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# A RING-H2 finger motif is essential for the function of Der3/Hrd1 in endoplasmic reticulum associated protein degradation in the yeast *Saccharomyces cerevisiae*

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**Abstract Der3/Hrd1p is a protein required for proper degradation of misfolded soluble and integral membrane proteins in the endoplasmic reticulum (ER) in the yeast *Saccharomyces cerevisiae*. It is located to the ER membrane and consists of a N-terminal hydrophobic region with several transmembrane domains and a large hydrophilic tail oriented to the ER lumen containing a RING finger motif of the H2 class. We had previously reported that a truncated version of Der3p, Der3ΔRp, lacking 111 residues of the lumenal domain including the RING finger motif is not functional, suggesting the involvement of this domain in the function of the protein in ER degradation. We substantiated this hypothesis by constructing a mutated form of Der3/Hrd1p replacing the last cysteine of the motif with a serine. This mutated Der3$_{C399S}$ protein maintains the correct localization and topology of the wild-type protein, however, is not able to support the degradation of soluble and integral membrane proteins. This point mutation altering the RING-H2 motif behaves as a dominant allele especially when overexpressed from a 2μ plasmid by this increasing the half-life of CPY* more than 6-fold when compared with a wild-type strain. Furthermore co-expression of *der3$_{C399S}$* with the wild-type allele is also able to partially suppress the temperature sensitive growth phenotype of a *sec61-2* strain. Finally we have shown that overexpression of Hrd3p suppresses the dominant effect of the *der3$_{C399S}$* mutation. These results could be explained by a competition between wild-type and mutant Der3 protein for the interaction with some other component of the ER degradation pathway, probably Hrd3p.**

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*Key words:* Endoplasmic reticulum degradation; Der3/Hrd1; RING-H2 finger motif; yeast

## 1. Introduction

The endoplasmic reticulum (ER) is the first organelle in the secretory pathway of all eukaryotic cells which is passed by plasma membrane, vacuolar, lysosomal and secretory proteins to their port of destination [1]. To ensure the delivery of only properly folded proteins to their site of action, a quality-control system operates in the ER, which is able to detect malfolded polypeptides or unassembled subunits of oligomeric proteins and block their further transport to the Golgi [2]. Misfolded proteins retained in the ER are finally transported back to the cytosol through a Sec61p containing channel [3,4], where they are degraded by the ubiquitin-proteasome system associated with the cytoplasmic face of the ER membrane [5,6]. Substrates for ER degradation include mutant forms of proteins responsible for severe diseases such as cystic fibrosis, pulmonary emphysema or Wilson's disease [7–9]. Also some viral proteins are able to induce abnormal degradation in the ER of proteins required for the immune response by this increasing the virulence of infection by HIV type I or human cytomegalovirus [10,11].

In the yeast *Saccharomyces cerevisiae* some components of the ER degradation pathway have been identified. Components of the import and folding machinery, Sec61p, Sec63p and Kar2p are also required for the retrograde transport of proteins to the cytosol [3,6]. Ubc6, Ubc7/Der2p and Cue1p are responsible for polyubiquitination of the substrate [5,6,12] and Rpn1p and other subunits of the 26S proteasome are necessary for degradation of the ER proteins in the cytosol [5,13].The function of three newly identified components of the ER membrane, Der1p, Der3/Hrd1p and Hrd3p, remained unknown [13–15].

Der3/Hrd1 is a protein required for ER degradation of a wide variety of proteins including misfolded lumenal CPY* [15] and integral membrane proteins, as are Sec61-2p [15], HMG-CoA reductase [13] and Pdr5* [16]. The protein exhibits a N-terminal domain with five putative transmembrane domains and a large hydrophilic tail oriented to the ER lumen containing a RING-H2 finger motif [15].

A RING-H2 finger domain is defined by the position and distance between six cysteines and two histidines and is able to bind two zinc atoms [17]. This class of motifs can be widely found in otherwise non-related families of yeast and mammalian proteins. In yeast, the RING-H2 domain of Ste5 plays a crucial role in Ste4-promoted oligomerization of the protein required for the pheromone response pathway [18] and a RING finger protein complex has been described as being essential in protein transport to the vacuole [19]. Recently the RING finger domain of the Vmw 110 protein of the herpes simplex virus type I has been shown to be essential for inducing degradation of the catalytic subunit of DNA-dependent protein kinase by the proteasome pathway [20].

We had previously shown that the integrity of the lumenal domain of Der3/Hrd1p is absolutely necessary for proper function of the ER degradation pathway [15]. Here we have constructed a point-mutated form of Der3/Hrd1p by eliminating one of the cysteine residues to demonstrate that the RING-H2 domain is essential for the activity of the protein and for unraveling genetic interactions with other components of the ER degradation system.

## 2. Materials and methods

### 2.1. Strains and growth conditions

*Saccharomyces cerevisiae* isogenic strains W303-1C (*MATα prc1-1 ade2-1 his3-11,15 leu2-3,112 trp1-1*) [14], W303-1CΔ3 (*MATα prc1-1*

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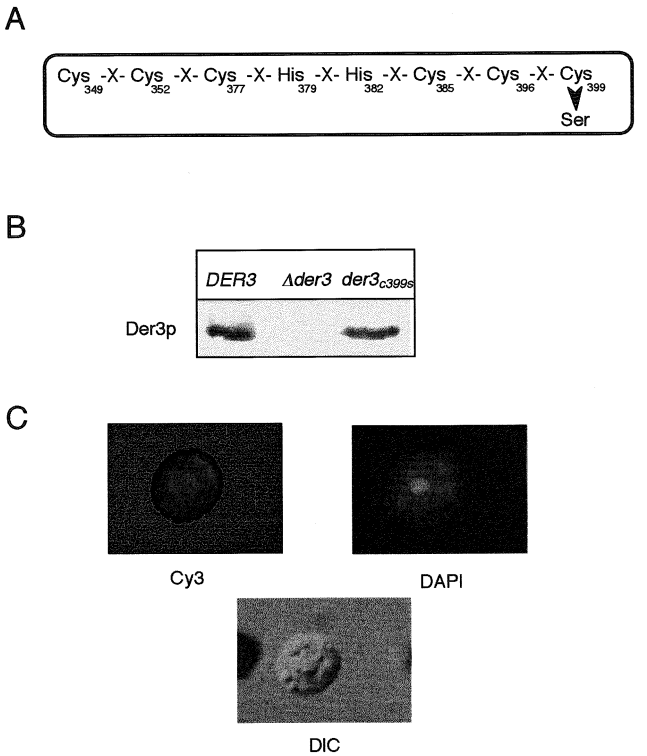
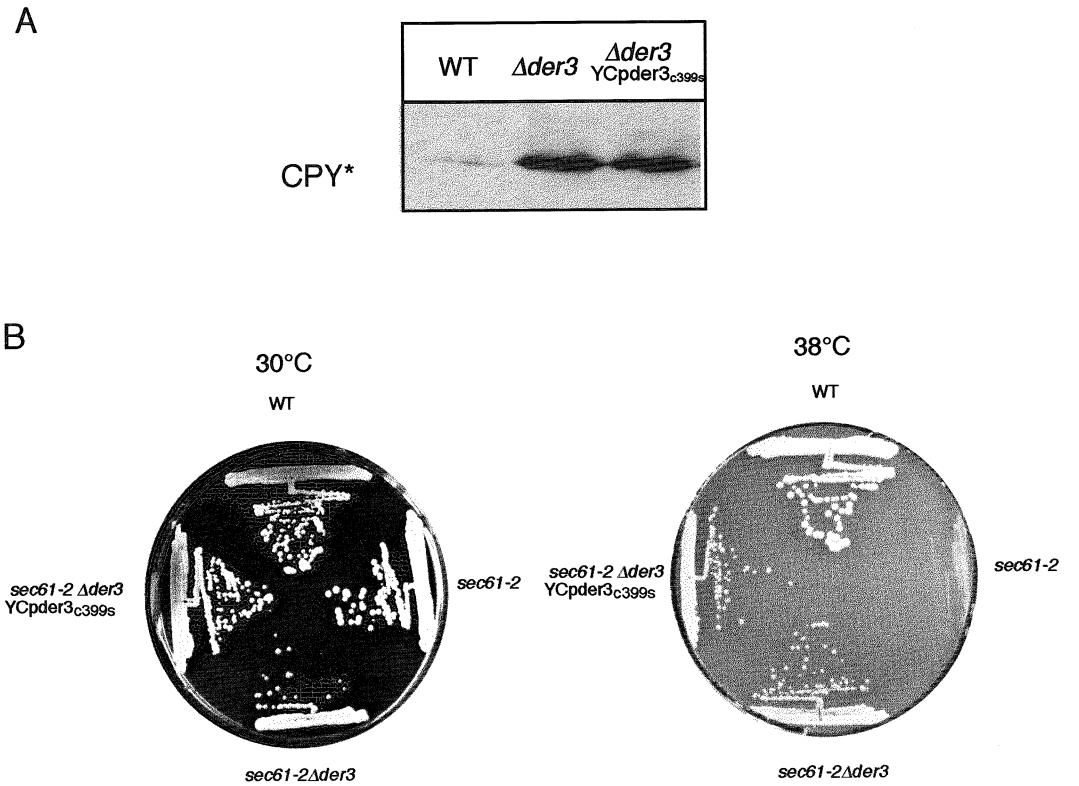



Fig. 1. Structure and localization of the $Der3_{C399S}$ mutant protein. A: The RING-H2 finger motif of Der3p. To construct the *der3*$_{C399S}$ mutant allele, Cys399 was replaced by a serine residue. B: Steady-state levels of Der3p and $Der3_{C399S}$p were measured by Western blotting in yeast cells from strains W303-1C (*DER3*), W303-1CΔ3 (Δ*der3*) and W303-1CΔ3 carrying the *der3c399s* allele on a centromeric plasmid (*der3c339s*). C: $Der3_{C399S}$ protein is localized in ER like structures. Yeast cells from strain W303-1CΔ3 carrying the 2μ plasmid pRS/$der3_{C399S}$ were stained with polyclonal anti-Der3 antibodies and goat anti-rabbit Cy3 antibody (Cy3) and 4,6-diamidino-2-phenylindole (DAPI); DIC: Nomarski optics. D: The C-terminal tail of Der3c399Sp remains oriented to the ER lumen. Yeast spheroplasts of the strain W303-1CΔ3 carrying the centromeric plasmid YCp/$der3_{C399S}$ were gently lysed and centrifugated to separate a soluble fraction (SN) from the pellet fraction (P) containing intact yeast microsomes. Aliquots of microsomes were incubated for 30 min on ice in the absence (−) or in the presence of trypsin (+T) or trypsin and Triton X-100 (T+X). After TCA precipitation pellets were resuspended in a urea buffer, immunoblotted after SDS-PAGE and stained with anti-Der3p or anti-CPY polyclonal antibodies.
←

Fig. 2. $Der3_{C399S}$ mutant protein is not able to support ER degradation. A: Steady-state levels of CPY* in crude extracts prepared from early stationary phase yeast cells grown on CM medium of the strains W303-1C (WT), W303-1CΔ3 (Δ*der3*) and W303-1CΔ3 carrying the *der3*$_{C399S}$ allele in a centromeric plasmid (Δ*der3* YCp/$der3_{C399S}$) were measured after Western blotting. B: The *der3*$_{C399S}$ mutant allele is not able to complement the suppression of the temperature sensitive growth phenotype of a *sec61-2* Δ*der3* strain. Strain W303-1C (WT), YRP086 (*sec61-2*) and YRP105 (*sec61-2* Δ*der3*) harboring the *LEU2* containing plasmid YCplac111 and YRP105 (*sec61-2* Δ*der3* YCp/$der3_{C399S}$) carrying the *der3*$_{C399S}$ allele on a centromeric plasmid were tested for growth at 30°C and 38°C on CM plates without leucine for 48 h.
↓



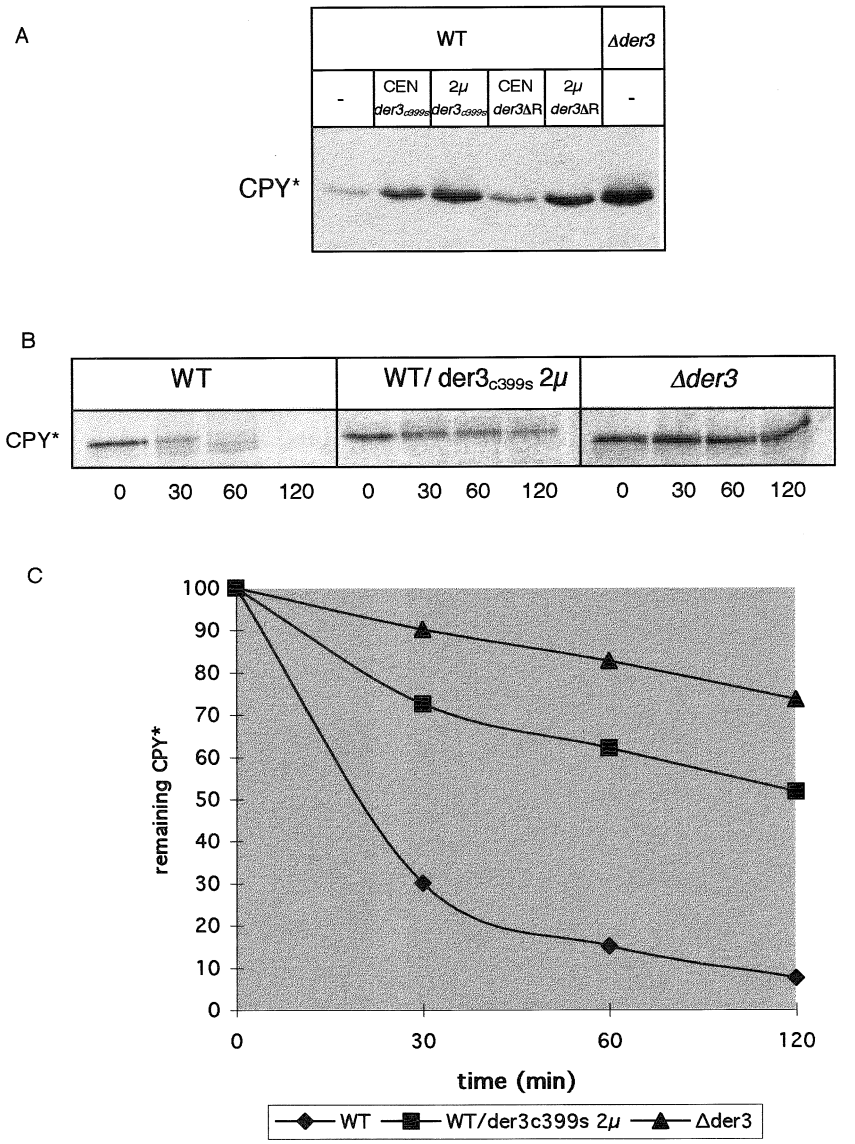



Fig. 3. Dosage-dependent dominance of *der3* mutant alleles affecting the RING-H2 motif region. A: Steady-state levels of CPY* in crude extracts prepared from strain W303-1C (WT) not expressing (−) or expressing $der3_{C399S}$ or *der3ΔR* from a centromeric (CEN) or a multicopy (2μ) plasmid and strain W303-1CΔ3 grown on CM medium were immunodetected with monoclonal anti-CPY antibodies after SDS-PAGE and Western blotting. B, C: Kinetics of degradation of CPY*. A pulse-chase experiment was performed using strain W303-1C (WT), W303-1C harboring the $der3_{C399S}$ allele on a multicopy plasmid (WT/$der3_{C399S}$ 2μ) and strain W303-1CΔ3. Exponentially growing cells on CM medium were labeled with [$^{35}$S]methionine as previously described [15]. After the chase aliquots of cells were taken at the indicated chase points and immunoprecipitated with polyclonal anti-CPY antibodies. Kinetics of CPY* degradation was measured using a Molecular Dynamics imaging system.

*ade2-1 his3-11,15 leu2-3,112 trp1-1 der3::HIS3*) [15], YRP086 (*MATα prc1-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 sec61-2*) [3] and YRP105 (*MATα prc1-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 sec61-2 der3::HIS3*) [3] were grown on CM media [21] at 30°C unless otherwise indicated.

### 2.2. Molecular biological techniques, site-directed mutagenesis and plasmid construction

Standard techniques of molecular biology have been previously described [22].

The $der3_{C399S}$ allele was constructed using the 'megaprimer' method [23]. The first round of PCR was performed with the primers 5′-ATCTTGGCACTATATCAA-3′ and 5′-CAATCTA**G**AAATAGGA-CA-3′ (whereby G in bold replaces a C from the wild-type gene) using as a template the plasmid pUC/DER3 [15]. For the second round the product of the first PCR reaction, digested with *Eco*RV and purified and the universal primer were used as primers. Conditions for both reactions have been previously described [23]. The final product of the reaction was digested with *Eco*RI and subcloned into the plasmid pUC/DER3 digested with *Eco*RI and *Eco*RV yielding the plasmid pUC/$der3_{C399S}$. The correctness of the recombinant clones containing the point mutation was checked by DNA sequencing. Plasmids YCp/$der3_{C399S}$ and pRS/$der_{C399S}$ were obtained by subcloning an *Eco*RI-*Hin*dIII fragment from pUC/$der_{C399S}$ into YCplac111 and pRS426 respectively.

### 2.3. Antibodies, Western blotting, immunofluorescence and pulse-chase experiments

Polyclonal anti-Der3p [15] was diluted 1:2500 for Western blotting and 1:250 for immunofluorescence. Monoclonal anti-CPY (Molecular Probes) was diluted 1:10 000 for Western blotting. Western blotting and immunodetection of CPY* and Der3p, immunofluorescence for intracellular localization of the $Der3_{C399S}$ protein, protease protection and pulse-chase experiments were performed as previously described [15].

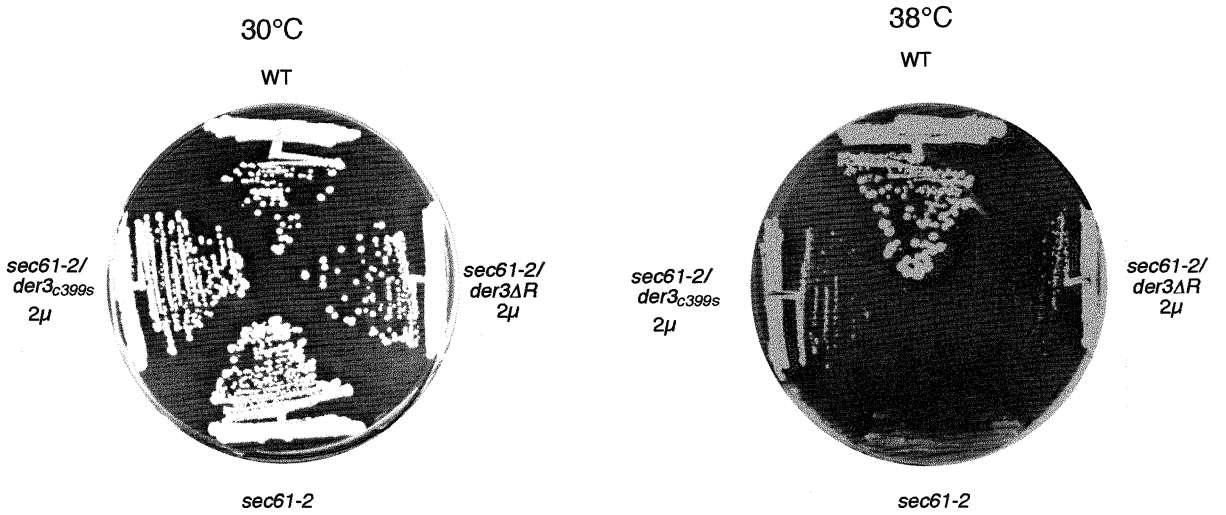



Fig. 4. Overexpression of *der3* mutant alleles afecting the RING-H2 domain partially suppresses the temperature sensitive growth phenotype of the *sec61-2* mutant. Strain W303-1C (WT) and YRP086 (*sec61-2*) harboring the *URA3*-based plasmid pRS426 and strain YRP086 expressing the $der3_{C399S}$ (*sec61-2* $der3_{C399S}$ 2μ) or the *der3ΔR* (*sec61-2 der3ΔR* 2μ) from a multicopy plasmid were tested for growth on CM plates at 30°C and 38°C for 72 h.

## 3. Results and discussion

We have constructed a mutant form of Der3/Hrd1p by replacing the last cysteine (Cys-399) of the RING-H2 finger domain by a serine (Fig. 1A). This mutant protein called $Der3_{C399S}$ was expressed in a Δ*der3* null mutant strain from a centromeric plasmid. Steady-state levels of the mutant protein were checked by Western blotting and immunodetection with anti-Der3 antibodies. As can be seen in Fig. 1B, $Der3_{C399S}$p can be easily detected as a single band with the same electrophoretic mobility and intensity than the Der3 wild-type protein. To confirm the correct intracellular localization of $Der3_{C399S}$ protein, we performed immunofluorescence with anti-Der3 antibodies in intact yeast cells from a strain expressing the mutant allele from a multicopy plasmid. As we have previously described for wild-type Der3p [15], a typical ER staining, perinuclear with some staining regions along the plasma membrane can be observed (Fig. 1C). We had reported that the C-terminal hydrophilic tail of Der3/Hrd1p is oriented to the ER lumen [15]. In order to confirm that this topology is not changed in the Der3c399S mutant protein, a protease protection experiment using isolated microsomes was performed (Fig. 1D). $Der3_{C399S}$p can be detected in the microsomes containing fraction (P) even in the presence of trypsin (+T) and only after adding a detergent (+T+X) the protein is accessible to the protease and is finally degraded. This result confirmed the lumenal orientation of the C-terminal domain of Der3c399Sp.

We investigated whether Der3c399Sp is able to support degradation of misfolded lumenal and integral membrane pro-

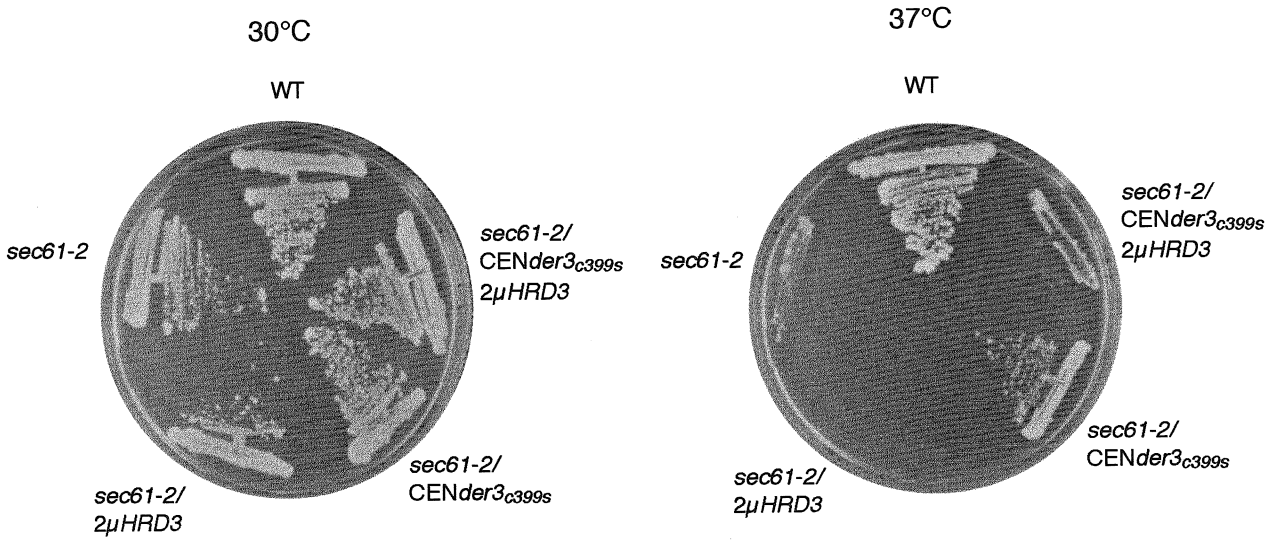



Fig. 5. Overexpression of the *HRD3* gene can partially revert the dominant effect of the *der3c399s* mutation. Strain W303-1C (WT) and YRP086 (*sec61-2*) carrying plasmids pRS426 and YCplac111, and YRP086 expressing *HRD3* from a multicopy plasmid (*sec61-2* 2μ *HRD3*) or $der3_{C399S}$ from a centromeric plasmid (*sec61-2* CEN $der3_{C399S}$) or both (*sec61-2* 2μ *HRD3* CEN $der3_{C399S}$) were grown on CM plates without leucine and uracil at 30°C and 37°C for 72 h.

teins of the ER. As example of a lumenal substrate, we measured steady-state levels of CPY* by Western blotting in yeast strains carrying *DER3* wild-type and mutant alleles. We found similar accumulation of CPY* in a *der3*$_{C399S}$ and a Δ*der3* null mutant strains (Fig. 2A). The *sec61-2* allele encodes a mutant form of the Sec61 protein, the major component of the protein import machinery of the ER. The *sec61-2* allele encoded protein at the restrictive temperature of 38°C is degraded by the 26S proteasome, leading to a temperature sensitive growth phenotype of cells [24]. We have described previously that this phenotype can be suppressed by a deletion of the *DER3*/*HRD1* gene [15]. As is shown in Fig. 2B this suppression cannot be reverted by the *der3*$_{C399S}$ allele. Thus, the RING-H2 finger domain is essential for the function of Der3p in ER associated degradation.

The RING-H2 finger domain of the Ste5 protein is required for interaction with Ste4 and subsequent oligomerization of the protein [18]. The RING-H2 finger domain of Der3p could play a similar role and mediate interactions with additional components of the ER degradation pathway. In order to investigate putative proteins interacting with Der3p through its RING finger motif, we used the *der3ΔR* and *der3*$_{C399S}$ mutant alleles as tools. As can be seen in Fig. 3A, co-expression of mutant and wild-type alleles leads to an increase in steady-state levels of CPY* due to a partial block of its degradation. This increase is more prominent in the case of the *der3*$_{C399S}$ point mutation and is dosage-dependent. Accumulation of CPY* is higher after overexpression of mutant alleles from multicopy plasmids as compared to expression on a CEN-plasmid (Fig. 3A). We have previously shown that *DER3* is able to support degradation of CPY* and Sec61-2p, even when expressed under the control of the GAL promoter [15]. In order to quantify the dominant effect of the *der3c399s* allele expressed from a 2μ plasmid on CPY* degradation over wild-type, we performed a pulse-chase experiment. As can be seen in Fig. 3B,C, the half-life of CPY* is increased by about 6-fold upon expression of the *der3*$_{C399S}$ in the wild-type background as compared to the corresponding strain carrying only the *DER3* wild-type allele. Not surprisingly co-expression of the *der3*$_{C399S}$ or the *der3ΔR* allele on a multicopy plasmid (Fig. 4) or a centromeric plasmid (not shown) in a *DER3* wild-type partially suppresses the temperature sensitive growth phenotype exerted by a mutated Sec61 protein (allele *sec61-2*).

Dosage-dependent dominance of the *der3*$_{C399S}$ allele can be explained by a competition between the wild-type and the mutant Der3 protein in the interaction with either itself or with some other component of the ER degradation pathway. The formation of non-functional hybrid dimers of wild-type and mutant molecules of Der3p could lead to the observed phenotype. We constructed a mutant form of Der3p in which the RING-H2 finger region was replaced by the oligomerization domain of glutathione *S*-transferase. However, this hybrid is not functional, suggesting that the RING-H2 finger domain is not involved in a putative dimerization of Der3p required for the function of the protein (data not shown). On the contrary, we found that overexpression of *HRD3*, another gene involved in ER degradation [13], is able to revert the dominance of *der3*$_{C399S}$. As can be seen in Fig. 5, suppression of the temperature sensitive growth phenotype of a *sec61-2* strain co-expressing both wild-type and mutant Der3p can be partially complemented after transformation with a multicopy plasmid carrying the *HRD3* gene.

We are currently investigating genetic and biochemical interactions of Der3p with Hrd3p and other components in order to explain their function in the ER degradation pathway.

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