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Modulation of Ubc4p/Ubc5p-Mediated Stress Responses by the RING-Finger-Dependent Ubiquitin-Protein Ligase Not4p in *Saccharomyces cerevisiae*

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ABSTRACT

The Ccr4-Not complex consists of nine subunits and acts as a regulator of mRNA biogenesis in *Saccharomyces cerevisiae*. The human ortholog of yeast *NOT4*, CNOT4, displays UbcH5B-dependent ubiquitin-protein ligase (E3 ligase) activity in a reconstituted *in vitro* system. However, an *in vivo* role for this enzymatic activity has not been identified. Site-directed mutagenesis of the RING finger of yeast Not4p identified residues required for interaction with Ubc4p and Ubc5p, the yeast orthologs of UbcH5B. Subsequent *in vitro* assays with purified Ccr4-Not complexes showed Not4p-mediated E3 ligase activity, which was dependent on the interaction with Ubc4p. To investigate the *in vivo* relevance of this activity, we performed synthetic genetic array (SGA) analyses using *not4*Δ and *not4L35A* alleles. This indicates involvement of the RING finger of Not4p in transcription, ubiquitylation, and DNA damage responses. In addition, we found a phenotypic overlap between deletions of *UBC4* and mutants encoding single-amino-acid substitutions of the RING finger of Not4p. Together, our results show that Not4p functions as an E3 ligase by modulating Ubc4p/Ubc5p-mediated stress responses *in vivo*.

In yeast, the Ccr4-Not complex is composed of nine subunits and has initially been identified as a transcriptional repressor (Collart and Struhl 1994). However, this evolutionary conserved complex is now known to regulate mRNA biogenesis at multiple levels (reviewed in Denis and Chen 2003; Collart and Timmers 2004). Several genetic and physical interactions between the Ccr4-Not complex and transcription initiation factors were described (Collart 1996; Lemaire and Collart 2000; Deluen *et al.* 2002). In addition, genetic interactions suggest a role for the Ccr4-Not complex in transcription elongation (Denis *et al.* 2001). Furthermore, the Ccr4p and Caf1p subunits of the complex are part of the major cytoplasmic mRNA deadenylase (Tucker *et al.* 2001; Chen *et al.* 2002).

Our previous work showed a requirement for the Ccr4-Not complex in transcriptional activation of genes encoding subunits of the ribonucleotide reductase (RNR) enzyme, *RNR1-4*. TBP, Set1p complex, and RNA polymerase II recruitment to the *RNR3* promoter was shown to be dependent on *NOT4* (Mulder *et al.* 2005). Moreover, deletion of genes encoding Ccr4-Not subunits resulted in derepression of several stress-response element (STRE)-regulated genes in an Msn2p-Msn4p-dependent manner (Lenssen *et al.* 2002). This function is controlled by the Glc7p-Bud14p phosphatase (Lenssen *et al.* 2005) and is responsible for expression of *HSP* genes following heat shock (Martinez-Pastor *et al.* 1996).

Several studies noted that *NOT4* encoded a protein containing a Zn-finger motif (Cade and Errede 1994; Irie *et al.* 1994), which was later found to be a RING finger in its human counterpart CNOT4 (Hanzawa *et al.* 2001). RING-finger-containing proteins constitute a subgroup of ubiquitin protein ligases (Lorick *et al.* 1999). Indeed, the human ortholog of Not4p, CNOT4, displays ubiquitylation activity *in vitro* (Albert *et al.* 2002). Covalent attachment of ubiquitin to a target lysine residue of a substrate requires a three-step cascade involving an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) (reviewed in Glickman and Ciechanover 2002). In addition, ubiquitylation of sequence-specific activators is a critical event in transcription activation (reviewed in Muratani and Tansey 2003). The E3 ligase activity of CNOT4 was shown to be dependent on the selective and specific interaction with UbcH5B, an ubiquitin conjugating enzyme (Winkler *et al.* 2004). In yeast, two orthologs of UbcH5B exist, *UBC4* and *UBC5*. These genes are redundant, but display

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TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Genotype	Source
EGY48	*MAT***a** *trp1 ura3 his3 LEU2::pLexAop1-LEU2*	Zervos *et al.* (1993)
W303-1B	*MATα leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 ura3-1*	Thomas and Rothstein (1989)
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
KMY58	Isogenic to BY4741 except *not4:KanMX*	EUROSCARF
2922	*MATα mfa1Δ::MFA1pr-HIS3 his3Δ1 ura3Δ0 lys2Δ0 can1Δ*	Gift from C. Boone (Tong *et al.* 2001)
KMY2	Isogenic to W303-1B except *not4:KanMX6*	Deluen *et al.* (2002)
KMY25	Isogenic to W303-1B except *not4:KanMX6 not5::KanMX6 + NOT5:URA3*	This work
KMY40	Isogenic to 2922 except *not4:URA3*	This work
KMY41	Isogenic to KMY40 except *not4L35A:URA3*	This work
KMY48	Isogenic to KMY40 except *not4K97R:URA3*	This work
KMY43	W303-1B *ubc4:HIS3*	This work
KMY45	W303-1B except *not4:KanMX6 ubc4:HIS3*	This work
KMY115	W303-1B except *CAF40-TAP:TRP1*	This work
KMY116	Isogenic to KMY2 except *CAF40-TAP:TRP1*	This work

partially distinct functions *in vivo* (Seufert and Jentsch 1990; Chuang and Madura 2005).

Here, we show that yeast Not4p displays E3 ligase activity *in vitro* and that this activity is important *in vivo* for Ubc4/5p-dependent stress responses. We identify residues in its RING finger that are critical for this activity by mediating interaction with Ubc4p and Ubc5p. Three independent approaches were taken to investigate the relevance of this activity *in vivo*. First, complementation of known phenotypes of *not4*Δ strains, by expressing RING-finger mutants, showed that not all processes involving Not4p require its E3 ligase activity. Second, genetic screens carried out to isolate novel synthetic lethal interactors of the *not4*Δ and the *not4L35A* allele reveal overlapping and distinct functions in transcription, ubiquitylation, and tolerance to DNA damage responses. Finally, we found that Ubc4/5p-interaction-deficient RING-finger mutants displayed sensitivity to hydroxyurea (HU), resistance to acute heat shock, and sensitivity to hygromycin B. These phenotypes are shared with deletions *UBC4* and *UBC5*. These data suggest that the RING-finger-mediated E3 ligase activity of Not4p modulates Ubc4p/Ubc5p-dependent stress responses *in vivo*.

MATERIALS AND METHODS

Strains and media: For strains, see Table 1. Cells were grown in YPD or SC lacking the appropriate amino acids and at the indicated temperatures.

Plasmids: Plasmid pRS314, containing HA-tagged wild-type *NOT4* under the control of the *DED1* promoter, was used for site-directed mutagenesis to generate the RING-finger mutant alleles. pRS306-*NOT4* (*NOT4* nt −473 to nt +1765 relative to the ATG) was constructed using a PCR fragment obtained from genomic DNA of strain W303-1A. pRS306-*not4L35A* and pRS306-*not4K97R* were obtained by site-directed mutagenesis of pRS306-*NOT4*. Constructs were generated using *Pfu* DNA polymerase and mutations were verified by DNA sequence analysis.

Yeast genetic techniques: Yeast deletion strains (ATG to STOP) were obtained using PCR-product-mediated gene replacement and were verified by PCR analysis. Genomic *not4* mutants were obtained by integrating the pRS306-*not4L35A* or pRS306-*not4K97R* plasmids into the genomic *NOT4* locus in a *not4*Δ background using the *Sma*I restriction site in the *NOT4* promoter region (nt −226 relative to +1 ATG).

Yeast two-hybrid analysis: EGY48 cells were transformed with the indicated B42-*NOT4* constructs (in the pJG4-5 vector) and LexA-*UBC4* or LexA-*UBC5* (in the pEG202 vector). Interactions were determined by spot assay on X-Gal indicator plates as described previously (Albert *et al.* 2000).

Protein purifications: TAP-tag-mediated protein purifications were performed essentially as described (Logie and Peterson 1999). Briefly, 20 liters of YPD culture were grown to OD600 ~2–3, washed, and lysed in E-buffer (20 mm HEPES-KOH pH 8, 350 mm NaCl, 10% glycerol, 0.1% Tween-20). Lysates were cleared by centrifugation in a Beckman 50.2Ti rotor (45,000 rpm, 45 min, 4°). An aliquot of lysate was used for purification over a 200-μl IgG sepharose column (IgG-sepharose fast flow; Pharmacia, Piscataway, NJ). Proteins were bound by rotating at 4° for 2 hr and subsequently washed with 35 ml E-buffer and 10 ml tobacco etch virus (TEV) protease cleavage buffer (10 mm Tris–HCl pH 8, 150 mm NaCl, 0.1% Tween-20, 0.5 mm EDTA, and 1 mm DTT). TEV protease (100 units) cleavage was performed in 1 ml at 18° for 2 hr. The TEV eluate was bound to 100 μl calmodulin affinity resin (Stratagene, La Jolla, CA) in binding buffer (10 mm Tris–HCl pH 8, 150 mm NaCl, 1 mm MgAc, 1 mm imidazole, 2 mm $CaCl_2$, 0.1% Tween-20, 10% glycerol, and 10 mm β-mercaptoethanol) while rotating at 4° for 1 hr. The column was washed with 25 ml binding buffer and bound proteins were recovered in elution buffer (10 mm Tris–HCl pH 8, 150 mm NaCl, 1 mm MgAc, 1 mm imidazole, 2 mm EGTA, 0.1% Tween-20, 10% glycerol, and 10 mm β-mercaptoethanol). His-Ubc4p was expressed from plasmid pQE32-*UBC4* (kind gift from T. K. Albert) and purified over Ni-NTA resin and eluted in lysis buffer (50 mm Tris–HCl pH 7.5, 50 mm KCl, 2.5 mm $MgCl_2$, 0.5 mm EDTA, 0.25 mm dithiotreitol, and 10% glycerol) containing 200 mm imidazole. Peak fractions were pooled and desalted using a PD10 column (Pharmacia) to lysis buffer without imidazole.

***In vitro* ubiquitylation assay:** Purified Ccr4-Not complexes (~500 ng total protein) were incubated with 250 ng his-Ubc4p, 50 ng rabbit E1 enzyme (Boston Biochem), and 500 ng ubiquitin (purified as described) (Winkler *et al.* 2004) in reaction buffer (50 mm Tris–HCl pH 8, 50 mm KCl, 2.5 mm

$MgCl_2$, 0.5 mM EDTA, 0.25 mM dithiotreitol, and 2 mM ATP) for 90 min at 30°. Reactions were stopped by addition of sample buffer and incubation at 95° for 5 min. Samples were separated by SDS–PAGE on a 10% gel, transferred to a nitrocellulose membrane, and analyzed using antibodies against ubiquitin (P4D1, Santa Cruz Biotechnoloy) or Not4p (rabbit polyclonal serum).

Plasmid shuffle assay: W303*not4*Δ*not5*Δ cells expressing *NOT5* from a *URA3* plasmid were transformed with the indicated plasmid-based *not4* alleles. Purified colonies were taken from plates, serially diluted (10-fold), and spotted onto SC-W and SC-W + 0.05% 5-fluoroorotic acid (5-FOA). Cells were grown for 3 days at 30°.

Phenotypic analysis: W303*not4*Δ cells were transformed with various *NOT4* constructs. Purified colonies were taken from plates, serially diluted (10-fold), and spotted onto the appropriate media. To test HU or hygromycin B sensitivity, cells were spotted on YPD plates containing the indicated concentrations of HU or hygromycin B. UV sensitivity was assessed by spotting cells on YPD plates and exposing to the indicated doses of UV light (Stratalinker, Stratagene). Cells were grown for 3–4 days at 30°. To test temperature sensitivity, cells were spotted on YPD plates and grown at 30° and 37°, respectively.

Synthetic genetic array analysis: Synthetic genetic array (SGA) was performed essentially as described previously (Tong *et al.* 2001). Briefly, BY4741 knockout collection strains were crossed with 2922*not4*Δ or 2922*not4L35A* strains. Diploids were selected on media containing G418 (200 μg/ml) lacking uracil and subsequently sporulated for 8 days at 22°. Haploid *MAT***a** progeny was selected on minimal medium supplemented with uracil, lysine, and canavanine (50 μg/ml). *MAT***a** URA^+ cells were subsequently selected on minimal medium supplemented with lysine and canavanine (50 μg/ml). Double deletion strains were isolated on minimal medium supplemented with lysine, canavanine (50 μg/ml), and G418 (200 μg/ml). Growth was assessed after 24, 48, and 72 hr by visual inspection.

Isolated potential genetic interactors were verified by repeating the SGA procedure as described above, except that double knockout cells were selected on SC–URA + G418 (200 μg/ml). A subset of the genetic interactions was validated using random spore analysis and/or tetrad dissection. Bar code sequencing was used to confirm the identity of the deletion strains showing synthetic growth phenotypes with the *not4*Δ or *not4L35A* alleles.

Heat-shock survival assay: W303*not4*Δ cells expressing mutant *not4* alleles or an empty vector (pRS314) were cultured in YPD and kept in exponential phase for at least 30 hr before heat shock at 50° for 10 min. An equivalent number of cells (~1000) were plated on YPD medium before and after heat shock to assess the percentage of survival. Before treatment, samples were taken for Northern blot analysis of *HSP* gene expression. Plasmids expressing *not4* alleles were not lost during growth in YPD (data not shown).

RNA extraction: Total RNA was purified using the hot phenol extraction procedure, as described previously (Mulder *et al.* 2005). Briefly, 40 ml yeast cultures (OD_{600} = 0.5–1) in YPD were added to 40 ml YPD containing 400 mM HU or 0.02% methyl methanesulfonate (MMS) to obtain the final concentration of 200 mM HU or 0.01% MMS. Samples (7.5–10 ml) were collected by centrifugation for 2 min at 5000 rpm. Cell pellets were frozen on dry ice. Frozen cells were resuspended in 500 μl of acid hot phenol:chloroform (5:1, pH 4.7, 65°) and 500 μl of TES buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5% SDS). Cells were incubated for 1 hr at 65° and vortexed every 10 min for 20 sec. The aqueous solution was extracted with phenol:chloroform and with chloroform: isoamyl alcohol (25:1). Finally, total RNA was collected by ethanol precipitation.

Northern blotting: RNA (10 μg) was separated by electrophoresis on a 1% agarose gel containing 10 mM Na–phosphate pH 6.7. Subsequently, RNA was transferred to a nylon membrane and crosslinked by UV-light irradiation. PCR product probes for full-length *RNR1*, *RNR2*, *RNR3*, *RNR4*, *HSP42*, *HSP78*, *HSP104*, *ACT1*, *TUB1*, and 18S rRNA were radiolabeled using the RediPrime II kit (Amersham Pharmacia Biotech). After prehybridization (1–4 hr), probes were added and membranes were incubated overnight at 42°. Blots were rinsed with 2× SSC at room temperature and sequentially washed with 2× SSC, 1× SSC, 0.5× SSC, and 0.3× SSC (twice) for 15 min at 65°. Membranes were either exposed to X-ray films or subjected to quantification using a Storm 820 PhosphorImager and ImageQuant software.

RESULTS

Not4p interacts with Ubc4p/Ubc5p and exhibits ubiquitin protein ligase activity *in vitro* in the context of the Ccr4-Not complex: CNOT4, the human ortholog of *NOT4*, displays ubiquitylation activity *in vitro* (Albert *et al.* 2002). This activity is dependent on the specific and selective interaction with UbcH5B (Winkler *et al.* 2004). In addition, Not4p was shown to interact with both Ubc4p and Ubc5p, the yeast orthologs of UbcH5B (Winkler *et al.* 2004; Krogan *et al.* 2006). To be able to investigate the role of this ubiquitylation activity *in vivo*, we took advantage of the high degree of homology between the RING-finger domains of human CNOT4 and yeast Not4p. Using NMR chemical perturbation data obtained from experiments using CNOT4 and UbcH5B (Albert *et al.* 2002) and the proposed structure of this complex (Dominguez *et al.* 2004), we designed RING-finger mutant alleles of *NOT4* (Figure 1A). The encoded proteins contained single-amino-acid substitutions at the surface of Not4p and were predicted to abolish the interaction between Not4p and Ubc4p/Ubc5p. Using a yeast two-hybrid setup, we determined the interaction with Ubc4p/Ubc5p. As shown in Figure 1B, substitution of the leucine residue at position 35 with alanine (L35A) resulted in a complete loss of interaction with both Ubc4p and Ubc5p. This was similar to substitution of I37 (I37A) and I64 to alanine (I64A) or tryptophan (I64W). Interestingly, we did not find that all mutations disrupted Ubc4p/Ubc5p interaction (Y61A, N63A, R78A, and K97R) (Figure 1B). The K97R substitution was included to inactivate a potential sumoylation site (LKME) located outside of the RING-finger domain (Figure 1A). Equal expression of the proteins was verified by immunoblotting (data not shown). Together, this analysis indicated that residues L35, I37, and I64 are critical for the interaction between Not4p and Ubc4p/Ubc5p.

Recombinant human CNOT4 was previously shown to display ubiquitylation activity in a reconstituted *in vitro* system (Albert *et al.* 2002), but this has not been shown for yeast Not4p. Moreover, these experiments

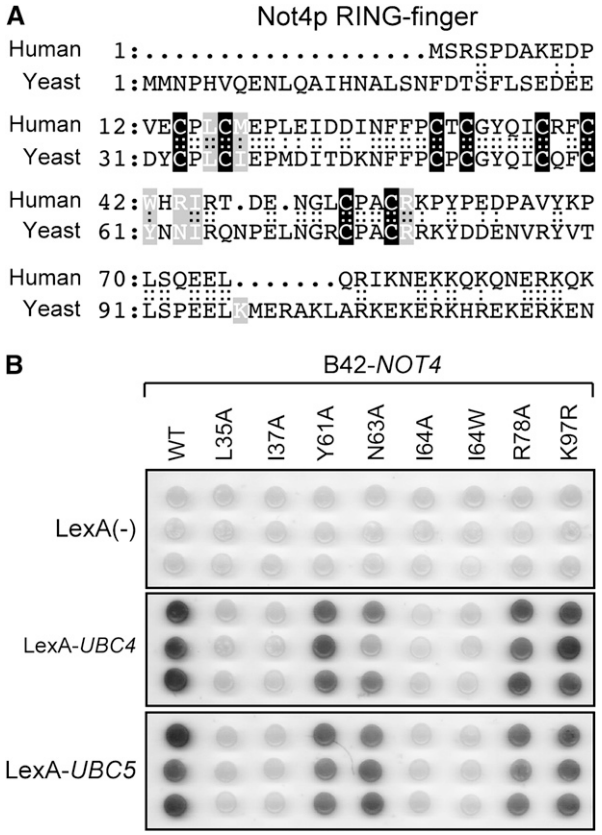

FIGURE 1.—Isolation of *NOT4* RING-finger point mutants disrupting the interaction of Not4p with Ubc4p/Ubc5p. (A) Alignment of the N-terminal region (residues 1–120) of human and yeast Not4p including the RING finger. Identical (::) and similar (:) residues are indicated. Solid and shaded residues specify the conserved cysteines and the residues targeted for substitution, respectively. (B) Yeast two-hybrid interaction between B42-Not4p variants and LexA-Ubc4p or LexA-Ubc5p. Three independent clones were spotted on X-Gal indicator plates and grown overnight at 30°.

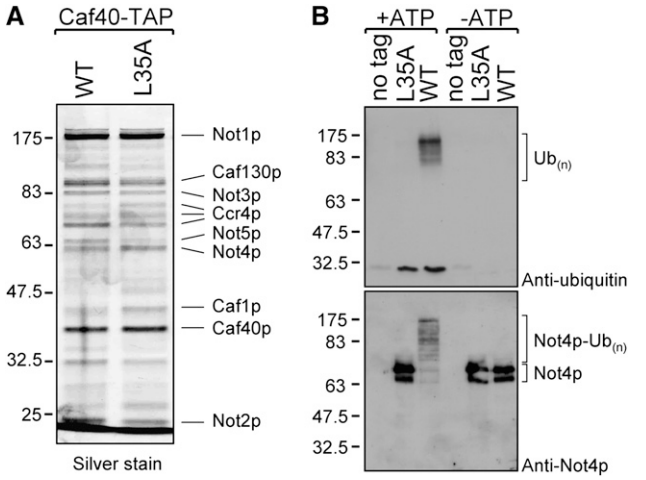

FIGURE 2.—The Ccr4-Not complex displays Ubc4p-dependent ubiquitylation activity *in vitro*, which requires an intact RING-finger domain of Not4p. (A) Tandem affinity-purified Ccr4-Not complexes containing either wild-type or L35A Not4p. Caf40-TAP strains expressing *NOT4* or *not4L35A* were used to isolate the Ccr4-Not complex. Purified proteins were separated by 10% SDS–PAGE and visualized by silver staining. (B) Not4p-mediated ubiquitylation activity depends on the interaction with Ubc4p. Purified Ccr4-Not complexes containing WT or L35A forms of Not4p were used in *in vitro* ubiquitylation reactions. Reactions were analyzed by Western blotting using antibodies against ubiquitin and Not4p.

have not been performed in the context of the complete Ccr4-Not complex. To purify the Ccr4-Not complex from yeast, we fused the TAP-tag to the 3′ end of the endogenous *CAF40* gene. Deletion of *NOT4* from this strain enabled subsequent reintroduction of either the wild-type or a mutant (*not4L35A*) allele of *NOT4* to its original locus (data not shown). Purification of TAP-tagged Caf40p from strains expressing either *NOT4* or *not4L35A* yielded Ccr4-Not complexes that were identical in subunit composition, as verified by silver staining (Figure 2A) and Western blotting (data not shown). We analyzed activity of both complexes in *in vitro* ubiquitylation assays supplied with E1 enzyme, recombinant Ubc4p, and ubiquitin. A mock purification from a non-tagged strain was included as a negative control in the experiment. We observed a robust auto-ubiquitylation activity in the lanes containing wild-type Not4p using antibodies directed against ubiquitin or Not4p (Figure 2B). In contrast, when the Ccr4-Not complexes contained the L35A form of Not4p, no auto-ubiquitylation could be observed (Figure 2B). As expected, Not4p-mediated ubiquitylation was dependent on addition of ATP and Ubc4p to the reaction (Figure 2B and data not shown). Together, these experiments show for the first time that the Ccr4-Not complex displays *in vitro* ubiquitylation activity. Moreover, this activity critically depends on the interaction between Ubc4p and Not4p. We note that in line with other multisubunit E3 ligase complexes Ubc4p and Ubc5p are not stable components of the Ccr4-Not complex as indicated by mass spectrometric analyses (data not shown).

Not4p has functions distinct from its ubiquitin protein ligase activity *in vivo*: To investigate the role for the ubiquitin protein ligase (E3) activity of Not4p *in vivo*, we tested complementation of several known *not4*Δ phenotypes by our collection of RING-finger mutants. Several genetic interactions within the Ccr4-Not complex are described (MAILLET *et al.* 2000), including synthetic lethality between deletions of *NOT4* and *NOT5*. We hypothesized that if this function of Not4p depends on its ubiquitylation activity, the *not4*Δ*not5*Δ synthetic lethality would not be complemented by introduction of E2-interaction-deficient mutants. *NOT4* RING-finger mutants were introduced into *not4*Δ*not5*Δ cells expressing *NOT5* from a plasmid carrying the *URA3* marker. Plasmid shuffle analysis showed that mutants disrupting the interaction between Not4p and Ubc4p/Ubc5p were capable in complementing the *not4*Δ*not5*Δ synthetic lethality, although a minor effect was observed with the R78A allele (Figure 3A). Next, complementation

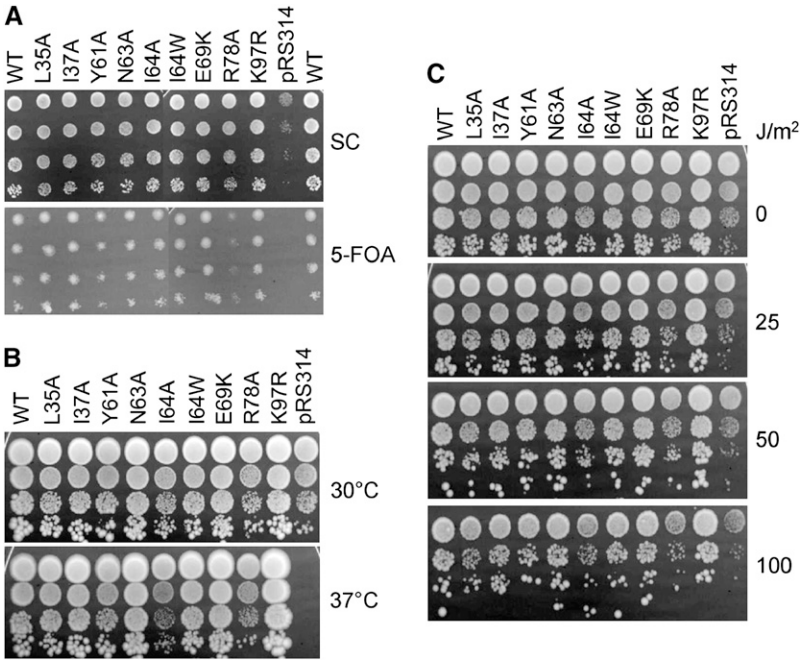

FIGURE 3.—The *in vivo* role of Not4p is not restricted to its E3 ligase function. (A) The *not4*Δ*not5*Δ synthetic lethality is independent of the RING finger of Not4p. W303 *not4*Δ*not5*Δ containing *NOT5* on a *URA3* plasmid were transformed with the indicated *not4* mutants on a *TRP1* plasmid or the empty vector. Colonies were taken from plates, serially diluted (5-fold), and spotted on plates with or without 0.05% 5-fluoroorotic acid (5-FOA). Growth was assessed after 4 days at 30°. (B) The temperature sensitivity of *not4*Δ cells is complemented by RING-finger mutant alleles of *NOT4*. W303 *not4*Δ cells were transformed with plasmids expressing RING-finger mutant alleles of *NOT4*. Tenfold serial dilutions were spotted on YPD and grown at 30° or 37° for 4 days. (C) The *not4*Δ UV sensitivity is complemented by RING-finger mutant alleles of *NOT4*. Strains from B were serially diluted (10-fold), spotted on YPD, and exposed to the indicated doses of UV light. Growth was assessed after 3 days at 30°.

of the temperature sensitivity of cells lacking *NOT4* was assessed. While *not4*Δ cells containing the control plasmid (pRS314) did not grow at the restrictive temperature, all RING-finger mutants and the wild type complemented the growth defect at 37° (Figure 3B). A third phenotype of *not4*Δ cells is sensitivity to UV irradiation (WESTMORELAND *et al.* 2004). We found that cells lacking *NOT4* were only mildly sensitive to this stress and that all tested mutants behaved as wild type (Figure 3C). In conclusion, mutation of the RING finger did not affect the complementation potential of Not4p. This indicates that the RING-finger-mediated E3 ligase activity is not involved in the phenotypes tested, but rather involves a separate function of Not4p.

SGA analysis: As a second approach to gain insight into the physiological role of Not4p, we initiated SGA experiments to identify novel genetic interactions with two *not4* alleles. To this end, query strains were constructed that were either *not4*Δ or expressed the *not4L35A* allele integrated into the *NOT4* genomic locus. These strains were subsequently used to perform SGA analysis on a collection of ~4800 deletion mutants (TONG *et al.* 2001). Initially, 200 genes were identified as potential genetic interactors using the *not4*Δ and the *not4L35A* RING-finger mutant. Repetition of the screens confirmed the genetic interaction for a total of 49 genes (Tables 2 and 3) with an overlap of 48% between the *not4*Δ and *not4L35A* screens (*P*-value = 1.8×10^{-20}; Figure 4A). Identity of the gene deletions in the library strains was determined independently by PCR analysis followed by sequencing of the "bar code." We selected 30 of the 49 interactors for random spore analysis and/or tetrad dissection. In all cases this confirmed the identified interaction with *not4*Δ and *not4(L35A)* alleles (Tables 2 and 3). Whereas the 13 interactors exclusively found with the *not4*Δ allele may reflect RING-finger-independent functions of Not4p, this is less clear for the 24 interactors found exclusively with the *not4L35A* allele (Figure 4).

The genes identified as genetic interactors were arranged into various functional categories (Saccharomyces Genome Database; Figure 4B). Genes involved in transcription or the ubiquitin conjugating system constituted a large portion of the identified genes (18/49, 37%). Surprisingly, multiple genes involved in the response to DNA damage genetically interacted with a deletion of *NOT4* and/or the *not4L35A* allele, suggesting a role for Not4p and its RING finger in this process. This is in agreement with our previous observations that the Ccr4-Not complex is involved in regulation of DNA-damage-induced gene expression (MULDER *et al.* 2005).

Interaction between Not4p and Ubc4p/Ubc5p is required for tolerance to hydroxyurea: Following the observation that the *not4L35A* allele genetically interacts with genes involved in DNA damage responses, strains expressing the *not4L35A* or *not4K97R* allele from the original *NOT4* locus were constructed and tested for HU sensitivity. The K97R mutation does not affect the RING-finger structure or the interaction with Ubc4p/Ubc5p (Figure 1B). Interestingly, cells expressing *not4L35A* were sensitive to HU at 200 mM, whereas the control mutation (*not4K97R*) was not (Figure 5A). To extend this observation, *not4*Δ cells were transformed with plasmids expressing other RING-finger mutants. Several mutants that could still interact with Ubc4p/Ubc5p (Y61A, N63A, and K97R) were included as controls. Cells expressing the L35A and I37A alleles, encoding proteins deficient in E2 interaction, displayed

TABLE 2

Synthetic genetic interactions with the *not4L35A* allele

ORF[a]	Name	SL/SS[b]	Function/process[c]
YDR069C[d,e]	*DOA4*	SL	Protein deubiquitination
YDR313C	*PIB1*	SL	Protein ubiquitination/E3 ligase activity
YFR010W	*UBP6*	SS	Protein deubiquitination
YGL058W[e]	*RAD6*	SS	DNA repair/protein ubiquitination
YMR275C[e]	*BUL1*	SL	Protein ubiquitination
YLR285W[e]	*NNT1*	SS	Chromatin silencing
YNL229C	*URE2*	SL	Transcription corepressor activity
YNL248C[e]	*RPA49*	SL	Transcription from Pol I promoter
YNL278W[e]	*CAF120*	SS	Transcription from RNA pol II promoter
YOL051W[e]	*MED15*	SS	Transcription from Pol II promoter
YOL054W[e]	*PSH1*	SL	RNA elongation from Pol II promoter
YOL148C[e]	*SPT20*	SS	Transcription/chromatin modification
YOR290C[e]	*SNF2*	SL	Chromatin remodeling
YJR060W[e]	*CBF1*	SS	DNA replication
YKL213C[e]	*DOA1*	SS	Double-strand break repair via NHEJ
YLR233C	*EST1*	SL	Telomere maintenance
YLR320W[e]	*MMS22*	SS	DNA repair (DSBR)
YAL002W	*VPS8*	SS	Late endosome to vacuole transport
YDL192W[e]	*ARF1*	SS	GTPase activity/ER to Golgi transport
YLR360W	*VPS38*	SL	Late endosome to vacuole transport
YNR006W[e]	*VPS27*	SL	Protein binding
YOR068C	*VAM10*	SS	Vacuole fusion
YOR332W[e]	*VMA4*	SL	Vacuolar ATPase
YPL234C	*TFP3*	SS	Vacuolar ATPase
YPR173C[e]	*VPS4*	SL	Late endosome to vacuole transport
YLR200W[e]	*YKE2*	SS	Protein folding/prefoldin complex
YKL009W[d,e]	*MRT4*	SL	rRNA processing
YOR302W[d,e]		SL	Regulation of protein biosynthesis
YLR289W[e]	*GUF1*	SL	GTPase activity
YBR133C[d,e]	*HSL7*	SS	Protein arginine N-methyltransferase
YJR104C[e]	*SOD1*	SS	Superoxide metabolism
YDR452W	*PHM5*	SL	Polyphosphate metabolism
YKL183W[e]	*LOT5*	SS	Unknown
YKR005C		SL	Unknown
YKR035C		SS	Unknown
YLR232W		SL	Unknown
YPL168W		SS	Unknown

NHEJ, nonhomologous end joining; DSBR, double-strand break repair.

[a] ORFs identified in both *not4L35A* and *not4*Δ screens are underlined.

[b] Synthetic lethal and synthetic sick, respectively.

[c] Annotations derived from the Saccharomyces genome database.

[d] These ORFs are frequently identified in SGA screens, even when performed against wild-type query strains (E. Cameroni and C. De Virgilio, unpublished results).

[e] Interactions confirmed by random spore analysis and/or tetrad dissection.

sensitivity to HU, whereas the interaction-proficient mutants behaved as wild type (Figures 5B and 1B). Interestingly, cells deleted for *UBC4* showed sensitivity to high concentrations of HU comparable to the L35A and I37A mutants (compare Figure 5B and 5C). In contrast, cells lacking *NOT4* are sensitive to low doses of HU. Nevertheless, a good correlation between HU sensitivity at 200 mM and interaction with Ubc4p/Ubc5p was observed, indicating that the ubiquitylation potential of Not4p is required for HU tolerance. Analysis of the *not4*Δ*ubc4*Δ double mutant revealed an epistatic relationship for the HU sensitivity phenotype (Figure 5D). This supports the notion that Not4p and Ubc4p constitute a functional E2–E3 pair *in vivo*.

Our previous work showed a requirement for *NOT4* in efficient HU-induced transcription of *RNR* genes (Mulder *et al.* 2005). Therefore, cells lacking *NOT4* or expressing WT or *not4L35A* from its original locus were

TABLE 3

Synthetic genetic interactions with the *not4*Δ allele

ORF[a]	Name	SL/SS[b]	Function/Process[c]
YBR170C[d]	*NPL4*	SL	ER-associated protein catabolism
YDR313C	*PIB1*	SL	Protein ubiquitination/E3 ligase activity
YFR010W	*UBP6*	SS	Protein deubiquitination/26S proteasome
YMR275C[d]	*BUL1*	SL	Protein ubiquitination
YPL084W[d]	*BRO1*	SL	Ubiquitin-dependent protein catabolism
YGL025C	*MED3*	SS	Transcription from RNA pol II promoter
YLR266W[d]	*BUR2*	SL	CDK/RNA pol II transcription
YOL051W[d]	*MED15*	SL	Transcription from RNA pol II promoter
YOL054W[d]	*PSH1*	SS	RNA elongation from Pol II promoter
YPR075W[d]	*NOT5*	SL	Transcription from RNA pol II promoter
YLR320W[d]	*MMS22*	SS	DNA repair (DSBR)
YAL002W	*VPS8*	SS	Late endosome to vacuole transport
YDL192W[d]	*ARF1*	SL	ER to Golgi transport
YJL188C	*BUD19*	SS	Bud site selection
YOR069W[d]	*VPS5*	SL	Retrograde transport
YOR332W[d]	*VMA4*	SL	Vacuolar ATPase
YPL234C	*TFP3*	SL	Vacuolar ATPase
YPR036W[d]	*VMA13*	SL	Vacuolar ATPase
YKR076W	*ECM4*	SS	Cell wall organization and biogenesis
YLR200W[d]	*YKE2*	SL	Protein folding/prefoldin complex
YOR302W		SS	Protein biosynthesis
YDR417C		SS	Unknown
YJL169W		SS	Unknown
YKL183W[d]	*LOT5*	SS	Unknown
YMR085W		SS	Unknown

DSBR, double-strand break repair.
[a] ORFs identified in both *not4L35A* and *not4*Δ screens are underlined.
[b] Synthetic lethal and synthetic sick, respectively.
[c] Annotations derived from the Saccharomyces genome database.
[d] Interactions confirmed by random spore analysis and/or tetrad dissection.

either mock treated or incubated with HU or MMS for 2 hr. Transcript levels of *RNR* genes were determined by Northern blot analysis. As expected, *not4*Δ cells show defects in expression of *RNR2*, *RNR3*, and *RNR4* mRNA. However, disruption of the interaction between Not4p and Ubc4p/Ubc5p did not significantly affect the transcriptional induction of the *RNR* genes (Figure 5E). Collectively, these experiments suggest that the ubiquitin protein ligase function of Not4p is involved in tolerance to high concentrations of HU in a manner distinct from transcriptional regulation of the *RNR* genes.

Disruption of the RING-finger-mediated interaction with Ubc4p/Ubc5p leads to resistance to acute heat shock and sensitivity to hygromycin B: *UBC4* and *UBC5* are shown to be involved in stress responses, such as proteasomal degradation of short-lived and abnormal proteins (Seufert and Jentsch 1990). One of the phenotypes associated with this function is resistance to acute heat shock. Indeed, *ubc4*Δ*ubc5*Δ double-mutant strains display tolerance to acute heat shock, whereas single deletions of *UBC4* or *UBC5* do not show this phenotype (Seufert and Jentsch 1990). Interestingly, deletion of *NOT4* or *NOT5* results in the same phenotype (Lenssen *et al.* 2002). We confirmed that deletion of *NOT4* induced a strong resistance to acute heat stress (Figure 6A). In addition, introduction of plasmids expressing RING-finger variants of *NOT4* showed that the L35A, I37A, I64A, and I64W alleles led to an increased survival (Figure 6A). In contrast, the Y61A, N63A, and E69K alleles, encoding proteins that retain the interaction with Ubc4p/Ubc5p, resulted in survival rates comparable to wild type. This suggests that the ubiquitin protein ligase activity of Not4p, together with Ubc4p and/or Ubc5p, is involved in regulation of this stress response. Tolerance to acute heat stress in *ubc4*Δ*ubc5*Δ, *not5*Δ, and *not4*Δ strains has been linked with increased levels of heat-shock gene expression under normal growth conditions (Seufert and Jentsch 1990; Lenssen *et al.* 2002, 2005). Therefore, Northern blot analysis was performed to determine the level of heat-shock gene expression in the RING-finger mutant strains. Notably, *HSP104* is the major heat-shock protein responsible for

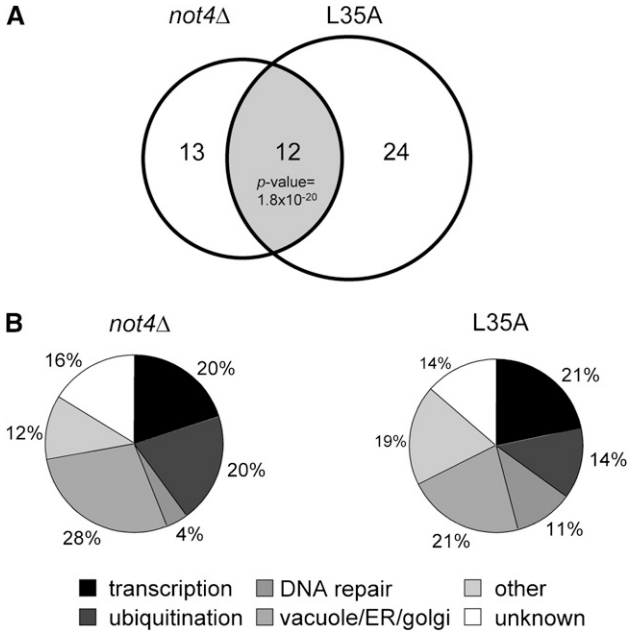

FIGURE 4.—Significant overlap between genes identified in *not4L35A* and *not4*Δ SGA screens. (A) Venn diagram displaying overlap in genes isolated in the SGA screens with *not4*Δ and *not4L35A*. (B) Genes involved in the same biological process (Gene Ontology annotations) are grouped and displayed in pie charts. Percentages of isolated genes in a functional group are given.

the response to acute heat stress (SANCHEZ and LINDQUIST 1990). As expected, *HSP104*, *HSP78*, and *HSP42* transcript levels were increased in the absence of *NOT4* (Figure 6B). Although interaction-deficient RING-finger mutants were resistant to acute heat stress, the basal levels of *HSP* gene expression were not affected (Figure 6B). In addition, heat-shock-induced transcription of these genes in these strains was comparable to wild type (data not shown).

Recently, Ubc4p was reported to associate to the 26S proteasome in the presence of hygromycin B (CHUANG and MADURA 2005), a compound that induces translation errors leading to misfolded proteins. Strains deleted for *UBC4* and *UBC5* were shown to be sensitive to hygromycin B. Interestingly, we found that the *not4L35A*-expressing strain was also sensitive to this drug (Figure 5D). In conclusion, strains expressing *NOT4* RING-finger mutants displayed similar phenotypes to strains deleted for *UBC4*, supporting the model that Ubc4p and Not4p form a functional E2/E3 pair *in vivo*.

DISCUSSION

Previous work showed that CNOT4, the human ortholog of Not4p, displays ubiquitin protein ligase (E3) activity *in vitro* (ALBERT *et al.* 2002; WINKLER *et al.* 2004). To facilitate *in vivo* analysis of the RING finger of Not4p we first obtained mutants preventing the interaction with Ubc4p and Ubc5p (Figure 1). Substitution of leucine 35 with alanine resulted in complete loss of *in vitro* E3 ligase activity of Not4p in the context of the Ccr4-Not complex (Figure 2). Second, using SGA analysis we identified various novel genetic interactions with either *not4*Δ or *not4L35A* alleles (Tables 2 and 3). Significant overlap between the screens was observed (Figure 4). Of the identified genes, several were previously suggested to play a role in DNA damage response pathways. Interestingly, we could show that disruption of the E3 ligase activity of Not4p resulted in sensitivity to HU (Figure 5), a phenotype shared by many genes involved in DNA damage responses. Finally, we found a strong correlation between the ability of Not4p to interact with Ubc4p/Ubc5p and tolerance to acute heat shock and sensitivity to hygromycin B (Figure 6). Together, our results indicate that the E3 activity of Not4p is required for an adequate response to replication stress and the presence of misfolded proteins *in vivo*.

Isolation of synthetic genetic interactors of *not4* alleles reveals links with transcription, ubiquitylation, and DNA damage responses: Complementation analysis of several known phenotypes of *not4*Δ cells did not yield insight into the physiological role of the RING finger of Not4p (Figure 3 and data not shown). Therefore, we performed genomewide screens to identify novel genetic interactions with either a null or a RING-finger mutant (L35A) allele of *NOT4* (Tables 2 and 3). The synthetic interactors isolated in these screens were categorized in four major groups consisting of genes involved in transcription, ubiquitylation, DNA damage response processes (discussed below), and organelle function (Figure 4).

A transcription function for NOT4: Mutant alleles of *NOT1*, *NOT3*, *NOT5*, and *CAF1* were previously found as suppressors of *srb4-138*, a temperature-sensitive mutant of *SRB4* (LEE *et al.* 1998), which suggested opposing roles for the Ccr4-Not and Srb/Mediator complexes. However, like the Ccr4-Not complex, the Srb/Mediator complex regulates transcription both positively and negatively (VAN DE PEPPEL *et al.* 2005). Strikingly, disruption of the tail module of the Srb/Mediator complex (by deletion of *MED15* or *MED3*), involved in interaction with transcription activators, results in synthetic lethality with a deletion of *NOT4* or the *not4L35A* allele (Tables 2 and 3). This suggests a functional overlap between the E3 ligase activity of Not4p and activation of transcription by the Srb/Mediator complex. Interestingly, the Med8p subunit, which resides in the tail module, has been shown to be part of an E3 ligase complex in mammals (BROWER *et al.* 2002). A recent report showed that cells lacking *MED3* or the N terminus of Srb7 are defective in transcriptional induction of *RNR3* after MMS treatment (ZHANG and REESE 2004). In agreement with this, we found that deletion of *MED15* or *MED3* resulted in sensitivity to HU (data not shown),

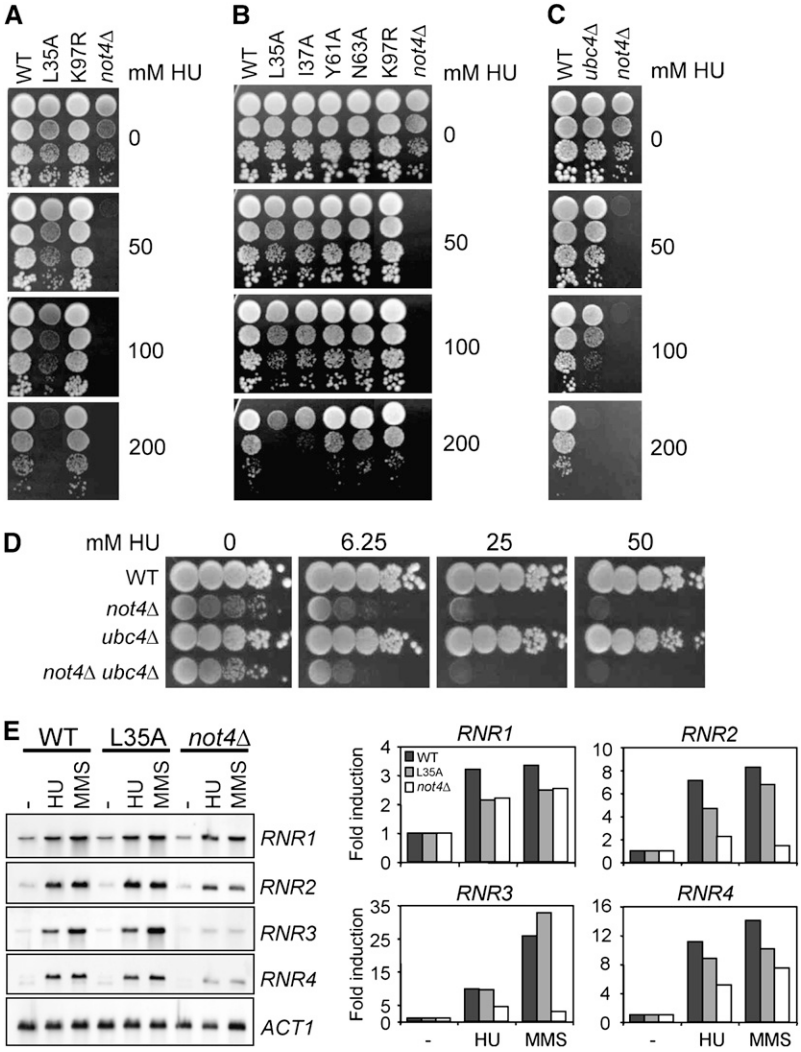

FIGURE 5.—The E3 ligase potential of Not4p is required for tolerance to high concentrations of HU. (A) The interaction between Not4p and Ubc4p/Ubc5p is important for tolerance to HU. W303 *not4*Δ cells expressing the indicated *not4* alleles from its endogenous locus were serially diluted (10-fold) and spotted on YPD plates containing the indicated concentration of hydroxy urea (HU). Cells were grown for 3 days at 30°. (B) Cells expressing pRS314-based E2-interaction-deficient RING-finger mutant alleles are sensitive to high concentrations of HU. W303 *not4*Δ cells were transformed with the indicated *not4* mutants on a *CEN TRP1* plasmid or the empty vector (pRS314). Analysis was performed as in A. We note that episomal expression of the *NOT4*, *not4L35A*, and *not4K97R* alleles results in an identical HU sensitivity as expression from the chromosomal locus. (C) Cells lacking *UBC4* are sensitive to high concentrations of HU. W303 *not4*Δ cells and W303 *ubc4*Δ cells were analyzed as in A. (D) Epistatic relationship between *NOT4* and *UBC4* in HU sensitivity. W303 WT, *not4*Δ, *ubc4*Δ, and *not4*Δ*ubc4*Δ cells were analyzed as in A. (E) The *not4L35A* allele-mediated HU sensitivity is independent of defects in *RNR* gene transcription. W303 cells expressing the indicated alleles, integrated into the *NOT4* genomic locus, were grown to exponential phase and treated for 120 min with 200 mM HU or 0.01% MMS, respectively. RNA was extracted and subjected to Northern blot analysis (left section). Probes were radiolabeled PCR products. *ACT1* was used as a loading control. Quantification of the Northern blots is shown in the right section.

a phenotype shared by cells lacking *NOT4* or expressing E2-interaction-deficient RING-finger mutants (Figure 4). We have tested other nonessential genes of the Srb/Mediator (*MED9*, *MED2*, *PGD1*, *ROX3*, *SIN4*, *SRB2*, *SRB5*, *NUT8*, *SRB10*, and *SRB11*), SAGA (*ADA1*, *ADA2*, *ADA3*, *GCN5*, *SPT3*, *SPT7*, *SGF73*, and *SGF29*), or SWI/SNF (*SNF5*, *SNF6*, *SNF11*, *SWI3*, and *TAF14*) complexes for genetic interaction with the *not4*Δ and *not4(L35A)* alleles, but we failed to observe any synthetic growth phenotypes (data not shown). In addition, several genes involved in transcription elongation showed a synthetic growth phenotype when deleted in combination with *NOT4*. Among these were *BUR2*, *PSH1*, and *YJL169W* (a dubious ORF partially overlapping with *SET2*). Psh1p (Pob3p-Spt16p-binding protein) was found to copurify with the FACT complex, which facilitates transcription elongation through chromatin (Ho *et al.* 2002; KROGAN *et al.* 2002). It is noteworthy that our SGA analysis could not uncover interactions with *POB3*, *SPT16*, or *NHP6A/B* since the former two genes are essential (and are not represented in the collection of single-gene knockouts) and the latter two (*NHP6A* and *NHP6B*) are functionally redundant. This suggests that isolation of *PSH1/YOL054W* could reflect further functional interactions between the Ccr4-Not and FACT complexes in transcription elongation. In agreement with this, it was recently found that the genes encoding the FACT complex subunit Nhp6p (FORMOSA *et al.* 2001) genetically interact with components of the Ccr4-Not complex (BISWAS *et al.* 2006). Finally, *NOT4* and *BUR2* display synthetic lethality, as does a deletion of *NOT4* combined with a temperature-sensitive allele of *BUR1* (K. W. MULDER, A. INAGAKI and H. Th. M. TIMMERS, unpublished results).

NOT4 is linked to the ubiquitin-26S proteasome pathway: As expected, a second group of genes displaying synthetic growth defects or lethality with *not4* alleles is linked to the ubiquitin proteasome pathway, confirming a role for the RING finger of Not4p in this process *in vivo*. For instance, Pib1p has been described to contain RING-finger-dependent E3 ligase activity *in vitro* (SHIN *et al.* 2001). Possibly, Pib1p constitutes a redundant E3 ligase for Not4p under certain conditions. However, this redundancy is not likely to be relevant for

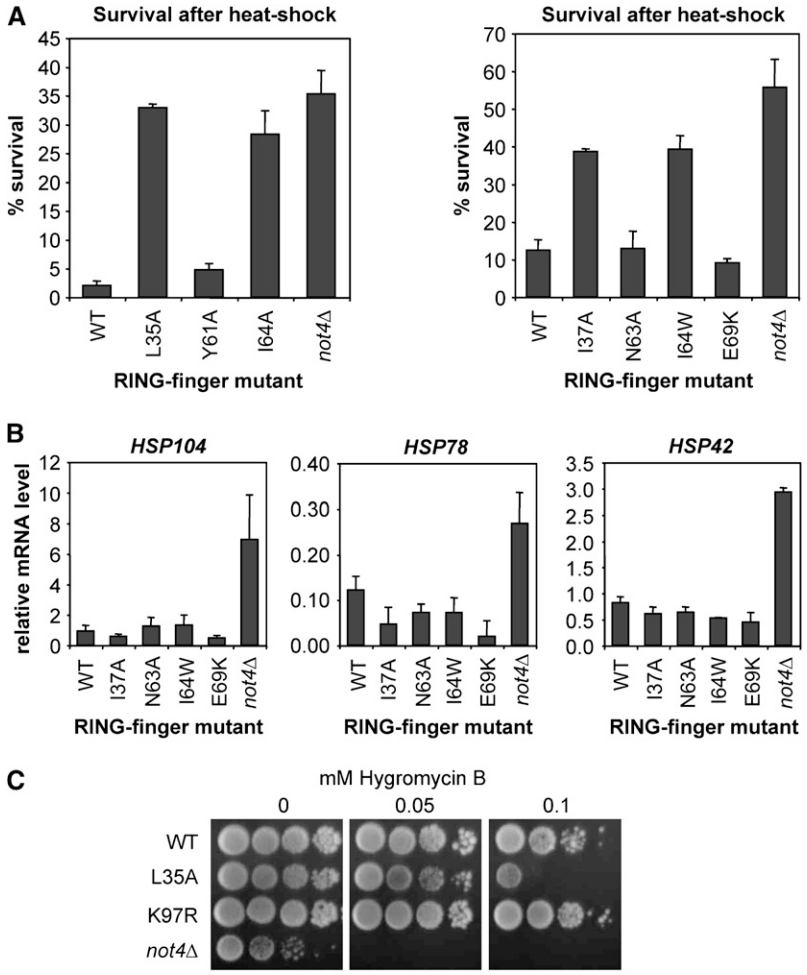

FIGURE 6.—Disruption of the RING-finger-mediated interaction of Not4p with Ubc4p/Ubc5p results in tolerance to acute heat shock. (A) E2 interaction-deficient RING-finger mutants are resistant to acute heat shock. W303 *not4*Δ cells expressing the indicated plasmid-based RING-finger mutants were grown exponentially for 2 days in YPD. Complementation of the *not4*Δ slow-growth phenotype by all mutant alleles prevented plasmid loss (data not shown). Identical cell numbers were plated on YPD before and after acute heat shock (10 min, 50°). Error bars indicate standard deviations of two experiments. (B) Heat-shock resistance of RING-finger mutants is independent of *HSP* gene expression under normal conditions. RNA was extracted before heat shock and subjected to Northern blotting. Quantification of *HSP42*, *HSP78*, and *HSP104* expression (after normalization to *TUB1* signals) is shown. Error bars indicate standard deviations of two experiments. (C) The E3 ligase activity of Not4p is required for tolerance to hygromycin B. Serial dilutions of W303 *not4*Δ strains expressing *NOT4*, *not4L35A*, or *not4K97R* from its original locus were spotted on YPD containing the indicated concentrations of hygromycin B.

the HU sensitivity of *not4*Δ and *not4L35A*, since *pib1*Δ cells are not sensitive to HU (data not shown). *DOA4* is a member of the family of deubiquitinating enzymes and interacts with the 26S proteasome (Papa and Hochstrasser 1993; Papa *et al.* 1999). Interestingly, deletion of *DOA4* results in sensitivity to HU in a manner epistatic to *rad9*Δ (Fiorani *et al.* 2004). Intriguingly, recent work shows that the HU sensitivity of cells lacking *CCR4* or *CAF1* is also epistatic to the pathway containing Rad9p (Traven *et al.* 2005), suggesting that the Ccr4-Not complex and Doa4p might be involved in this pathway in parallel to each other. In addition, Ubc4p has recently been shown to interact with the 26S proteasome in the presence of hygromycin B, inducing translational misreading (Chuang and Madura 2005). This is in concert with the observation that *UBC4* and *UBC5* are required for degradation of abnormal and short-lived proteins (Seufert and Jentsch 1990).

NOT4 and DNA damage responses: Interestingly, 11% (*not4L35A*) and 4% (*not4*Δ) of the genetic interactors are involved in DNA damage responses (Tables 2 and 3 and Figure 4). Our screens revealed interactions with *mms22*Δ and *rad6*Δ, suggesting a role for the E3 ligase activity of Not4p in the cellular response to DNA damage. Notably, expression of RING-finger mutant alleles complemented the mild UV sensitivity phenotype of *not4*Δ cells (Figure 3). Subsequent phenotypic analysis showed HU sensitivity of strains expressing RING-finger variants of Not4p that were disrupted in their interaction with Ubc4p and Ubc5p (Figures 1 and 4). Moreover, it has been shown that components of the Ccr4-Not complex are required for cell cycle progression after ionizing radiation in a manner epistatic to the *RAD9*-dependent DNA-damage checkpoint (Westmoreland *et al.* 2004). Recent data suggest that *MMS22* forms a genetic module (*MMS22*m) together with *MMS21*, *RTT101*, and *RTT107* (Pan *et al.* 2006). In addition, *RAD6* is part of both the *BRE1*m and the postreplication repair (PRR) pathway. Both these modules are genetically distinct from the *RAD9*m, supporting the hypothesis that *NOT4* functions in the *RAD9* pathway (Westmoreland *et al.* 2004; Traven *et al.* 2005).

The E3 ligase activity of Not4p is involved in regulation of Ubc4p/Ubc5p-mediated stress tolerance: Previous work showed that integrity of the Ccr4-Not complex is required for survival in the presence of HU (Mulder *et al.* 2005). This drug is known to directly target the RNR complex. RNR is responsible for

conversion of NDPs to dNDPs, which is the rate-limiting step in dNTP production (Jordan and Reichard 1998). HU treatment results in a decreased replication rate by limiting the dNTP pools. Deletion of genes involved in DNA-damage repair pathways also leads to sensitivity to HU. The observation that disruption of the interaction between Ubc4p/Ubc5p and Not4p confers HU sensitivity indicates a role for its E3 ligase activity in tolerance to replication stress. Interestingly, deletion of *UBC4* resulted in a similar HU sensitivity (Figure 5). In addition, deletion of both *UBC4* and *UBC5* leads to a severe slow-growth phenotype (Seufert and Jentsch 1990), indicating functional redundancy between these genes. However, the regulation of expression of these genes differs significantly (Seufert and Jentsch 1990). For instance, *UBC4* transcript levels drop significantly during stationary phase, whereas *UBC5* levels are increased. Differential regulation of transcription might provide an explanation for the observation that some phenotypes that are associated with deletion of *UBC4* are not evident in *ubc5*Δ cells, although these genes are functionally redundant during normal growth. Furthermore, *UBC4* and *UBC5* expression is increased by heat shock. Interestingly, strains deleted for both *UBC4* and *UBC5* display tolerance to acute heat shock (Seufert and Jentsch 1990). This treatment is thought to produce aberrantly folded proteins *in vivo*. Interestingly, deletion of *NOT4* also gives rise to resistance to heat shock, which is most likely to result from derepression of *HSP* gene expression under noninducing conditions (Lenssen *et al.* 2005). We found that the role of the RING finger of Not4p in tolerance to acute heat shock is independent of *HSP* gene expression (Figure 6). The functional redundancy between *UBC4* and *UBC5* in mediating tolerance to acute heat shock and the observation that RING-finger mutants of Not4p display an identical phenotype indicate that Not4p is the critical E3 ligase in this process. Strikingly, *not4*Δ as well as *ubc4*Δ*ubc5*Δ cells are sensitive to hygromycin B (Chuang and Madura 2005). This drug induces misreading of the mRNA template in the ribosome, resulting in misfolding of the newly synthesized proteins. This sensitivity is, at least in part, dependent on the E3 ligase activity of Not4p, since the *not4L35A* allele could not fully complement this phenotype of *not4*Δ cells (Figure 6D). This suggests that the Ubc4p/Ubc5p-mediated response to abnormal and translationally damaged proteins is dependent on the interaction of Not4p with Ubc4p/Ubc5p. In agreement with this suggestion, we found a genetic interaction between *not4*Δ, *not4L35A*, and a deletion of *YKE2* (Tables 2 and 3). This gene encodes a subunit of the prefolding/GimC cytoplasmic chaperone complex, involved in cellular protection against protein aggregation.

It is clear that the RING finger of Not4p is involved in tolerance to various stresses, including tolerance to misfolded proteins *in vivo*. However, its exact role in this process and identification of its substrates require further investigation. Collectively, our results provide the first evidence that Not4p functions as a RING-finger-dependent E3 ligase *in vitro* and identified involvement of this activity in several processes *in vivo*.

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