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Phenotypes Associated with *Saccharomyces cerevisiae* Hug1 Protein, a Putative Negative Regulator of dNTP Levels, Reveal Similarities and Differences with Sequence-Related Dif1[§]

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Saccharomyces cerevisiae **Hug1 is a small protein of unknown function that is highly inducible following replication stress and DNA damage. Its deletion suppresses the lethality of deletion of checkpoint kinase Mec1. Although DNA damage responses were largely normal in the *HUG1* deletion mutant, we found enhanced resistance towards heat in logarithmic phase. In response to simultaneous carbon and replication stress, overall growth delay and less pseudohyphal filament formation were evident. These novel phenotypes are shared with deletion mutants of the negative regulators of ribonucleotide reductase, Dif1 and Sml1. Microarray analysis showed the influence of Hug1 on the expression of a large number of transcripts, including stress-related transcripts. Elevated dNTP levels in *hug1Δ* cells may result in a stress response reflected by the observed phenotypes and transcript profiles. However, in contrast to a deletion of structurally related Dif1, Rnr2-Rnr4 subcellular localization is not grossly altered in a Hug1 deletion mutant. Thus, although Hug1 appears to be derived from the Rnr2-Rnr4 binding region of Dif1, its mechanism of action must be independent of determining the localization of Rnr2-Rnr4.**

Keywords**:** *S. cerevisiae*, dNTP pools, ribonucleotide reductase, stress responses

In eukaryotic cells, DNA damage and replication stress elicit a variety of checkpoint-associated regulatory responses that improve DNA damage repair and tolerance, stabilize replication forks, arrest cell cycle progression or trigger apoptosis and senescence (Kastan and Bartek, 2004; Friedberg *et al.*, 2006; Lazzaro *et al.*, 2009). Such checkpoint responses include many changes in transcript levels. In the yeast *Saccharomyces cerevisiae*, Crt1-Ssn6-Tup1 represses several transcripts that are inducible by agents that damage DNA or inhibit replication (Huang *et al.*, 1998; Zaim *et al.*, 2005). The repression is released through degradation of Crt1 following phosphorylation by Dun1 kinase which itself is activated by Rad53 and, further upstream, by Mec1 (Huang *et al.*, 1998). Both Rad53 and Mec1 kinases play key roles in checkpoint arrests and are also essential for viability (Nyberg *et al.*, 2002). Within this pathway, genes encoding ribonucleotide reductase (RNR) subunits were found to be highly inducible. Their transcriptional up-regulation is one of several mechanisms for transiently elevating dNTP levels in response to replication stress.

One of the most enigmatic genes regulated by Crt1 is *HUG1*. *HUG1* encodes an initially overlooked protein of only 68 amino acids and is among the most DNA-damage inducible yeast genes, irrespective of the type of DNA damage (Basrai *et al.*, 1999; Benton *et al.*, 2007; Mizukami-Murata *et al.*, 2010). The high transcript induction ratios are reflected at the level of the protein (Lee *et al.*, 2007). Remarkably, its deletion rescues the lethality of *MEC1* deletion (Basrai *et al.*, 1999) and elevation of dNTP pools is currently the only known mechanism for this phenomenon. The *HUG1* single deletion mutant, however, has no readily identifiable phenotype.

RNR activity is increased in response to replicational stress on various levels - by transcriptional up-regulation through Crt1, by subunit relocalization, by modifying inhibitor interaction as well as inhibitor degradation, and by reducing dNTP feedback inhibition (Huang *et al.*, 1998; Zhao *et al.*, 2001; Chabes *et al.*, 2003; Yao *et al.*, 2003; Lee and Elledge, 2006). In its active, cytoplasmic form, RNR holoenzyme of *S. cerevisiae* exists as a heterotetramer consisting of Rnr1 homodimers and Rnr2-Rnr4 heterodimers (Fig. 1). Dif1 (for *d*amage-regulated *i*mport *f*actor) regulates dNTP levels negatively by mediating nuclear import of the co-transported small RNR subunits Rnr2-Rnr4 (Lee and Elledge, 2006; Wu and Huang, 2008) (Fig. 1). In response to replication stress, Dif1 is inactivated by checkpoint- kinase dependent phosphorylation and subsequent degradation.

An evolutionary perspective has provided additional important clues to the possible function of Hug1. In *S. cerevisiae*, the *DIF1* region of chromosome XII was duplicated and its paralog is found split into the two separately transcribed genes *HUG1* and *SML1* on chromosome XIII (Fig. 1). *SML1* encodes an Rnr1 inhibitor that is inactivated and degraded following phosphorylation by checkpoint kinase Dun1 (Zhao and Rothstein, 2002). The responsible phosphodegron region of Sml1 is conserved in Dif1. As indicated by moderate se-

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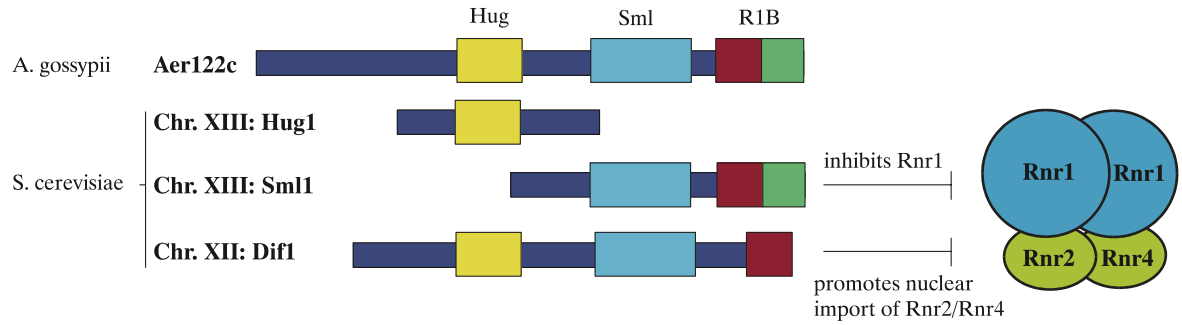

Fig. 1. Dif1 and its relationship to proteins involved in negative regulation of RNR activity (figure adapted from (Lee *et al.*, 2008). In this alignment, *Ashbya gossypii* Aer122c is depicted as the prototype ancestral protein containing three domains: Hug (putative Rnr2-Rnr4 binding domain), Sml (a phosphodegron essential for Sml1 degradation after replication stress) and R1B (Rnr1 binding domain, divided into two subdomains; inactive in Dif1). *S. cerevisiae* ortholog Dif1 acts by facilitating nuclear import of Rnr2-Rnr4. Nuclear sequestration prevents the formation of cytoplasmically active RNR. A *DIF1* paralog originated from chromosome duplication, which is split into two genes, *SML1* and *HUG1*. *SML1*, encoding an Rnr1-binding inhibitor, is transcribed separately from the *HUG1* gene that is located immediately upstream.

quence similarity, the Hug1-homologous region of Dif1 maps to its Rnr2-Rnr4 binding domain (Lee and Elledge, 2006; Wu and Huang, 2008) (Fig. 1). Therefore, one may assume a role of Hug1 in dNTP metabolism by binding to Rnr2-Rnr4.

In this study, we have evaluated the phenotype conferred by a *HUG1* deletion. While we did not find any major sensitivity towards DNA damage, we detected altered responses to other stresses that correlate with a changed transcript profile. However, unlike Dif1, Hug1 did not greatly affect Rnr2-Rnr4 localization. Given the observed phenotypic similarities with Sml1 and Dif1 we suggest that Hug1 negatively regulates RNR activity, albeit by means other than changing Rnr2-Rnr4 location.

Materials and Methods

Yeast strains

All strains were derived from BY4741 (MAT*a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Isogenic deletion strains (*hug1Δ::kanMX4*, *sml1Δ::kanMX4*, *dif1Δ::kanMX4*, *ecm7Δ::kanMX4*) were purchased from OpenBiosystems. *HUG1* deletion was introduced through microhomology-mediated recombination with a PCR product derived from the commercial deletion strain (Rothstein, 1989). A strain containing a GFP-Rnr4 fusion was obtained from Invitrogen.

Survival assays

Cells were precultured to late-logarithmic phase in YPD overnight (1% yeast extract, 2% peptone, 2% dextrose). To obtain mid-logarithmic-phase culture, this culture was diluted in fresh YPD and grown for 4 h at 30°C. Stationary-phase cultures were harvested after 7 days of incubation.

For UV exposure, appropriate dilutions were spread on YPD plates and irradiated with a 254 nm germicidal UV lamp. To determine temperature sensitivity, cultures were incubated at 43°C before plating. Colonies were counted following 3-5 days incubation at 30°C and surviving fractions (relative to an untreated culture) were calculated.

Hydroxyurea assays

In order to study pseudohyphal filamentation and growth responses to hydroxyurea (HU), different dilutions of a logarithmic phase cell culture were spread on YPD plates with reduced dextrose content (0-0.2%), containing 100 mM HU (US Biological). Filamentation was best observed at 12 h after plating. To evaluate growth on dextrose-free medium, plates were incubated for up to 7 days.

To detect Rnr4 localization during recovery from S-phase arrest, early-logarithmic phase cells were exposed to α-factor (synthesized by GeneScript Corp.) as described (Pabla *et al.*, 2006). G1 synchronized cells were washed, briefly sonicated and incubated in fresh YPD containing 100 mM HU for 1.5 h, then resuspended in fresh YPD without HU.

RNA preparation and microarray analysis

Cells were pre-grown overnight to logarithmic phase, plated and cultured at 30°C for 12 h on plate. Plates contained normal YPD (2% dextrose), YPD with reduced dextrose content (0.2% dextrose), with or without 100 mM HU. Cells were scraped from the plate and collected, washed with sterile water, then total RNA extraction was performed using acidic hot phenol and a Mini Bead Beater (Biospec Products) according to published protocols. RNA was further purified by use of RNA Midi kits (QIAGEN, USA). Microarray analysis was performed by the microarray facility of University of Texas Southwestern Medical Center at Dallas using Affymetrix GeneChip Yeast Genome 2.0 arrays.

Results

Sensitivity of *hug1Δ* towards DNA-damage and stress-inducing agents

We re-evaluated the possible phenotype of a *HUG1* deletion towards DNA damaging agents and other stressors. In terms of colony forming ability, we compared cells in the logarithmic growth phase and stationary phase cells (harvested after 7 days of incubation). Using ultraviolet light (254 nm), no significantly increased sensitivity was detected irrespective of culture age (Figs. 2A and B). Duration of checkpoint arrests for various DNA-damaging agents and cell cycle stages was similar in wild type and *hug1Δ* (data not shown).

However, for heat treatment (43°C), a higher resistance of *hug1* cells was found in logarithmic phase (Fig. 2C) but not

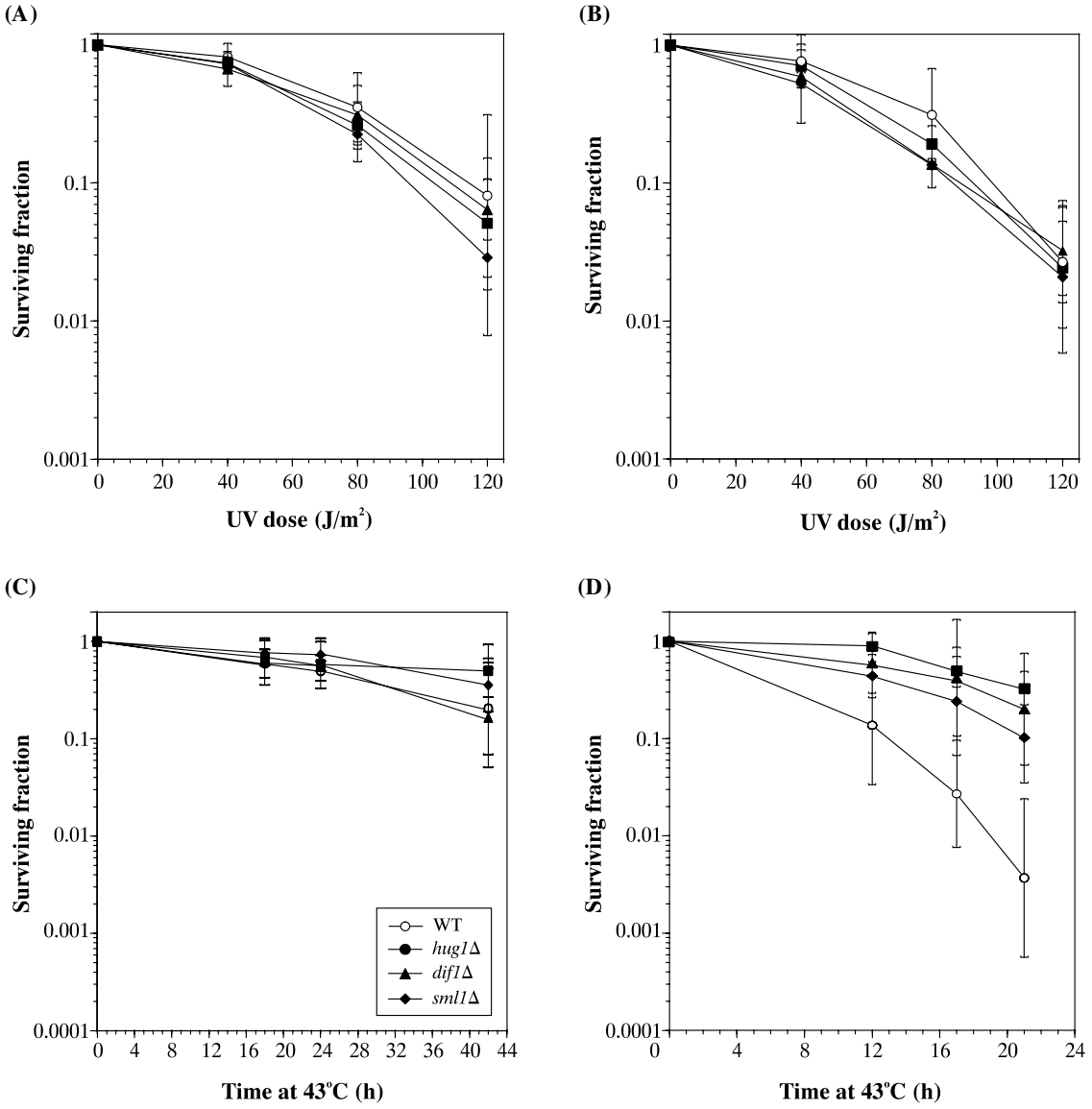

Fig. 2. Sensitivity of colony formation of wild-type (○), *hug1*Δ (■), *dif1*Δ (▲), and *sml1*Δ (◆) cells of (A, C) logarithmic-phase or (B, D) 7 day cultures (=stationary phase) following treatment with (A, B) 254 nm UV radiation or (C, D) incubation at 43°C. Average and standard deviations of 3-4 independent experiments are shown.

in stationary phase (Fig. 2D). The well-known overall increase in resistance from logarithmic to stationary phase towards heat was evident for both wild type and mutant. Importantly, the phenotypes of normal UV response but increased resistance towards 43°C in logarithmic phase are shared with mutants of other negative regulators of dNTP levels (*dif1*, *sml1*) (Figs. 2A-D).

Growth and filamentation response to hydroxyurea

The *hug1*Δ strain is not HU sensitive in normal YPD media (Basrai *et al.*, 1999) (Fig. 3A). Additional carbon stress was induced by reducing the dextrose content from 2% down to 0% which still permits very slow growth. Interestingly, at dextrose concentrations of less than 0.02% in the presence of 100 mM HU, a growth delay was apparent in *hug1*Δ as compared to wild type (Fig. 3A). Deletion mutants of *DIF1* and *SML1* showed HU sensitivity on dextrose-free plates similar to *hug1*Δ (Fig. 3A).

When under moderate carbon stress (0.2% dextrose), we found transient pseudohyphae formation if wild-type cells were exposed on plates for about 12 h to 100 mM HU. The *hug1*Δ strain showed less pronounced pseudohyphae formation under the same conditions (Fig. 3B). Since a polar budding pattern was observed in *hug1*Δ, the main reason for the defect appeared to be the absence of cell elongation. Once again, deletion mutants of *DIF1* or *SML1* exhibited a very similar phenotype as *hug1*Δ (Fig. 3B).

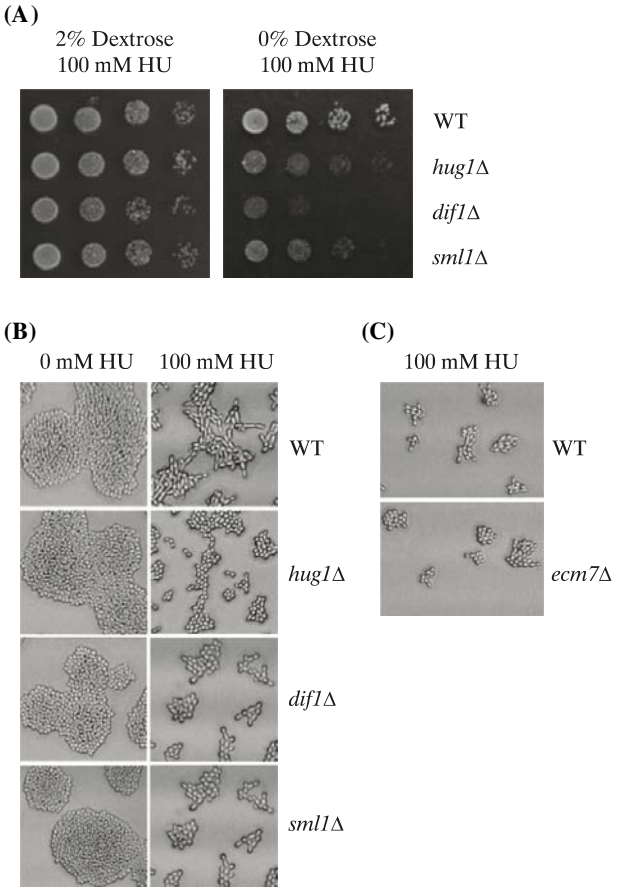

Fig. 3. Phenotypes of wild type, *hug1*Δ, *dif1*Δ, *sml1*Δ and *ecm7*Δ in response to hydroxyurea under conditions of reduced dextrose content. (A) Serial dilutions were plated to determine growth on YPD plates, containing 100 mM HU and normal dextrose content (2%) or no dextrose. Photographs were taken following 2 or 7 days of incubation at 30°C, respectively. (B, C) Pseudohyphae formation was determined after 12 h of incubation on YPD plates containing 100 mM HU and 0.2% dextrose.

Major influence of Hug1 on transcript profiles

We performed RNA microarray analysis comparing transcript profiles of wild type and *hug1*Δ under the various replication conditions studied previously. Relative transcript abundance was examined for clues to the reason for the published phenotypes of *hug1* mutants and those discovered in this study. We compared transcript profiles of *hug1*Δ strain and isogenic wild type grown on plates containing 2% dextrose (YPD) as well as 0.2% dextrose, after 12 h of incubation following plating of logarithmic-phase cells. Low dextrose content was a precondition to detect HU-induced filamentation and transcript profiles were thus also compared in the presence or absence of HU at 0.2% dextrose. Lists of all transcripts ranked by ratio of increase or decrease are available as Supplementary Data (Supplementary data Tables 1-5). The complete set of data can be accessed through http://sites.google.com/site/siedelab/Home/affymetrix-data.

Although Hug1 is hard to detect in cells without replication stress or DNA damage, its suppressor effect on *mec1* lethality must be connected with its constitutive and not its inducible level since a *mec1* deletion would preclude inactivation of the Crt1 repressor and thus inducibility of *HUG1*. Under normal logarithmic growth conditions, without replication stress, the transcript profile of a *hug1* deletion mutant is indeed strikingly different from that of the isogenic wild type, with more than 10% of all genes more than 3 fold up- or down-regulated. A high percentage of stress-related genes among all genes with altered transcript levels were noted (approximately 19%). This significantly exceeds the frequency of such genes in the genome and suggests a specific function of Hug1 in stress responses.

To illustrate some of our findings, we have listed the transcripts with the highest degree of overexpression in *hug1*Δ vs. wild-type at both 2% and 0.2% dextrose (Table 1A). The changes do not seem to follow simple rules, perhaps reflecting the complex phenotypic alterations described above. In Table 1B, transcripts that are highly up-regulated in the wild type in the presence of 100 mM HU are listed. The corresponding transcript profiles were derived from cells incubated for 12 h

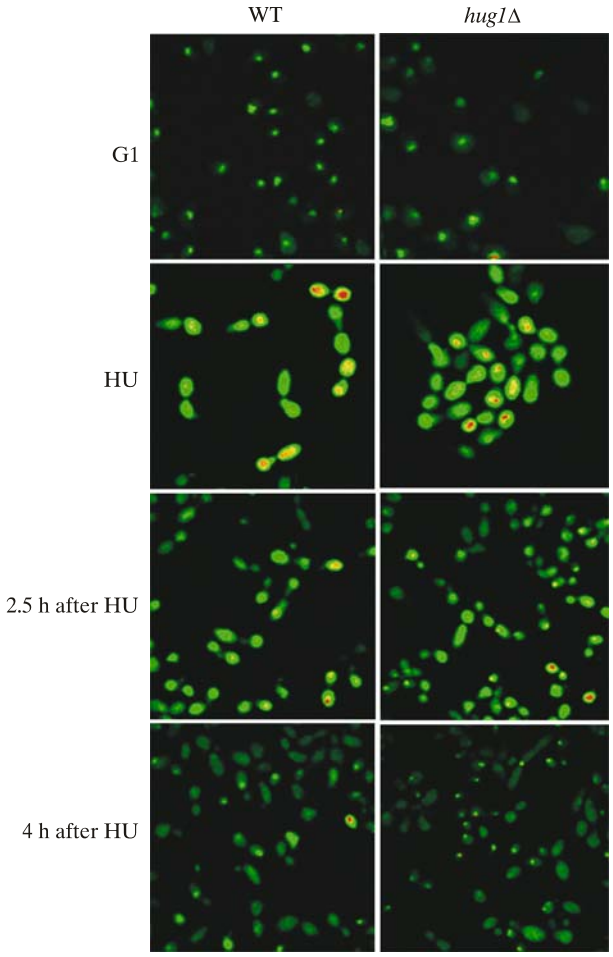

Fig. 4. Localization of GFP-Rnr4 in HU treated wild type and *hug1*Δ during recovery from S-phase arrest. Following release from G1 arrest and treatment with 100 mM HU, cells were incubated for the periods indicated in fresh YPD without HU.

Table 1. List of transcripts and their putative roles with at least 9 fold higher expression levels between two tested strains or conditions. n (A), *hug1*Δ and wild type transcript profiles are compared. Only transcripts with higher expression levels in *hug1*Δ under both 2% and 0.2% dextrose conditions are listed. In (B), the wild-type transcript profile in response to 100 mM HU is compared to the wild type profile without HU (at 0.2% dextrose). In (B), notable differences in regulation of these transcripts between wild type and *hug1*Δ are indicated by asterisks, as outlined below.

Gene	Putative role of encoded product
A. *hug1*Δ vs. wild type	
AQY2	Aquaporin
ARG80	Transcription factor of arginine-responsive genes
CDC13	Single-stranded telomeric DNA binding protein
CHS1	Chitin synthase
DED1	DEAD box RNA helicase
DEP1	Transcriptional modulator of histone deacetylase
FLO1	Cell wall protein
HBT1	Required for polarized cell morphogenesis
IMP2	Transcriptional activator with role in iron homeostasis
MET13	Methylene tetrahydrofolate reductase
MPS1	Nuclear envelope protein
NAB3	Single stranded RNA-binding protein
PAM18	Constituent of mitochondrial inner membrane translocase
PRY3	Unknown, homolog of plant PR-1 antifungal proteins
RIP1	Ubiquinol-cytochrome-C reductase
RIX1	Required for ribosome RNA processing
RPL15A	Ribosome subunit
RRP42	Involved in ribosome RNA processing
SCS3	Inositol metabolism
VHT1	Vitamin H transporter
YBL112C	Unknown
YIH1	Cytoplasmic protein of unknown function, punctate distribution pattern
B. Wild type 100 mM HU vs. 0 mM HU	
APA1	Diadenosine tetraphosphate phosphorylase
APL1	Beta-adaptine
CDA1	Chitin deacetylase
CDA2	Chitin deacetylase
COS12	Subtelomerically encoded protein, possibly required for membrane trafficking
COS4	Subtelomerically encoded protein, possibly required for membrane trafficking
DAK2	Dihydroxyacetone kinase, role in stress adaptation
DAL80	Transcription factor, downregulating nitrogen degradation pathways
ECM6	Membrane protein
ECM7[a]	Membrane protein, inducible by zinc deficiency
FRQ1	Calcium sensor, frequenine homolog
HSP26[b]	Small heat shock protein with chaperone activity.
HXK1	Hexokinase
ILM1[a]	Mitochondrial DNA maintenance, required for filamentous growth during slowed DNA synthesis.
IMD1	Homology with IMP dehydrogenase (pseudogene?)
NCE102[b]	Transmembrane protein involved in secretion
PES4	Poly(A) binding protein
PIC2	Mitochondrial phosphate carrier
PMA2	Plasma membrane H^+ ATPase
RIM4	RNA binding protein required for expression of sporulation genes
RNP1[a]	RNA binding protein
RPA135	RNA polymerase I subunit
SEC63	Required for ER import
SGA1	Sporulation-specific glucoamylase
SMA1	Role in spore membrane assembly
SNO1	Role in pyridoxine metabolism, inducible during stationary phase
SNO4	Chaperone and cysteine protease
SPS1	Meiotic serine/threonine kinase
URA3[a]	Orotidine-5′-phosphate decarboxylase

[a] Not HU inducible in *hug1*Δ.
[b] Not HU inducible in *hug1*Δ but higher expression than in wild type without HU.

under conditions of 0.2% dextrose/100 mM HU and thus at a time when cells had adapted and resumed growth (Fig. 3). RNR gene expression was no longer greatly elevated. *DAK2*, typically overexpressed in stress-adapted cells, was overexpressed under these conditions (Table 1B). Although a haploid strain was used, a number of up-regulated transcripts usually associated with sporulation were noted.

When up-regulated transcripts following HU treatment were compared to *hug1*Δ, the majority of inducible wild-type genes remained inducible. However, some were not up-regulated (such as *ILM1*) or resembled derepressed genes (such as *HSP26*), i.e. characterized by a high expression level already without HU (Table 1B).

When individual transcripts were examined for changes possibly corresponding to defective filamentation, we found *ILM1* and *ECM7* to be grossly altered in regulation. The gene *ECM7,* encoding a cell wall protein (Lussier *et al.*, 1997), was highly inducible by HU in the wild type (20 fold) while in *hug1*Δ its level actually decreased 6 fold in response to HU from a somewhat higher constitutive level. Consequently, we studied the filamentation phenotype of an *ECM7* deletion mutant and detected a defect in cell elongation very similar to *hug1*Δ (Fig. 3C). *ECM7* may thus represent a gene relevant for stress-induced filamentation whose expression is abrogated in *hug1*Δ.

No major role of Hug1 in localization of Rnr4

Since a portion of Hug1 protein appears to have been derived from the Rnr2-Rnr4 binding domain of a common ancestor protein of Dif1 and Hug1 (Lee *et al.*, 2008) (Fig. 1), we asked if Hug1 has a similar mechanism of action as Dif1. Active RNR is cytoplasmic. A *DIF1* deletion mutant is not able to import (co-transported) Rnr2-Rnr4 into the nucleus where these subunits normally reside in unstressed cells (if not in S phase) (Lee *et al.*, 2008; Wu and Huang, 2008). However, unlike *dif1*Δ cells, *hug1*Δ cells synchronized in G1 exhibited a strong nuclear Rnr4-GFP signal (Fig. 4). Upon release into HU-containing medium, pronounced cytoplasmic localization was found in both wild type and *hug1*Δ. General levels of GFP-Rnr4 were unaffected by Hug1 as demonstrated by flow-cytometry (data not shown). During recovery in HU-free medium, nuclear localization, which is restored in wild-type cells (outside of S phase) after about 4 h, may be somewhat accelerated in *hug1*Δ cells (Fig. 4). As others have speculated (Lee *et al.*, 2008), Hug1 may compete with Dif1 for Rnr2-Rnr4 binding and thus delay Rnr2-Rnr4 nuclear re-import in wild type. However, overall kinetics of localization appeared to be unaltered.

Discussion

Although *HUG1* is highly inducible by DNA-damaging agents, no sensitivity phenotype of *hug1* mutants has previously been uncovered (Basrai *et al.*, 1999). We confirmed the absence of UV sensitivity in both logarithmic and stationary phase. However, we detected significant resistance of logarithmic-phase *hug1*Δ cells to extended incubation under heat stress conditions (43°C) as compared to the isogenic wild type. With regard to HU, we confirmed that *hug1*Δ cells show normal resistance under high-dextrose conditions. However, growth is more impaired in *hug1*Δ than in wild type if HU is combined with carbon stress (YPD medium with <0.02% dextrose).

Yeast can respond to various nutritional stresses and slowed replication by pseudohyphal filament formation, characterized by polarized budding and cell elongation (Jiang and Kang, 2003). The chosen haploid strain background (BY4741) has not been a preferred genetic background for such studies due to a relatively poor response. If HU is applied under conditions of lowered dextrose content (0.2% instead of 2% dextrose), transient pseudohyphae formation is observed in wild type. This response is more muted in *hug1*Δ, primarily because of reduced cell elongation.

As assumed for Hug1, Dif1, and Sml1 are negative regulators of RNR activity and, as found for *HUG1*, their deletion rescues the lethality of *MEC1* deletion (Basrai *et al.*, 1999; Zhao *et al.*, 2000; Lee *et al.*, 2008). Additionally, overexpression of either Hug1 or Dif1 is lethal for *mec1 sml1* cells. HU sensitivity of *dun1*Δ is suppressed by *HUG1* deletion and, similarly, *DIF1* overexpression renders *dun1*Δ more HU sensitive (Basrai *et al.*, 1999; Lee *et al.*, 2008). We discovered that these similarities between Hug1, Dif1 and Sml1 extend to all the novel phenotypes described in this study.

These data reaffirm that Hug1 acts as a negative RNR regulator, comparable to Dif1 and Sml1. We assume that constitutively elevated dNTP levels in the *HUG1* deletion mutant represent a stress situation. The possible consequence of reduced NTP/dNTP ratios may affect many areas of metabolism, i.e. RNA synthesis or other metabolic pathways dependent on ATP, GTP or NAD. The observed altered transcript pattern in *hug1*Δ is interpreted as indicative of such a stress response. The generated transcript profile may be beneficial for certain stresses (such as heat) but not for others (such as absence of dextrose in the presence of HU).

Hug1 has also been identified as a substrate of the cyclin-dependent kinase Pho85 (Ptacek *et al.*, 2005). Reminiscent of certain Hug1 phenotypes described here, a main role of this kinase is the phosphorylation of stress-response proteins to keep them in an inactive state if environmental conditions are satisfactory (Huang *et al.*, 2007). However, a comparison of the available transcript profiles shows little overlap between *hug1*Δ and cells with downregulated Pho85 (Carroll *et al.*, 2001). There is also little overlap in transcript profile changes associated with the general stress response of yeast (Gasch and Werner-Washburne, 2002).

However, the phenotype of less pronounced pseudohyphae formation in response to HU was convincingly correlated with lowered expression of *ECM7*. Additionally, the levels of heat shock gene transcripts were examined since heat resistance in logarithmic phase is a phenotype of *hug1*Δ. The heat shock genes *HSP26*, *HSP30* and *HSP32* were all expressed at 4-6 fold higher levels in *hug1*Δ than wild type (2% dextrose); *ECM10*, however, encoding a heat shock protein of the Hsp70 family located in mitochondria, was 3.5 fold underexpressed (Supplementary data Table 1).

Given the sequence similarity of Dif1 and Hug1 (Fig. 1), are both proteins applying a similar mechanism of RNR regulation? In contrast to a *dif1*Δ mutant, Rnr2-Rnr4 localization in *hug1*Δ is virtually unaffected in G1 cells, in HU treated cells and in those recovering from replication stress. To account for its negative regulator function, we hypothesize that Hug1 binds to Rnr2-Rnr4 as predicted and inhibit in some other

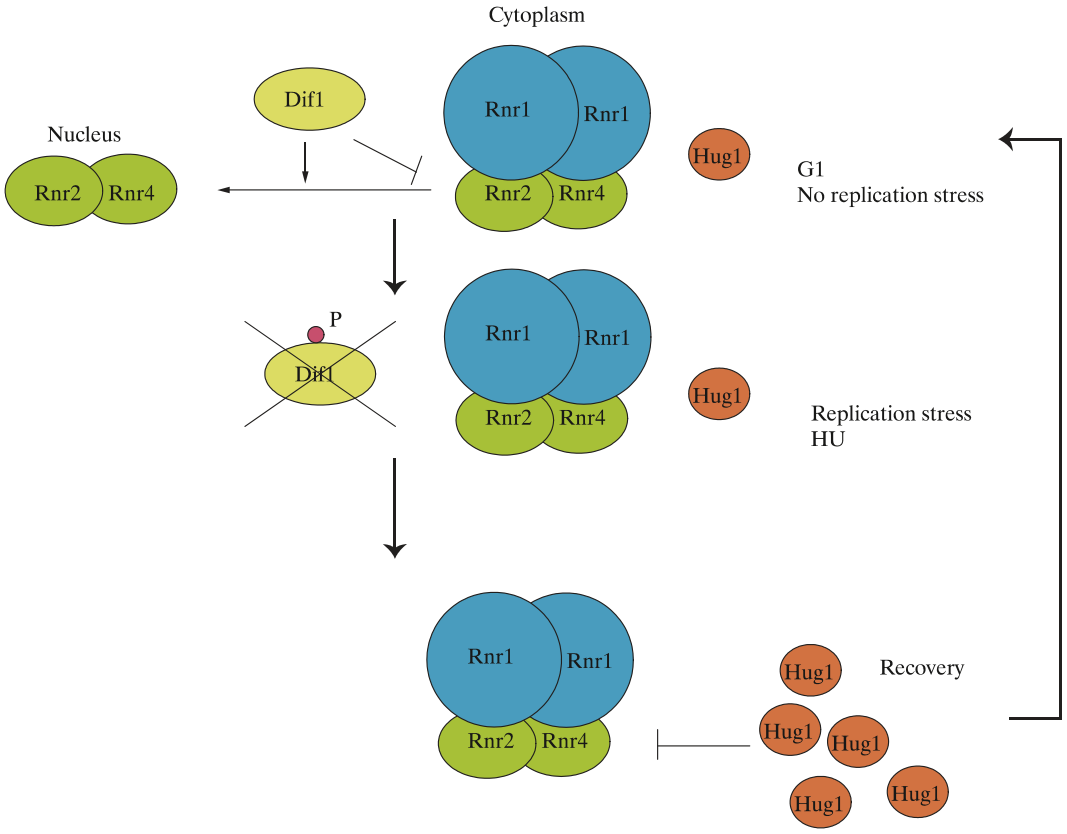

Fig. 5. Scheme for the roles of Dif1 and Hug1 during replication stress and recovery. If replication stress occurs, phosphorylation-mediated Dif1 degradation results in elevated cytoplasmic localization of Rnr2-Rnr4 and enhanced RNR activity. Hug1 is highly expressed at a later stage associated with recovery, leading to RNR inhibition.

way RNR activity, but not by subunit localization. In support of this assumption, mild inhibition of RNR activity *in vitro* has previously been noted for recombinant Dif1 (Lee *et al.*, 2008). Most recently, a detailed characterization of mutants of the *Schizosaccharomyces pombe* Dif1 homolog Spd1 revealed that its roles in nuclear import and restraining RNR activity could be separated (Nestoras *et al.*, 2010). The Hug domain was required for both functions. Spd1 interacts with both Rnr1 and Rnr2 and its Hug domain may mediate this interaction. It was suggested that Spd1 could modulate RNR complex architecture.

The regulation of the highly HU-inducible Hug1 is opposite to that of Dif1 which is inactivated following replication stress. This discrepancy can be rationalized by assuming a role of Hug1 in reducing and resetting dNTP levels during the recovery stage following insult (Basrai *et al.*, 1999) (Fig. 5). In agreement with this assumption, a delayed increase of *HUG1* mRNA as compared to other Crt1-regulated transcripts has been noted after HU stress (Basrai *et al.*, 1999).

In summary, we assume that the gene duplication of *DIF1* generated two negative regulators of dNTP levels that use interaction with Rnr2-Rnr4 as a common feature but employ different mechanisms of RNR downregulation - subunit localization as opposed to inhibitory binding. However, note that if the *S. pombe* paradigm holds, the relevant mechanism may be quite similar and may also involve Rnr1 (Nestoras *et al.*, 2010). Divergent evolution resulted in opposing regulation and a differently timed response to replication stress so that Dif1 is downregulated as soon as stress occurs and Hug1 is upregulated during the adaptation/recovery phase (Fig. 5). A careful study of a time course of interaction of Rnr2 with Dif1 vs. Hug1 may address this issue in the future, preferably by using a sensitive method like fluorescence quenching (FRET) (Nestoras *et al.*, 2010).

Acknowledgements

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References

Basrai, M.A., V.E. Velculescu, K.W. Kinzler, and P. Hieter. 1999. *NORF5/HUG1* is a component of the *MEC1*-mediated checkpoint response to DNA damage and replication arrest in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 19, 7041-7049.

Benton, M.G., N.R. Glasser, and S.P. Palecek. 2007. The utilization of a *Saccharomyces cerevisiae HUG1P*-GFP promoter-reporter construct for the selective detection of DNA damage. *Mutat. Res.* 633, 21-34.

Carroll, A.S., A.C. Bishop, J.L. DeRisi, K.M. Shokat, and E.K. O'Shea. 2001. Chemical inhibition of the Pho85 cyclin-dependent kinase reveals a role in the environmental stress response. *Proc. Natl. Acad. Sci. USA* 98, 12578-12583.

Chabes, A., B. Georgieva, V. Domkin, X. Zhao, R. Rothstein, and L. Thelander. 2003. Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell.* 112, 391-401.

Friedberg, E.C., G.C. Walker, W. Siede, R.D. Wood, R.A. Schultz, and T. Ellenberger. 2006. DNA Repair and Mutagenesis, 2nd (ed.). American Society of Microbiology Press, Washington, D.C., USA.

Gasch, A.P. and M. Werner-Washburne. 2002. The genomics of yeast responses to environmental stress and starvation. *Funct. Integr. Genomics* 2, 181-192.

Huang, D., H. Friesen, and B. Andrews. 2007. Pho85, a multifunctional cyclin-dependent protein kinase in budding yeast. *Mol. Microbiol.* 66, 303-314.

Huang, M., Z. Zhou, and S.J. Elledge. 1998. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell.* 94, 595-605.

Jiang, Y.W. and C.M. Kang. 2003. Induction of *S. cerevisiae* filamentous differentiation by slowed DNA synthesis involves Mec1, Rad53 and Swe1 checkpoint proteins. *Mol. Biol. Cell.* 14, 5116-5124.

Kastan, M.B. and J. Bartek. 2004. Cell-cycle checkpoints and cancer. *Nature* 432, 316-323.

Lazzaro, F., M. Giannattasio, F. Puddu, M. Granata, A. Pellicioli, P. Plevani, and M. Muzi-Falconi. 2009. Checkpoint mechanisms at the intersection between DNA damage and repair. *DNA Repair (Amst).* 8, 1055-1067.

Lee, Y.D. and S.J. Elledge. 2006. Control of ribonucleotide reductase localization through an anchoring mechanism involving Wtm1. *Genes Dev.* 20, 334-344.

Lee, M.W., B.J. Kim, H.K. Choi, M.J. Ryu, S.B. Kim, K.M. Kang, E.J. Cho, H.D. Youn, W.K. Huh, and S.T. Kim. 2007. Global protein expression profiling of budding yeast in response to DNA damage. *Yeast* 24, 145-154.

Lee, Y.D., J. Wang, J. Stubbe, and S.J. Elledge. 2008. Dif1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase. *Mol. Cell.* 32, 70-80.

Lussier, M., A.M. White, J. Sheraton, T. di Paolo, J. Treadwell, S.B. Southard, C.I. Horenstein, and *et al*. 1997. Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* 147, 435-450.

Mizukami-Murata, S., H. Iwahashi, S. Kimura, K. Nojima, Y. Sakurai, T. Saitou, N. Fujii, and *et al*. 2010. Genome-wide expression changes in *Saccharomyces cerevisiae* in response to high-LET ionizing radiation. *Appl. Biochem. Biotechnol.* 162, 855-870.

Nestoras, K., A.H. Mohammed, A.S. Schreurs, O. Fleck, A.T. Watson, M. Poitelea, C. O'Shea, and *et al.* 2010. Regulation of ribonucleotide reductase by Spd1 involves multiple mechanisms. *Genes Dev.* 24, 1145-1159.

Nyberg, K.A., R.J. Michelson, C.W. Putnam, and T.A. Weinert. 2002. Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.* 36, 617-656.

Pabla, R., V. Pawar, H. Zhang, and W. Siede. 2006. Characterization of checkpoint responses to DNA damage in *Saccharomyces cerevisiae*: basic protocols. *Meth. Enzymol.* 409, 101-117.

Ptacek, J., G. Devgan, G. Michaud, H. Zhu, X. Zhu, J. Fasolo, H. Guo, and *et al*. 2005. Global analysis of protein phosphorylation in yeast. *Nature* 438, 679-684.

Rothstein, R. 1989. Targeting, disruption, replacement, and allelic rescue: integrative DNA transformation in yeast. *Meth. Enzymol.* 194, 281-301.

Wu, X. and M. Huang. 2008. Dif1 controls subcellular localization of ribonucleotide reductase by mediating nuclear import of the R2 subunit. *Mol. Cell. Biol.* 28, 7156-7167.

Yao, R., Z. Zhang, X. An, B. Bucci, D.L. Perlstein, J. Stubbe, and M. Huang. 2003. Subcellular localization of yeast ribonucleotide reductase regulated by the DNA replication and damage checkpoint pathways. *Genetics* 100, 6628-6633.

Zaim, J., E. Speina, and A.M. Kierzek. 2005. Identification of new genes regulated by the Crt1 transcription factor, an effector of the DNA damage checkpoint pathway in *Saccharomyces cerevisiae. J. Biol. Chem.* 280, 28-37.

Zhao, X., A. Chabes, V. Domkin, L. Thelander, and R. Rothstein. 2001. The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *EMBO J.* 20, 3544-3553.

Zhao, X., B. Georgieva, A. Chabes, V. Domkin, J.H. Ippel, J. Schleucher, S. Wijmenga, L. Thelander, and R. Rothstein. 2000. Mutational and structural analyses of the ribonucleotide reductase inhibitor Sml1 define its Rnr1 interaction domain whose inactivation allows suppression of *mec1* and *rad53* lethality. *Mol. Cell. Biol.* 20, 9076-9083.

Zhao, X. and R. Rothstein. 2002. The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. *Proc. Natl. Acad. Sci. USA* 99, 3746-3751.