Supplemental Methods

Yeast strain construction

The indicated yeast deletion strains were created using homologous recombination with PCR-amplified knockout cassettes and confirmed by PCR. The Sth1 Anchor-Away (Sth1-AA) strain was constructed in yeast strain FH001 (Hodges et al. 2019) by homologous recombination with a PCR product amplified from pFA6a-FRB-HIS3MX6 (gift from Dr. Frank Holstege), consisting of the FKBP12-rapamycin-binding (FRB) domain of the human mTOR protein, as well as GFP and a selectable marker (His3MX6). The Tet-off Sth1 yeast strain was previously described (Czaja et al. 2014).

UV sensitivity assay

Strains were grown in Yeast extract Peptone Dextrose (YPD) media at 30^{o}C overnight. 1:10 dilutions were made based on the OD_{600} reading, with the first dilution containing approximately 1×10^{8} cells/mL and the last containing approximately 1×10^{3}. 5uL of each dilution was then spotted on either SC (synthetic complete) or YPD plates and exposed to the indicated dose of UVC light. Plates were then incubated at 30^{o}C in the dark until imaged (3 and 5 days post-UV exposure).

Alkaline gel assay of bulk repair of CPD lesions

Wild-type and mutant strains were grown in 50mL YPD until OD_{600} reached ~0.65 (mid-log phase). Cells were then pelleted and resuspended in dH_2O. 10mL of cells were collected for a “No UV” control. The remaining cells were exposed to 100J/m^2 UVC light. 10mL of cells were collected immediately following UV exposure as a “0hr UV”

timepoint. The remaining cells were pelleted, resuspended in 30mL YPD, and allowed to repair for 1, 2, or 3 hours post-UV treatment. Cell pellets were stored at -80^{o}C until DNA isolation.

Genomic DNA (gDNA) was isolated using cell lysis buffer, acid-washed glass beads, and phenol:chloroform:isoamylalcohol (PCI, 25:24:1), and was then RNase A treated for at least 15 minutes at 37^{o}C. gDNA concentration was measured via NanoDrop spectrophotometer and was either treated with T4 endonuclease V (T4 endoV from Epicentre or gift from Dr. Steven Roberts) or mock treated for 2 hours at 37^{o}C to cleave DNA at sites of CPD lesions (5ug per treatment). The T4 endoV reaction was stopped using 6x alkaline loading dye and run on a 1.2% alkaline gel at 30V for 20 hours to denature and separate nicked single stranded DNA. The gel was neutralized for 1 hour using a neutralization buffer, stained with SYBR gold (Thermo Fisher Scientific) for 1 hour, and washed twice with dH_2O, 1 hour per wash. Gel was then analyzed via Typhoon FLA biomolecular imager (GE Healthcare). ImageQuant v5.2 was used to determine the intensity of each lane. The median intensity of each lane was used to determine the average fragment size based on known fragment sizes of the HindIII-digested λ phage DNA ladder and the distance of band migration. The Poisson distribution was used to determine the approximate number of CPDs per kb at each timepoint, and each timepoint was compared to the “0hr UV” timepoint, as previously described (Bespalov et al. 2001; Hodges et al. 2019). The resulting repair data was analyzed using GraphPad Prism.

CPD-seq experiments

Briefly, WT or mutant yeast cultures were grown to an OD_{600} of approximately 0.8 in YPD, pelleted, and resuspended in dH_2O. Anchor away strains (indicated by -AA) were grown to OD_{600} ~0.8 for mutants and ~0.4 for WT and treated with 50mg/mL rapamycin for 3 hours to conditionally deplete the tagged nuclear proteins. Cells were collected for a "No UV" sample, and the remaining cells were treated with 125J/m^2 UVC light. Cells were collected immediately following exposure ("0hr UV"), and the remaining cells were resuspended in YPD (+rapamycin for -AA strains) and allowed to repair for 2 hours at 30^{o}C. Genomic DNA was isolated via phenol:chloroform:isoamylalcohol (PCI) extraction and ethanol precipitation, and was RNase A treated for at least 30 minutes. DNA was then sonicated into fragments between 200 and 700bp in length using a Bioruptor 300 Sonicator (15 cycles: 30 seconds on, 30 seconds off). DNA fragments were end-repaired and dA-tailed, and a double stranded trP1 adapter was ligated to both ends of the fragments. trP1 adapter ligation was PCR confirmed using primers complimentary to the trP1 adapter. Free 3'-OH groups were blocked with Terminal Transferase (NEB) and either dideoxyATP (ddATP) or ddGTP.

Samples were then treated with T4 endonuclease V (i.e., T4 PDG, NEB) and AP endonuclease APE1 (NEB) to cleave CPD lesions and create an abasic site, respectively. 5' phosphate groups were then removed using shrimp alkaline phosphatase (Affymetrix), and DNA was subsequently denatured at 95^{o}C for 5 minutes and snap-cooled on ice. A second double stranded adapter, the A adapter, was then ligated to the 3'-OH created immediately upstream of the cleaved UV lesion (NEBNext Quick Ligation Module). Second adapter ligation was confirmed by PCR using an unlabeled primer complementary to the trP1 adapter and a Cy3-labeled primer

complementary to the A adapter (A primer). Each A adapter contains a unique barcode that allows for the creation of different libraries to be pooled and analyzed through multiplexed DNA sequencing techniques, as well as a biotin-labeled strand. DNA containing the biotin label was purified using Streptavidin beads (Thermo Fisher Scientific), the non-biotinylated DNA strand was released using 0.15M NaOH, and then used as a template for second strand synthesis, using the A primer. Libraries were PCR amplified for 5-6 cycles and combined at equal volumes to be submitted for Ion Proton sequencing (Life Technologies). Sequencing reads were aligned to the SacCer3 yeast genome via Bowtie 2 software, and the corresponding dinucleotide damage site was determined as previously described (Mao et al. 2016; Mao and Wyrick 2020).

CPD-seq data analysis

CPD-seq data analysis was performed as previously described (Mao et al. 2016; Duan et al. 2020; Mao et al. 2020). Briefly, sequencing reads were separated based on the A-adapter barcode sequences, and barcode sequences were trimmed. Sequences were then aligned to the *Saccharomyces cerevisiae* SacCer3 genome using Bowtie 2 software. Alignment files were then converted to BED files using SAMtools and BEDTools software. The CPD lesion site was determined by extracting the dinucleotide sequences immediately upstream of the 5' end of the sequencing read, on the opposite strand. All reads were analyzed and the dinucleotide distribution among reads was visualized via GraphPad Prism (Figures 2B, S4B-C, S9). Non-dipyrimidine reads were filtered from further analysis, and the resulting CPD-containing BED files were split into positive and negative strand reads. Each strand read was then sorted and counted

using IGVtools, generating WIG files. Background files were generated to give a value of zero to each dipyrimidine in the genome that did not map a CPD lesion. For each CPD-seq experiment, the 2hr repair time point was analyzed relative to the matched 0hr control to determine the fraction of unrepaired CPDs remaining following 2hr repair. Since there was some variability in the overall fraction of unrepaired CPDs between replicates for the *snf6*Δ and *rad16*Δ Sth1-AA experiments, the CPD-seq reads from the two independent replicates were combined for each of these experiments prior to analysis. Perl scripts used for processing and analyzing the CPD-seq and NMP-seq (see below) data are available in the Supplemental Code.

TC-NER and gene plot analysis

High resolution analysis of repair along the NTS and TS calculated the fraction of unrepaired CPD lesions remaining after two hours of repair at every position surrounding transcription start sites (TSS, 0bp) from -500bp to +640bp across 5205 yeast genes, as previously described (Duan et al. 2020; Mao et al. 2020). Custom Perl scripts (see Supplemental Code) gave a CPD lesion count for every position in every gene for the 0hr control and 2hr repair time point (Figures S2A-C, 5A-C, S6A-C, S8A-D). Nucleosome coordinates for Anchor Away strains were obtained from WT-AA and Sth1-AA strains (Kubik et al. 2018), and nucleosome coordinates for non-Anchor Away cells were obtained from WT cells (Weiner et al. 2015).

Repair was also analyzed for bins along the TS and NTS strands of ~5000 genes (Figures 2C-E, 3F-H, S4D-E, 4D-E), as previously described (Mao et al. 2016; Mao et al. 2020). For transcriptional asymmetry analysis, the $\log_2$ ratio of unrepaired CPDs in

each bin was calculated for the TS relative to the NTS (Figures 4A-B, 4F, S4F-G, S7B-D).

For gene plot analysis (Figures S3A-C, 3I-J, S5, 4G right), the fraction of CPDs remaining was calculated for 6 bins spanning the transcribed region (TSS to transcription termination site (TTS), as well as 501 bp flanking the TSS and TTS (3 bins each). Genes were organized based on transcription levels (or another specified parameter), and repair patterns were visualized using TreeView (Saldanha 2004). Gene plot analysis was performed as previously described (Duan et al. 2020), except that genes with identical transcription levels were ordered alphabetically (instead of ordered randomly). Changes in RNA polymerase II occupancy following Sth1 depletion, based on published Rpb3 ChIP-seq data for Sth1-AA cells with or without rapamycin (Kubik et al. 2018), were analyzed in a similar manner for comparison (Figures 4G, S7A).

Analysis of repair in nucleosomes

The fraction of CPDs remaining along the NTS was plotted against the known locations of strongly positioned nucleosomes in genes, using nucleosome positions determined for WT cells (Weiner et al. 2015) or Sth1-AA cells (Kubik et al. 2018). The fraction of CPDs (or NMPs) remaining were determined from -90bp to +90bp from the nucleosome dyad (Figures 2F-H, 5E-G, 6E-F). The NTS nucleosomal DNA was oriented in the 5'-to-3' direction, as previously described (Mao et al. 2020), either for just the +1, +2, and +3 nucleosomes relative to the TSS or all coding nucleosomes.

NMP-seq experiments

NMP-seq (N-methylpurine-seq) was used to map the formation and repair of alkylation damage following treatment with MMS (methylmethane sulfonate), following our previously published protocol (Mao et al. 2017). Yeast cultures were grown in YPD to an OD_{600} of approximately 0.3 and treated with 1X nocodozale for 3 hours to arrest cell cycle progression, and thereby prevent dilution of NMP lesions by ongoing DNA replication. Anchor away (-AA) cells were treated with 50mg/mL rapamycin for 3 hours in conjunction with nocodozale treatment. Following incubation, cells were collected for a “No MMS” control. The remaining cells were treated with 0.4% MMS for 10 minutes at room temperature in a hood, and were inverted every 2 minutes. Cells were then pelleted and YPD+MMS was removed. Cell pellets were washed twice with sterile dH_2O before collection (“0hr MMS”) or resuspension in YPD + rapamycin for 2 hours of repair (“2hr MMS”). Genomic DNA was then isolated and prepared into libraries as described in our CPD-seq method, with the following exception: instead of treatment with T4 endoV, gDNA was treated with recombinant human AAG glycosylase (AAG 1-79Δ, construct received from Dr. Leona Samson at MIT) to cleave NMP lesions. APE1 was again used to generate new 3’-OH groups. Following library preparation, samples were combined in equal volumes and submitted for Ion Proton sequencing (Life Technologies). Sequencing reads were aligned to the SacCer3 yeast genome via Bowtie 2 software, and the corresponding alkylation damage site was counted as previously described (Mao et al. 2017). Yeast strains lacking the *MAG1* DNA glycosylase were used for the 0hr control experiments, in order to eliminate repair during the 10 minute MMS exposure.

NMP-seq data analysis

NMP-seq data analysis conducted as previously described (Mao et al. 2017). Briefly, sequencing reads were trimmed of their barcodes and the 3' nucleotide, and subsequently aligned to the SacCer3 yeast genome using Bowtie 2. Alignment files were then converted to BED files using SAMtools and BEDTools software. The NMP lesion site was determined by extracting the single nucleotide immediately upstream of the 5' end, on the opposite strand. All reads were analyzed for the nucleotide distribution analysis (Figures S9A-B). From there, only G-reads were retained as putative 7-methylguanine (7meG) lesions and analyzed. Analysis of 7meG repair in gene coding regions (Figures 6B-D) and nucleosomes (Figures 6E-F) was performed as described above.

RNA isolation protocol for RT-qPCR

Wild-type (BY4741) and *rsc2*Δ (KB004) yeast were grown in YPD media to a cell density between 0.3 and 0.6 OD_{600}. A total of 7 OD units were collected in a 50ml conical tube and centrifuged at 3500 rpm for 5 minutes at 4^{o}C to pellet cells. Media was poured off, and pellet was resuspended in 900uL nuclease-free water to be transferred to three 1.5mL microcentrifuge tubes; each containing 300uL of cell suspension so as to not exceed capacity of the NEB columns used later. Cells were spun at 6000 rpm for 1.5 minutes at 4^{o}C. Water was then pipetted off, and cells were flash frozen in liquid nitrogen and incubated at -80^{o}C for at least 10 minutes. Following incubation, one tube of each strain was thawed on ice and resuspended in 400uL TES buffer (10mM Tris-HCl pH 7.5, 10mM EDTA, 0.5% SDS). 400uL of acid phenol chloroform (pH ~4.3) was

added and the tubes vortexed for 10 seconds. Tubes were incubated at 65^{o}C for 30 minutes, with quick vortexes every 10 minutes. Cells were placed on ice for 5 minutes, then spun at the highest speed for 5 minutes at 4^{o}C. Aqueous layer was transferred to a new 1.5mL, and phenol chloroform extraction was repeated twice. Aqueous layer was placed in a new 1.5mL tube. For every 50uL of aqueous layer, 100uL of NEB Monarch RNA Lysis Buffer (#T2012-1) was added and mixed. Equal volumes of (>95%) ethanol were added and tubes were inverted. The mixture was then added to a NEB Monarch RNA binding column and spun at 16,000xg for 30 seconds, continuing with the NEB Monarch Total RNA Miniprep Kit protocol from part 2 step 4 (#T2010). After the 2 minute spin with the RNA wash buffer, samples were spun an additional 1 minute to ensure ethanol was not carried through to the final elution. Samples were eluted in 50uL nuclease-free water and stored at -80^{o}C until use.

cDNA generation and qPCR

cDNA was generated using the NEB ProtoScript II kit (#E6560S). Reagents were combined and incubated at 42^{o}C for 1 hour, followed by 80^{o}C for 5 minutes to heat kill the reaction. No RT controls followed the same protocol, except no enzyme mix was added and the incubation did not contain the 80^{o}C heat kill step.

qPCR was set up using the following primers:

UBC6 forward: CCTGTCGTGGCTTCATCACT

UBC6 reverse: GGAATCCTGGCTGGTCTGTC

TFB1 forward: CACAGCACGTTGTGTGTAGC

TFB1 reverse: TGTCGACAACACTTCTCAAGCA

UBC6 was selected as the control gene based on a previous study (Teste et al. 2009). The qPCR cycle went as follows: 1) 95^{o}C for 1 min., 2) 95^{o}C for 10 sec., 3) 61^{o}C for 30 sec., (steps 2-3 repeated 39 times), 4) 65^{o}C to 95^{o}C in 0.5^{o}C increments for 5 sec. to produce a melt curve. Three biological replicates were run in triplicate for each strain and primer set. qPCR analysis calculated the $\Delta\Delta$ct values for each replicate. Values from three replicates were visualized using GraphPad Prism.

Supplemental References

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