Cross-Species Complementation of Nonessential Yeast Genes Establishes Platforms for Testing Inhibitors of Human Proteins

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ABSTRACT Cross-species complementation can be used to generate humanized yeast, which is a valuable resource with which to model and study human biology. Humanized yeast can be used as an *in vivo* platform to screen for chemical inhibition of human protein drug targets. To this end, we report the systematic complementation of nonessential yeast genes implicated in chromosome instability (CIN) with their human homologs. We identified 20 human–yeast complementation pairs that are replaceable in 44 assays that test rescue of chemical sensitivity and/or CIN defects. We selected a human–yeast pair (h*FEN1*/y*RAD27*), which is frequently overexpressed in cancer and is an anticancer therapeutic target, to perform *in vivo* inhibitor assays using a humanized yeast cell-based platform. In agreement with published *in vitro* assays, we demonstrate that HU-based PTPD is a species-specific hFEN1 inhibitor. In contrast, another reported hFEN1 inhibitor, the arylstibonic acid derivative NSC-13755, was determined to have off-target effects resulting in a synthetic lethal phenotype with y*RAD27*-deficient strains. Our study expands the list of human–yeast complementation pairs to nonessential genes by defining novel cell-based assays that can be utilized as a broad resource to study human drug targets.

KEYWORDS human–yeast cross-species complementation; chromosome instability; chemical inhibitors; FEN1; cancer targets

OFF-TARGET effects are a major cause of clinical trial failures for cancer therapeutics (Lin *et al.* 2019). As such, establishing additional preclinical models can contribute to the translation of more effective clinical outcomes. One such model is the humanized yeast system, which has been used as an *in vivo* platform for studying chemical inhibition of human protein targets [reviewed in Simon and Bedalov (2004), Mager and Winderickx (2005), and Zimmermann *et al.* (2018)]. Yeast can be humanized using two different approaches: heterologous expression in which a human gene is expressed ectopically in yeast or cross-species complementation in which the human gene complements a mutation in the cognate yeast gene [reviewed in Dunham and Fowler (2013) and Laurent *et al.* (2016)]. Irrespective of orthology, heterologous expression of human genes that induce a phenotypic readout in wild-type yeast cells (such as growth inhibition) can be leveraged to elucidate the pathological functions of disease genes (Cooper *et al.* 2006), identify drug targets (Jo *et al.* 2017), and screen for chemical inhibitors that rescue the growth defect (Perkins *et al.* 2001; Tugendreich *et al.* 2001; Sekigawa *et al.* 2010). In cases where a yeast homolog can be identified for a human gene, cross-species complementation of yeast mutations by human genes can be utilized to elucidate the functional homology between human and yeast proteins (Lee and Nurse 1987), characterize human disease variants (Marini *et al.* 2008; Trevisson *et al.* 2009; Mayfield *et al.* 2012; Sun *et al.* 2016; Yang *et al.* 2017), evaluate tumor-specific mutations (Shaag *et al.* 2005; Hamza *et al.* 2015), and screen for chemical inhibitors (Marjanovic *et al.* 2010). Several large-scale studies have systematically tested the ability of single human genes to replace their yeast orthologs (Zhang *et al.* 2003; Hamza *et al.* 2015; Kachroo *et al.* 2015; Sun *et al.* 2016) and paralogs (Hamza *et al.* 2015; Yang *et al.* 2017; Garge *et al.* 2019; Laurent *et al.* 2019). However, the focus of these complementation screens was restricted to essential yeast

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genes whose mutation allowed for testing the rescue of lethal growth defects. In contrast, nonessential yeast genes, the majority of which cause minimal growth defects when disrupted, can only be screened for complementation of visible phenotypes or in conditional assays that induce measurable growth phenotypes. Conditional assays could involve growing the nonessential gene mutants in restrictive media conditions [*e.g.*, alternate sugar sources (Guimier *et al.* 2016) or a limiting metabolite (Agmon *et al.* 2019)], adding chemicals to sensitize the yeast strain, or converting the nonessential yeast gene to an essential gene by disrupting a synthetic lethal partner (Greene *et al.* 1999).

Chromosome instability (CIN) mutants are of particular interest for human complementation in yeast. CIN is an enabling characteristic of cancer development and progression, and is a major contributor to the heterogeneity of tumors (Negrini *et al.* 2010; Hanahan and Weinberg 2011). The simplicity and genetic tractability of the budding yeast, *Saccharomyces cerevisiae*, make it a model experimental system to delineate conserved biological pathways and processes such as those involved in CIN (Measday and Stirling 2016). Large-scale yeast screens have generated a comprehensive list of genes whose mutation (Myung *et al.* 2001; Smith *et al.* 2004; Kanellis *et al.* 2007; Yuen *et al.* 2007; Andersen *et al.* 2008; Stirling *et al.* 2011) or overexpression (Zhu *et al.* 2015; Ang *et al.* 2016; Duffy *et al.* 2016; Frumkin *et al.* 2016; Tutaj *et al.* 2019) contribute to CIN. Yeast can also be utilized to identify chemical sensitivities to cytotoxic agents caused by CIN gene mutations that may be exploited to selectively target tumor cells (O'Neil *et al.* 2017). For instance, genotoxic agents that act by alkylation are common cancer chemotherapy drugs and yeast mutants that are sensitive to these agents identify candidate human genes required for the DNA damage response (Svensson *et al.* 2012).

Proteins required for chromosome stability are also attractive targets for therapeutic inhibition in cancer cells (Tanaka and Hirota 2016). Indeed, the yeast CIN gene list identifies candidate human CIN genes whose mutation or overexpression may contribute to tumorigenesis (Barber *et al.* 2008; Stirling *et al.* 2011; Duffy *et al.* 2016). One such attractive target is the human DNA flap endonuclease 1 (h*FEN1*). Yeast assays have demonstrated that deletion or overexpression of y*RAD27* (ortholog of h*FEN1*) causes CIN and DNA damage in yeast (Greene *et al.* 1999; Yuen *et al.* 2007; Duffy *et al.* 2016), while studies using human cells have confirmed that depletion or overexpression of h*FEN1* causes DNA damage (Jimeno *et al.* 2017; Becker *et al.* 2018; Mengwasser *et al.* 2019). FEN1 functions in DNA replication and repair, and is required for Okazaki fragment maturation through removal of 5′ flaps during lagging-strand synthesis (Balakrishnan and Bambara 2013). Due to its key role in DNA replication, h*FEN1* has been shown to support rapid proliferation of cancer cells and is overexpressed in breast (Singh *et al.* 2008; Abdel-Fatah *et al.* 2014; He *et al.* 2016), lung (Nikolova *et al.* 2009; He *et al.* 2017), prostate (Lam *et al.* 2006), gastric (Wang *et al.* 2014), brain (Krause *et al.* 2005), and pancreatic (Iacobuzio-Donahue *et al.* 2003) cancer. Further, based on synthetic lethal genetic interaction relationships, h*FEN1* is a second-site target for the selective killing of homologous recombination (HR)-defective and cohesin-mutated cancer cells (McManus *et al.* 2009; van Pel *et al.* 2013). As such, many studies have reported the screening and development of hFEN1 inhibitors as potential anticancer therapeutics (Tumey *et al.* 2005; Dorjsuren *et al.* 2011; McWhirter *et al.* 2013; van Pel *et al.* 2013; Exell *et al.* 2016; He *et al.* 2016; Deshmukh *et al.* 2017).

A humanized yeast system based on cross-species complementation can be utilized as an *in vivo* platform for inhibitor screening. Human complementation of essential yeast gene mutants has been used as a phenotypic readout to screen for inhibitors of human proteins that induce growth inhibition in yeast (Meczes *et al.* 1997; Wider *et al.* 2009; Marjanovic *et al.* 2010; Mayi *et al.* 2015). To expand the list of yeast cell-based platforms beyond those discovered for essential yeast genes, we screened a subset of 112 nonessential yeast CIN genes for complementation by candidate human homologs. We then demonstrated that complementation of nonessential yeast genes in conditional assays that induce measurable growth phenotypes is a viable strategy to test inhibitors of hFEN1.

Materials and Methods

Complementation assays

Generating expression vectors and yeast strains for complementation assays: A list of human homologs of nonessential yeast CIN genes (Yuen *et al.* 2007; Stirling *et al.* 2011) was generated from Yeastmine (Balakrishnan *et al.* 2012). Human complementary DNAs (cDNAs) in Gateway-compatible entry clones (Yang *et al.* 2011) were shuttled into the yeast destination vector pAG416GPD-ccdB+6Stop (*URA3*, CEN, constitutive GPD promoter, and 6-amino acid C-terminal extension) (Alberti *et al.* 2007; Kachroo *et al.* 2015) using LR Clonase II (Invitrogen, Carlsbad, CA) to generate expression clones. Expression vectors and the vector control pRS416 (*URA3*) (Sikorski and Hieter 1989) were transformed into the corresponding *MAT*α yeast haploid knockout strain (Giaever *et al.* 2002), and wild-type strain *MAT*α BY4742 (Brachmann *et al.* 1998) and transformants were selected on SC-Ura media.

To create yeast strains with integrated human cDNAs, donor DNA generated by PCR was cotransformed into BY4742 along with linear fragments encoding Cas9 and a single guide RNA targeted to the coding region of either y*RAD1*, y*RAD10*, y*MMS4*, or y*MUS81* (Supplemental Material, Table S1), following the protocol described by William Shaw (see https://benchling.com/pub/ellis-crispr-tools). Donor DNA for h*MUS81* was obtained by PCR using the entry clone from hORFeome V8.1 (Yang *et al.* 2011) as template and primers were designed to include a stop codon. Donor DNA for h*ERCC4* and h*EME1* was obtained by PCR using clones from the Mammalian Gene Collection (Dharmacon) as template. Donor DNA for h*ERCC1* was generated using

pAG416GPD-h*ERCC1*+6Stop as template in the PCR resulting in a PCR product that also contained the *CYC1* terminator. The double-deletion strains *mus81*Δ *mms4*Δ and *rad1*Δ *rad10*Δ were made by clustered regularly interspaced short palindromic repeats (CRISPR)-mediated deletion (Table S1) of *MMS4* and *RAD10* in the *mus81*Δ::kanMX and *rad1*Δ::kanMX *MAT*α deletion strains, respectively. For the deletions, donor DNA was constructed by annealing two complimentary oligos composed of flanking homology to the left and right of the deletion site. To confirm CRISPR-mediated insertions or deletions, yeast transformants were screened by PCR using primers that flank the region of homology on the donor DNA and verified by sequencing.

Sequence identity (%) in relation to the yeast gene was determined for all human–yeast pairs using NWalign (Y. Zhang, http://zhanglab.ccmb.med.umich.edu/NW-align), and significance between the % sequence identity of complementation pairs and % sequence identity of tested human–yeast pairs was calculated using a Mann–Whitney *U*-test. Yeast gene size was obtained from Yeastmine and significant differences were calculated using the hypergeometric distribution.

Growth assays to assess rescue of chemical sensitivities: Chemical sensitivity complementation assays for yeast strains with *URA3*-marked vectors were carried out in SC-Ura media (± chemical) at 30°, while assays for yeast strains with integrated human cDNAs were carried out in SC media (± chemical) at 30°. For spot assays, wild-type and mutant strains from saturated cultures were serially diluted in 10-fold increments, and plated onto media without or with chemicals at the following concentrations: 0.01% MMS, 200 mM HU, 15 μg/ml benomyl, 8% ethanol, 100 ng/ml cycloheximide, 5 μg/ml camptothecin (CPT), and 10 μg/ml bleomycin. For growth curve validations, cultures were grown to midlog phase then diluted to OD_{600} = 0.1 in 200 μl media ± chemical at the indicated concentrations. OD_{600} readings were measured every 30 min over a period of 24–48 hr in a TECAN M200 plate reader and plates were shaken for 10 min before each reading. Strains were tested in three replicates per plate per condition and the area under the curve (AUC) was calculated for each replicate. Strain fitness was defined as the AUC of each yeast strain relative to the AUC of the control strain (BY4742 ± pRS416) grown on the same plate in the same media condition.

A-like faker assays: Following the method of Yuen *et al.* (2007), isolates of wild-type *MAT*α BY4742 and *MAT*α deletion strains containing *URA3*-marked vectors were patched on each plate in 1-cm^2 squares on SC-Ura, and incubated at 30° for 2 days. Patches were mated to a *MAT*α *his1* tester lawn by replica plating on YPD followed by incubation at 30° for 24 hr. The mated lawn was replica-plated to SC-6 (−Ura, −Lys, −Ade, −His, −Trp, and −Leu) media and incubated for 2 days at 30° to select for His+ products. Complementation of the a-like faker (ALF) phenotype was assessed by comparing the number of colonies per patch to the wild-type control patch on the same plate.

Testing inhibitors of hFEN1

Yeast strains: All strains used for testing inhibitors were *pdr1*Δ *pdr3*Δ, and were constructed by CRISPR-mediated deletion of yeast *PDR1* and *PDR3* in *MAT***a** wild-type and *rad27*Δ::kanMX strains as described previously in the *Materials and Methods*. The h*FEN1* ORF was integrated by CRISPR into the y*RAD27* locus using donor DNA obtained from pAG416GPD-h*FEN1*+6Stop as template in a PCR. The resultant integrated h*FEN1* ORF was flanked by the endogenous y*RAD27* promoter and terminator. Yeast *RAD52* was then deleted in the following two strains: *pdr1*Δ *pdr3*Δ and *pdr1*Δ *pdr3*Δ *rad27*Δ::h*FEN1* by CRISPR, as previously described in the *Materials and Methods* (Table S1).

Growth assays to test hFEN1 inhibitors: PTPD (catalog number: AOB3872) and NSC-13755 (catalog number: AOB3879) were obtained from AOBIOUS INC. For liquid growth curve assays, yeast strains from midlog phase cultures were diluted to OD_{600} = 0.1 in 200 μl media ± 0.01% MMS and the inhibitors were added at the indicated concentrations (or a 0.5% DMSO control). All growth assays were performed in SC media at 30°. OD_{600} readings were measured every 30 min over a period of 24 hr in a TECAN M200 plate reader and plates were shaken for 10 min before each reading. Strains were tested in three replicates per plate per condition and the AUC was calculated for each replicate. Strain fitness was defined as the AUC of each yeast strain relative to the AUC of the control strain (*pdr1*Δ *pdr3*Δ + 0.5% DMSO control) grown on the same plate ± MMS treatment.

Overexpression of hFEN1: Human *FEN1* in an entry clone was shuttled into the yeast destination vector pAG425GAL-ccdB+6Stop (*LEU2*, 2μ, inducible GAL promoter, and 6-amino acid C-terminal extension) (Alberti *et al.* 2007; Kachroo *et al.* 2015) as previously described in the *Materials and Methods*. The overexpression vector and the vector control pRS425 (Christianson *et al.* 1992) were transformed into BY4742, and transformants were selected on SC-Leu media. Generated strains were grown to midlog phase in both dextrose or galactose SC-Leu media before dilution to OD_{600} = 0.1 in the same media for growth curve analysis as previously described in the *Materials and Methods*.

Data availability

Strains and plasmids are available upon request. Figure S1 contains complementation assays for the nonessential CIN genes. Figure S2 contains complementation assays of two-subunit yeast complexes. Figure S3 contains growth curve assays for testing hFEN1 inhibitors in MMS. Figure S4 contains growth curve assays for testing hFEN1 inhibitors in HR-deficient strains. Figure S5 contains growth curve assays assessing overexpression of h*FEN1* in yeast. Table S1 lists primers used for CRISPR-mediated insertion and deletion. Table S2 lists plasmids and strains used in study. Table S3 lists all candidate complementation pairs tested in study.

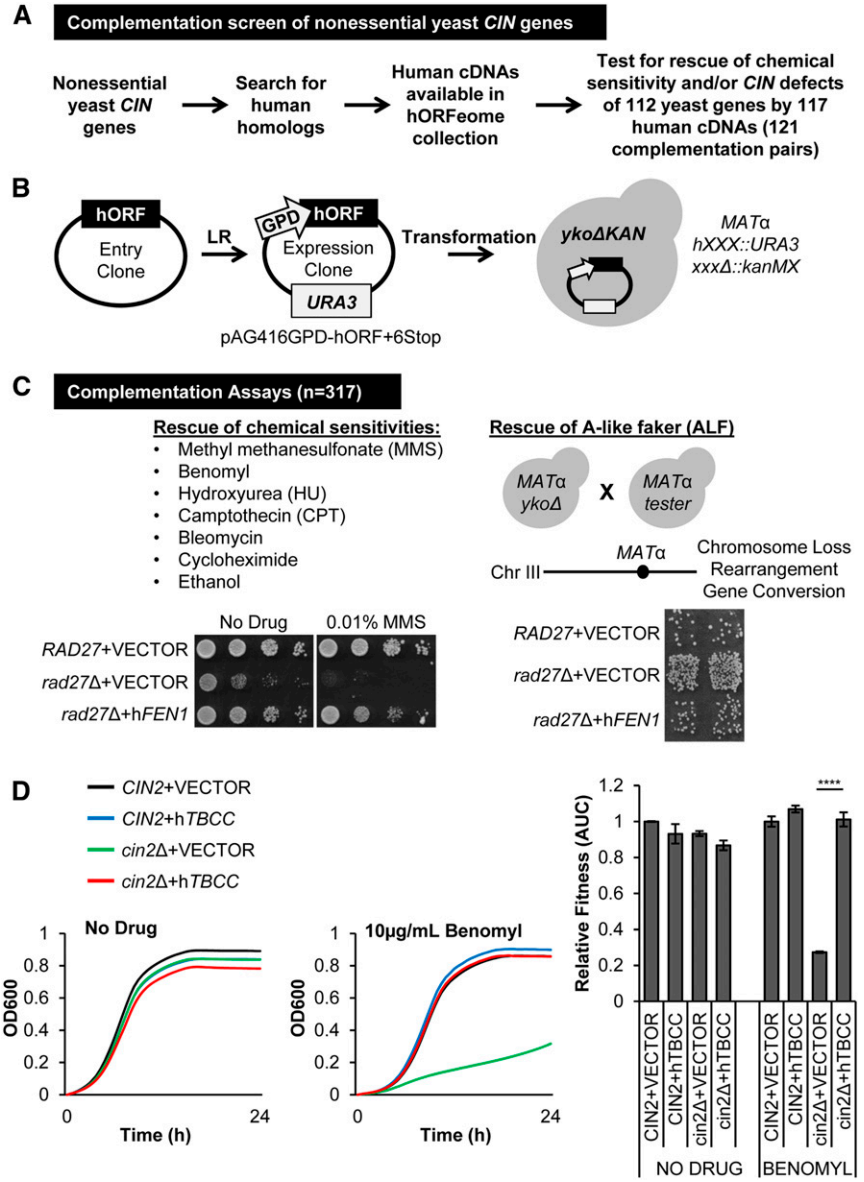

Figure 1 Overview of the complementation screen for the nonessential yeast genes. (A) Pipeline outlining which human–yeast pairs were included in the complementation screen. (B) Human cDNAs cloned in the indicated yeast expression vector or a vector control were transformed into the corresponding haploid yeast knockout mutant (*yko*Δ), and maintained on −Ura media. (C) Yeast strains were spotted in 10-fold dilution on media ± chemical based on the reported sensitivity of the yeast mutant to the seven chemicals. Complementation was scored based on the ability of human cDNA expression to rescue fitness defects of the yeast knockout strain. In the presented example, h*FEN1* expression rescues *rad27*Δ sensitivity to MMS. Growth curve validations for identified hits are shown in Figure S1. For ALFs, α-type mutant strains containing *URA3*-marked vectors were mated to a *MAT*α tester strain and growth of diploid progeny was assessed on selective media. Loss, deletion, or inactivation of the *MAT*α locus allows *MAT*α cells to mate as **a**-type cells. Complementation was scored based on the ability of human cDNA expression to decrease the ALF frequency of the yeast knockout strain. In the presented example (two independent isolates per strain), h*FEN1* expression decreases the elevated frequency of ALF cells that results from deletion of y*RAD27*. (D) Liquid growth curve assays were used to validate complementation observed in spot assays. In the presented example, h*TBCC* expression rescues *cin2*Δ sensitivity to benomyl. Each represented curve is the average of three replicates per media condition. Fitness of each strain was quantified by calculating the AUC of each replicate independently. Strain fitness was defined as the AUC of each yeast strain relative to the AUC of the wild-type strain containing the vector control and grown in the same media condition (mean ± SD). Student's *t*-test. **** $P < 0.0001$. ALF, a-like faker; AUC, area under the curve; cDNA, complementary DNA; Chr, chromosome; CIN, chromosome instability.

Table S4 compares our list of complementation pairs to literature sources. Supplemental material available at figshare: https://doi.org/10.25386/genetics.11573229.

Results

Identification of human–yeast complementation pairs for the nonessential yeast CIN genes

A comprehensive list of yeast CIN genes revealed that of the ∼1100 essential yeast genes, 323 are mutable to a CIN phenotype (Stirling *et al.* 2011). Likewise, of the ∼4800 nonessential yeast genes, 369 are mutable to a CIN phenotype as deletion alleles (Yuen *et al.* 2007; Stirling *et al.* 2011). Previously, we screened the essential yeast CIN genes for complementation by corresponding human cDNAs by assaying rescue-of-lethality of the haploid yeast null allele derived from sporulation of heterozygous diploid strains (Hamza *et al.* 2015). In this study, we identified human–yeast complementation pairs for the nonessential yeast CIN genes. In contrast to essential genes, most haploid deletion strains for nonessential genes display no growth defects when grown under standard laboratory conditions (Giaever *et al.* 2002). To establish our complementation assays, we tested the ability of human gene expression to rescue the chemical sensitivity and/or CIN defects of the nonessential yeast deletion mutant. The chemicals utilized to induce growth defects included MMS (alkylating agent) (Beranek 1990), benomyl (destabilizes microtubules) (Gupta *et al.* 2004), HU (impedes DNA replication) (Koç *et al.* 2004), CPT (topoisomerase inhibitor) (Hsiang *et al.* 1989), bleomycin (induces DNA strand breaks) (Chen *et al.* 2008), cycloheximide (protein synthesis inhibitor) (Schneider-Poetsch *et al.* 2010), and ethanol (impacts many cellular pathways including the cell cycle and morphogenesis) (Stanley *et al.* 2010). To test rescue of CIN defects, we

Table 1 Human genes that complement nonessential yeast deletion mutants

Yeast systematic name	Yeast standard name	Human Entrez gene identifier	Human standard name	Complementation assay[a]	Yeast gene brief description[b]
YAL016W	*TPD3*	5518	*PPP2R1A*	MMS, HU, ALF	Regulatory subunit A of the heterotrimeric PP2A complex
YBR026C	*ETR1*	51102	*MECR*	Ethanol, cycloheximide	2-enoyl thioester reductase
YDR226W	*ADK1*	204	*AK2*	Ethanol	Adenylate kinase, required for purine metabolism
YDR363W-A	*SEM1*	7979	*SHFM1*	HU, ethanol, ALF	19S proteasome regulatory particle lid subcomplex component
YEL003W	*GIM4*	5202	*PFDN2*	Benomyl, ethanol	Subunit of the heterohexameric cochaperone prefoldin complex
YEL029C	*BUD16*	8566	*PDXK*	Ethanol	Putative pyridoxal kinase
YGL058W	*RAD6*	7320	*UBE2B*	MMS, HU, bleomycin, ALF	Ubiquitin-conjugating enzyme (E2)
YGR078C	*PAC10*	7411	*VBP1*	Ethanol, cycloheximide, ALF	Part of the heteromeric cochaperone GimC/ prefoldin complex
YGR180C	*RNR4*	6241	*RRM2*	MMS, HU, bleomycin	Ribonucleotide-diphosphate reductase (RNR) small subunit
YIL052C	*RPL34B*	6164	*RPL34*	Ethanol	Ribosomal 60S subunit protein L34B
YJL115W	*ASF1*	55723	*ASF1B*	MMS, CPT, ALF	Nucleosome assembly factor
YJL140W	*RPB4*	5433	*POLR2D*	HU, MMS	RNA polymerase II subunit B32
YKL113C	*RAD27*	2237	*FEN1*	MMS, ethanol, cycloheximide, ALF	5′ to 3′ exonuclease, 5′ flap endonuclease
YLR418C	*CDC73*	79577	*CDC73*	HU	Component of the Paf1p complex
YML094W	*GIM5*	5204	*PFDN5*	Ethanol, cycloheximide	Subunit of the heterohexameric cochaperone prefoldin complex
YML095C	*RAD10*	2067	*ERCC1*	MMS, HU	Single-stranded DNA endonuclease (with Rad1p)
YOL012C	*HTZ1*	3015	*H2AFZ*	MMS, HU, ethanol	Histone variant H2AZ
YOR002W	*ALG6*	29929	*ALG6*	Ethanol	α 1,3 glucosyltransferase
YPL022W	*RAD1*	2072	*ERCC4*	MMS, HU	Single-stranded DNA endonuclease (with Rad10p)
YPL241C	*CIN2*	6903	*TBCC*	Benomyl	GTPase-activating protein (GAP) for Cin4p

ALF, a-like faker.
[a] Complementation assays are shown in Figure S1.
[b] Brief description obtained from Yeastmine.

used the ALF assay, which measures loss of the *MAT*α locus leading to dedifferentiation to an **a**-mating phenotype and subsequent mating to a *MAT*α tester strain (Yuen *et al.* 2007; Stirling *et al.* 2011). In this assay, the ability of haploid cells to mate with a tester strain of the same mating type and form diploids reflects loss, deletion, or inactivation of the *MAT*α locus.

To set up the complementation assays, we generated a list of human sequence homologs of nonessential yeast CIN genes using the Yeastmine database (Balakrishnan *et al.* 2012) (Figure 1A). This included orthologs, which are genes that diverged by speciation and typically perform the same biological function across species, and paralogs, which diverged by duplication and generally perform biologically distinct yet mechanistically related functions (Koonin 2005). Each human ORF was shuttled via Gateway cloning into a yeast expression vector (single-copy centromeric plasmid and constitutive GPD promoter) (Alberti *et al.* 2007; Kachroo *et al.* 2015) (Figure 1B). For each yeast strain, we queried the *Saccharomyces* Genome Database (SGD) to determine if the yeast deletion mutant has fitness defects in the presence of at least one of the indicated chemicals. We also searched previously published reports (Yuen *et al.* 2007; Stirling *et al.* 2011) to identify mutants that display increased diploid mating products in ALF assays compared to the wild-type strain. Overall, this established assayable phenotypes to test 112 nonessential yeast CIN deletion mutants for complementation by 117 human cDNAs (121 candidate complementation pairs tested across 317 complementation assays) (Figure 1C and Table S3). Our screens identified 20 human cDNAs that rescue the chemical sensitivity and/or CIN defects of 20 nonessential yeast mutants (Table 1). Successful complementation pairs were validated by growth curves and encompassed 44 assays that ranged from one to four assays per pair (Figures S1 and S2). For instance, we demonstrated that h*TBCC* expression rescues *cin2*Δ sensitivity to benomyl (Figure 1D), and that h*FEN1* expression rescues *rad27*Δ sensitivity to MMS, ethanol, and cycloheximide, as well as rescuing CIN defects of *rad27*Δ strains in the ALF assay (Figure S1). Based on a curated list of complementation pairs available from the Yeastmine database, our work identified 13 novel complementation pairs (Table S4).

We assessed features that predict complementation of nonessential yeast gene deletion mutants. Previous screens that tested complementation of essential yeast genes determined that the strongest predictive feature of complementation is that genes in the same pathway, process, or complex tended to be similarly replaceable or nonreplaceable (Hamza *et al.* 2015; Kachroo *et al.* 2015, 2017; Sun *et al.* 2016).

Human genes were more likely to complement if they had a higher proportion of interacting partners that also complemented and vice versa. We observed that this property also applies to the nonessential yeast genes. For example, we determined that each member of the y*RAD1*/y*RAD10* endonuclease complex was replaceable by their respective human orthologs, h*ERCC4*/h*ERCC1*, individually. Conversely, for another endonuclease complex composed of y*MUS81*/y*MMS4*, neither subunit was replaceable by either h*MUS81* or h*EME1* individually. To examine if both members of the complex were required for a successful complementation, we used CRISPR/Cas9-based genomic engineering to replace both members of the yeast complex with the human orthologs. This generated yeast strains in which the human gene ORFs are integrated in the genome and under control of the native yeast gene regulation. A CRISPR/Cas9-mediated ORF replacement strategy ensures stable and endogenously regulated human gene expression that accounts for stoichiometric balance of complex subunits (Ryan *et al.* 2014), while addressing the limitations on the number of yeast selection markers needed for inactivation of multiple yeast genes and simultaneous expression of multiple human genes in yeast. Unlike h*ERCC4*/h*ERCC1*, which could be successfully replaced as a complex (Figure 2A), the combined h*MUS81*/h*EME1* complex failed to complement its yeast heterodimer counterpart (Figure 2B). Other prominent features that were determined to be in common between replaceable essential and nonessential yeast genes are yeast gene size and human/yeast protein sequence similarity. Replaceable nonessential yeast genes were more likely to be shorter in length (Figure 3A), while human/yeast protein sequence identity was found to be a poor predictor of replaceability (Figure 3B). Even though complementation pairs tend to have higher than average sequence similarity, sequence features alone cannot predict a successful complementation. Overall, our results indicate that features that predict complementation of nonessential yeast genes are similar to those observed for essential yeast genes.

Cross-species complementation as a platform for testing hFEN1 inhibitors

Our human–yeast complementation screen demonstrated that h*FEN1* can replace y*RAD27*, thereby presenting an *in vivo* yeast cell-based platform to test chemical inhibition of the human protein. We selected two commercially available compounds reported to inhibit enzymatic activity of hFEN1 *in vitro* to test in our complementation assays: the N-hydroxyurea-based PTPD and the arylstibonic acid derivative NSC-13755 (Dorjsuren *et al.* 2011). To increase membrane permeability, CRISPR/Cas9-based genomic engineering was utilized to construct yeast strains deficient in y*PDR1* and y*PDR3*, which regulate the multidrug resistance pathway (Balzi and Goffeau 1995). Rather than expressing h*FEN1* from a plasmid with the GPD promoter, the CRISPR system was used to replace the genomic y*RAD27* ORF with the h*FEN1* ORF so that h*FEN1* was expressed from the y*RAD27* promoter.

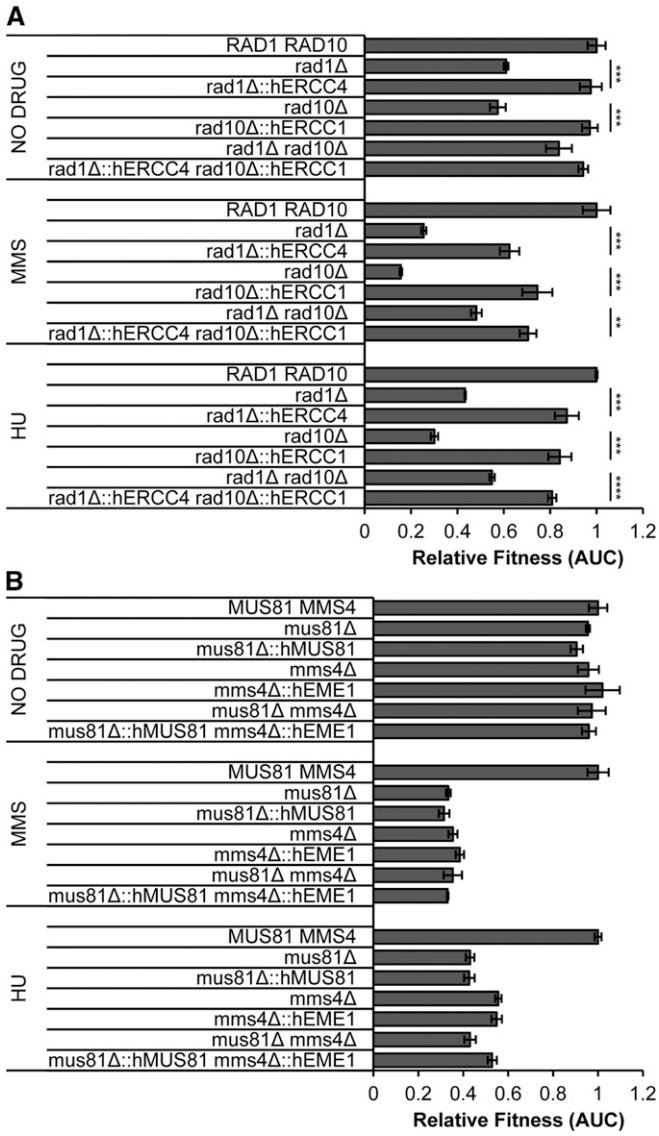

Figure 2 Testing complementation of two-subunit yeast complexes. (A) h*ERCC4*/h*ERCC1* expression (separately or together) rescues *rad1*Δ/*rad10*Δ sensitivity to MMS (0.01%) and HU (150 mM). Student's *t*-test. ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$. (B) h*MUS81*/h*EME1* expression (separately or together) does not rescue *mus81*Δ/*mms4*Δ sensitivity to MMS and HU. Each strain was tested in three replicates per condition and the AUC value was calculated for each replicate independently. Strain fitness was defined as the AUC of each yeast strain relative to the AUC of the wild-type (BY4742) strain grown in the same media condition (mean ± SD). Corresponding growth curves are shown in Figure S2. AUC, area under the curve.

In the absence of added hFEN1 inhibitor, the yeast *rad27*Δ null mutant displays a near wild-type growth rate but is sensitive to treatment with 0.01% MMS (Figure 4, black bars). Expression of h*FEN1* (*rad27*Δ::h*FEN1*) fully rescues the MMS sensitivity (Figure 4). We tested the ability of the two candidate hFEN1 inhibitors to cause growth inhibition of the *rad27*Δ::h*FEN1* yeast strain in the presence of MMS.

PTPD elicited concentration-dependent inhibition of hFEN1 as measured by fitness of the *rad27*Δ::h*FEN1* yeast

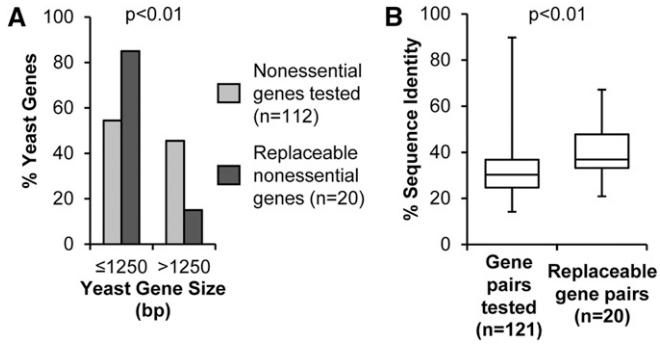

Figure 3 Analyzing features of nonessential yeast genes that predict replaceability. (A) Replaceable nonessential yeast genes are more likely to be shorter in length. Yeast genes were binned by gene length (base pair) and represented as a proportion of the total number of genes input for each set. (B) Amino acid sequence identity is not a strong predictive feature of complementation. The box plot highlights the medians and ranges of sequence identity for each set of human–yeast gene pairs.

strain in MMS (Figure 4A). PTPD did not have an effect on the fitness of the y*RAD27* strain in MMS, indicating that this inhibitor is species-specific to the hFEN1 protein. However, even in the absence of MMS, we did observe a minor concentration-dependent growth inhibition of the *rad27*Δ mutant, which may indicate inhibition of additional off-target yeast protein(s) resulting in negative genetic interactions that impact *rad27*Δ strain fitness. In contrast, NSC-13755 elicited phenotypes inconsistent with hFEN1 inhibition. NSC-13755 did not elicit concentration-dependent inhibition of growth as measured by fitness of the *rad27*Δ::h*FEN1* yeast strain in MMS (Figure 4B). At the highest concentration of NSC-13755 tested (1 mM), rescue of *rad27*Δ sensitivity to MMS was observed to the same extent by either y*RAD27* or h*FEN1*. Furthermore, in the absence of MMS, NSC-13755 caused severe fitness defects in the *rad27*Δ mutant (at the highest concentration tested, 1 mM), and these defects were rescued to the same extent by either y*RAD27* or h*FEN1*, indicating NSC-13755 inhibition of off-target yeast protein(s) that exhibit synthetic lethality with the *rad27*Δ mutant (Figure 4B). To confirm these results, we generated HR-deficient strains by CRISPR-mediated deletion of y*RAD52* in y*RAD27* and *rad27*Δ::h*FEN1* yeast strains. Given that the yeast *rad27*Δ *rad52*Δ double mutant shows a synthetic lethal phenotype (Symington 1998), hFEN1 inhibition can be assessed by inhibition of growth in the absence of MMS. While PTPD caused mild fitness defects in the y*RAD27 rad52*Δ strain, PTPD caused severe concentration-dependent growth defects in the *rad27*Δ::h*FEN1 rad52*Δ strain (Figure 5A), confirming that PTPD inhibits hFEN1 *in vivo*. In contrast, NSC-13755 did not impact fitness of the *rad27*Δ::hFEN1 *rad52*Δ strain relative to the y*RAD27 rad52*Δ strain (Figure 5B). Notably, the differential sensitivity of the *rad27*Δ mutant (relative to y*RAD27* wild-type) caused by NSC-13755 (Figure 4B) was not observed for the *rad52*Δ mutant (relative to y*RAD52* wild-type), suggesting that the off-target effects of this compound may be restricted to yeast protein(s) that are synthetic lethal with the *rad27*Δ null allele. Overall, these results demonstrate the utility and feasibility of using complementation in conditional assays to test chemical inhibition of human protein targets in a cell-based platform.

Discussion

The development of high-throughput and large-scale technologies have expanded the screening capacity for human–yeast complementation pairs. As a result, several systematic screens have reported testing the essential yeast genes for replaceability (Zhang *et al.* 2003; Hamza *et al.* 2015; Kachroo *et al.* 2015; Sun *et al.* 2016; Yang *et al.* 2017; Garge *et al.* 2019; Laurent *et al.* 2019). These studies generated overlapping lists of human–yeast complementation pairs and arrived at similar conclusions regarding features that predict the replaceability of essential yeast genes. However, compared to the essential yeast genes, the nonessential genes are a much larger set and have a variety of different phenotypic readouts, making them more difficult to screen systematically for complementation. In this study, we have started this process by focusing on a subset of the nonessential yeast genes, specifically those required for chromosome maintenance. We identified 20 complementation pairs that are replaceable in 44 assays that test rescue of chemical sensitivity and/or CIN defects. For some human–yeast pairs, we demonstrated that the human gene can complement the yeast gene in multiple complementation assays. Although we did not identify any in this study, there are reported cases of complementation pairs that complement some but not all mutant phenotypes (Yamagata *et al.* 1998; Tamburini *et al.* 2005; Davey *et al.* 2011). For instance, h*WRN* (homolog of y*SGS1*) suppressed the increased rate of illegitimate recombination of *sgs1*Δ but could not rescue *sgs1*Δ sensitivity to HU (Yamagata *et al.* 1998). Our study defines 44 complementation assays that provide yeast cell-based platforms to elucidate human protein function, characterize human gene variants, and study conserved protein domains based on human–yeast complementation relationships. The utility of these yeast cell-based platforms (Dunham and Fowler 2013; Laurent *et al.* 2016) and the accelerated pace of discovery for human–yeast complementation pairs has led the SGD to curate complementation data in their Yeastmine database (Balakrishnan *et al.* 2012).

Our analysis of complementing/noncomplementing human–yeast gene pairs found that features predicting the replaceability of nonessential yeast genes were similar to those observed for essential yeast genes. In general, a major limitation to cross-species complementation involving multiprotein complexes is the potential for interactions of the human protein subunits with the cognate yeast interaction partners. We presented the example of humanizing two related endonucleases that each form heterodimers and function in similar DNA repair processes with some functional overlap (Kikuchi *et al.* 2013; Dehé and Gaillard 2017). In one case, each subunit of the yRad1/yRad10 endonuclease was replaceable, indicating that

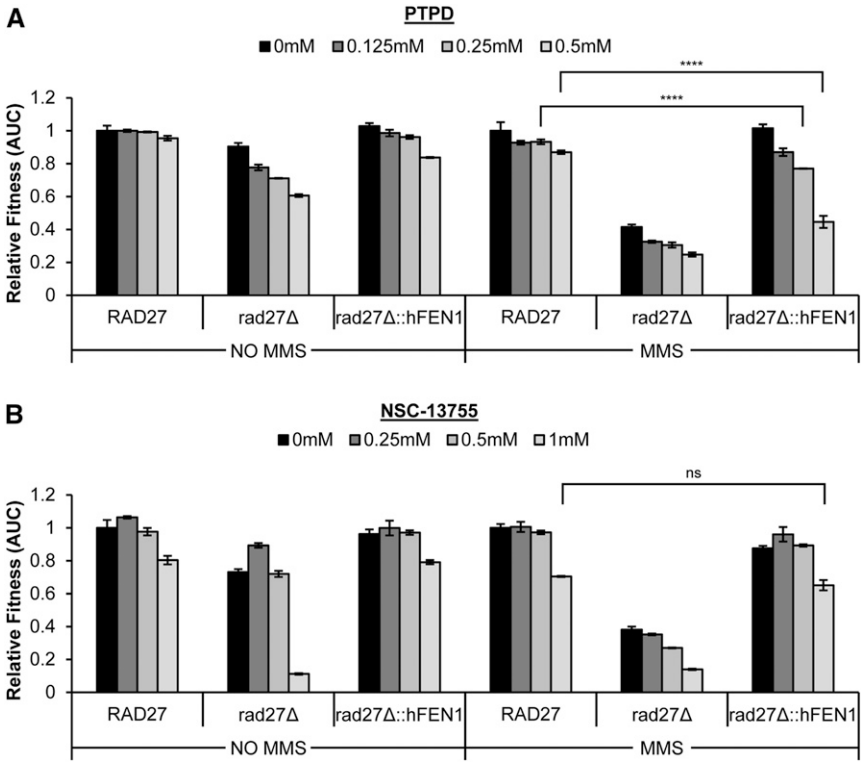

Figure 4 Utilizing complementation in yeast as an *in vivo* platform to test hFEN1 inhibitors. (A) PTPD inhibits hFEN1. (B) NSC-13755 is not a potent hFEN1 inhibitor. All strains in this figure are in a *pdr1*Δ *pdr3*Δ background. Each strain was tested in three replicates per condition and the AUC value was calculated for each replicate independently. Strain fitness was defined as the AUC of each yeast strain relative to the AUC of the control strain (*pdr1*Δ *pdr3*Δ + 0.5% DMSO) grown in the same media condition (mean ± SD). "No MMS" refers to SC media. "MMS" refers to SC + 0.01% MMS media. Corresponding growth curves are shown in Figure S3. Student's *t*-test. **** $P < 0.0001$. AUC, area under the curve.

each human ortholog was able to form a heterodimer with the other yeast subunit. In contrast, yMus81/yMms4 was nonreplaceable even when combining both human subunits in the yeast strain. While these results are consistent with previous findings that showed that genes in the same complex tend to be similarly replaceable or nonreplaceable (Hamza *et al.* 2015; Kachroo *et al.* 2015, 2017; Sun *et al.* 2016), there are reported cases of two-subunit yeast complexes that are only replaceable when both human protein orthologs are expressed (Katahira *et al.* 1999; Gao *et al.* 2005; Ozanick *et al.* 2005; Arnesen *et al.* 2009; Davey *et al.* 2011; Paul *et al.* 2015). In another study, the four-subunit yeast nucleosome was shown to be replaceable by the human nucleosome only after a rare event that allowed yeast cells to adapt and acquire suppressor mutations (Truong and Boeke 2017). A more robust complementation was observed after converting five human histone amino acid residues to the amino acids found in their yeast counterparts, thus revealing the importance of interactions in the success of a human complementation experiment. Overall, our results suggest that the major limitation to predicting the ability of some yeast complexes to be humanized is the lack of information on the minimum complex/pathway members that need to be replaced simultaneously. While replacing one subunit of yRad1/yRad10 was sufficient for complementation, humanization of yMus81/yMms4 may require the replacement of additional yeast genes by human proteins to regulate the functions of the humanized endonuclease (Dehé and Gaillard 2017). Thus, attempts to discover and test the minimum requirements for humanization can provide new avenues to study important properties of human proteins.

We demonstrated an application of humanized yeast in which cross-species complementation of chemical sensitivity facilitated testing candidate inhibitors of hFEN1. Generally, there are two main approaches to screen for inhibitors of human targets in a humanized yeast system: (i) inhibitors that rescue yeast growth defects caused by ectopic heterologous expression of the human gene in yeast and (ii) inhibitors that are screened against the ability of human gene expression to rescue growth defects of the yeast mutant. As a platform for inhibitor screening, human complementation of the yeast null mutant has several benefits over ectopic heterologous expression of human genes in yeast. Human proteins that complement yeast mutants are functional in a cellular system and in the context of other biological pathways, and inhibitors that prevent complementation may better reflect inhibition of the human protein activity in a cellular context. Screening in a null mutant background further eliminates the potential of nonspecific inhibition of the cognate yeast protein and further facilitates the identification of inhibitors that are species-specific (Wider *et al.* 2009; Bilsland *et al.* 2016). Moreover, severe growth defects caused by ectopic heterologous expression of human genes in yeast may be reversed by suppressor mutations that bypass the fitness defects and, as such, large-scale screens for inhibitors that rescue growth defects may have high backgrounds due to false positives. Cross-species complementation may also provide an alternative phenotypic readout for human proteins that do not induce severe toxicity in yeast. For example, overexpression of h*FEN1* in wild-type yeast causes a minimal growth defect (~15%) (Figure S5), which is not enough of a differential sensitivity for a

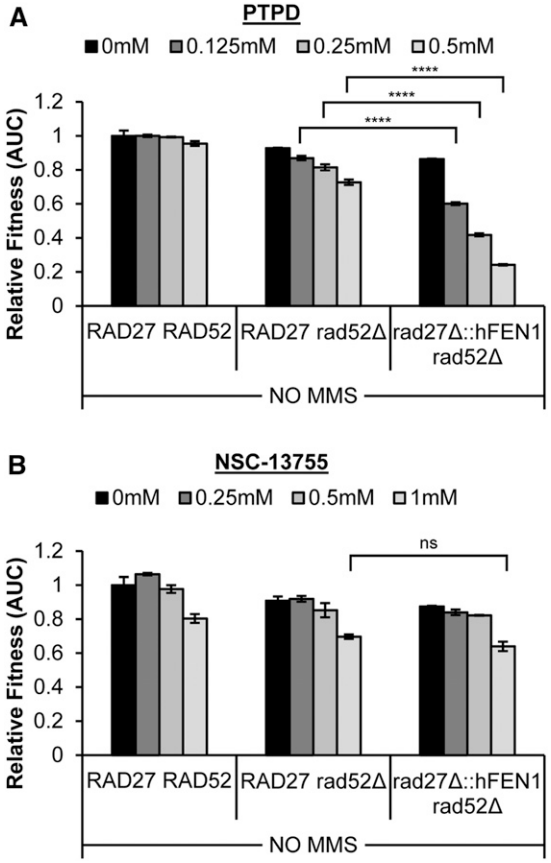

Figure 5 Utilizing complementation to test hFEN1 inhibitors in homologous recombination-deficient yeast. (A) PTPD reduces fitness of a h*FEN1*-integrated strain deficient in y*RAD52*. (B) NSC-13755 does not impact fitness of y*RAD52*-deficient yeast. All strains in this figure are in a *pdr1*Δ *pdr3*Δ background. Each strain was tested in three replicates per condition and the AUC value was calculated for each replicate independently. Strain fitness was defined as the AUC of each yeast strain relative to the AUC of the control strain (*pdr1*Δ *pdr3*Δ + 0.5% DMSO) grown in the same media condition (mean ± SD). The control strains presented in this figure are the same as in Figure 4 since the experiments were performed in the same plate. "No MMS" refers to SC media. Corresponding growth curves are shown in Figure S4. Student's *t*-test. **** $P < 0.0001$. AUC, area under the curve.

restoration-of-growth screening strategy. However, complementation of growth inhibition of the yeast *rad27*Δ null allele in MMS media by h*FEN1* results in an ~60% rescue of growth as measured by liquid growth assays. In the case of essential yeast genes or nonessential yeast genes disrupted in conjunction with a synthetic lethal partner, inhibition of the complementing human protein may result in lethality. Of course, one of the main limitations to cross-species human gene complementation using *S. cerevisiae* as the host is that not all human genes can complement a yeast loss-of-function mutant. For such cases, heterologous expression of human genes in yeast is advantageous if experiments can be designed based on an ability to induce a phenotypic readout.

Our results underscore the necessity of coupling *in vitro* enzymatic assays with *in vivo* assays when assessing inhibitor target activity and specificity. The *in vitro* assays identified PTPD as a more potent inhibitor of hFEN1 than NSC-13755 (Dorjsuren *et al.* 2011). This result may be reflected in our *in vivo* growth assays that determined that NSC-13755 is not potent enough to inhibit hFEN1 activity to a level that causes growth inhibition comparable to the *rad27*Δ mutant. Instead, NSC-13755 seemed to be a potent inhibitor of other yeast protein(s) that are synthetic lethal with the *rad27*Δ mutant. Consistent with this observation, *in vitro* assays have classified NSC-13755 and other arylstibonic acid derivatives as enzymatic inhibitors of the human apurinic/apyrimidinic endonuclease hAPE1 (Seiple *et al.* 2008; Simeonov *et al.* 2009), the regulatory phosphatases hPTEN and hCDC25 (Mak *et al.* 2012), and topoisomerase IB (hTOP1) (Kim *et al.* 2008), in addition to hFEN1. Based on the *in vivo* assays presented in our study, we cannot deduce which yeast homolog(s) of these human protein *in vitro* targets, or other potential yeast protein(s), are the *in vivo* target(s) of NSC-13755. However, a humanized yeast system in which ectopic expression of these human proteins in yeast results in measurable phenotypic readouts may prove to be a suitable *in vivo* platform to determine the target most sensitive to the inhibitory activity of NSC-13755. The effectiveness of such a system is reflected in our *in vivo* assays for the HU-based PTPD. While this inhibitor did display some mild off-target effects, it appeared to be a potent hFEN1 inhibitor. In support of our results, other HU-based derivatives have been shown to increase human cell line sensitivity to MMS treatment (Tumey *et al.* 2005; Exell *et al.* 2016) and selectively impair the proliferation of HR-defective cancer cell lines (Exell *et al.* 2016; He *et al.* 2016; Ward *et al.* 2017). Further, *in vitro* assays have demonstrated that some HU-based compounds are able to enzymatically inhibit the related endonucleases hXPG (Tumey *et al.* 2005) and hEXO1 (Exell *et al.* 2016), but showed species specificity upon testing with bacteriophage T5 FEN and *Kluyveromyces lactis* XRN1 (Exell *et al.* 2016). These results may explain the mild off-target effects observed for the *rad27*Δ and *rad52*Δ mutants in our *in vivo* assays. PTPD appears to be a species-specific hFEN1 inhibitor, but it may exhibit weak inhibition of other related yeast endonucleases that in turn causes negative genetic interactions in the *rad27*Δ and *rad52*Δ mutant strains. Accordingly, a humanized yeast system may be utilized to study the off-target effects of the HU-based compounds to filter those inhibitors that are most potent and specific to the desired target in an *in vivo* setting. The yeast cell-based assays presented in this study can be utilized to screen inhibitors of other cancer-relevant human targets such as the hERCC1/hERRC4 endonuclease complex (Elmenoufy *et al.* 2019), the ribonucleotide reductase hRRM2 (Zhou *et al.* 2013), the histone chaperone hASF1B (Seol *et al.* 2015), and the ubiquitin-conjugating enzyme hUBE2B (Sanders *et al.* 2013). Overall, these results highlight the utility of humanized yeast as a surrogate system and promote the necessity to complete testing of remaining yeast genes for complementation.

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