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The Yeast DNA Damage Checkpoint Kinase Rad53 Targets the Exoribonuclease, Xrn1

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ABSTRACT The highly conserved DNA damage response (DDR) pathway monitors the genomic integrity of the cell and protects against genotoxic stresses. The apical kinases, Mec1 and Tel1 (ATR and ATM in human, respectively), initiate the DNA damage signaling cascade through the effector kinases, Rad53 and Chk1, to regulate a variety of cellular processes including cell cycle progression, DNA damage repair, chromatin remodeling, and transcription. The DDR also regulates other cellular pathways, but direct substrates and mechanisms are still lacking. Using a mass spectrometry-based phosphoproteomics screen in Saccharomyces cerevisiae, we identified novel targets of Rad53, many of which are proteins that are involved in RNA metabolism. Of the 33 novel substrates identified, we verified that 12 are directly phosphorylated by Rad53 in vitro: Xrn1, Gcd11, Rps7b, Ded1, Cho2, Pus1, Hst1, Snv2, Set3, Snu23, Alb1, and Scp160. We further characterized Xrn1, a highly conserved 5’ exoribonuclease that functions in RNA degradation and the most enriched in our phosphoproteomics screen. Phosphorylation of Xrn1 by Rad53 does not appear to affect Xrn1’s intrinsic nuclease activity in vitro, but may affect its activity or specificity in vivo.

KEYWORDS DNA Damage Response checkpoint Xrn1 Rad53 phosphoproteomics

Cells incur DNA damage from both endogenous and exogenous sources. To ensure faithful cell division, the highly conserved DNA damage response (DDR) pathway monitors genomic integrity and protects against genotoxic stresses. Genome instability is a common characteristic of aging cells and cancer cells and components of the DDR machinery are often mutated in cancer (Jackson and Bartek 2009; Negrini et al. 2010; Ashworth et al. 2011). In Saccharomyces cerevisiae, DNA damage activates the sensor kinases, Mec1 and Tel1 (ATR and ATM in human, respectively) (Melo and Toczyski 2002; Ciccia and Elledge 2009; Blackford and Jackson 2017). The response is further amplified by activation of the effector kinases, Rad53 and Chk1, to regulate a variety of cellular processes including cell cycle progression, DNA damage repair, chromatin remodeling, and transcription.

The DDR induces a number of physiological changes within the cell, including changes in gene expression and protein levels. At the gene expression level, microarray-based transcriptomic analyses identified transcripts that are up- or down-regulated in a Mec1/Tel1 dependent manner (Jelinsky and Samson 1999; Gasch et al. 2001). In addition, proteomics analyses identified targets of ATM/ATR through the enrichment of phosphopeptides (Matsuoka et al. 2007; Smolka et al. 2007). Many of these DDR regulated-transcripts and protein targets have known roles in DNA damage repair and cell cycle regulation, but the significance of other targets has not been characterized. In addition, the DDR affects other cellular pathways for which direct targets are not known. For example, Mec1 has been shown to induce expression of genes involved in carbohydrate metabolism and reactive oxygen species (ROS) detoxification, and down regulates the expression of ribosomal protein genes in DNA damage (Gasch et al. 2001). Putative substrates of ATM and ATR include proteins involved in RNA modification and cell structure (Matsuoka et al. 2007). Several studies also reveal the involvement of ATM in insulin signaling, AKT signaling, and the pentose phosphate pathway (Khalil et al. 2011; Cosentino et al. 2011; Fraser et al. 2011). Thus, novel substrates of the DDR remain to be discovered.
One area of regulation that is not well understood is the direct effect of the DDR on post-transcriptional regulation of gene expression. As an intermediate between genes and proteins, altering the abundance of mRNAs would effectively affect protein levels as well. One of the key players of mRNA dynamics is Xrn1. Xrn1 is a conserved 5′-3′ exoribonuclease that preferentially degrades 5′ monophosphorylated single-stranded RNA (Jones et al. 2012; Nagarajan et al. 2013). This arises in the cell when mRNAs are decapped prior to degradation or during processing of rRNA or tRNA (Chernyakov et al. 2008; Whipple et al. 2011; Harigaya and Parker 2012; Braun et al. 2012; Wu and Hopper 2014). Xrn1 is a component of the cytoplasmic processing (P) bodies and stress granules that are involved in mRNA sequestration and decay, and is responsible for the majority of mRNA degradation in the cell (Stevens et al. 1991; Bashikrov et al. 1997; He et al. 2003; Kedersha et al. 2005; Newbury 2006; Lindahl et al. 2009). Involvement of Xrn1 in DNA damage repair comes from the observation that xrn1Δ cells are sensitive to DNA damaging agents, but the mechanism for how this occurs is not known (Page et al. 1998; Manfrini et al. 2014).

Here we identified 33 novel substrates of Rad53 using a phosphoproteomic screen, and confirmed that Rad53 directly phosphorylates 12 of them in vitro. Many of these substrates are involved in mRNA and rRNA processing and turnover. Specifically, we show that Rad53 phosphorylates Xrn1 in vivo and in vitro, linking the DNA damage response to the regulation of RNA metabolism. We found that phosphorylation does not affect the nucleolytic activity of Xrn1. Mutations in the C terminus of Xrn1, where this occurs is not known (Page et al. 1998; Manfrini et al. 2014).

Mass spectrometry

Cells were grown to mid-log phase in C-lysine-arginine media, supplemented with heavy labeled lysine and arginine or unlabeled control lysine and arginine at a concentration of 0.06 mg/mL. Cell pellets were washed three times with a denaturing urea buffer (8 M urea, 0.1 M Tris pH 8, 150 mM NaCl, 1 Roche mini protease inhibitor tablet without EDTA/10 mM, 10 mM sodium butyrate and 10 mM nicotinamide) in a BioSpec bead-beater. Extracts were treated with 1 M TCEP (Sigma C4706-2), then 0.5 M iodoacetamide (Sigma L1149-5G, prepared fresh in water), followed by 10 mM DTT to quench excess iodoacetamide. Samples were diluted ~4 fold (to less than 2 M urea) with 0.1 M Tris pH 8 and digested overnight with 1 mg trypsin to 100 mg protein (Promega V511A, dissolved in 50 mM acetic acid). TFA was added to a final concentration of 0.3–0.1% TFA and the peptides were loaded onto the Sep Pak C18 column, washed, and eluted with 1 mL 40% ACN/0.1% TFA prior to lyophilization.

For protein abundance analysis samples were resuspended in 90% HILIC buffer B and injected onto a TSKgel amide-80 column (Tosoh Biosciences, 2.0 mm × 15 cm packed with 5 μm particles) equilibrated with 10% HILIC buffer A (2% ACN, 0.1% TFA) and 90% HILIC buffer B (98% ACN, 0.1% TFA) using an AKTA P10 purifier system. The samples were then separated by a one-hour gradient from 90% HILIC buffer B to 55% HILIC buffer B at a flow rate of 0.3 mL/min. Fractions were collected every 1.5 min, combined into 10 fractions, evaporated to dryness, and reconstituted in 0.1% formic acid for mass spectrometry analysis. For phosphorylation analysis, phosphopeptides were further purified using Fe3+-IMAC as described previously (Costa et al. 2015). Purified phosphopeptides were desalted using C18 STAGE tips, evaporated to dryness, and reconstituted in 0.1% formic acid for mass spectrometry analysis.

Mass spectrometry samples were analyzed in technical duplicate on a Thermo Scientific LTQ Orbitrap Elite mass spectrometry system equipped with a Proxeon EASY nLC 1000 ultra high-pressure liquid chromatography and autosampler system. Raw mass spectrometry data were analyzed using the MaxQuant software package (version 1.3.0.5) (Cox and Mann 2008). Data were matched to SwissProt reviewed entries for S. cerevisiae in the UniProt protein database. MaxQuant was configured to generate and search against a reverse sequence database for false discovery rate calculations.

Western Blot

Standard TCA precipitations were preformed to extract proteins. Samples were resuspended in SDS-PAGE sample buffer, boiled for 10 min, and supernatants were transferred to new tubes. Proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore). Western blotting was performed with the following antibodies. Primary antibodies: α–GF (Clontech #632381); α–Rad53 (Abcam ab104232); α–Flag (Sigma-Aldrich, F3165); α–Myc (BioLegend, #626802); α–Dbf4 (Santa Cruz Biotechnology sc5705); α–eIF2α S51-P (Cell Signaling Technology #97215). All primary antibodies were used at 1:1,000. Secondary antibodies: gom (BioRad #172-1011), grr (BioRad #170-6515), dog (Santa Cruz Biotechnology sc2033) were used at 1:10,000. Blots were visualized by film or LiCor’s Odyssey Imaging System.
In vitro kinase assay
Cells were collected, washed with water, and resuspended in lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA) supplemented with 0.174 mg/ml PMSF, 5 mM sodium fluoride, 10 mM sodium orthovanadate, 5 mM 2-phosphoglycerol, 1 μg/ml leupeptin, 1 μg/ml bestatin, and 1 μg/ml pepstatin. Cells were lysed with bead beater and separated from beads. Extracts were clarified by centrifugation at 4°C (2X). Extracts were quantitated using a BCA protein assay kit (Pierce). Immunoprecipitations were carried out in volumes of 500-600 μl with 0.5 μl of anti-GFP antibody (Abcam, ab190) overnight. Precipitated protein complexes were then recovered with 20 μl Protein A beads (Invitrogen, Dynabeads) for 40 min. Beads were washed with lysis buffer with inhibitors 3X followed by two washes with kinase buffer (20 mM Tris-HCl pH 7.5, 20 mM MgCl2, 2 mM MnCl2, 1 mM DTT, 25 μM ATP). Beads are then incubated with γ-32P-ATP (Perkin Elmer) plus kinase buffer pre-incubated with 150 nM purified activated Rad53 (30 min at 30°C) or no Rad53 for 60 min at 30°C. Beads were then washed with lysis buffer 3X to remove kinase, and eluted in 40 μl SDS-PAGE sample buffer with 0.1 M DTT at 65°C for 10 min with periodic agitation. Samples were boiled for 5 min prior to loading onto SDS-PAGE gel and transferred to PVDF membrane. Blots were visualized by phosphorimagery screen, scanned with a Typhoon phosphorimager (GE Healthcare), and quantified with ImageJ. Westerns were performed to determine protein loading and visualized by LiCor’s Odyssey Imaging System.

Nuclease assay
Nuclease assay was modified from Pellegrini et al. (2008). pRS303, which contains a T7 promoter, was linearized with AlwNI (NEB) and used as a substrate for in vitro transcription by PCR using T7 RNA polymerase (NEB) as follows: 5 μl T7 buffer; 22 μl H2O, 2 μl ATP, 2 μl UTP, 2 μl CTP, 0.5 μl GTP, 0.5 μl GTP*, 3 μl GMP, 2 μl RNasefree (Ambion), 7 μl DNA. Reactions were incubated at 60°C for 10 min and cooled to room temperature. 2 μl T7 RNA polymerase was added and reactions were incubated at 37°C for 4 hr. After addition of 2 μl DNase, reactions were incubated for another 30 min RNAs were purified with G50 columns twice to remove unincorporated labels, and purified RNA transcripts were added to nuclease reaction buffer (30 mM Tris HCl, pH 8.0, 2 mM MgCl2, 50 mM NaCl, 0.5 mM DTT, 20 μg/ml BSA). Flag purified Xrn1 were added and reactions were allowed to proceed at 37°C. Samples were collected after 15, 30, and 60 min samples were quenched with equal amount of 6X urea loading buffer, incubated at 80°C for 10 min, followed by incubation on ice. Samples were loaded onto 10% TBE-Urea gels (Invitrogen; Novex TBE-Urea Gels), and ran at 180V for 35 min. The gels were visualized by a Typhoon Phosphoimager (GE Healthcare).

Northern Blot
RNA was extracted with Qiagen RNeasy kit. Samples were run on 1% agarose gel containing 1.2 M formaldehyde and transferred to Amersham HYBOND+ Nylon membrane overnight, then crosslinked with Stratagene Stratalinker at 254 nm setting. Radioactive probe was made using PCR amplified 5.85 rDNA, Prime It II labeling kit and dCTP* (Perkin-Elmer). Blots were hybridized with Amersham Alkaline Phosphatase Hybridization as indicated, and visualized with a Typhoon phosphorimager.

S35-methionine incorporation
Cells were grown to mid-log phase in C-methionine media and each culture was divided in two. To one half of the cultures, 4-NQO was added to 2 μg/mL for 15 min. Subsequently, S35-methionine (Perkin Elmers) was added and samples taken at 1 min and 5 min. Cells were wash with water, pelleted, and flash frozen on dry ice. Frozen pellets were resuspended in 1X sample buffer and boiled at 100°C for 10 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and visualized by film.

Ribosome Profiling
Illumina’s ARTseq Ribosome Profiling Kit for yeast (RPYS12116) was used for ribosome profiling experiments. The size and integrity of the RNA samples were confirmed by Agilent’s Bioanalyzer and submitted to UCSF Center for Advanced Technology for sequencing.

qPCR
RNA was extracted with Qiagen RNeasy kit and DNase treated with Zymo “DNA-free RNA” kit. cDNA library was generated with BioRad’s iScript RT Supermix for RT-qPCR as instructed and qPCR reaction was performed with BioRad’s SsoFast EvaGreen Supermix as instructed. Primers used: GAL1_FW: TGGTTGTAAACATGGCGGTA; GAL1_RV: GGCGGTTTCAAATCTGTTA; ACT1_FW: AGGTATCA-TGTCGCTATGGG; ACT1_RV: ACAAGGCAAAACGGCTTG; Hst3qPCR_FW: CACAAGTCTATTGGCCG; Hst3qPCR_RV: CTGCTCCAGGAAAAGTCTG.

Spot test
Yeast strains were inoculated into 3-5 ml YPD grown overnight with aeration at 30°C. Tenfold dilution series were set up in 96-well plates, and 3 μl aliquots of the dilution series were transferred to plates. Plates were incubated 2-3 days at 30°C until colonies formed and then were photographed.

Data availability
Strains and plasmids are available upon request (Table S1). Supplemental material is available on figshare. Supplemental material of Supplemental data contains a table and four figures. Supplemental Methods contains an additional Materials and Methods section. File S1 contains the phosphopeptidase dataset comparing GAL1pr-RAD53 TEF1pr-DUN1 and rad53Δ cells. File S2 contains the SILAC protein abundance dataset comparing GAL1pr-RAD53 TEF1pr-DUN1 and rad53Δ cells. File S3 contains the ribosomal profiling data comparing rad53Δ cells in HU and rad53Δ cells untreated. File S4 contains the ribosomal profiling data comparing wild type cells in HU and wild type cells untreated. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7176383.

RESULTS

Identification of Rad53 substrates
While previous screens for targets of the DDR kinases, Mec1, Tel1, and Rad53, identified novel targets, a number of known substrates of Rad53, such as Sld3 and Ndd1, were not identified in these screens (Smolka et al. 2007; Chen et al. 2010; Lopez-Mosqueda et al. 2010 p.; Zegerman and Dillfe 2010; Edenberg et al. 2014; Zhou et al. 2016). Therefore, we sought to improve the limit of detection of Rad53 substrates by mass spectrometry by designing a system to saturate the phosphorylation of Rad53 substrates. Most Rad53 substrates that have been characterized have a large number of phosphorylated residues. However, examination of the phosphoproteins by electrophoresis shows a heterogeneous set of shifted bands, suggesting that the sites are not saturated in vivo. First, we expressed Rad53 using the inducible galactose (GAL1) promoter to allow for transient overexpression. We found that constitutive...
Figure 1 Phosphoproteomic screen for Rad53 targets. A. Western blots showing mobility shift of Rad53 substrates: Sld3, Ndd1, and Dbf4 in the strain indicated. pppΔ refers to ptc3Δ ptc2Δ pph3Δ. Sld3 and Ndd1 are respectively tagged with MYC and Flag epitope tags and visualized with antibodies against the tag. Dbf4 is visualized with antibodies against Dbf4. B. Schematic of the experiment for phosphopeptide mass spectrometry. Both strains are in the sml1Δ background. Asynchronous yeast cultures were treated with galactose for 1 hr to induce Rad53 expression followed by the addition of 2 μg/mL 4-NQO for 15 min before harvesting. C. Comparison of fold changes for a phosphopeptide between replicates. Box region shows phosphopeptides that are ≥25 fold enriched in both replicates in GAL1pr-RAD53 TEF1pr-DUN1 strains. D. Enriched biological process-associated GO terms using DAVID for the top 54 proteins.
overexpression of Rad53 by the TEF1 or ADH1 promoters is detrimental to cells, in agreement with published data (Sun et al. 1996; Marsolier et al. 2000). Additionally, Rad53 in these cells runs at a higher mobility on SDS-PAGE gels indicating a constitutive activation of the checkpoint (data not shown). In addition to Gal1pr-Rad53, we also placed Dun1, a checkpoint kinase that requires Rad53 for activation, under the TEF1 constitutive promoter, and we deleted PTC2, PTC3, and PHH2, which are phosphatases known to counteract checkpoint phosphorylation (Leroy et al. 2003; O’Neill et al. 2007; Travesa et al. 2008). We expect this to stabilize substrates in the phosphorylated state.

Driving Rad53 expression from the GAL1 promoter leads to phosphorylation of Rad53 substrates Sld3, Ndd1, and Dbf4, even in the absence of DNA damage, indicating that overexpressing Rad53 activates the checkpoint independently of DNA damage (Figure 1A; compare lanes 19 to 20). In contrast, deletion of the phosphatases alone does not lead to phosphorylation of Rad53 substrates in the absence of DNA damage (Figure 1A; lanes 3 and 4). Furthermore, overexpression of Rad53 leads to modification of Rad53 substrates specifically, and not a general DNA damage response. For example, H2AX, a substrate of Mec1, is not significantly phosphorylated when Rad53 is overexpressed (data not shown). In general, Rad53 targets appear much more highly phosphorylated in this system than in wild type cells (compare lane 2 to lane 20 for each substrate). This is consistent with the hypothesis that most Rad53 substrates are only targeted on a subset of their sites upon DNA damage.

For the screen, we used a very short exposure to the DNA damaging agent, 4-nitroquinoil oxide (4-NQO), to avoid accumulation of phosphorylation events downstream of Rad53 due to alteration of cell cycle position (Figure 1B). In contrast, previous proteomics screens for Rad53 substrates treated cells for 3-3 hr in methyl methanesulfonate (MMS) (Smolka et al. 2007; Chen et al. 2010; Zhou et al. 2016). 4-NQO, a UV mimetic drug that distorts DNA by the addition of bulky adducts, rapidly activates the DNA damage checkpoint as detected by Rad53 mobility shift. Cells overexpressing Rad53 and Dun1 (GAL1pr-RAD53 TEF1pr-DUN1) and rad53Δ cells were treated with 4-NQO for 15 min before harvesting. Proteins were extracted, digested with trypsin, and phosphopeptides were enriched using a column based method and identified by mass spectrometry. Two replicates of each experiments were performed. In total, we identified over 29,000 phosphorylated peptides. After filtering out missing values, we quantified more than 13,000 phosphopeptides in all four samples (Figure 1C and Supplementary File S1). In addition to the expected Dun1 phosphopeptides, an Xrn1 exoribonuclease phosphopeptide was highly enriched (Figure 1C). Xrn1 exoribonuclease cDNA was strongly reduced by 4-NQO, but not MMS (Figure 3B and C). However, this was unaffected by deletion of Gcn2, RAD53, or MEC1 and TEL1, suggesting it might represent a physical block to the translation machinery due to mRNA damage caused by 4-NQO (Figure 3C and D). To control for any potential damage to mRNA caused by chemical agents, we use the cdc13-1 mutation, which, at the non-permissive temperature, causes the accumulation of single-stranded DNA near the telomeres that activates the DNA damage checkpoint (Garvik et al. 1995; Lydall and Weinert 1995). This mutation caused very little effect on methionine incorporation, although the heat shock required to inactivate the cdc13-1 allele induced some eIF2 alpha phosphorylation. However, the presence of the cdc13-1 allele did not further increase the level of phosphorylated eIF2 alpha (Figure 3E).

As we saw no effect of the DDR on the global translation rate, we performed ribosome profiling to determine whether specific transcripts, or classes of transcripts, were being regulated by Rad53. In humans, DNA damage is thought to reprogram translation to allow the selective translation of a subset of messages (Jousset et al. 2003; Holcik and Sonenberg 2005; Kruiswijk et al. 2012). We used ribosome profiling to examine ribosome occupancy in Rad53Δ vs. rad53Δ cells to determine whether the DNA damage checkpoint pathway affected translation directly. To avoid any possible negative effect of 4-NQO on mRNA structure, we used hydroxyurea (HU) to deplete the dNTP pool, which causes accumulation of ssDNA and activation of the DDR (Santocanale and Diffley 1998). Cells were arrested in G1 and released in HU for 45 min, and both total mRNA and ribosome-protected footprints were sequenced. We examined the translation rate for each message by normalizing ribosome-bound transcript to total mRNA for each gene. Figure 3E compares this translation rate for each transcript between rad53Δ vs. wild type (y-axis) as compared to the mean transcript level (x-axis). We found no significant evidence for translational regulation by Rad53 after normalization of RAD53-dependent transcriptional effects in either the presence or absence of HU (Figure 3E; Supplementary File S3 and S4). Thus, although there is an enrichment of proteins involved in cytoplasmic RNA maintenance and degradation in our list of Rad53-dependent phosphopeptides, Rad53 does not seem to be generally required for normal RNA processing or translation.
Xrn1 is an in vitro and in vivo substrate of Rad53

We identified 54 proteins corresponding to phosphopeptides that were at least 25-fold enriched in GAL1pr-RAD53 compared to rad53Δ cells (Table 1). A 25-fold enrichment cutoff was selected arbitrarily for a workable set of protein candidates to follow up in our in vitro kinase assay. 21 of these 54 proteins are previously known or identified substrates of Rad53 and 33 of these are novel substrates as indicated in bold (Table 1). The observation that some of the identified phosphopeptides are within previously known Rad53 substrates validates our screening technique. For example, the nucleoporins, Nup1, Nup2, and Nup60 are often observed in screens for Rad53 and DDR targets, which points to a role of the DDR in regulating mRNA export, as suggested by Zhou et al. 2016 (Smolka et al. 2007; Chen et al. 2010; Zhou et al. 2016). Pin4, on the other hand, is a known DDR target that is phosphorylated by Mec1 and Tel1 (Pike et al. 2004). Zhou et al., 2016 identified Pin4 phosphopeptides enriched in wild type vs. rad53Δ cells treated with MMS. Our data also suggest that Pin4 is also a direct substrate of Rad53 (Table 1 and Figure 4). Ded1, on the other hand, was only twofold enriched (L/H ratio of 0.4969) in the published rad53Δ/wild type dataset and fell below the fourfold enrichment cutoff that was set (Zhou et al. 2016). Comparing our dataset to those of Smolka et al., 2007, Chen et al., 2010, and Zhou et al., 2016 as shown in Table 1, there are few proteins identified in three or more of the four datasets. This suggests that each dataset is subject to its own caveats, reinforcing the value of conducting this screen multiple times in subtly different ways. Furthermore, given that our screen was done with 4-NQO, as opposed to MMS, and our cells were subjected to only fifteen minutes of DNA damage vs. two to three hours, we would expect some differences between the datasets. For example, our analysis may miss sites that accumulate over time, but will also avoid potential indirect effects associated with RAD53-dependent cell cycle arrest after long time points. To this end, we followed up with in vitro kinase assays to determine which substrates were direct targets of Rad53 or Dun1 (described below).

In 8/54 cases, multiple phosphosites were identified for the same substrate. The enrichment of these peptides is not due to increased protein abundance in GAL1pr-RAD53 cells, as indicated by the SILAC ratio between GAL1pr-RAD53 and rad53Δ. In order to determine whether these were direct substrates of Rad53 or Dun1, we examined their phosphorylation by these kinases in vitro. Activated epitope tagged Rad53 and Dun1 were purified from cells treated with 4-NQO. As a control for Dun1 phosphorylation, we used the Dun1 substrate Sml1. Sml1 was strongly phosphorylated by Dun1 and poorly phosphorylated by Rad53 in vitro (Figure 4; top left panel). We purified 44 substrates that were available in the GFP collection and found that 21 were moderate to strong Rad53 substrates in vitro. No substrate tested was phosphorylated by Dun1, suggesting that this kinase may have a much more limited set of targets (Figure 4). Specifically, Xrn1 is a strong in vitro substrate of Rad53. Xrn1 was previously found in the set of over 13,000 peptides identified in a SILAC screen comparing phosphopeptides between rad53Δ cells and wild type cells treated with MMS (Zhou et al. 2016). However, Xrn1, along with a very large number of other proteins, was not within their fourfold enrichment cutoff and was therefore not considered significant. In our phosphoproteomics screen, Xrn1 is over 200-fold enriched in GAL1pr-RAD53 vs. rad53Δ cells.

Because we identified Xrn1 in a screen in which Rad53 was overexpressed, we wished to confirm Xrn1 phosphorylation in vivo. We epitope tagged Xrn1 on its C terminus with either GFP or Flag and examined its electrophoretic mobility after treatment with damaging agents. Phosphorylation of Xrn1 is DNA damage-dependent in vivo, as detected by a mobility shift. Consistent with Xrn1 being a direct substrate of Rad53, its modification in DNA damage is dependent on Rad53 and the upstream sensor kinases, Mec1 and Tel1 (Figure 5A, B). In contrast, Dun1 is not required for Xrn1 mobility shift in DNA damage (Figure 5B, C). This modification is independent of DNA damage type as Xrn1 is a strong in vitro substrate of Rad53. Xrn1 was strongly phosphorylated by Dun1 and poorly phosphorylated by Rad53 in vitro. The nucleoporins, Nup1, Nup2, and Nup60 are proteins whose phosphopeptides were ≥25 fold enriched in our phosphoproteomics are indicated in blue (in bold).

Phosphorylation of Xrn1 does not noticeably alter its nuclease activity

Next, we sought to determine how this modification of Xrn1 affects Xrn1 function. Post-translational modification of a protein can alter its biochemical activity, localization, or stability. To examine whether
phosphorylation altered Xrn1’s nuclease activity, we purified Xrn1 from untreated cells or cells treated with 4-NQO and examined its activity in an in vitro nuclease assay. We found that Xrn1 purified from untreated wild type cells, cells treated with 4-NQO, and rad53Δ cells had similar nuclease activity in vitro (Figure 6A, B, C). Similarly, phosphorylation of Xrn1 in vitro by Rad53 had no effect on Xrn1’s nuclease activity (data not shown). Thus, we are not able to determine the effect of Rad53 phosphorylation on the in vitro nuclease activity of Xrn1.

We wondered whether the effect of Rad53 phosphorylation on Xrn1 might not be observable in an in vitro system. To overcome the possibility that purification of Xrn1 may perturb its regulatory mechanism, we examined turnover of transcripts in vivo. First, we followed mRNA degradation of the GAL1 transcript in 4-NQO-treated cells. GAL1 transcript is strongly expressed when cells are grown in galactose, but is quickly repressed when glucose is available. GAL1 mRNA had both a higher steady state level and a longer half-life in xrn1Δ cells (33 min) compared to wild type cells (17.1 min) (Figure 6D). However, RAD53 deletion did not strongly affect the mRNA half-life (15.5 min) (Figure 6D). We also examined mRNA turnover for HST3 message. The HST3 gene is regulated by the DDR transcriptionally and

Figure 3 Downregulation of rRNA processing or protein synthesis is independent of the DNA damage checkpoint. A. Wild type, mec1Δ, and rad53Δ cells are untreated or treated with 2 μg/ml 4-NQO. Samples were collected at 30 min and 60 min after treatment for RNA extraction. The full length 35S pre-rRNA transcript is processed to yield mature 18S, 5.8S, and 25S rRNA. Northern blot with probe against full-length 5.8S sequence allows detection of the 32/33S and 27S intermediates, and the fully processed 5.8S rRNA. B. Film of S35-methionine incorporation. Wild type, rad53Δ, and mec1Δ tel1Δ cells were untreated or treated with 2 μg/ml 4-NQO for 15 min S35-methionine was added and samples were taken after 1 min and 5 min. C. Film of S35-methionine incorporation. Left panel: wild type and gcn2Δ cells were untreated or treated with 2 μg/ml 4-NQO for 60 min Middle panel: G1 arrested wild type and gcn2Δ cells were untreated or treated with 0.05% MMS for 60 min Right panel: G1 arrested wild type and cdc13-1 cells were grown at 23°C or 32°C for 2 hr. S35-methionine was added and samples were taken after 1 min and 5 min. D. Western blot of eIF2 alpha phosphorylation. Left panel: wild type and gcn2Δ cells were untreated or treated with 2 μg/ml 4-NQO for 60 min Right panel: G1 arrested wild type and cdc13-1 cells were grown at 23°C or 32°C for 2 hr. Samples were blotted for phosphorylated eIF2 alpha-S51 and Dbf4. E. Wild type and rad53Δ cells were arrested in G1 with a factor and released into rich media or rich media with 200 mM HU for 45 min before harvesting. Total RNA and ribosome footprints were purified using Illumina’s ARTseq kit before sequencing. Left panel is an M-A plot showing the average expression for a gene between rad53 Δ and wild type cells (x-axis) vs. the fold change between rad53Δ and wild type cells (y-axis) in the absence of DNA damage. Right panel is an M-A plot showing the average expression for a gene between rad53Δ and wild type cells (x-axis) vs. the fold change between rad53Δ and wild type cells (y-axis) in the presence of HU.
### Table 1: Unique phosphopeptides that are enriched ≥25 fold in GAL1-RAD53/rad53Δ.

| Protein | Phospho-site | OX/Δ 1 | OX/Δ 2 | SILAC OX/Δ | IVK | References                          |
|---------|--------------|--------|--------|-------------|-----|-------------------------------------|
| Xrn1    | S1467        | 277    | 206    | 1           | +   | This study                          |
| Pin4    | S189, S190, S191 | 146    | 263    | 1           | -   | This study; Zhou et al., 2016 (Asynchronous) |
| Pab1    | S299         | 79     | 144    | 1           | ND  | This study                          |
| Nup2    | S316         | 82     | 138    | 1           | ++  | This study; Smolka et al, 2007       |
| Pab1    | S565         | 64     | 161    | 1           | ND  | This study                          |
| Npl3    | S224         | 81     | 114    | 1           | ND  | This study; Smolka et al, 2007       |
| Pgm2    | S2           | 64     | 127    | 2           | +   | This study; Smolka et al, 2007       |
| Gcd11   | S258         | 122    | 62     | 1           | +   | This study                          |
| Npl3    | S224         | 73     | 88     | 1           | ND  | This study; Smolka et al, 2007       |
| Fun30   | S98          | 75     | 79     | 1           | ++  | This study; Chen et al, 2010         |
| Nsr1    | S405, S409   | 86     | 60     | 1           | ND  | This study; Zhou et al., 2016        |
| Rps7B   | S168         | 40     | 97     | 1           | ++  | This study                          |
| Net1    | T838         | 43     | 91     | 1           | ++  | This study; Smolka et al, 2007; Zhou et al., 2016 |
| Rp7     | S4           | 55     | 65     | 1           | ND  | This study                          |
| Gga1    | S185         | 45     | 72     | 1           | -   | This study                          |
| Def1    | S273         | 53     | 61     | 1           | ++  | This study; Smolka et al, 2007       |
| Nop56   | S317         | 70     | 45     | 1           | -   | This study                          |
| Sec7    | S215, S218   | 66     | 46     | 1           | -   | This study                          |
| Nsp1    | S285         | 55     | 49     | 1           | +   | This study; Smolka et al, 2007; Zhou et al., 2016 |
| Ded1    | S218         | 77     | 32     | ND          | +   | This study                          |
| Cho2    | S598         | 30     | 73     | 1           | +   | This study                          |
| Nsp1    | S532         | 70     | 30     | 1           | +   | This study; Smolka et al, 2007; Zhou et al., 2016 |
| Hxt2    | S11, S13     | 24     | 86     | 1           | -   | This study; Zhou et al., 2016 (S-phase) |
| Nsp1    | S323         | 34     | 53     | 1           | +   | This study; Smolka et al, 2007; Zhou et al., 2016 |
| Rp7     | S278         | 31     | 55     | 1           | ND  | This study; Zhou et al., 2016 (S-phase) |
| Nsp1    | S551         | 35     | 47     | 1           | +   | This study; Smolka et al, 2007; Zhou et al., 2016 |
| Ski7    | S88, S90     | 32     | 51     | ND          | -   | This study; Zhou et al., 2016        |
| Ubc4    | S12          | 31     | 50     | 1           | ND  | This study                          |
| Pan1    | S745         | 39     | 39     | 1           | ND  | This study                          |
| Rpn8    | S314         | 49     | 31     | 1           | -   | This study                          |
| Prp45   | S370         | 40     | 37     | ND          | -   | This study                          |
| Net1    | S747         | 36     | 42     | 1           | ++  | This study; Smolka et al, 2007; Zhou et al., 2016 |
| YLR257W | S137, S139   | 40     | 37     | 1           | ND  | This study; Zhou et al., 2016 (Asynchronous 1X) |
| Nup60   | S63          | 28     | 52     | 1           | ++  | This study; Smolka et al, 2007; Zhou et al., 2016 |
| Rpg1    | S872         | 36     | 37     | 1           | ND  | This study; Zhou et al., 2016        |
| Nsr1    | S405         | 26     | 50     | 1           | ND  | This study; Zhou et al., 2016        |
| Rrd1    | S385         | 34     | 38     | ND          | ND  | This study                          |
| Hsp42   | S213, S214   | 401    | 3      | 1           | ND  | This study; Zhou et al., 2016        |
| Nup1    | S754         | 43     | 29     | 1           | ++  | This study; Smolka et al, 2007; Chen et al., 2010 |
| Inh1    | S38          | 28     | 43     | 1           | -   | This study                          |
| Nup2    | S68          | 35     | 34     | 1           | ++  | This study; Smolka et al, 2007; Zhou et al., 2016 (Asynchronous 1X) |
| Enp1    | S404         | 23     | 50     | 1           | ++  | This study; Chen et al., 2010        |
| Pus1    | S478         | 13     | 83     | 1           | +   | This study                          |
| Net1    | S785         | 27     | 39     | 1           | ++  | This study; Smolka et al, 2007; Chen et al., 2010 |
| Hst1    | S143         | 40     | 26     | ND          | +   | This study                          |
| Tub2    | S280         | 40     | 25     | 1           | ND  | This study                          |
| Crn1    | S462         | 25     | 39     | 1           | -   | This study                          |
| Yta7    | S1290        | 20     | 49     | 1           | ND  | This study; Zhou et al., 2016 (Asynchronous 1X) |
| Gle1    | S108         | 33     | 29     | ND          | ND  | This study; Zhou et al., 2016 (Asynchronous 1X) |
| Aro9    | S502         | 55     | 16     | ND          | -   | This study                          |
| Nop12   | S2           | 41     | 22     | 1           | -   | This study                          |
| Ecm32   | S206         | 25     | 34     | ND          | -   | This study; Zhou et al., 2016        |
| Ubp13   | S198         | 39     | 22     | ND          | ND  | This study                          |
| Srv2    | S346         | 6      | 142    | 1           | +   | This study                          |
| Set3    | S741         | 30     | 28     | ND          | +   | This study                          |
| Smu23   | S233         | 26     | 32     | ND          | -   | This study                          |
| Srv2    | S343         | 20     | 41     | 1           | +   | This study                          |
| Alb1    | S41          | 26     | 31     | ND          | +   | This study                          |
| Rba50   | S233         | 34     | 23     | 1           | -   | This study                          |
| Las1    | S467         | 24     | 30     | ND          | +   | This study                          |
| Yap1    | S503         | 24     | 30     | 1           | -   | This study; Zhou et al., 2016 (Asynchronous 1X) |

(showed Yta7 binds Rad53 in MMS)
post-translationally, and thus it was a reasonable candidate for a message that would be targeted by the DDR (Edenberg et al. 2014). We removed the ability of the DDR to regulate HST3 transcription by replacing the HST3 promoter with the GAL1 promoter. Upon shifting the cells to glucose media to inactivate the GAL1 promoter, we found that the turnover kinetics of the HST3 transcript were similar in wild type (50.8 min) and rad53Δ cells (44 min) exposed to 4-NQO (Figure 6E). Next, we tested another mRNA candidate that might be targeted by Xrn1. Histone gene deletions have been shown to rescue rad53Δ and lsm1Δ mutants (Gunjan and Verreault 2003; Herrero and Moreno 2011). We wondered if this is mediated by Xrn1. We found that histone gene deletion did not rescue the slow growth of xrn1Δ cells or their DNA damage sensitivity (Figure 6F), suggesting that histone genes are not the critical target of Xrn1.

Xrn1 is required for normal levels of RNA in the cell and xrn1Δ cells have significantly higher levels of RNA, especially small cryptic RNA species (van Dijk et al. 2011; Sun et al. 2013). We wondered whether excess RNAs might promote formation of RNA:DNA hybrids. If Xrn1 lost the promotion of accumulation of RNA:DNA hybrids, overexpressing RNAse H (Rnh1), which specifically degrades RNA:DNA hybrids, should rescue the DNA damage sensitivity of xrn1Δ cells. To examine this, we integrated an additional copy of Rnh1, driven by the TEF1 promoter. However, this did not rescue the sensitivity of xrn1Δ cells to phleomycin (Figure 6G), indicating that it is unlikely that the DNA damage sensitivity of xrn1Δ is due to high levels of RNA:DNA hybrids.

Since Xrn1 is a component of the P-bodies, we asked whether the phosphorylation of Xrn1 affects its ability to be incorporated into P-bodies during DNA damage (Sheth and Parker 2006; Parker and Sheth 2007; Teixeira and Parker 2007; Buchan et al. 2008; Frankis and Lykke-Andersen 2008). We treated wild type and rad53Δ cells with 4-NQO to determine whether Rad53 affects the localization of Xrn1-GFP upon DNA damage. We found that Xrn1 forms distinct GFP foci during DNA damage, and that this is independent of Rad53 (Figure S1). To determine whether DNA damage affects the interaction of Xrn1 with P-body components, we performed co-IPs of Flag-tagged Xrn1 with a number of P-body proteins that are available in the yeast GFP collection in the absence or presence of MMS. Interaction of Xrn1 with Pat1, Ede3, Scd6, Nrp1, Pbp1, Rpg1, YGR250C/Rie1, Npl3, and Ngr1 do not change during DNA damage or in the absence of Rad53 (Figure S2). However, the level of YGR250C/Rie1 increased in DNA damage, as previously described (Tkach et al. 2012). Of note, we found that in the absence of Rad53, the interaction between Xrn1 and Lsm3 is reduced, but this does not appear to depend on DNA damage (Figure 7A).

Because a candidate based approach to determine the effect of Rad53 phosphorylation on Xrn1 proved inconclusive, we sought to identify changes in interaction partners using SILAC IP followed by mass spectrometry. After treatment with 4-NQO to induce DNA damage, we purified Xrn1-Flag from wild type cells grown in light labeled arginine and lysine and rad53Δ cells grown in heavy labeled arginine and lysine. A replicate was done in the reciprocal direction, where wild type cells were grown in heavy labeled arginine and lysine and rad53Δ cells were grown in light labeled arginine and lysine. We found five proteins that show a reciprocal change in Xrn1 association: YMR196W, Hsp26, Eap1, Mss116, and Arx1 (Figure 7B). We examined each of these interactions by co-immunoprecipitation of GFP-tagged alleles of candidates with Xrn1-Flag. We could not detect a DNA damage-dependent change in the interaction of Hsp26, Eap1, or Arx1 with Xrn1 (Figure S3). Interestingly, similar to Lsm3, interaction of Xrn1 with Mss116 is altered in rad53Δ independently of DNA damage (Figure 7C). In contrast, expression of YMR196W appears to be strongly induced by DNA damage in a RAD53-dependent manner (Figure 7C).

To understand the role of Xrn1 during DNA damage, we sought to identify the residues that are phosphorylated by Rad53. Our original screen identified a single site on Xrn1 (S1467) that was enriched in GAL1pr-RAD53 cells (Table 1). Deletion of a small portion of the C terminus (1396-1528) that includes this residue was not sufficient to abolish the Xrn1 shift in DNA damage, nor did it result in any damage sensitivity (Figure S4A and S4B). To further characterize the phosphorylation sites, we phosphorylated purified Xrn1 with Rad53 in vitro and mapped the in vitro sites by mass spectrometry. Six sites in the C-terminal region corresponding to amino acids 1155 – 1330 showed strong enrichments (S1155, T1268, S1306, S1328, S1329). However, within this 175 amino acid region, there are 32 serine and threonine residues. Since Rad53 does not have a strong consensus sequence and our coverage in this region of the protein was poor, we mutated all serines and threonines to alanines disrupted the nuclease function of Xrn1. We performed an in vitro assay and determined that the nuclease activity itself is defective in these mutants (Figure S4C and S4D).

We sought to determine the effect of Rad53 phosphorylation on Xrn1 function, and determined that the phosphorylation does not affect the inherent nuclease activity of Xrn1, nor does it appear to affect Xrn1’s interaction with any of the P-bodies proteins that we have tested. Xrn1 is also involved in rRNA processing and removal of tRNA introns (Stevens et al. 1991; Wu and Hopper 2014). Our Northern blot result in Figure 3A suggests that it is unlikely that Rad53 is targeting Xrn1 to alter rRNA processing. However, the effect of Rad53-dependent phosphorylation of Xrn1 on tRNA introns remains to be tested. Our attempt to study specifically the Rad53-dependent phosphorylation residues in Xrn1 was inhibited by the fact that changing 32 serines and threonines to alanines disrupted the nuclease function of Xrn1.

### Table 1, continued

| Protein | Phospho-site | OX/Δ 1 | OX/Δ 2 | SILAC OX/Δ | IVK | References |
|---------|--------------|--------|--------|------------|-----|------------|
| Rrp4A   | T60          | 31     | 22     | 1          | ND  | This study; Zhou et al., 2016 (S-phase) |
| Ecm32   | S206         | 27     | 25     | ND         | -   | This study; Zhou et al., 2016 (S-phase) |
| Nsp1    | S221         | 31     | 21     | 1          | +   | This study; Zhou et al., 2016 (S-phase) |
| Sec53   | S54          | 18     | 35     | 1          | ND  | This study |
| Scp160  | S325         | 21     | 29     | 1          | ++  | This study |
DISCUSSION
The DDR regulates RNA metabolism through multiple substrates and pathways

Of the 54 substrates that we have identified in our screen, 24 are involved in some aspect of RNA biology. In particular, several distinct substrates in the mRNA decay pathway were identified in our screen (Figure 2A). Similarly, processing of the 35S pre-rRNA also appears to be targeted by the DDR through multiple distinct substrates (Figure 2B). Nup1, Nup2, Nup60, and Npl3 have previously been implicated in the DNA damage response and contribute to genome stability (Santos-Pereira et al. 2014; Ibarra and Hetzer 2015). Targeting multiple proteins in the same pathway suggests that there is a general functional rewiring of these pathways in response to DNA damage by the checkpoint.

Figure 4 Validation by in vitro kinase assay of proteins associated with enriched phosphopeptides in the screen. A. In vitro kinase assay for 44 proteins whose phosphopeptides were enriched by ≥25 fold and where the GFP-tagged strains were available in the GFP collection. For each protein set, - indicates no kinase control, R indicates Rad53, and D indicates Dun1. Top panels are autorads showing $^{32}\text{P}$ signal indicating transfer of $^{32}\text{P}$-ATP to substrate. * indicates Rad53 autophosphorylation signal. † indicates contaminating kinase in IP. Bottom panels show Western blot for GFP as loading control for each lane. Dcp2 (20X enriched) and Pbp1 (2X enriched) were selected because of their connection to Xrn1 in P body biology.

Figure 5 Rad53 is required for Xrn1 mobility shift during DNA damage. A. Western blot showing Xrn1-GFP shift upon DNA damage, and is dependent on Rad53 and Mec1/Tel1. Asynchronous yeast cultures were treated with 2 μg/mL 4-NQO for 90 min before harvesting. B. Western blot showing Xrn1-GFP shift is independent of Dun1. Asynchronous cells were treated with 0.05% MMS for 3 hr before harvesting. C. Western blot showing Xrn1-Flag shift is dependent on Rad53, but independent of Dun1. Asynchronous cells were treated with 2 μg/mL 4-NQO for 90 min. Rad53 is shown as a control for DNA damage treatment, because it hyper-shifts in response to DNA damage.
Figure 6  Xrn1 phosphorylation does not interfere with its nuclease activity in vitro, or its ability to degrade endogenous transcripts. A. Nuclease assay using in vitro transcribed RNA as substrate with purified Xrn1 from cells untreated (-) and treated (+) with 2 μg/mL 4-NQO for 1 hr. Nuclease assay was allowed to proceed for 60 min and samples were collected at the indicated time. Mock IP sample was allowed to proceed for 60 min B. Quantification of product (NMP) formation normalized to t = 0 signal. C. Western blot showing relative Xrn1 abundance used in experiment in A. D. Normalized mRNA levels of GAL1 transcript turnover. The GAL1 promoter is rapidly repressed when galactose in the media is replaced with glucose. 4-NQO was added to a concentration of 2 μg/mL concurrently with glucose and samples were collected at the indicated time.
Xrn1 is involved in key aspects of the mRNA life cycle and the cellular dynamics of mRNAs are tightly regulated. In cells, actively translating mRNAs are associated with ribosomes and the translation initiation machineries, while non-translating mRNAs are sequestered into cytoplasmic P-bodies or stress granules (Decker and Parker 2012). These messenger ribonucleoprotein (mRNP) aggregates differ in the fate of their mRNAs and in their composition, and our screen identified several proteins involved in this dynamic (Figure 2A). P-bodies typically contain mRNAs that are targeted for decay, while stress granules consist of mRNAs that are stalled or paused in translation, contain translation initiation proteins, and can reinitiate translation (Kedersha et al. 2005; Decker and Parker 2012). However, there is a dynamic between translating mRNAs, P-bodies, and stress granules and mRNAs can transition between these processes. In addition to Xrn1, our screen uncovered other factors involved in mRNA dynamics: Ded1, Pab1, Rpg1, and Ecm32. Gle1, in human cells, is also found to be a component of stress granules (Aditi et al. 2015). Ded1, Pab1, and Rpg1 are required for re-initiation of translation from stress granules. Collectively, this suggests that Rad53 is generally regulating mRNA dynamics. The Longhese lab recently showed that cells deleted for RNA processing proteins, including xrn1Δ, are defective in processing single-stranded DNA that is necessary for checkpoint activation and suggested that this, in part, explains the DNA damage sensitivity of xrn1Δ cells (Manfrini et al. 2014). Our data show that the cell directly targets Xrn1 during DNA damage.

The functional role of Xrn1 during DNA damage

Post-translational modification is a common mode of regulation in the cell, as it quickly and transiently affects the functional role of the protein being modified. The sensitivity of xrn1Δ cells is consistent with a model in which phosphorylation by Rad53 activates Xrn1. Xrn1 phosphorylation does not appear to affect its stability, localization, or core activity in vitro. It is possible that the in vivo effect of Xrn1 phosphorylation may not be detectable by the methods used here, or it may target specific substrates that were not examined. However, there is no evidence that Xrn1 itself has sequence preferences or specificity, and given its diverse role in processing of mRNA, rRNA, and tRNA, it is unlikely that any sequence preferences are due solely to Xrn1. However, there is precedent for Xrn1 being responsible for degradation of specific class of substrates. In response to glucose starvation, Snf1 phosphorylates the C terminus of Xrn1 and this is involved in degradation of Snf1-targeted transcripts (Braun et al. 2014). One of the sites of Snf1 phosphorylation, S1330, is immediately adjacent to S1329, which we identified as a Rad53 phosphosite. This suggests that phosphorylation of the C-terminus is a general mechanism for regulating Xrn1 function, and in DNA damage, Rad53 may regulate Xrn1 to promote cellular survival. In addition, human Xrn1 has also been shown to degrade initiator tRNA under heat stress and one ortholog of Arabidopsis Xrn1 shows some sequence specificity (Rymarquis et al. 2011; Watanabe et al. 2013). However, the mechanism for how Xrn1 is recruited to these transcripts has not been determined. Therefore, it is possible that changes in binding partners may allow Xrn1 access to specific transcripts. These binding partners are likely to include other Rad53 substrates, given the number of substrates we identified in pathways associated with RNA metabolism. Thus, it may be difficult to detect phenotypes in individual phosphosite mutants.

In summary, we have found evidence that the DDR regulates RNA metabolism in response to DNA damage. Of the 33 novel substrates identified, we verified that 12 are directly phosphorylated by Rad53 in vitro: Xrn1, Gcd11, Rps7b, Ded1, Cho2, Pus1, Hst1, Srv2, Set3, Snu23, Alb1, and Scp160 (Figure 2). Of these, half are involved in RNA processes: Xrn1, Ded1, and Scp160 are mRNA binding proteins; Snu23 and Alb1 are rRNA processing proteins; and Pus1 is a tRNA: pseudouridine synthase. Together, these data suggest a concerted effort on the part of the DNA damage checkpoint pathway to alter gene expression post-transcriptionally.

E. Normalized mRNA levels of HST3 transcript turnover. HST3 is placed under the GAL1 promoter, which is downregulated in glucose and the rate of degradation of HST3 was determined. 4-NQO was added to a concentration of 2 μg/mL concurrently with glucose and samples were collected at the indicated time. F. Tenfold serial dilutions of the indicated strains grown on YPD, YPD + 100 mM HU, or YPD + 1 μg/mL phleomycin (or YPD + 1.5 μg/mL phleomycin). Plates were grown at 30°C for 2-3 days before scanning. G. Tenfold serial dilutions of the indicated strains grown on YPD, YPD + 100 mM HU, or YPD + 1 μg/mL phleomycin. Plates were grown at 30°C for 2-3 days before scanning.
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