A New Method, “Reverse Yeast Two-Hybrid Array” (RYTHA), Identifies Mutants that Dissociate the Physical Interaction Between Elg1 and Slx5

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ABSTRACT The vast majority of processes within the cell are carried out by proteins working in conjunction. The Yeast Two-Hybrid (Y2H) methodology allows the detection of physical interactions between any two interacting proteins. Here, we describe a novel systematic genetic methodology, “Reverse Yeast Two-Hybrid Array” (RYTHA), that allows the identification of proteins required for modulating the physical interaction between two given proteins. Our assay starts with a yeast strain in which the physical interaction of interest can be detected by growth on media lacking histidine, in the context of the Y2H methodology. By combining the synthetic genetic array technology, we can systematically screen mutant libraries of the yeast *Saccharomyces cerevisiae* to identify *trans*-acting mutations that disrupt the physical interaction of interest. We apply this novel method in a screen for mutants that disrupt the interaction between the N-terminus of Elg1 and the Slx5 protein. Elg1 is part of an alternative replication factor C-like complex that unloads PCNA during DNA replication and repair. Slx5 forms, together with Slx8, a SUMO-targeted ubiquitin ligase (STUbL) believed to send proteins to degradation. Our results show that the interaction requires both the STUbL activity and the PCNA unloading by Elg1, and identify topoisomerase I DNA–protein cross-links as a major factor in separating the two activities. Thus, we demonstrate that RYTHA can be applied to gain insights about particular pathways in yeast, by uncovering the connection between the proteasomal ubiquitin-dependent degradation pathway, DNA replication, and repair machinery, which can be separated by the topoisomerase-mediated cross-links to DNA.

KEYWORDS SGA; clamp unloader; SUMO-targeted ubiquitin ligase (STUbL); PCNA

PROTEINS control all biological systems in the cell, and while some perform their functions independently, the vast majority of proteins interact with others for proper biological activity. Protein–protein interactions (PPIs) facilitate most biological processes including the formation of cellular macromolecular structures and enzymatic complexes, gene expression, cell growth, proliferation, nutrient uptake, morphology, motility, intercellular communication, and

more. The importance of PPIs led to the development of many technologies to detect them, and to the first system-level maps of the protein interactomes. For eukaryotes, the most popular experimental platform for large-scale analysis of PPIs is the yeast, *Saccharomyces cerevisiae*. Protein complexes have been characterized in yeast using affinity purification followed by mass spectrometry (Ho *et al.* 2002). Other approaches, such as fluorescence resonance energy transfer (Jares-Erijman and Jovin 2006), protein-fragment complementation assay (PCA) (Michnick *et al.* 2010), and high-throughput yeast two-hybrid (Y2H) analyses (Uetz *et al.* 2000) have been used to identify binary interactions. The systematic unbiased utilization of these methods led to various maps of the protein interactome of yeast, and later of several additional model organisms (Uetz *et al.* 2000; Tarassov *et al.* 2008; Babu *et al.* 2009).

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Whereas all these methodologies enable the detection of interactions between any pair of proteins, comparable methods to identify mutants that cause dissociation of particular protein interactions are harder to find. The identification of *trans*-acting mutants that dissociate a particular PPI is valuable for unraveling important regulatory mechanisms, and for defining the biological effect of a specific perturbation. To address this issue, we recently developed a systematic approach termed reverse PCA (rPCA), that allows the identification of such dissociation events for genes that were specifically identified to interact by the PCA (Lev *et al.* 2013, 2014; Keren-Kaplan *et al.* 2016). However, since the PCA or the Y2H are not compatible, and for the same query proteins there is only ~30% overlap between the list of physical interactors obtained by the two methods (Yu *et al.* 2008), it would not be effective to identify the mutants that reverse the PPIs that were specifically identified in a Y2H assay by rPCA.

In this report, we describe the “Reverse Yeast Two-Hybrid Array” (RYTHA), which combines the Y2H and the synthetic genetic array (SGA) methodologies (Tong and Boone 2006).

The Y2H, which was first devised by the Fields lab (Fields and Song 1989; Uetz *et al.* 2000), uses the transcription factor *GAL4* (necessary for activating *GAL* genes, which are required for utilizing galactose as a carbon source). Two different plasmids were engineered to produce protein products in which the *GAL4* DNA-binding domain (BD) fragment is fused to one protein, while another plasmid is engineered to produce a protein product fused to the *GAL4* activation domain (AD). The protein fused to the BD is referred to as the “bait,” and the protein fused to the AD as the “prey.” If the “bait” and “prey” proteins interact, then the AD and BD of the transcription factor are indirectly connected, bringing the AD in proximity to the transcription start site, and the transcription of a reporter gene (*e.g.*, *HIS3*) can occur (Fields and Song 1989). In this way, a successful interaction between the fused proteins is linked to a change in the ability to grow on medium lacking histidine.

The SGA methodology, which was designed by the Boone lab, allows the selection of particular *MATa* meiotic progeny from a sporulating diploid culture (Tong and Boone 2006). Specifically, if both *MATa* and *MATα* meiotic progeny (haploid spores) are induced to germinate, then haploid cells can mate with one another and generate heterozygote diploids. The presence of the haploid selection marker (HSM) ensures the germination of a single mating type by fusing a reporter open reading frame (ORF) (*URA3* in our case) to a haploid mating type-specific promoter (*STE1pr-URA3*), which, in our case, has been integrated at the *CAN1* locus (*can1Δ::STE1pr-URA3*). *MATa* cells carrying *STE1pr-URA3* are able to grow on medium lacking uracil, whereas *MATα* and *MATa/α* cells carrying *STE1pr-URA3* are unable to do so because the expression of *STE1pr-URA3* is repressed in these cells. Because only a fraction (~10%) of the heterozygous diploids enter meiosis, rare mitotic crossover events can contribute to false negative scores, as a *MATa/a* diploid (derived from a *MATa/α*

cell) behaves like a *MATa* haploid, expresses *STE1pr-URA3*, and carries other selected markers. To avoid this complication, two recessive markers that confer drug resistance, *can1Δ* and *lyp1Δ*, were added. The *CAN1* gene encodes an arginine permease that allows canavanine, a toxic analog for arginine, to enter and kill cells. Similarly, the *LYP1* gene encodes a lysine permease that allows thialysine, a toxic analog for lysine, to enter and kill cells. Including *can1Δ* and *lyp1Δ* in the query strain means that *MATa/a* diploid cells are killed by canavanine and thialysine because they carry a wild-type copy of the *CAN1* and *LYP1* genes (Kuzmin *et al.* 2014).

The combination of the Y2H and SGA approaches allows the systematic screening of mutant libraries of the yeast *S. cerevisiae* to identify those mutations that disrupt the physical interaction of interest. We demonstrate the feasibility of this approach by applying it to the discovery of mutants that dissociate the interaction between Elg1, a subunit of an alternative replication factor C (RFC) complex (Kupiec 2016), and Slx5, a subunit of the Slx5–Slx8 small ubiquitin-like modifier (SUMO)-targeted ubiquitin ligase (STUbL) (Ii *et al.* 2007a). Analysis of the screen’s results assigned Elg1 and Slx5 to the topoisomerase I (Top1)-mediated DNA-protein cross-link repair process, a role that was unknown for both of these genes until today. This example demonstrates that RYTHA can be applied to gain insights about particular pathways in yeast.

Materials and Methods

Strains and plasmids

All the strains used in this study are isogenic to BY4741, BY4742, or BY4743 (Brachmann *et al.* 1998). The relevant genotypes are presented in Supplemental Material, Table S1, together with all plasmids used. Gene deletions were generated using one-step PCR-mediated homologous recombination as previously described (Longtine *et al.* 1998; Goldstein and McCusker 1999). To construct the YSB49 query strain, we replaced the genes *GAL4* and *GAL80* with HygB and NatMX markers (*gal4::HygB gal80::NatMX*) from the strain Y9230 (*MATα Δcan1::STE2pr-URA3 Δlyp1ura3Δ0 leu2Δ0 his3Δ1*). A cross to BY4741 resulted in the formation of YSB28 (*MATα Δcan1::STE2pr-URA3 Δlyp1 his3Δ1 Δleuura3Δ0 Δmet15 gal80::NatMX gal4::HygB*). To add the *LYS2::GAL1pr-HIS3* Y2H reporter gene, YSB28 was mated to the original Y2H strain pJ69 (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 Δgal4 Δgal80 LYS2::GALL-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (James *et al.* 1996). Diploid selection, sporulation, and tetrad dissection resulted in YSB49 [*MATα lyp1Δ his3Δ leu2Δ0 ura3Δ met15Δ LYS2::GAL1p-HIS3* (verified by PCR) *gal80::NatMX gal4::HygB trp1Δ901 CAN1*]. Prior to mating with the RYTHA mutant collection (RMC), YSB49 is transformed with the Y2H *LEU2* and *TRP1* plasmids expressing the interacting *GAL4* AD and BD fusion proteins of interest.

To generate the RMC strains, we systematically mated the commercial deletion collection strains (*MATa his3Δ leu2Δ*

met15Δ ura3Δ zzz::KanMX) to YSB110 (*MATα can1Δ::ste1pr-URA3 his3Δ leu2Δ ura3Δ met15Δ trp1::MET15 gal80::clonNAT lys2::ble zzz::KanMX*), and used the SGA approach to obtain the intermediate RMC library strains (*MATα his3Δ leu2Δ ura3Δ trp1::MET15 gal80::clonNAT lys2::ble can1Δ::ste1pr-URA3*). Mating of this library with YSB111 (*MATα his3Δ leu2Δ ura3Δ lys2::ble met15Δ trp1::MET15 gal80::clonNAT gal4::HygB*), and a second round of SGA enabled the selection of the RMC strains (*MATα his3Δ leu2Δ ura3Δ lys2::ble met15Δ trp1::MET15 gal80::clonNAT gal4::HygB zzz::KanMX can1Δ::ste1pr-URA3*).

Two-hybrid assay

To detect two-hybrid interactions, yeast strain PJ69 (Fields and Song 1989; James *et al.* 1996) was cotransformed with a *LEU2*-marked plasmid containing genes fused to the *GAL4* activating domain (pGAD424) and a plasmid containing genes fused to the *GAL4* DNA-binding domain (pGBU9). Yeast cultures were grown in synthetic defined (SD)-*URA-LEU* medium and spotted on SD-*URA-LEU* plates, and SD-*HIS* plates containing different concentrations of the histidine antagonist 3-amino-1,2,4-triazole (3AT): 0.5, 0.8, and 1 mM. Cells were incubated for 3 days at 30°.

Media and growth conditions

S. cerevisiae strains were grown at 30°, unless otherwise specified. Standard YEP medium (1% yeast extract, 2% Bacto Peptone), supplemented with 2% galactose (YEPGal) or 2% dextrose (YEPD), was used for nonselective growth.

The medium used in the RYTHA analysis was a modification of the medium used for SGA (Tong and Boone 2006, 2007). Drugs were added to the following final concentrations: canavanine (50 µg/ml; Sigma [Sigma Chemical], St. Louis, MO); thialysine (50 µg/ml; Sigma); clonNAT (100 µg/ml; Werner Bioagents); G418 (Geneticin) (200 µg/ml; Invitrogen, Carlsbad, CA); and Hygromycin B (100 µg/ml; Calbiochem, San Diego, CA). Because ammonium sulfate impedes the function of G418 and clonNAT, synthetic medium containing these antibiotics was prepared with monosodium glutamic acid (MSG; Sigma) as a nitrogen source. Synthetic medium contained 0.1% yeast nitrogen base w/o amino acid and ammonium sulfate, 0.1% glutamic acid, 2% dextrose, 0.2% amino acid mix, and 2% agar (SD).

Data analysis, filtering, and quality assessment

Plates were scanned using an HP Scanjet G4010 scanner and converted to JPG images with the resolution of 300 dpi. The “BALONY” automated computer-based scoring system was used to analyze digital images of colonies to generate an estimate of the relative growth rate based on pixel density (Young and Loewen 2013). Colony size on the control histidine-containing medium depends on the growth rate of the individual mutant strains. Control colonies of size < 80 pixels were disregarded as being too small to be a reliable control reference. Next, the scores for each deletion mutant were estimated by calculating the ratio of the colony size grown on the

medium lacking histidine (with or without 3AT), divided by the value obtained on histidine-containing medium (termed as: “–HIS ratio” and “3AT ratio”). The ratio scores were normalized by the mean of each plate to eliminate systematic plate-to-plate effects. Normalized ratio scores were sorted in ascending order obtaining a RANK for each normalized ratio score. The median rank was calculated out of all valued repeats of “–HIS” and “3AT” experiments. If there were < 2 valid repeats, the score of the gene was discarded as not reliable. Mean values between “–HIS” and “3AT” final scores were calculated and ranked in ascending order. All dubious ORFs were excluded from the list. Top-rated candidates were considered to affect the studied PPI (~1.5% of the assayed genes). As a cutoff, we considered all candidates above the last of the histidine biosynthesis genes in our list. Other cutoffs are possible (e.g., by percentile).

Betweenness values (BV) were calculated as directed in the EasyNetwork database: <http://www.esyn.org>.

Western blotting

Western blotting and quantification were performed as described previously. Antibodies used for western blotting were mouse polyclonal anti-HA (sc-7392, Santa Cruz).

Data availability

All the data are available at <https://www.benaroyalab.com/rytha>.

Results and Discussion

In the Y2H system, the interaction between two given proteins is selected by the ability of strains to grow on a medium lacking histidine (SD-*HIS*). RYTHA combines the Y2H (Fields and Song 1989) and the SGA methodologies (Tong *et al.* 2001), and enables a system-level detection of *trans*-acting proteins that, when mutated, cause a dissociation of this interaction. Starting with two proteins found as interactors in the Y2H assay, we identify mutants that cause a reduction in the expression of the reporter gene *HIS3*, and thus a growth defect SD-*HIS* medium.

The RYTHA query strain and the RMC

The *MATα* query strain (YSB49) carries two plasmids, which express the fragments of the *GAL4* DNA-BD and the AD, fused at the N-terminus of two interacting proteins (X and Y), and marked with the auxotrophic markers, *TRP1* and *LEU2*, respectively. We also replaced the genes *GAL4* and *GAL80* with *HygB* and *NatMX* markers (*gal4::HygB gal80::NatMX*), and added a construct of the Y2H reporter gene *GAL1-HIS3* linked to the *LYS2* locus, and the Δ *lyp1* recessive markers, that confers resistance to thialysine (Figure 1A). Since the genetic background of YSB49 is a modified version of the original Y2H strain (PJ69) (James *et al.* 1996), we show that the two well-characterized Y2H-interacting proteins AD-*MEC3* and BD-*RAD17* (Kondo *et al.* 1999) can activate the reporter gene *HIS3* in both strains. (Figure 1C).

A MAT α query strain

B MAT α RMC strain

C

Figure 1 (A) Schematic representation and the relevant genetic markers of the RYTHA query strain (YSB49), and (B) the modified mutant collection (RMC). The MAT α query strain (YSB49) carries two plasmids that express fragments of GAL4 DNA-BD and the AD, fused at the N-terminus of the proteins of interest (X and Y), and linked to auxotrophic markers, TRP1 and LEU2, respectively. YSB49 also contains the Y2H construct with the reporter HIS3 gene linked to LYS2. This gene is under the control of the GAL1 promoter (GAL1p) which is activated by the GAL4 transcription factor. GAL80 (another protein in the galactose utilization pathway) can bind to GAL4 and block transcriptional activation. We thus deleted the endogenous copies of the genes GAL4 and GAL80 (*gal4Δ::NatMX* and *gal80Δ::Hygβ*). These genes were deleted from both the query and the RMC strains, to facilitate their selection during the RYTHA final selection step (for details, see Figure 2). Each of the RMC strains carries a gene deletion mutation linked to a kanMX marker (*zzz::KanMX*). To enable selection of the LYS2-GAL1pr-HIS3 Y2H reporter and the GAL4-BD-X-TRP1 plasmid, we deleted the genes LYS2 and TRP1 with the genes *ble* (*lys2::ble*), and MET15 (*trp1::MET15*). Additionally, the RMC strains contain the haploid selection marker integrated and

The query strain was crossed to an ordered array of a modified version of the commercial yeast deletion mutant collection (see below for more details). The yeast deletion mutant collection consists of ~4700 strains, each carrying the strain BY4741 auxotrophic markers (*MATa/ura3Δ0/leu2Δ0/his3Δ1/met15Δ0*), and a gene deletion mutation linked to a *KanMX* marker, which confers resistance to the antibiotic G418 (Brachmann *et al.* 1998; Giaever *et al.* 2002). To make the deletion collection compatible with RYTHA, we generated the RMC (Figure 1B). To this end, we systematically modified each of the 4700 deletion strains as follows. Firstly, to enable selection of the *TRP1*-marked *GAL4-BD* plasmid we replaced the *TRP1* gene (the BY4741 strain is prototroph to tryptophan) with the gene *MET15* (*trp1::MET15*). Secondly, the YSB49 query strain contains the Y2H construct with the reporter *HIS3* gene linked to the *LYS2* gene. To enable selection of this construct during the RYTHA final selection step (see below), we replaced the *LYS2* gene (the BY4741 strain is prototroph to lysine) with the gene *ble* (Gatignol *et al.* 1987), which confers resistance to the antibiotic phleomycin, (*lys2::ble*). Next, as mentioned above, YSB49 carries deletions in the genes *GAL4* and *GAL80*. To facilitate the RYTHA's final selection step, we deleted these genes from the deletion collection (*gal4::HygB* and *gal80::NatMX*). Finally, the query strain YSB49 is deleted for the *LYP1* gene (*lyp1Δ*), a useful SGA selection tool. We added the other SGA marker *can1Δ::STE1pr-URA3* to the RMC strains.

These four genetic modifications were introduced into each one of the 4700 RMC strains by crossing the yeast deletion collection (via SGA) with appropriate intermediate strains (see *Materials and Methods* for details).

RYTHA: a method for detecting trans-acting mutations dissociating a specific PPI

Using the SGA methodology, the *MATa* query strain (YSB49) is crossed to the ordered array of RMC, selecting on SD+HIS+URA+G418 plates (Figure 2, step 1). The resulting array of heterozygous diploids (selected on SD-TRP-LEU+G418 plates), is then induced to undergo meiosis on sporulation (SPO) medium (3% K acetate plates) (Figure 2, step 2), and the set of desired *MATa* haploid meiotic progeny cells can be subsequently selected on the haploid selection media (HSMed), exploiting the SGA HSM (Figure 2, step 3). These steps allow the recovery of a library of ~4500 haploid meiotic progeny, each harboring both X-BD and Y-AD fusion proteins, on the background of a mutation

in a single yeast gene ("*zzz::KanMX*"). This array is transferred to a control HSMed supplemented with histidine ("*+HIS*"), which indicates the effect of the *zzz::KanMX* mutation *per se* on growth rate (Figure 2, step 4, left). The selected haploids are also transferred to a second set of HSMed lacking histidine ("*-HIS*") to select for impaired activation of the reporter gene *GAL1p-HIS3* (Figure 2, step 4, right). Additional sets of HSMed plates lacking histidine and carrying increased levels of the histidine competitor 3AT can also be used for higher stringency of selection.

To assess the level of cell growth, plates are scanned and colony growth is assessed by using an automated computer-based scoring system (BALONY). This system analyzes digital images of colonies to generate an estimate of the relative growth rate based on pixel density (Young and Loewen 2013). Impaired PPI is scored when the colony size on the *-HIS* medium is significantly smaller than that on the control (*+HIS*) array (Figure 2, step 4, indicated by black arrows, and red circles in Figure 2B). Although not carried out here, it is also possible to look for mutants that promote the interaction between the two proteins studied, and thus rank last in the interaction score described.

Genome-wide RYTHA screen

To demonstrate the feasibility and the specificity of the RYTHA methodology, we performed two systematic RYTHA screens:

A control *HIS3* RYTHA screen: This first control screen was performed with a query strain that contains a fully functional *HIS3* gene, and thus can grow on *-HIS* plates independently of the Y2H *HIS3* reporter gene. This screen thus aimed to uncover possible false positive hits. The only mutants identified in the control *HIS3* screen were the expected genes involved in the histidine biosynthesis pathway (*HIS1-HIS7*), which represent the set of false positives that should be identified in all RYTHA future screens.

RYTHA enables systematic identification of mutants that mediate the interaction between *Elg1-BD* and *Slx5-AD*:

The *Slx5/Slx8* heterodimer is a STUbL (Cook *et al.* 2009). Mutations in *SLX5* or *SLX8* lead to the accumulation of high-molecular weight SUMOylated substrates, suggesting a role for this complex in marking SUMOylated proteins for degradation (Wang *et al.* 2006; Li *et al.* 2007b; Uzunova *et al.* 2007). *Δslx5* and *Δslx8* cells exhibit increased genomic instability, manifested by an increase in gross chromosomal

replacing the *CAN1* gene (*can1Δ::STE1pr-URA3*), which allows for selective germination of *MATa* meiotic progeny, since only these cells express the *STE1pr-URA3* reporter. Deletion of the gene *LYP1* (*lyp1Δ*) in YSB49, and *CAN1* in the RMC strains, allows for selective killing of *MATa/a* diploid cells by canavanine and thialysine in the heterozygote diploids. (C) The reporter gene *HIS3* can be expressed in the query strain YSB49 genetic background. The Y2H plasmids expressing the interacting proteins Mec3 and Rad17 fused to the *GAL4* AD and BD, respectively, were transformed into the RYTHA query strain YSB49 and the Y2H original strain PJ69. The combination of the pOBD empty plasmid and AD-MEC3 was used as the negative control. Ten-fold serial dilutions of the indicated strains were plated on medium that selects for the presence of the plasmids (SD-TRP-LEU), and for the expression of the reporter gene *HIS3* (SD-TRP-LEU-HIS). AD, activation domain; BD, binding domain; RMC, RYTHA mutant collection; RYTHA, Reverse Yeast Two-Hybrid Array; SD, synthetic defined medium; Y2H, yeast two-hybrid.

Figure 2 (A and B) General scheme of RYTHA, a systematic method for detecting *trans*-acting mutations that dissociate a specific PPI in *Saccharomyces cerevisiae*. (A) Step 1: the *MAT α* query strain is crossed to an ordered array of the *MAT α* RMC strains, each strain carrying a gene deletion mutation linked to a kanMX marker (*zzz::KanMX*). Step 2: the growth of the resultant *zzz::KanMX/ZZZ* heterozygous diploids is selected on a synthetic medium lacking leucine and tryptophan and supplemented with G418 (SD-LEU-TRP+G418). Step 3: the heterozygous diploids are transferred to medium with reduced levels of carbon and nitrogen to induce sporulation and the formation of haploid meiotic progeny. Step 4: using the *can1 Δ ::MFA1pr-URA3* and *Δ lyp1* SGA HSM (not shown for simplicity), spores are transferred to a HSMed, i.e., SD lacking uracil, which allows for selective germination of *MAT α* meiotic progeny, and supplemented with canavanine and thialysine, which allows for selective germination of meiotic progeny that carry the *Δ can1* and *Δ lyp1* HSMs. To select for the *GAL4-BD-X-TRP1/GAL4-AD-LEU2* plasmids, the HSMed lacks leucine and tryptophan. G418 was added to

rearrangements, sensitivity to DNA-damaging agents, increased mutation rates, and cell cycle delay (Wang *et al.* 2006; Zhang *et al.* 2006; Burgess *et al.* 2007; Nagai *et al.* 2011). The human ortholog of Slx5/Slx8, hRNF4, undergoes dimerization and activates its E3 activity in the presence of SUMO chains (Rojas-Fernandez *et al.* 2014). Thus, the ubiquitin E3 ligase activity of Rnf4 is directly linked to the availability of its polySUMO substrates.

Ubiquitin and SUMO also play a role in the choice of DNA repair pathway: PCNA, the ring that slides along the DNA strand during replication, undergoes either ubiquitination or SUMOylation. These modifications have a role in directing the cell toward one of the DNA damage bypass or repair pathways [Moldovan *et al.* 2007; reviewed in Gazy and Kupiec (2012)].

PCNA is loaded and unloaded from the DNA by the RFC complex, a protein complex composed of five RFC subunits (Rfc1-5) (Gazy *et al.* 2015; Kupiec 2016). Elg1 resembles the large subunit of RFC, and forms an alternative clamp loader/unloader in which it replaces Rfc1 and interacts with the other four RFC subunits (Ben-Aroya *et al.* 2003). The Elg1 RFC-Like Complex (RLC) interacts preferentially with SUMOylated PCNA and unloads modified and unmodified PCNA from chromatin (Parnas *et al.* 2011; Kubota *et al.* 2013; Shiomi and Nishitani 2013).

ELG1 plays a role in many aspects of genome stability maintenance in yeast: deletion of the gene causes an increased rate of spontaneous recombination, gross chromosomal rearrangements, increased MMS sensitivity, and elongated telomeres (Ben-Aroya *et al.* 2003; Smith *et al.* 2004; Smolikov *et al.* 2004). In addition, it exhibits physical and genetic interactions with a variety of genes from the replication and repair pathways, as well as the SUMO pathway (Parnas *et al.* 2009, 2011). The human *ELG1* ortholog, ATAD5, was shown to be involved in the deubiquitination of PCNA and of the Fanconi Anemia FANCI/FANCD2 heterodimer, through its interactions with the deubiquitinating complex USP1/UAF1 (Kee and D'Andrea 2010; Lee *et al.* 2013).

An unbiased Y2H screen, using Elg1's N-terminus as bait, identified Slx5 as a physical interactor of Elg1 (Parnas *et al.* 2011). We have previously established that the physical interaction between Slx5 and the N-terminus of Elg1 depends on the presence of intact SIMs (SUMO-interacting motifs) in the two proteins, and is abolished by deletion of Siz2, the SUMO E3 ligase, or by expressing a SUMO protein that is unable to undergo polySUMOylation (*smt3-3R*) (Parnas

et al. 2011). Taken together, these results imply that the physical interaction between the N-terminus of Elg1 and Slx5 depends on the formation of polySUMO chains to which both proteins attach through their SIMs. Further examination of the requirements for the physical interaction between the Slx5/8 complex and Elg1 might be a way to elucidate the complex physical and functional interactions between the replication fork and DNA repair mechanisms.

We carried out a RYTHA screen (Figure 1) to search for genes that affect the interactions between Elg1 and Slx5. Using SGA technology (Tong *et al.* 2001), we crossed strain MK14562, a derivative of SBY49 carrying the Elg1 N-terminus and Slx5 Y2H plasmids, to the RMC collection of all the nonessential yeast mutants. After meiosis and appropriate selections (see *Materials and Methods*), we looked for haploid strains carrying both plasmids that exhibited a His[−] phenotype. As expected from any RYTHA screen, the resulting list of mutants included those that affect the histidine biosynthesis pathway. Using these mutants to establish a cutoff threshold, our screen identified 38 genes that, when deleted, resulted in a His[−] phenotype (Table 1 and Table 2). Importantly, this list included the deletion of *SIZ2*, which has already been shown to affect the Elg1-Slx5 interaction (Parnas *et al.* 2011). We divided the list of candidates into functional groups (Table 1). This analysis revealed proteins that play a role in DNA replication and repair pathways, chromosome segregation and integrity, and protein modification. Additionally, we have identified genes that have a general role in protein transport, translation, RNA processing, and the stress response.

Next, we analyzed our candidate list by employing an unbiased bioinformatic tool, YEASTMINE (YM) (Balakrishnan *et al.* 2012), to identify hub-interacting genes/proteins that interact (physically or genetically) with a significant number of genes/proteins from our candidate list. After discarding six “sticky” proteins (with > 100 partners), YM revealed 14 hub proteins (Table S2).

As expected, the hub proteins are particularly enriched for those involved in DNA metabolism and genome stability. These proteins interact with each other and with the RYTHA hits to form a tight network (Figure 3) that includes both Slx5, Slx8, and Elg1. To find the most central node in this protein interaction network, we calculated the BV, a numeric value that reflects the importance of a certain node within a network (Dunn *et al.* 2005; Joy *et al.* 2005). The protein that received the highest BV, and is thus considered as the most

select for the gene deletion mutation. Step 5: the germinated spores are transferred to the same medium described in step 4 (left), and similar medium lacking histidine (right). Medium supplemented with histidine is used as a control, and provides an indication of mutants that affect growth rate *per se* under normal conditions (indicated by a dashed black arrow). The haploids that were selected for further analysis showed impaired growth on the experimental medium lacking histidine when compared to the control array (indicated by black arrows). (B) Flatbed scanner is used to scan the plates from step 5. The images represent colonies obtained 2 days after pinning of a single 1536-density array plate. The BALONY software is used to analyze colony growth rate based on pixel density. The colonies encircled in red and yellow are similar to those indicated in black and dashed arrows, respectively, shown in step 5. G418, geneticin; HSM, haploid selection markers; HSMed, haploid selection medium; PPI, protein-protein interaction; RMC, RYTHA mutant collection; RYTHA, Reverse Yeast Two-Hybrid Array; SD, SD, synthetic defined medium; SGA, synthetic genetic array.

Table 1 Genes that, when mutated, affect the physical interaction between Elg1 and Slx5, divided according to function

Systematic Name	Standard Name	Description
DNA Replication and repair		
YNL072W	<i>RNH201</i>	Ribonuclease H2 catalytic subunit; removes RNA primers during Okazaki fragment synthesis and misincorporated ribonucleotides during DNA replication
YER070W	<i>RNR1</i>	Large subunit of ribonucleotide-reductase; the RNR complex catalyzes a rate-limiting step in dNTP synthesis; regulated by DNA replication and DNA damage
YBR223C	<i>TDP1</i>	Tyrosyl-DNA phosphodiesterase I; involved in the repair of DNA lesions created by topoisomerase I and topoisomerase II
YKR056W	<i>TRM2</i>	tRNA methyltransferase and endonuclease with a role in DNA repair
YHR134W	<i>WSS1</i>	Metalloprotease involved in DNA repair, removes DNA–protein cross-links at stalled replication forks during replication of damaged DNA
YMR284W	<i>YKU70</i>	Subunit of the telomeric Ku complex; involved in nonhomologous end joining and telomere length maintenance
YPR062W	<i>FCY1</i>	Cytosine deaminase
Chromosome segregation and genome integrity		
YOL004W	<i>SIN3</i>	Component of histone deacetylase complexes; involved in transcriptional repression and activation of diverse processes, involved in the maintenance of chromosomal integrity
YBR039W	<i>ATP3</i>	ATP synthase, decreased chromosome/plasmid maintenance
YEL029C	<i>BUD16</i>	Putative pyridoxal kinase; required for genome integrity
YDR254W	<i>CHL4</i>	Outer kinetochore protein required for chromosome stability; involved in new kinetochore assembly and sister chromatid cohesion
YBR010W	<i>HHT1</i>	Histone H3; core histone protein required for chromatin assembly
YBR157C	<i>ICS2</i>	Unknown function, null mutant shows decreased chromosome maintenance
YDR532C	<i>KRE28</i>	Subunit of a kinetochore–microtubule-binding complex
Protein modification		
YLR361C	<i>DCR2</i>	Protein Phosphatase. Dosage-dependent positive regulator of the G1/S phase transition
YOR156C	<i>SIZ2</i>	SUMO E3 ligase
YDL190C	<i>UFD2</i>	Ubiquitin chain assembly factor (E4)
YAL005C	<i>SSA1</i>	Member of the HSP70 family; required for ubiquitin-dependent degradation of short-lived proteins
YBR101C	<i>FES1</i>	Factor exchange for SSA1. Hsp70 nucleotide exchange factor; protein abundance increases in response to DNA replication stress
YDR503C	<i>LPP1</i>	Lipid phosphate phosphatase
YDR219C	<i>MFB1</i>	Mitochondria-associated F-box protein
Transport		
YKR093W	<i>PTR2</i>	Integral membrane peptide transporter
YBR172C	<i>SMY2</i>	ER to Golgi vesicle-mediated transport
YOR357C	<i>SNX3</i>	Sorting nexin for late-Golgi enzymes
YJR135W-A	<i>TIM8</i>	Mitochondrial intermembrane space protein
Translation and RNA processing		
YGL135W	<i>RPL1B</i>	Subunit of the cytosolic large ribosomal subunit
YKL156W	<i>RPS27A</i>	Ribosomal Protein of the Small subunit, protein abundance increases in response to DNA replication stress
YGR276C	<i>RNH70</i>	3'–5' exoribonuclease
YLR405W	<i>DUS4</i>	Dihydrouridine synthase, tRNA biosynthesis
YOR076C	<i>SKI7</i>	GTP-binding protein that couples the Ski complex and exosome
Stress response		
YNR074C	<i>AIF1</i>	Apoptosis-inducing factor
YIL111W	<i>COX5B</i>	Subunit Vb of cytochrome c oxidase
YBR159W	<i>IFA38</i>	Sphingolipid biosynthesis
YER118C	<i>SHO1</i>	Transmembrane osmosensor for filamentous growth and osmoregulatory pathways
YGL096W	<i>TOS8</i>	Putative transcription factor; found associated with chromatin, induced during meiosis and under cell-damaging conditions
YDR346C	<i>SVF1</i>	Protein with a potential role in cell survival pathways
Others		
YLR023C	<i>IZH3</i>	Membrane protein involved in zinc ion homeostasis
YDR393W	<i>SHE9</i>	Protein required for normal mitochondrial morphology

RNR, Ribonucleotide Reductase; SUMO, small ubiquitin-like modifier; HOG, .

important protein in this network, is Top1 (Figure 3 and Table S2).

Top1 is a highly conserved enzyme, which resolves DNA supercoils associated with transcription and replication (Cho

et al. 2013). To do so, Top1 covalently binds DNA and, after relieving the supercoil tension, religates the DNA ends. Occasionally, the transient intermediate fails to be resolved, resulting in a DNA–protein cross-link (DPC) that

Table 2 Final ranking of mutants that screened positive in a RYTHA assay for Slx5 and Elg1

Gene Name	ORF Name	Score	Final Rank
<i>HIS5^a</i>	<i>YIL116W</i>	4	1
<i>HIS2^a</i>	<i>YFR025C</i>	11	2
<i>SHO1</i>	<i>YER118C</i>	11	3
<i>HIS1^a</i>	<i>YER055C</i>	12	4
<i>SKI7</i>	<i>YOR076C</i>	14.5	5
<i>HIS1^a</i>	<i>YER055C</i>	15.5	6
<i>HIS1^a</i>	<i>YER055C</i>	16.5	7
<i>RPL1B</i>	<i>YGL135W</i>	22	8
<i>IFA38</i>	<i>YBR159W</i>	22.5	9
<i>BUD16</i>	<i>YEL029C</i>	24	10
<i>RPS27A</i>	<i>YKL156W</i>	26.5	11
<i>AIF1</i>	<i>YNR074C</i>	27	12
<i>HIS7^a</i>	<i>YBR248C</i>	32	13
<i>HIS6^a</i>	<i>YIL020C</i>	32.5	14
<i>SIZ2</i>	<i>YOR156C</i>	33	15
<i>RNR1</i>	<i>YER070W</i>	34	16
<i>SMY2</i>	<i>YBR172C</i>	35	17
<i>DUS4</i>	<i>YLR405W</i>	36	18
<i>CHL4</i>	<i>YDR254W</i>	42	19
<i>ATP3</i>	<i>YBR039W</i>	42	20
<i>SIN3</i>	<i>YOL004W</i>	46	21
<i>TIM8</i>	<i>YJR135W-A</i>	46	22
<i>RNH70</i>	<i>YGR276C</i>	47.5	23
<i>IZH3</i>	<i>YLR023C</i>	51.5	24
<i>ICS2</i>	<i>YBR157C</i>	54	25
<i>TRM2</i>	<i>YKR056W</i>	61.5	26
<i>SNX3</i>	<i>YOR357C</i>	66.5	27
<i>FCY1</i>	<i>YPR062W</i>	71	28
<i>TOS8</i>	<i>YGL096W</i>	74	29
<i>YDR532C</i>	<i>YDR532C</i>	74	30
<i>FES1</i>	<i>YBR101C</i>	74	31
<i>COX5B</i>	<i>YIL111W</i>	74	32
<i>TDP1</i>	<i>YBR223C</i>	78.5	33
<i>HHT1</i>	<i>YBR010W</i>	78.5	34
<i>DCR2</i>	<i>YLR361C</i>	79	35
<i>LPP1</i>	<i>YDR503C</i>	79	36
<i>YKU70</i>	<i>YMR284W</i>	82	37
<i>SVF1</i>	<i>YDR346C</i>	82	38
<i>MFB1</i>	<i>YDR219C</i>	84	39
<i>WSS1</i>	<i>YHR134W</i>	85	40
<i>SSA1</i>	<i>YAL005C</i>	90	41
<i>RNH201</i>	<i>YNL072W</i>	94	42
<i>PTR2</i>	<i>YKR093W</i>	95	43
<i>SHE9</i>	<i>YDR393W</i>	98.5	44
<i>UFD2</i>	<i>YDL190C</i>	120.5	45
<i>HIS4^a</i>	<i>YCL030C</i>	135.5	46

^a Genes involved in histidine biosynthesis, expected in every screen.

needs to be processed by DNA repair mechanisms to allow DNA replication (Pommier *et al.* 2003). In addition, Top1 was also shown to participate in the removal of ribonucleotides from genomic DNA, particularly in the absence of the main enzymatic complex involved in that process, RNase H2 (Cho *et al.* 2013; Williams *et al.* 2013; Amon and Koshland 2016). In this process, Top1 serves as an endonuclease that cleaves the DNA strand where the ribonucleotide is incorporated (Kim *et al.* 2011). These two Top1-mediated processes result in noncanonical (“dirty”) DNA ends, which then need to be processed by additional DNA repair mechanisms.

Finding the connection between Elg1-Slx5 interaction and Top1-mediated DPC repair

To validate the results obtained by RYTHA, we deleted a subset of genes found in the RYTHA screen in a naïve Y2H strain (that was not created as part of the RYTHA procedure) carrying Elg1- and Slx5-containing Y2H plasmids. Figure 4A shows representative drop assays on plates that lack histidine with and without different concentrations of the histidine antagonist 3AT (see *Materials and Methods*). Deletion of *SIZ2*, *UFD2*, *WSS1*, *TDP1*, *RNH201*, and *BUD16* impair the interaction between the N-terminus of Elg1 and Slx5, and hence confirm the screen’s results. Below, we discuss the genes found in the screen and their possible connection with *ELG1*, *SLX5*, and *TOP1*.

SIZ2: As explained above, *SIZ2* is an E3 ubiquitin ligase with physical and genetic interactions with Elg1, Slx5, and Slx8 (Wang *et al.* 2006; Mullen and Brill 2008; Parnas *et al.* 2011). Deletion of *SIZ2* was already found to abolish the interaction between Elg1 and Slx5 (Parnas *et al.* 2011). Siz2 probably mediates the interaction between Elg1 and Slx5 through its functional role as an E3, by SUMOylating a substrate that could be the mediator of the interaction.

WSS1: Wss1 is a metalloprotease that was identified in a screen for high-copy number suppressors of a temperature-sensitive mutant allele of the SUMO protein, *SMT3* (Biggins *et al.* 2001). *WSS1* was shown to interact both physically and genetically with Slx5 and Slx8 (Mullen *et al.* 2010). It has two SIMs and it also gets SUMOylated (Hannich *et al.* 2005). Wss1 has been shown to be involved in the repair of DPCs that are created as a by-product of the activity of Top1 (Stinglele *et al.* 2014). To resolve Top1-mediated DPCs, Wss1 interacts with Cdc48, a chaperone-like ATPase that binds ubiquitin- or SUMO-modified proteins and segregates them from their environment (protein complexes, membranes, or chromatin) (Jentsch and Rumpf 2007; Baek *et al.* 2013). The trapped Top1 DPC gets SUMOylated and interacts with Wss1 and Cdc48 (Stinglele *et al.* 2014). It is still unclear whether Cdc48 is involved in the degradation of peptides that remain after Wss1-mediated proteolysis or in preparing the DPC for Wss1 proteolytic activity.

WSS1 may be essential to allow Elg1-Slx5 interactions in two possible ways: physically (for example, the interaction between Elg1 and Slx5 may be mediated by a poly SUMOylated Wss1) and functionally (*i.e.*, the catalytic activity of Wss1 may be required to allow Elg1-Slx5 interaction).

To differentiate between these two options, we used a protease-deficient mutant of *WSS1* (Mullen *et al.* 2010), in which two point mutations abolish the protease activity of this enzyme (*wss1-PD*).

Figure 4B shows that a plasmid carrying the wild-type version of *WSS1* is able to complement the $\Delta wss1$ mutant, restoring growth on plates without histidine. In contrast, when Wss1 is inactive (*wss1-PD*), it can no longer mediate

Figure 3 A dense network of proteins interact with the identified RYTHA hits. Proteins identified by YEASTMINE as interacting with the SLX STUBL and Elg1 form a dense interactive network among themselves and with the RYTHA hits. In green: genetic interactions. In brown: physical interactions. RYTHA, Reverse Yeast Two-Hybrid Array.

the interaction between Slx5 and Elg1, despite similar levels of expression (Figure 4C). Thus, Wss1 protease activity is required for the Elg1-Slx5 interaction to take place.

UFD2: A ubiquitin chain assembly factor that promotes the formation of polyubiquitin chains (Bohm *et al.* 2011). Similarly to Wss1, Ufd2 was shown to interact with the desegregase Cdc48 and also with Rad23, a protein that interacts both with the ubiquitination machinery and with the proteasome (Richly *et al.* 2005; Hanzelmann *et al.* 2010). Cdc48 binding to Ufd2 releases the interaction between Ufd2 and Rad23, and therefore releases the polyubiquitinated substrate from the ubiquitination machinery and into the pathway of

proteasomal degradation (Baek *et al.* 2011). Since proteasomal degradation is a crucial step in Wss1-mediated DPC repair, a deletion of Ufd2 should lead to an accumulation of unrepaired DPCs, much like a deletion of Wss1.

TDP1: Tdp1 is a Tyrosyl-DNA phosphodiesterase that catalyzes the hydrolysis of proteins that are covalently linked to the 3'-phosphate of DNA, including Top1-derived peptides (Pouliot *et al.* 1999). *TDP1* is conserved throughout evolution (Gajewski *et al.* 2012) and inhibitors of the human enzyme are of major interest in cancer therapeutics. Inhibitors of Top1 that result in Top1-dependent DPCs are commonly used against cancer, and therefore Tdp1 inhibitors are likely

Figure 4 Validation of RYTHA. (A) A Y2H strain bearing an activating domain plasmid (pACT) expressing Slx5 and a binding domain plasmid (pGBU) expressing the N-terminal part of Elg1 was deleted for various genes, and 10-fold dilutions were plated on control SD-URA-LEU plates and the same plates without histidine (–HIS), with or without the indicated concentrations of 3AT. A Y2H strain bearing an activating domain plasmid (pACT) expressing Slx5 and a binding domain plasmid (pGBU) expressing the N-terminal part of Elg1 or an empty vector (ev) were used. (B) Wss1 catalytic activity is important for the interaction between Elg1 and Slx5. A Y2H strain bearing an activating domain plasmid (pACT) expressing Slx5 and a binding domain plasmid (pGBU) expressing the N-terminal part of Elg1 was deleted for *WSS1* and transformed with plasmids carrying either WT *WSS1* or the PD *wss1* allele. (C) The protein expression levels of the WT Wss1 and the phosphatase mutant Wss1 PD are similar. Protein lysates from $\Delta wss1$ cells exogenously expressing *WSS1*, or *wss1*-PD fused to HA (*pWss1*:HA and *pWss1*-PD:HA, respectively), were separated by SDS-PAGE, and immunoblotted with anti-HA and anti-tubulin (loading control) antibodies. (D) A model of how the various mutants may be affecting the level of DPC occurrence. 3AT, 3-amino-1,2,4-triazole; DPC, DNA–protein cross-link; PD, protease-deficient; RYTHA, Reverse Yeast Two-Hybrid Array; SD, synthetic defined medium; WT, wild-type; Y2H, yeast two-hybrid.

to increase the efficiency of this kind of chemotherapy (Huang *et al.* 2011; Pommier 2013).

A deletion of *TDP1* is synthetically lethal with $\Delta wss1$ because they work in parallel in resolving the Top1-mediated DPCs (Stingle *et al.* 2014). As $\Delta wss1$, *TDP1* deletion also reduced the interaction between Elg1 and Slx5. However, the reduction was much milder in $\Delta tdp1$ than in $\Delta wss1$ strains, indicating that Wss1 plays a more important contribution to the interaction between Elg1 and Slx5.

RNH201: Rnh201 is the catalytic subunit of the RNase H2 complex, which protects genome integrity by removing RNA nucleotides incorporated into DNA during replication and/or Okazaki fragment synthesis (Nguyen *et al.* 2011; Wahba *et al.* 2011; Amon and Koshland 2016). Rnh201 plays a key role in DNA damage response and DNA replication processes (Allen-Soltero *et al.* 2014). It has been shown that, in the absence of RNase H2 activity, the resolution of the DNA–RNA hybrids is performed by Top1 and hence, in the absence of Rnh201, there will be a higher level of ribonucleotide incorporation into DNA, and thus a higher probability of Top1 activity and of the formation of Top1 adducts (Potenski *et al.* 2014).

BUD16: Bud16 is a key enzyme in the metabolism of the active form of vitamin B6. Mutations in this gene disturb the dTMP synthesis pathway, which in turn causes an increased rate of dUTP incorporation into DNA strands (Kanellis *et al.* 2007). This leads to an increased rate of DNA–RNA hybrid formation and genome instability. DNA–RNA hybrids require the

activity of Top1 for repair; therefore, in the absence of Bud16, there will probably be more Top1 activity that will in turn lead to more Top1 mediated DPCs.

To summarize (Figure 4D), the deletion of either *UFD2*, *WSS1*, *TDP1*, *RNH201*, or *BUD16* is predicted to increase the rate of Top1-mediated DPC occurrence.

Top1 dependency

We reasoned that if the physical interaction between Slx5 and Elg1 takes place in the context of DPC repair, the phenotype monitored in our screen (abolishment of interaction between the two proteins) should be dependent on Top1 activity. We thus deleted *TOP1* from the strains obtained in our screen and tested the Y2H interactions in the double mutants. Indeed, a deletion of *TOP1* suppressed the reduced interaction phenotype of $\Delta wss1$, $\Delta bud16$, and $\Delta ufd2$ (Figure 5A). The phenotype of the $\Delta tdp1$ deletion was very weak and therefore we could not see a clear suppression effect in the double mutant (data not shown). Interestingly, deletion of *TOP1* had no effect on the $\Delta siz2$ and $\Delta rnh201$ strains.

We conclude that the reduced interaction between Elg1 and Slx5 that is observed on the background of $\Delta wss1$, $\Delta bud16$, and $\Delta ufd2$ depends on Top1 activity.

The interaction between Elg1 and Slx5 depends on SLX STUB1 and Elg1 activity, and on PCNA modifications

To further elucidate the nature of the interaction between Elg1 and Slx5, we carried out additional analyses. Since the RYTHA screen and the Y2H validations were performed with

Figure 5 The effect of Top1, Slx8, Elg1, and PCNA on the interaction between Elg1 and Slx5. (A) A Y2H strain bearing an activating domain plasmid (pACT) expressing Slx5 and a binding domain plasmid (pGBU) expressing the N-terminal part of Elg1 was deleted for various genes, and 10-fold dilutions were plated on control plates (UL) and plates without histidine (ULH) with the indicated concentrations of 3AT. The second lane carries an empty pGBU vector. (B, C) The interaction between Elg1 N-terminus and Slx5 depends on the activity of Slx5/8 and Elg1 (B) and on PCNA SUMOylation (C). Deletion of *ELG1*, *SLX8*, or mutation of the two lysines (K127, K164) to arginine in *POL30* (*pol30-RR*) abolishes the Y2H interaction between Elg1 and Slx5. (D) A model of how Top1 DPC affect the interaction between Elg1 and Slx5. Upon encounter of a DNA-protein complex (such as Topo1-DNA), PCNA is unloaded, Top1 is digested by proteases such as Wss1 and Tdp1, and the peptides further sent for degradation by the Slx5/Slx8 STUbL. If Top1 DPC repair is impaired, the two proteins separate (either because the STUbL is not recruited, the DPC is moved toward the nuclear envelope, or for topological reasons). 3AT, 3-amino-1,2,4-triazole; DPC, DNA-protein cross-link; STUbL, SUMO-targeted ubiquitin ligase; SUMO, small ubiquitin-like modifier; Top1, topoisomerase 1; Y2H, yeast two-hybrid.

the N-terminal part of Elg1, we deleted the entire *ELG1* gene from the genome of the Y2H strain. This deletion abolished the interaction between the Elg1 N-terminus and Slx5, implying that the interaction is not merely structural (for example, through the SIMs at the N-terminus of Elg1), but that it depends on the activity of the entire Elg1 protein. We also deleted Slx8, the partner of Slx5 in the SLX STUbL complex, and this deletion also abolished the interaction between Slx5 and Elg1's N-terminus (Figure 5B) (the strain carrying a deletion of *SLX8* was absent from our deletion collection and thus was not obtained as a hit in the RYTHA screen). Taken together, the results imply that the interaction between Elg1 and Slx5 depends on Elg1 activity (possibly as a PCNA unloader) and on Slx5's STUbL activity, performed by the heterodimer Slx5-Slx8. Deletion of *SLX5* had no effect, probably because the null allele was complemented by the full-length *SLX5* gene expressed as a fusion from the Y2H plasmid.

Another important factor in figuring out the nature of the interaction between Elg1 and Slx5 is PCNA (Parnas *et al.*

2010, 2011). Slx5 was shown to interact with PCNA (Parnas *et al.* 2011) and Slx5-Slx8 was shown to be recruited to DNA damage sites and to localize to replication forks (Cook *et al.* 2009; Nie and Boddy 2016). This may suggest a role for the SLX complex in the DNA repair process. Therefore, we decided to examine the effect of PCNA modifications on the Elg1-Slx5 interaction. Figure 5C shows that the Elg1-Slx5 interaction was impaired in the presence of a PCNA allele that is unable to undergo modifications (*pol30-RR*), suggesting that PCNA modifications are important for Elg1 and Slx5 interaction.

Taking all the observations together, our results suggest the following model (Figure 5D). During DNA replication, the SLX and Elg1-RLC complexes meet, probably at replication forks, where they may collaborate in normal Okazaki fragment processing or in dealing with stalled replication forks. The activity of both the Elg1 RLC and the SLX STUbL are necessary for the interaction. Another requirement for this interaction is PCNA SUMOylation, which seems to be a prerequisite for efficient unloading by the Elg1 RLC (Parnas *et al.*

2010) (Figure 5). PCNA unloading by Elg1 may facilitate repair, whereas the SLX complex may play a role in completing it, probably by sending remaining peptides to degradation.

However, DPCs created by Top1 activity relocate at least one of the two proteins, leading to their dissociation. It has been proposed that the Slx5/8 STUbL may participate in the relocation of broken chromosomes to the nuclear periphery (Nagai *et al.* 2008). Alternatively, the separation could be due to topological changes caused by the DPCs, which interfere with passage of the fork. Mutations that prevent the repair of DPCs (*e.g.*, $\Delta wss1$, $\Delta tdp1$, and $\Delta ufd2$), or those that cause an increase in the level of DPCs (*e.g.*, $\Delta rnh201$ and $\Delta bud16$) thus lead to a dissociation between the two complexes (Figure 5D). The interaction is restored, at least in the case of $\Delta wss1$, $\Delta tdp1$, and $\Delta ufd2$, upon removal (deletion) of Top1 (Figure 4A). Thus, as long as Top1 DPCs are not formed (in a $\Delta top1$ strain) or are rapidly processed (in a wild-type strain), Elg1 and Slx5 colocalize. Upon creation of DPCs, the two proteins separate.

Conclusions

In this paper, we present a new methodology (RYTHA) that allows the systematic screening of a yeast deletion mutant library for mutants that lead to the dissociation of a physical interaction between any two proteins of interest. Our methodology is easy to implement, and can be modified to allow more sophisticated features in the future, such as conditional (*e.g.*, temperature, pH, or osmotic pressure) abolishment of particular interactions. The anticipated results of the RYTHA approach represent the many changes in protein complexes that could arise from several nonexclusive genetic and biochemical perturbations. For example, the deletion of a certain gene ("C"), could lead to disruption of the interaction between two given proteins (A and B), if protein C represents a scaffold protein for the A-B-C protein complex, or stabilizes protein A and/or B. "C" could also regulate the expression levels of A and/or B, or represent a gene required for a specific post-translational modification, required for the PPI. RYTHA cannot distinguish between these possibilities, because in all these cases, the output will be impaired growth on a medium lacking histidine. The mechanism can be identified when combining Gene Ontology term finder annotations, and further genetic and biochemical analyses. Indeed, using these approaches, we have discovered new relationships between pathways in yeast, which has lead us to establish a connection between the proteasomal ubiquitin-dependent degradation pathway and the DNA replication and repair machinery. The Y2H methodology requires the two interacting proteins to be in the nucleus (to affect the transcription of the reporter gene) even if the proteins are naturally located at the cytoplasm. Our list of candidates includes both nuclear and cytoplasmic proteins, despite the fact that the query proteins were nuclear; thus, RYTHA can be used to study any pair of interacting proteins. We believe that our research may lay the foundation for future comprehensive studies to study the effect of genetic perturbations on

in vivo PPI networks, and thus, is expected to promote further understanding of the eukaryotic interactome.

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Literature Cited

- Allen-Soltero, S., S. L. Martinez, C. D. Putnam, and R. D. Kolodner, 2014 A *Saccharomyces cerevisiae* RNase H2 interaction network functions to suppress genome instability. *Mol. Cell. Biol.* 34: 1521–1534.
- Amon, J. D., and D. Koshland, 2016 RNase H enables efficient repair of R-loop induced DNA damage. *Elife* 5: e20533.
- Babu, M., N. J. Krogan, D. E. Awrey, A. Emili, and J. F. Greenblatt, 2009 Systematic characterization of the protein interaction network and protein complexes in *Saccharomyces cerevisiae* using tandem affinity purification and mass spectrometry. *Methods Mol. Biol.* 548: 187–207.
- Baek, G. H., I. Kim, and H. Rao, 2011 The Cdc48 ATPase modulates the interaction between two proteolytic factors Ufd2 and Rad23. *Proc. Natl. Acad. Sci. USA* 108: 13558–13563.
- Baek, G. H., H. Cheng, V. Choe, X. Bao, J. Shao *et al.*, 2013 Cdc48: a swiss army knife of cell biology. *J. Amino Acids* 2013: 183421.
- Balakrishnan, R., J. Park, K. Karra, B. C. Hitz, G. Binkley *et al.*, 2012 YeastMine—an integrated data warehouse for *Saccharomyces cerevisiae* data as a multipurpose tool-kit. *Database (Oxford)* 2012: bar062.
- Ben-Aroya, S., A. Koren, B. Liefshitz, R. Steinlauf, and M. Kupiec, 2003 ELG1, a yeast gene required for genome stability, forms a complex related to replication factor C. *Proc. Natl. Acad. Sci. USA* 100: 9906–9911.
- Biggins, S., N. Bhalla, A. Chang, D. L. Smith, and A. W. Murray, 2001 Genes involved in sister chromatid separation and segregation in the budding yeast *Saccharomyces cerevisiae*. *Genetics* 159: 453–470.
- Bohm, S., G. Lamberti, V. Fernandez-Saiz, C. Stapf, and A. Buchberger, 2011 Cellular functions of Ufd2 and Ufd3 in proteasomal protein degradation depend on Cdc48 binding. *Mol. Cell. Biol.* 31: 1528–1539.
- Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li *et al.*, 1998 Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14: 115–132.
- Burgess, R. C., S. Rahman, M. Lisby, R. Rothstein, and X. Zhao, 2007 The Slx5-Slx8 complex affects sumoylation of DNA repair proteins and negatively regulates recombination. *Mol. Cell. Biol.* 27: 6153–6162.
- Cho, J. E., N. Kim, Y. C. Li, and S. Jinks-Robertson, 2013 Two distinct mechanisms of Topoisomerase 1-dependent mutagenesis in yeast. *DNA Repair (Amst.)* 12: 205–211.
- Cook, C. E., M. Hochstrasser, and O. Kerscher, 2009 The SUMO-targeted ubiquitin ligase subunit Slx5 resides in nuclear foci and at sites of DNA breaks. *Cell Cycle* 8: 1080–1089.

- Dunn, R., F. Dudbridge, and C. M. Sanderson, 2005 The use of edge-betweenness clustering to investigate biological function in protein interaction networks. *BMC Bioinformatics* 6: 39.
- Fields, S., and O. Song, 1989 A novel genetic system to detect protein-protein interactions. *Nature* 340: 245–246.
- Gajewski, S., E. Q. Comeaux, N. Jafari, N. Bharatham, D. Bashford *et al.*, 2012 Analysis of the active-site mechanism of tyrosyl-DNA phosphodiesterase I: a member of the phospholipase D superfamily. *J. Mol. Biol.* 415: 741–758.
- Gatignol, A., M. Baron, and G. Tiraby, 1987 Phleomycin resistance encoded by the ble gene from transposon Tn 5 as a dominant selectable marker in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 207: 342–348.
- Gazy, I., and M. Kupiec, 2012 The importance of being modified: PCNA modification and DNA damage response. *Cell Cycle* 11: 2620–2623.
- Gazy, I., B. Liefshitz, O. Parnas, and M. Kupiec, 2015 Elg1, a central player in genome stability. *Mutat. Res. Rev. Mutat. Res.* 763: 267–279.
- Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles *et al.*, 2002 Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 418: 387–391.
- Goldstein, A. L., and J. H. McCusker, 1999 Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15: 1541–1553.
- Hannich, J. T., A. Lewis, M. B. Kroetz, S. J. Li, H. Heide *et al.*, 2005 Defining the SUMO-modified proteome by multiple approaches in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280: 4102–4110.
- Hanzelmann, P., J. Stingle, K. Hofmann, H. Schindelin, and S. Raasi, 2010 The yeast E4 ubiquitin ligase Ufd2 interacts with the ubiquitin-like domains of Rad23 and Dsk2 via a novel and distinct ubiquitin-like binding domain. *J. Biol. Chem.* 285: 20390–20398.
- Ho, Y., A. Gruhler, A. Heilbut, G. D. Bader, L. Moore *et al.*, 2002 Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415: 180–183.
- Huang, S. N., Y. Pommier, and C. Marchand, 2011 Tyrosyl-DNA Phosphodiesterase 1 (Tdp1) inhibitors. *Expert Opin. Ther. Pat.* 21: 1285–1292.
- Ii, T., J. Fung, J. R. Mullen, and S. J. Brill, 2007a The yeast Slx5-Slx8 DNA integrity complex displays ubiquitin ligase activity. *Cell Cycle* 6: 2800–2809.
- Ii, T., J. R. Mullen, C. E. Slagle, and S. J. Brill, 2007b Stimulation of in vitro sumoylation by Slx5-Slx8: evidence for a functional interaction with the SUMO pathway. *DNA Repair (Amst.)* 6: 1679–1691.
- James, P., J. Halladay, and E. A. Craig, 1996 Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* 144: 1425–1436.
- Jares-Erijman, E. A., and T. M. Jovin, 2006 Imaging molecular interactions in living cells by FRET microscopy. *Curr. Opin. Chem. Biol.* 10: 409–416.
- Jentsch, S., and S. Rumpf, 2007 Cdc48 (p97): a “molecular gearbox” in the ubiquitin pathway? *Trends Biochem. Sci.* 32: 6–11.
- Joy, M. P., A. Brock, D. E. Ingber, and S. Huang, 2005 High-betweenness proteins in the yeast protein interaction network. *J. Biomed. Biotechnol.* 2005: 96–103.
- Kanellis, P., M. Gagliardi, J. P. Banath, R. K. Szilard, S. Nakada *et al.*, 2007 A screen for suppressors of gross chromosomal rearrangements identifies a conserved role for PLP in preventing DNA lesions. *PLoS Genet.* 3: e134.
- Kee, Y., and A. D. D’Andrea, 2010 Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes Dev.* 24: 1680–1694.
- Keren-Kaplan, T., L. Zeev Peters, O. Levin-Kravets, I. Attali, O. Kleinfeld *et al.*, 2016 Structure of ubiquitylated-Rpn10 provides insight into its autoregulation mechanism. *Nat. Commun.* 7: 12960.
- Kim, N., S. N. Huang, J. S. Williams, Y. C. Li, A. B. Clark *et al.*, 2011 Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. *Science* 332: 1561–1564.
- Kondo, T., K. Matsumoto, and K. Sugimoto, 1999 Role of a complex containing Rad17, Mec3, and Ddc1 in the yeast DNA damage checkpoint pathway. *Mol. Cell. Biol.* 19: 1136–1143.
- Kubota, T., K. Nishimura, M. T. Kanemaki, and A. D. Donaldson, 2013 The Elg1 replication factor C-like complex functions in PCNA unloading during DNA replication. *Mol. Cell* 50: 273–280.
- Kupiec, M., 2016 Alternative clamp loaders/unloaders. *FEMS Yeast Res.* 16: fow084.
- Kuzmin, E., S. Sharifpoor, A. Baryshnikova, M. Costanzo, C. L. Myers *et al.*, 2014 Synthetic genetic array analysis for global mapping of genetic networks in yeast. *Methods Mol. Biol.* 1205: 143–168.
- Lee, K. Y., H. Fu, M. I. Aladjem, and K. Myung, 2013 ATAD5 regulates the lifespan of DNA replication factories by modulating PCNA level on the chromatin. *J. Cell Biol.* 200: 31–44.
- Lev, I., M. Volpe, L. Goor, N. Levinton, L. Emuna *et al.*, 2013 Reverse PCA, a systematic approach for identifying genes important for the physical interaction between protein pairs. *PLoS Genet.* 9: e1003838.
- Lev, I., M. Volpe, and S. Ben-Aroya, 2014 Identification of genes important for the physical interaction between protein pairs through reverse PCA (rPCA). *Curr. Protoc. Cell Biol.* 64: 17.15.1–17.15.11.
- Longtine, M. S., A. McKenzie, 3rd, D. J. Demarini, N. G. Shah, A. Wach *et al.*, 1998 Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14: 953–961.
- Michnick, S. W., P. H. Ear, C. Landry, M. K. Malleshaiah, and V. Messier, 2010 A toolkit of protein-fragment complementation assays for studying and dissecting large-scale and dynamic protein-protein interactions in living cells. *Methods Enzymol.* 470: 335–368.
- Moldovan, G. L., B. Pfander, and S. Jentsch, 2007 PCNA, the maestro of the replication fork. *Cell* 129: 665–679.
- Mullen, J. R., and S. J. Brill, 2008 Activation of the Slx5-Slx8 ubiquitin ligase by poly-small ubiquitin-like modifier conjugates. *J. Biol. Chem.* 283: 19912–19921.
- Mullen, J. R., C. F. Chen, and S. J. Brill, 2010 Wss1 is a SUMO-dependent isopeptidase that interacts genetically with the Slx5-Slx8 SUMO-targeted ubiquitin ligase. *Mol. Cell. Biol.* 30: 3737–3748.
- Nagai, S., K. Dubrana, M. Tsai-Pflugfelder, M. B. Davidson, T. M. Roberts *et al.*, 2008 Functional targeting of DNA damage to a nuclear pore-associated SUMO-dependent ubiquitin ligase. *Science* 322: 597–602.
- Nagai, S., N. Davoodi, and S. M. Gasser, 2011 Nuclear organization in genome stability: SUMO connections. *Cell Res.* 21: 474–485.
- Nguyen, T. A., Y. S. Tak, C. H. Lee, Y. H. Kang, I. T. Cho *et al.*, 2011 Analysis of subunit assembly and function of the *Saccharomyces cerevisiae* RNase H2 complex. *FEBS J.* 278: 4927–4942.
- Nie, M., and M. N. Boddy, 2016 Cooperativity of the SUMO and Ubiquitin pathways in genome stability. *Biomolecules* 6: 14.
- Parnas, O., A. Zipin-Roitman, Y. Mazor, B. Liefshitz, S. Ben-Aroya *et al.*, 2009 The ELG1 clamp loader plays a role in sister chromatid cohesion. *PLoS One* 4: e5497.
- Parnas, O., A. Zipin-Roitman, B. Pfander, B. Liefshitz, Y. Mazor *et al.*, 2010 Elg1, an alternative subunit of the RFC clamp loader, preferentially interacts with SUMOylated PCNA. *EMBO J.* 29: 2611–2622.

- Parnas, O., R. Amishay, B. Liefshitz, A. Zipin-Roitman, and M. Kupiec, 2011 Elg1, the major subunit of an alternative RFC complex, interacts with SUMO-processing proteins. *Cell Cycle* 10: 2894–2903.
- Pommier, Y., 2013 Drugging topoisomerases: lessons and challenges. *ACS Chem. Biol.* 8: 82–95.
- Pommier, Y., C. Redon, V. A. Rao, J. A. Seiler, O. Sordet *et al.*, 2003 Repair of and checkpoint response to topoisomerase I-mediated DNA damage. *Mutat. Res.* 532: 173–203.
- Potenski, C. J., H. Niu, P. Sung, and H. L. Klein, 2014 Avoidance of ribonucleotide-induced mutations by RNase H2 and Srs2-Exo1 mechanisms. *Nature* 511: 251–254.
- Pouliot, J. J., K. C. Yao, C. A. Robertson, and H. A. Nash, 1999 Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. *Science* 286: 552–555.
- Richly, H., M. Rape, S. Braun, S. Rumpf, C. Hoege *et al.*, 2005 A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell* 120: 73–84.
- Rojas-Fernandez, A., A. Plechanovova, N. Hattersley, E. Jaffray, M. H. Tatham *et al.*, 2014 SUMO chain-induced dimerization activates RNF4. *Mol. Cell* 53: 880–892.
- Shemesh, K., M. Sebesta, M. Pacesa, S. Sau, A. Bronstein *et al.*, 2017 A structure-function analysis of the yeast Elg1 protein reveals the importance of PCNA unloading in genome stability maintenance. *Nucleic Acids Res.* 45: 3189–3203.
- Shiomi, Y., and H. Nishitani, 2013 Alternative replication factor C protein, Elg1, maintains chromosome stability by regulating PCNA levels on chromatin. *Genes Cells* 18: 946–959.
- Smith, S., J. Y. Hwang, S. Banerjee, A. Majeed, A. Gupta *et al.*, 2004 Mutator genes for suppression of gross chromosomal rearrangements identified by a genome-wide screening in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 101: 9039–9044.
- Smolikov, S., Y. Mazor, and A. Krauskopf, 2004 ELG1, a regulator of genome stability, has a role in telomere length regulation and in silencing. *Proc. Natl. Acad. Sci. USA* 101: 1656–1661.
- Stingele, J., M. S. Schwarz, N. Bloemeke, P. G. Wolf, and S. Jentsch, 2014 A DNA-dependent protease involved in DNA-protein crosslink repair. *Cell* 158: 327–338.
- Tarassov, K., V. Messier, C. R. Landry, S. Radinovic, M. M. Serna Molina *et al.*, 2008 An in vivo map of the yeast protein interactome. *Science* 320: 1465–1470.
- Tong, A. H., and C. Boone, 2006 Synthetic genetic array analysis in *Saccharomyces cerevisiae*. *Methods Mol. Biol.* 313: 171–192.
- Tong, A. H. Y., and C. Boone, 2007 16 High-throughput strain construction and systematic synthetic lethal screening in *Saccharomyces cerevisiae*. *Methods Microbiol.* 36: 369–386.
- Tong, A. H., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader *et al.*, 2001 Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* 294: 2364–2368.
- Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson *et al.*, 2000 A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature* 403: 623–627.
- Uzunova, K., K. Gottsche, M. Miteva, S. R. Weisshaar, C. Glanemann *et al.*, 2007 Ubiquitin-dependent proteolytic control of SUMO conjugates. *J. Biol. Chem.* 282: 34167–34175.
- Wahba, L., J. D. Amon, D. Koshland, and M. Vuica-Ross, 2011 RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. *Mol. Cell* 44: 978–988.
- Wang, Z., G. M. Jones, and G. Prelich, 2006 Genetic analysis connects SLX5 and SLX8 to the SUMO pathway in *Saccharomyces cerevisiae*. *Genetics* 172: 1499–1509.
- Williams, J. S., D. J. Smith, L. Marjavaara, S. A. Lujan, A. Chabes *et al.*, 2013 Topoisomerase 1-mediated removal of ribonucleotides from nascent leading-strand DNA. *Mol. Cell* 49: 1010–1015.
- Young, B. P., and C. J. Loewen, 2013 Balony: a software package for analysis of data generated by synthetic genetic array experiments. *BMC Bioinformatics* 14: 354.
- Yu, H., P. Braun, M. A. Yildirim, I. Lemmens, K. Venkatesan *et al.*, 2008 High-quality binary protein interaction map of the yeast interactome network. *Science* 322: 104–110.
- Zhang, C., T. M. Roberts, J. Yang, R. Desai, and G. W. Brown, 2006 Suppression of genomic instability by SLX5 and SLX8 in *Saccharomyces cerevisiae*. *DNA Repair (Amst.)* 5: 336–346.

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