addition to nuclear transport proteins (10), proteins involved in chromatin remodeling and DNA replication/repair are among the newly identified Rad53-specific targets (see Fig. 2B and Table 1). Dun1-specific targets are classified as having a partial or complete dependence on Dun1 (see supplemental Table S3). A partial Dun1 dependence was detected for Ecm21, Rnr3, Hpc2, and Sec3, suggesting that these proteins may be indirect rather than direct substrates of Dun1. A complete Dun1 dependence was found for Gle1, Npl3, Nup159, Mlp8, Rco1, Rlp7, and Mlp1 (see Table 1 and supplemental Table S3), suggesting that these proteins may be potential Dun1 substrates. Because the consensus phosphorylation sites for Rad53 or Dun1 are not well defined (10, 38), the establishment of bona fide substrates of Rad53 and Dun1 requires further studies.

Protein-protein interactions often mediate substrate phosphorylation by protein kinases in vivo. A summary of the known physical interactions between the kinase-specific targets identified here is shown (see Fig. 2C). Among Mec1 and Tel1 specific targets, Taf9, Taf10, and Taf12 are components of the TFIIID complex, which interacts with Sp7 in the SAGA complex and the Paf1 complex including Cdc73 and Hpr1. Dad1 and Dad3 are components of the Dam1 complex involved in chromosome segregation. Rco1, Ea3, and Sin3 are subunits of the histone deacetylase Rpd3S complex. Dpb4 and Isw2 are involved in chromatin remodeling, along with several other histone remodelers (see Table 1). Thus there appear to be extensive physical associations between Mec1/Tel1-specific targets. Interestingly, multiple checkpoint kinases can also phosphorylate the same protein complex. Rpd3S is a histone deacetylase complex whose Rco1 subunit shows Dun1-specific phosphorylation, whereas Sin3 and Ea3 subunits undergo Mec1/Tel1-dependent phosphorylation. Moreover, Mec1/Tel1 and Rad53 appear to phosphorylate different residues of Mrc1, Rtt107, and Mcd1. Thus the large-scale quantitative MS screens have uncovered a complex phosphorylation regulation of these targets.

TABLE 1

| Functions | Mec1/Tel1 | Rad53 | Dun1 |
|-----------|-----------|-------|------|
| DNA damage checkpoint | Rfa1, Rfa2, Psy4, Mec1, Mrc1, Rad9, Rad53 | Mrc1, Dun1, Rx1, Rad53 | Dun1, Rnr3 |
| RNA PolII transcription | Rdb1, Ta9, Ta10, Ta12, Sp7, Cdc73, Hpr1, Dst1, Toa2, Spn1 | Rph1, Hhf1 | Rco1 |
| Histone modification | Sin3, Ea3, Hta1 | Hpc2, Esc1 | Hpc2 |
| Chromatin remodeling | Swi3, Ioc2, Iss2, Dpb4, Ies4 | Eap1 | Npl3 |
| mRNA processing | Prp19, Cbf5, Pwp2, Ysh1, Abd1, Bcd1 | Mcd1, Ycg1, Cep3 | Mcd1, Ycg1, Cep3 |
| Cell cycle regulation | Sap185, Ndd1, Bir1, Dad1, Dad3, Chfl, Mcd1 | Rad54, Rti107, Rad27, Dbf4, Pol1 | Nup1, Nup2, Nup60 |
| DNA replication and repair | Pol31, Msh6, Rtt107, Rad23, Rad26, Srx4, Sir4, Sum1 | Nup1, Nup2, Nup60 | Gle1, Nup159, Mlp1 |
| Nuclear transport | Nup60 | Plm2 | Plm2 |
| Stress response | Dak1, Hsp26, Hsp12, Zpr1, Asg1, Nma111 | Pho81, Fun30, Nug1 | Ecm21, Sec3, Mrp8 |
| Others | Lsc2, Spe4, Asc2, Met12, Pfk1 | | |

Identify and Characterize Substrates in DNA Damage Response
Mec1/Tel1 Substrates May Transiently Associate with RPA via Chromatin—Mec1 is recruited to the site of DNA damage via its interaction with RPA-coated single-stranded DNA (17, 18), which may mediate the phosphorylation of Mec1 and/or Tel1 substrates in vivo. To test this hypothesis, a quantitative MS approach was used to identify RPA-associated proteins (see Fig. 3A). Silver staining of the one-step purified RPA sample reveals two specific bands corresponding to Rfa1 and Rfa2 (see Fig. 3B). No other specific bands could be detected by silver staining, suggesting that other RPA-associated proteins are less abundant and likely to interact with RPA in a more transient manner. To identify the lower abundant RPA-associated proteins, the remainder of the sample was analyzed directly using quantitative MS. Three examples of MS data of a representative peptide from Mec1, Taf14, and Isw2 are shown (see Fig. 3C). In each case, it is identified as exclusively...
labeled by heavy Lys/Arg and is thus from the RPA purification, suggesting that it is specifically associated with RPA. The same criterion is used to determine other RPA-associated proteins (see Fig. 3D). Among the RPA-specific associated proteins, subunits of histones were found along with subunits of chromatin remodelers including Isw1, Isw2, Ies3, Ino80, Taf14, Rtt102, and Rsc8 (49). These findings suggest that RPA may associate with these proteins via DNA/nucleosome structures with DNA possibly bridging their interactions. As expected, known direct RPA-binding proteins including Mec1, Dna2, and Rad52 are also detected as RPA-specific. Interestingly, there are extensive physical interactions between the RPA-specific associated proteins identified here (indicated by open circles in Fig. 3D) and the Mec1/Tel1-specific targets identified in the large-scale MS screens (indicated by a star). Solid lines indicate known physical interactions between them. Filled circles indicate the proteins found as both RPA-specific and Mec1/Tel1 substrates. Open circles indicate those only found as RPA-specific associated proteins.

RPA, TFIID-SAGA, and Chromatin Remodeling Complexes Undergo MMS-induced Phosphorylation of SQ/TQ Sites—To further examine the role of RPA in mediating the phosphorylation of Mec1/Tel1 substrates, we chose to purify RPA,
TFIID-SAGA, and chromatin remodeling complexes including ISW1, ISW2, SWI/SNF, RSC, SWR1, and INO80 from both untreated and MMS-treated cells. Using SILAC and quantitative MS, MMS-induced phosphorylation of the above protein complexes were identified and quantified. To address whether there are any MMS-induced changes in protein abundance, the immobilized metal affinity column-unbound fractions containing the unphosphorylated peptides were also analyzed. Unless noted otherwise, no MMS-induced change in the abundance of these proteins was found (results not shown), thus the observed changes in phosphorylation are not due to protein abundance changes. Because Mec1 and Tel1 prefer to phosphorylate SQ/TQ sites, MMS-induced SQ/TQ phosphorylation of these proteins are shown, together with results from the above large-scale MS screens (see Fig. 4 and supplemental Table S6).

The analysis of purified RPA identifies the MMS-induced SQ/TQ phosphorylation of all three subunits of RPA as well as its associated proteins including Mec1, Msh6, Ies1, and Dna2.
The analysis of purified TFIID-SAGA identifies the same MMS-induced SQ/TQ phosphorylation sites of Taf9, Taf10, Taf12, and Hsp26 as found in large-scale MS screens, in addition to a new MMS-induced SQ/TQ phosphorylation of Sgf73 (see Fig. 4B and also supplemental Table S6). Among the chromatin remodelers, analysis of the ISW1 complex reveals two MMS-induced SQ/TQ phosphorylation sites of the Ioc2 subunit. In addition to the known phosphorylation of histone H2A on Ser129, a new MMS-induced TQ phosphorylation of H2B (Thr129) is identified (see Fig. 4C). For the ISW2 complex, the same SQ/TQ phosphorylation sites on Isw2, Dpb4, and Rfa1 are found to be both MMS-induced and Mec1/Tel1-specific (see Fig. 4D). Thus, the ISWI family of chromatin remodeling complexes appears to be phosphorylated by Mec1 and Tel1.

The INO80 and SWR1 complexes are known to be involved in the DNA DSB response and recruited to the site of DNA damage (50). Ies4, a subunit of the INO80 complex, was previously found to be a target of Mec1 and Tel1 (26). Our analysis of the INO80 complex identifies MMS-induced SQ/TQ phosphorylation of Ino80 and Ies1, in addition to Ies4 (see Fig. 4E). Furthermore, several MMS-induced SQ/TQ phosphorylation sites are found in proteins that may be transiently associated with INO80, including Bdf1, Rfa1, Leo1, Msh6, Hta1, Prp19, Dpb4, and Spn1. SWR1, another INO80 family chromatin remodeling complex in yeast, was also purified and analyzed (49). Analysis of SWR1 identifies the same SQ/TQ phosphorylation of Bdf1 and a new MMS-induced SQ/TQ phosphorylation of Swr1 (see Fig. 4F). RSC is an essential chromatin remodeling complex in yeast (49). Previous studies have implicated its role in DNA DSB response (51–54). MMS-induced SQ/TQ phosphorylation on five subunits of RSC, including Sth1, Rsc2, Rsc4, Rsc30, and Arp7, are identified from purified RSC (see Fig. 4G). Again, MMS-induced SQ/TQ phosphorylation of RSC-associated proteins including Rfa1, Dpb4, Hta1, Hsp26, Leo1, and Yta7 are found. Analysis of purified SWI/SNF identifies MMS-induced SQ/TQ phosphorylation of Swi3, as well as Arp7, Prp19, and H2A (Fig. 4H). Taken together, both INO80 and RSC families of chromatin remodelers appear to be targeted by Mec1 and Tel1, and they show extensive interactions with histones, RPA, and each other.

In summary, the results from the large-scale MS screens and the analysis of purified protein complexes show that RPA, TFIID-SAGA, histones, and multiple chromatin remodeling complexes form a large protein interaction network targeted by Mec1 and Tel1. Although using purified protein complexes will give more information about their phosphorylation, a significant percentage of these proteins are already identified from the large-scale MS screens; illustrating the scope and depth of these screens (see Fig. 4).

Involvement of Chromatin Remodeling Complexes in the DNA Damage Response—To further examine the role of RPA-associated proteins, in particular the chromatin remodeling complexes, in the DNA damage response, we examined the DNA damage sensitivities of null mutants of non-essential subunits of these complexes. DNA damage hypersensitivities have been reported previously for mutations to various subunits of RSC and INO80 (51, 54). A total of 35 deletion mutants are examined (see supplemental Fig. S1). Among them, deletion of TAF14, ADA1, SPT7, SPT20, ARPS, ARP8, SWI3, SNF5, and SNF6 causes strong sensitivities to both MMS and hydroxyurea (see Fig. 5). On the other hand, mutants with deletion of ISW1 or ISW2 have relatively minor DNA damage sensitivities under the same conditions. Ada1, Spt7, and Spt20 are subunits of the SAGA complex. Thus it is not surprising that similar patterns of DNA damage hypersensitivity are observed for mutants devoid of these proteins. Arp5 and Arp8 are subunits of the INO80 complex and their mutation also causes similar DNA damage hypersensitivities. Last, Swi3, Snf5, and Snf6 are subunits of the SWI/SNF complex and their mutations lead to strong DNA hypersensitivities.
damage hypersensitivities. These observations are consistent with the idea that these chromatin remodelers are involved in the DNA damage response.

**DISCUSSION**

The DNA damage checkpoint regulates cellular responses to DNA damage (1–3), yet its molecular basis is far from being understood. Therefore it is necessary to identify and characterize substrates of the checkpoint kinases. In the present study, several quantitative MS screens are used to determine the kinase-substrate relationships in the DNA damage response on a proteome-wide scale. This leads to the identification of 53 new targets in addition to 34 previously known substrates of these checkpoint kinases; thus greatly expanding the present knowledge. Quantitative analysis of RPA-associated proteins uncovers a large protein-protein interaction network involving RPA, histones, TFIID-SAGA, and multiple chromatin remodeling complexes. In particular, the analysis of chromatin remodeling complexes identifies them as a new family of Mec1 and Tel1 substrates. These results strongly support that RPA-associated chromatin may mediate the phosphorylation of Mec1 and Tel1 substrates in vivo. The identification of multiple chromatin remodelers as Mec1 and Tel1 substrates also raises the possibility that these proteins, together with other Mec1 and Tel1 substrates, could contribute to the genome maintenance functions of Mec1 and Tel1, which requires further functional studies with a more focused scope (15, 55).

Global analysis of the substrates of the DNA damage checkpoint kinases reveals several interesting features. First, Mec1/Tel1, Rad53, and Dun1 have functionally distinct targets in vivo. Although most Mec1- and Tel1-specific targets participate in major DNA and RNA metabolisms such as mRNA transcription, DNA replication and repair, many targets of Rad53 and Dun1 function in nuclear transport. Second, there exists a complex phosphorylation regulation of some of these kinase targets. For example, phosphorylation of Mrc1, Mcd1, Rfx1, Hpc2, and Rtt107 are controlled by multiple checkpoint kinases. In addition, phosphorylation of multiple subunits of the same protein complex is observed for TFIID-SAGA, INO80, RSC, and others. The complex phosphorylation regulation identified here should help to direct functional studies of these proteins in future studies. Third, the identification of RPA-associated proteins suggests that RPA-associated chromatin mediates Mec1 and Tel1 signaling in vivo. This notion is reinforced by the observation that phosphorylation of Rfa1 and H2A is often detected in multiple protein complexes including RPA itself, INO80, ISW2, and RSC complexes (see Fig. 4). The extensive physical associations between RPA and chromatin remodeling complexes, which may be mediated by DNA structures, are also consistent with the known recruitment of INO80, RSC, and SWR1 to the site of DNA damage (51–54). On the other hand, not all Mec1/Tel1 substrates associate with RPA. They could be phosphorylated in an RPA-independent manner, which remains to be determined. Finally, aside from chromatin remodeling complexes, Mec1 and Tel1 also control the phosphorylation of proteins involved in DNA replication, DNA repair, mRNA transcription and processing, chromosome segregation, and many others (see Table 1). In most cases, their phosphorylation by Mec1 and Tel1 has not been previously described. We emphasize here that like chromatin remodelers, they are also excellent candidates for further investigations as bona fide substrates of Mec1 and Tel1. The findings here thus open many new opportunities for further mechanistic and functional studies.

The DNA alkylating agent MMS used here likely causes DNA damage throughout the genome and can broadly induce phosphorylation of many targets of the DNA damage checkpoint kinases. Although the use of MMS treatment in this study allows us to identify a broad spectrum of potential substrates of the checkpoint kinases, it is necessary to determine whether distinct substrates are phosphorylated in a DNA damage-specific and temporal manner in future studies. The present study thus provides a starting point for many future investigations. Finally, the quantitative MS technology used here is expected to be widely applicable to study numerous phosphorylation-mediated processes in cells on a proteome-wide scale.

Acknowledgments—We thank Drs. Chris Putnam and Richard Kolodner for providing yeast deletion strains obtained from Open Biosystems.

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