Role of SSD1 in Phenotypic Variation of Saccharomyces cerevisiae Strains Lacking DEG1-Dependent Pseudouridylation

Bahar Khonsari, Roland Klassen * and Raffael Schaffrath *

Institut für Biologie, Fachgebiet Mikrobiologie, Universität Kassel, Heinrich-Plett-Str. 40, D-34132 Kassel, Germany; bahar.khonsari@gmail.com
* Correspondence: roland.klassen@uni-kassel.de (R.K.); schaffrath@uni-kassel.de (R.S.)

Abstract: Yeast phenotypes associated with the lack of wobble uridine (U34) modifications in tRNA were shown to be modulated by an allelic variation of SSD1, a gene encoding an mRNA-binding protein. We demonstrate that phenotypes caused by the loss of Deg1-dependent tRNA pseudouridylation are similarly affected by SSD1 allelic status. Temperature sensitivity and protein aggregation are elevated in deg1 mutants and further increased in the presence of the ssd1-d allele, which encodes a truncated form of Ssd1. In addition, chronological lifespan is reduced in a deg1 ssd1-d double mutant, and the negative genetic interactions of the U34 modifier genes ELP3 and URM1 with DEG1 are aggravated by ssd1-d. A loss of function mutation in SSD1, ELP3, and DEG1 induces pleiotropic and overlapping phenotypes, including sensitivity against target of rapamycin (TOR) inhibitor drug and cell wall stress by calcofluor white. Additivity in ssd1 deg1 double mutant phenotypes suggests independent roles of Ssd1 and tRNA modifications in TOR signaling and cell wall integrity. However, other tRNA modification defects cause growth and drug sensitivity phenotypes, which are not further intensified in tandem with ssd1-d. Thus, we observed a modification-specific rather than general effect of SSD1 status on phenotypic variation in tRNA modification mutants. Our results highlight how the cellular consequences of tRNA modification loss can be influenced by protein targeting specific mRNAs.

Keywords: yeast; SSD1; pseudouridine; tRNA modification

1. Introduction

Post-transcriptional RNA modifications are abundant in tRNA, where they may support stability, integrity, and translational efficiency [1–3]. Different modifications are introduced at different positions of specific tRNAs. Some modifications are installed in a sequential order, and several modification genes show strong genetic interactions because independent modifications may contribute to the same tRNA functional aspect [4,5]. Extensive functional redundancy may partly explain why no loss-of-function phenotypes are observable for many of the conserved modification genes. However, some specific modification genes are linked to growth phenotypes in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe [6]. An tRNA modification that is important for the normal growth of yeast cells is pseudouridylation at positions 38 and 39 (Ψ38/39), which is introduced by pseudouridine synthase Deg1. The tRNA \textsuperscript{Gln} \textsuperscript{UUG} overexpression suppresses the general growth defects of deg1 mutants, revealing a functional dependency of this tRNA on Deg1-dependent Ψ38/39 [7,8]. Pseudouridylation is a frequent tRNA modification that can be found in all parts of tRNA [9–12]. There are several additional \textsuperscript{Ψ} synthases responsible for pseudouridylation at other tRNA positions, and defects in some of these enzymes (including the Deg1 orthologue Pus3) are linked to neurodegenerative diseases such as intellectual disability in humans [13,14]. In yeast, the absence of Deg1 also influences neutral lipid content, amino acid levels, and sensitivity against rapamycin (an inhibitor of...
the TORC1 (target of rapamycin) kinase complex [15–18]), which might contribute to the general growth defect.

Yeast DEG1 exhibits strong negative genetic interactions with various genes involved in the formation of 5-methoxycarboxymethyl-2-thiouridine (mcm\textsuperscript{5}S\textsuperscript{2}U\textsubscript{34}). The latter tRNA modification is found in the wobble positions of tRNA\textsuperscript{GlnUUG}, tRNA\textsuperscript{GluUUC}, and tRNA\textsuperscript{LysUUU}, and two separate pathways are required for its synthesis [19–21]. The Elongator complex (composed of proteins Elp1-Elp6) and additional accessory proteins introduce the methoxycarbonylmethyl (mcm\textsuperscript{5}) residue at the C5 position of the wobble uridine (U\textsubscript{34}) [22]. An independent pathway involving Uba4, Urm1, and Ncs2/Ncs6 is responsible for the U\textsubscript{34} thiolation at C2, completing the mcm\textsuperscript{5}S\textsuperscript{2}U\textsubscript{34} composite [19,20,23]. Combining defects in either U\textsubscript{34} thiolation or C5 modification with a deg1 mutation results in strong synthetic growth and/or temperature sensitivities that are, in part, suppressible by tRNA\textsuperscript{GlnUUG} overexpression [7,8]. This points to a functionally relevant collaboration of the anticodon loop modifications at U\textsubscript{34} and U\textsubscript{38} in tRNA\textsuperscript{GlnUUG}. Similar functional collaborations between modified U\textsubscript{34} and other anticodon loop modifications have been demonstrated [7,24,25]. A strong negative genetic interaction occurs even among the genes involved in the C2 and C5 modifications of U\textsubscript{34} itself [26–28]. Several of the combined anticodon loop modification defects result in synthetic temperature sensitivities occurring in tandem with an accumulation of protein aggregates, which may form in response to ribosome slow-down at the individual hard-to-translate codons [7,28,29]. It is assumed that the protein aggregate induction contributes to the growth and temperature sensitivity phenotypes of the combined modification mutants.

Interestingly, the strength of double mutant phenotypes for the C2 and C5 hypomodification of U\textsubscript{34} is modulatable by the allelic variant of a gene (SSD1) encoding an mRNA-binding protein [30]. Ssd1 binds mRNAs encoding cell wall proteins and represses their translation under stress conditions [31,32]. The latter involves the association of Ssd1 and bound mRNA with cytoplasmic processing bodies (P-bodies) and requires the prion-like protein domain of Ssd1 [33]. Under favorable growth conditions, Ssd1 is thought to mediate the delivery of cell wall protein-encoding mRNA to sites of polarized growth [33]. Common laboratory yeast strains such as S288C and W303-1B [34] carry the allelic variants termed SSD1-\textsuperscript{v} or ssd1-\textsuperscript{d}, the latter of which encodes a truncated defective Ssd1 form. SSD1-\textsuperscript{v} (suppressor of SIT4 deletion) suppresses the lethal effect of SIT4 phosphatase gene deletion [35], while ssd1-\textsuperscript{d} cannot but shortens chronological lifespan [36] and enhances the phenotypes of Elongator mutants [30], including a more pronounced genetic interaction of ELP3 and the thiolase gene NCS2, as well as the enhanced accumulation of protein aggregates in the combined elp3 ncs2 mutant [30]. It remained unknown, however, whether phenotypic modulation by SSD1 status is specific to Elongator-dependent U\textsubscript{34} modifications or can be generalized to phenotypes from other tRNA modification mutants. In this study, we reveal a modulation of deg1 mutant phenotypes by SSD1 and demonstrate that other tRNA modification defects are not similarly affected. A possible relevance of independent cell wall integrity defects induced by ssd1-\textsuperscript{d}, elp3, and deg1 is discussed.

2. Results

2.1. Comparison of elp3 and deg1 Mutant Phenotypes in ssd1-\textsuperscript{d} and SSD1-\textsuperscript{v} Strains

To analyze whether the SSD1 allelic variants influence phenotypes induced by lack of pseudouridine synthase Deg1 in the yeast S. cerevisiae, we generated deg1 mutants in both SSD1-\textsuperscript{v} (BY4741) and ssd1-\textsuperscript{d} (W303-1B) strain backgrounds. For comparison, we deleted ELP3 in both backgrounds and analyzed the growth of all strains in response to elevated cultivation temperatures. In both cases, the modification defects were found to cause a fitness defect at elevated temperatures (Figure 1A).
As previously observed, the growth defect of deg1 mutants was more pronounced than that of elp3. The difference was observable in both ssd1-d and SSD1-ν (Figure 1A). However, in the ssd1-d strain, the growth defect of both tRNA modification mutants at 37 °C was enhanced compared to that with the SSD1-ν background. At 37 °C, only the ssd1-d elp3 and deg1 mutants, not the SSD1-ν counterparts, displayed a substantial growth defect. At 39 °C, however, all ssd1-d strains, including the wild-type control, were growth-impaired in comparison to their respective SSD1-ν counterparts (Figure 1A). Of note: the ssd1-d allele was previously shown to restrict growth at elevated temperatures [37].

To further test whether the difference in growth phenotypes was due to the allelic variation of SSD1, we introduced the SSD1-ν allele into the ssd1-d strains and analyzed phenotypic complementation. Both ssd1-d and ssd1-d deg1 strains were transformed with either an empty vector or a plasmid containing SSD1-ν ([SSD1-ν]). As a control, a plasmid carrying ssd1-d [ssd1-d] was introduced in parallel. As shown in Figure 1B, the thermosensitive growth of wild-type ssd1-d and the deg1 mutant could indeed be suppressed by SSD1-ν. Growth at elevated temperatures was improved for both the ssd1-d and ssd1-d deg1 strains upon the expression of SSD1-ν but not with ssd1-d or empty vector controls. Thus, the enhanced thermosensitivity of ssd1-d strains can be solely attributed to the ssd1-d allele, and SSD1-ν positively affects temperature resistance in the wild type and the tRNA modification mutant. Therefore, the enhancement of elp3 and deg1 phenotypes by the ssd1-d allele could reflect an additive effect being caused by two independent mechanisms increasing thermosensitivity. Interestingly, the temperature phenotype of an ssd1-d elp3 mutant was shown to be suppressible by osmotic stabilization [38], but it remained unknown whether this extends to SSD1-ν elp3 or deg1 mutants. Therefore, we tested the growth of elp3 and deg1 in both strain backgrounds at elevated temperatures in the presence of sorbitol.
(Figure S1). As a result, we observed a mild suppression of temperature phenotypes of the ssd1-d strains but not of the SSD1-v strains.

In addition to temperature sensitivity, we tested the effect of SSD1 variation on the rapamycin phenotype of elp3 and deg1 mutants. As expected, deg1 and elp3 mutants displayed increased rapamycin sensitivity compared to the respective wild-type control [16,30] in both genetic backgrounds (Figure 2). In comparison, however, elp3 displayed stronger rapamycin sensitivity than deg1. Rapamycin sensitivity was generally increased in ssd1-d strains. Importantly, the wild-type ssd1-d strain also exhibited enhanced sensitivity compared to the SSD1-v wild-type strain (Figure 2). To test whether the observed difference in rapamycin sensitivity between the ssd1-d and SSD1-v strains was due to the loss of SSD1 function, we analyzed the rapamycin phenotype in an ssd1 deletion strain and tested whether the ssd1-d phenotype could be complemented by the ectopic expression of SSD1-v. We found that ssd1 deletion in the SSD1-v strain increased drug sensitivity and SSD1-v expression in the ssd1-d strain suppressed drug sensitivity (Figure S2). In addition, a complete deletion of SSD1 in the SSD1-v strain background increased temperature and rapamycin sensitivity, both of which were further increased upon the deletion of DEG1 (Figure S3). Hence, the SSD1 status itself influences rapamycin resistance. Therefore, increased rapamycin sensitivity in ssd1-d tRNA modification mutants may have resulted from an additive effect being caused by independent consequences of tRNA modification loss and Ssd1 defects.

Figure 2. Comparison of the rapamycin sensitivity of tRNA modification mutants in SSD1-v and ssd1-d backgrounds. WT, elp3, and deg1 mutants of both background strains were serially diluted and spotted on YPD plates containing the indicated amounts of rapamycin. Plates were incubated at 30 °C for 48 h.

2.2. Impact of SSD1 on Genetic Interaction of DEG1 with mcm5s2U34-Relevant Genes

Since ssd1-d individually modulated the growth phenotypes of elp3 and deg1 tRNA modification mutants, we sought to test the effect of SSD1 variation on the negative genetic interaction between U34 and U38/39 modifiers. A strong synergistic growth retardation occurs upon the combination of U34 modification defects caused by mutations of either URM1 or ELP3 and the deletion of DEG1 [7]. An elp3 deg1 double mutant is viable in the SSD1-v background but displays a very pronounced growth defect at 30 °C [7]. An urm1 deg1 double mutant is similarly viable and displays near normal growth at 30 °C but severely delayed growth at 37 °C [7].

To test for the phenotypes of the same double mutants in the ssd1-d background, we used the plasmid shuffle approach previously employed to generate the SSD1-v double mutant strains. First, elp3 and urm1 mutations were each complemented with an appropriate 5-fluoroorotic acid (5-FOA) counterselectable plasmid that provided either ELP3 or URM1. DEG1 was subsequently deleted, and the ability to lose the urm1- and elp3-complementing plasmids was monitored by checking growth on 5-FOA-supplemented media (Figure 3A,B). In contrast to the control strains lacking the additional deg1 deletion, both elp3 deg1 and urm1 deg1 double mutants in the ssd1-d background were unable to
grow on 5-FOA media (Figure 3C,D). Thus, both elp3 deg1 and urm1 deg1 double mutants were inviable in the ssd1-d strain background. Hence, the observed synthetic sick genetic interaction of urm1/elp3 and deg1 in SSD1-v was further aggravated in the ssd1-d strain, in which a synthetic lethal interaction was observed (Figure 3C,D). This result supports the assumption that both, elp3 and deg1 mutations cause more severe phenotypic consequences in the ssd1-d strain; consequently, the double mutant is inviable in this background.

Figure 3. Plasmid shuffle assay to determine genetic interaction between DEG1 and ELP3 or URM1 in the ssd1-d strain. (A) Scheme indicating position and required genes for mcm5 s2U34 and Ψ38/39 modifications in tRNA. (B) Principle of plasmid shuffle assay involving elp3 deg1 or urm1 deg1 double mutants carrying URA3-CEN plasmids that provide for ELP3 or URM1 wild-type gene functions, respectively. 5-FOA medium (FOA) counterselects against the URA3-based plasmids and thus uncovers the double mutant phenotype. (C) Result of plasmid shuffle assay in the deg1 urm1 strain. (D) Result of plasmid shuffle assay in the deg1 elp3 strain. WT and indicated mutants with and without URA3-based plasmids were serially diluted and spotted on YPD, URA, and FOA plates. YPD and URA plates were incubated for 48 h, and FOA plates were incubated for 72 h at 30 °C.

Negative growth phenotypes of SSD1-v deg1 urm1 and deg1 elp3 double mutant strains were previously shown to be partially suppressible by the overexpression of tRNA^Gln^UUG [7]. A functional defect of this tRNA is thought to account for the negative phenotypes of the double mutants, and the higher-than-normal levels of the defective tRNA can likely compensate for functional impairment. To test whether the synthetic lethal interaction between elp3/urm1 and deg1 in the ssd1-d background can similarly be suppressed, we repeated the plasmid shuffle approach shown in Figure 3 in the presence of an overexpression construct for tRNA^Gln^UUG or an appropriate empty vector control. As
shown in Figure S4, the growth of ssd1-d elp3 deg1 and urm1 deg1 strains was rescued in the presence of the tRNA overexpression construct but not the empty vector control. In both double mutants, however, colony formation on 5-FOA media required prolonged incubation times and was thus significantly delayed compared to the wild type or the respective single tRNA modification mutants. Hence, tRNA\textsubscript{Gln}\textsubscript{UUG} overexpression provides a partial, rather than complete, rescue towards the negative genetic interactions between ELP3/URM1 and DEG1 in the ssd1-d strain (Figure S4).

2.3. Expression of the Gln-Rich Protein Rnq1 in deg1 Mutants

Multiple lines of evidence indicate a critical dependence of tRNA\textsubscript{Gln}\textsubscript{UUG}-decoding efficiency on \(\Psi_{38}\) [4,8,12]. Hence, aggravated phenotypes of ssd1-d deg1 in comparison to ssd1-v deg1 could be related to a further elevated decoding defect of tRNA\textsubscript{Gln}\textsubscript{UUG} in the ssd1-d strain. To compare the expression defect related to tRNA\textsubscript{Gln}\textsubscript{UUG} in the absence of pseudouridylation at position 38/39, we introduced a gene encoding the GFP-tagged Gln-rich prion protein Rnq1 [39] into WT and deg1 mutants of both SSD1 backgrounds. Both strains are [PIN+], implying the conversion of Rnq1-GFP into amyloid aggregates. Rnq1 contains a high number of Gln codons, and the translation of its mRNA was shown to be reduced in the absence of \(\Psi_{38/39}\) [7]. As described before, we observed a clear downregulation of Rnq1-GFP protein levels in the ssd1-v deg1 mutant in comparison to ssd1-v. Surprisingly, however, Rnq1-GFP levels were similar in the ssd1-d and ssd1-d deg1 strains (Figure 4). Thus, the expression of the Gln-rich Rnq1 was found to be improved rather than impaired in the ssd1-d deg1 mutant compared to the ssd1-v strain. Hence, a generally exacerbated translational defect of the \(\Psi_{38}\) deficient tRNA appears unlikely to account for the more severe phenotypes observed above in the genetic background of ssd1-d cells.

![Figure 4. Comparison of protein levels of the glutamine-rich Rnq1-GFP fusion protein in absence of \(\Psi_{38/39}\) in SSD1-v and ssd1-d backgrounds. The total protein extract from indicated strains expressing Rnq1-GFP was used for Western analysis with anti-GFP and anti-Cdc19 antibodies. GFP and Cdc19 signal intensities were normalized to the respective WT intensity.](image)

2.4. Role of SSD1 Status Variation in Protein Aggregation

Previous work has revealed disturbed protein homeostasis as a major consequence observable in yeast strains lacking mcm\(^5\)s\(^2\)U\(_{34}\) modification and/or \(\Psi_{38/39}\) [7,27,29]. The effect is most pronounced in double mutants lacking parts of the mcm\(^5\)s\(^2\)U\(_{34}\) modification in combination with the deg1 mutation or in the complete absence of the mcm\(^5\)s\(^2\)U\(_{34}\) modification [7]. The protein homeostasis defect is thought to contribute to mutant growth defects at elevated temperatures since temperature stress-challenges the proteostasis machinery. Given the increased the thermosensitivity of ssd1-d and ssd1-d deg1 strains compared to their SSD1-v variants, we investigated potential changes in the content of cellular protein aggregates. We considered the possibility that enhanced growth phenotypes in ssd1-d deg1 might occur along with enhanced protein aggregation, since a similar effect was observed for an elp3 ncs2 mutant [30].
Total protein and protein aggregate contents were extracted from wild-type and deg1 mutants (SSD1-v and ssd1-d) and then analyzed on Nu-PAGE gradient gels. As shown in Figure 5A, a deg1 mutation increased the protein aggregate content in both SSD1-v and ssd1-d backgrounds. ssd1-d displayed slightly more aggregates than SSD1-v, and ssd1-d deg1 showed slightly more aggregates as than SSD1-v deg1 (Figure 5A and Figure S5).

![Figure 5](image)

Figure 5. Impact of deg1 and ssd1-d on protein aggregation. Total protein and aggregate contents were extracted from (A) WT and deg1 mutants in both SSD1-v and ssd1-d backgrounds. (B) Total protein and aggregate contents from ssd1-d WT and the ssd1-d deg1 mutant in the presence (+) and absence (−) of plasmid-based SSD1-v [SSD1-v]. Samples were analyzed by Nu-PAGE and Coomassie staining.

To test whether the differences in protein aggregation were due to SSD1 loss of function, we introduced SSD1-v plasmids into the ssd1-d and ssd1-d deg1 strains and compared protein aggregation between them. As shown in Figure 5B and Figure S5, the presence of the SSD1-v plasmid [SSD1-v] suppressed both thermosensitivity and protein aggregation, strongly suggesting a functional correlation.

2.5. Chronological Aging in deg1 Mutants

In addition to its effect on temperature tolerance, SSD1 has also been implicated in the process of chronological aging. ssd1-d was correlated with a shorter chronological lifespan than SSD1-v [36]. Since both ssd1-d and deg1 mutations independently appeared to increase protein aggregation and because this effect might be relevant for long-term stationary phase survival, we tested the effects of ssd1-d and deg1 alone and in combination on chronological aging. A chronological aging assay was performed for the SSD1-v and ssd1-d strains with and without the deg1 mutation over a time range of 17 days in the stationary phase (Figure 6A). As expected, the ssd1-d strain exhibited a faster decline in viability than the SSD1-v strain. At 7 days in the stationary phase, less than 50% viability was retained in the ssd1-d cultures, whereas the SSD1-v cultures took 10–12 days to reach this point. The SSD1-v deg1 mutant showed a similar decline in viability over time as the wild-type SSD1-v, suggesting that in this background, DEG1 does not represent a major aging factor. However, the ssd1-d deg1 mutant showed a strongly accelerated loss of viability in comparison to the wild-type ssd1-d control (Figure 6A). Hence, in contrast to
the SSD1-\(v\) strain background, the DEG1 loss-of function appears to contribute to aging in the background of an ssd1-d strain.

![Figure 6](image_url)

**Figure 6.** Influence of SSD1 and DEG1 on chronological aging. (A) Chronological aging was analyzed for the SSD1-\(v\) WT and the ssd1-d deg1 mutant in comparison to the ssd1-d and ssd1-d deg1 strains over a time range of 17 days. Viability (%) represents the determined colony forming units (CFU) per ml normalized to the respective value at day 0. The mean of three independent cultures and the standard deviation is given. (B) As in (A) but with indicated strains in the presence or absence of plasmid-based SSD1-\(v\) [SSD1-\(v\)].

To test whether the differential effects of deg1 mutation in the ssd1-d and SSD1-\(v\) strains were exclusively due to the difference in the allelic variant of SSD1, we introduced SSD1-\(v\) plasmids into the wild-type ssd1-d and deg1 mutant and analyzed their chronological aging. As shown in Figure 6B, the presence of the SSD1-\(v\) plasmid [SSD1-\(v\)] improved viability over time in the stationary phase, consistent with the established role of SSD1 in stationary phase survival. Importantly, however, ssd1-d deg1 [SSD1-\(v\)] still displayed an accelerated loss in viability over time compared to the ssd1-d [SSD1-\(v\)] control. Hence, the observation that DEG1 represents an aging-relevant gene in the ssd1-d but not SSD1-\(v\) strain background is likely due to differences between the two strains other than their none similar SSD1 locus.

2.6. Phenotypic Diversity of Other tRNA Modification Defects in ssd1-d and SSD1-\(v\) Strains

Besides elp3 and deg1, other tRNA modification genes are linked to the mutant phenotype of retarded growth at elevated temperatures. To test more generally whether temperature-sensitive growth phenotypes of tRNA modification mutants are aggravated by ssd1-d, we selected the tRNA pseudouridine synthase Pus1 and the tRNA methyltransferases Trm1 and Ncl1, and we compared their mutant phenotypes in ssd1-d and SSD1-\(v\) backgrounds. In SSD1-\(v\), all three mutants (pus1, trm1, and ncl1) displayed robust temperature sensitivity compared to the wild-type SSD1-\(v\) (Figure 7A), confirming earlier reports [40–42]. Interestingly, however, while the ssd1-d strain again showed an elevated temperature sensitivity compared to the SSD1-\(v\) strain, only a modest further enhancement (relative to ssd1-d) was seen for ssd1-d pus1. Unexpectedly, ssd1-d ncl1 and ssd1-d trm1 mutants were not significantly more temperature-sensitive than the ssd1-d control (Figure 7A). To further study genetic interaction strength in ssd1-d and SSD1-\(v\) backgrounds, we additionally deleted the TRM8 methyltransferase gene [43]. In ncl1 trm8 mutants, a strong negative genetic interaction is well-described and mechanistically linked to the rapid tRNA decay of tRNA\(^{Val}_{AAC}\) at 37 °C [44]. This results in the complete absence of growth of an SSD1-\(v\) ncl1 trm8 double mutant at 37 °C but not at 30 °C. In the ssd1-d ncl1 trm8 strain, a robust synthetic temperature sensitivity was observed at 37 °C, but
compared to the SSD1-v ncl1 trm8 mutant, the defect was ameliorated rather than enhanced (Figure 7B).

An additional phenotype described for SSD1-v trm1 and pus1 mutants is an enhanced sensitivity against the anticancer drug 5-fluorouracil (5-FU) [40]. A combination of mild heat stress was further shown to strongly increase the efficiency of the drug, potentially by a destabilizing effect on the hypomodified tRNAs. Given the unexpected absence of temperature-sensitive phenotypes for ssd1-d trm1 and pus1 mutants, we sought to analyze the 5-FU phenotype in the two strain backgrounds. Compared to the wild-type SSD1-v, the SSD1-v trm1 and pus1 strains displayed an increased sensitivity to 10 mg/mL 5-FU at 30 °C, but this was not the case for the corresponding ssd1-d strains (Figure S6). However, when the drug was applied at 37 °C, both ssd1-d trm1/pus1 and SSD1-v trm1/pus1 mutants became strongly sensitized relative to the wild-type controls, thus confirming the expected single mutant phenotypes. Interestingly, the ssd1-d pus1 and trm1 mutants appeared to be slightly more resistant to this effect compared to the SSD1-v strains (Figure S6). Hence, 5-FU phenotypes of ssd1-d trm1 and pus1 in general are confirmed, but they are ameliorated in comparison to the SSD1-v strain background.

In conclusion, while elp3 and deg1 mutants exhibit more severe growth phenotypes in ssd1-d compared to the SSD1-v background, this is not generally true for other tRNA modification mutants. In at least three cases (pus1, trm1, and ncl1), the opposite effect of less severe growth defects in the ssd1-d strain can be observed.
3. Discussion

In the yeast *S. cerevisiae*, the RNA-binding Ssd1 protein plays prominent roles in cell wall remodeling through the delivery of cell wall protein-encoding mRNA to polarized growth sites [31]. Hence, *ssd1* mutations leading to the loss of Ssd1 function are linked to growth defects at elevated temperatures [37] and in the presence of cell-wall-stress-inducing drugs such as calcofluor white (CFW) [45,46]. More recently, *SSD1* was implicated in the phenotypic variation of *elp3* mutants lacking the catalytic subunit of the tRNA modification complex, Elongator [30]. Pleiotropic *elp3* phenotypes [38,47] are enhanced by the *ssd1-d* allele present in common laboratory strains derived from W303 yeast [30]. *SSD1* is evolutionarily conserved in the fungal kingdom and its orthologue in the distantly related fission yeast *S. pombe* similarly encodes a P-body associated RNA-binding protein [48,49]. Thus, the functional conservation of Ssd1 in the fungal kingdom is likely, but phenotypic variation of tRNA modification defects in other fungi remain to be studied.

Mechanistically, a functional Elongator complex is required to maintain the translational capacity of tRNA$^{\text{LysU}}_{\text{UUU}}$ and tRNA$^{\text{GlnU}}_{\text{UGC}}$ [47,50–52]. Deg1 was also shown to be required for the functionality of tRNA$^{\text{GlnU}}_{\text{UGC}}$ and the strong negative genetic interactions of *DEG1* with Elongator-related genes are due to an additive functional impairment of this tRNA [7,8,16,29]. However, prior to this study, it remained unclear whether *DEG1* also shares the recently described negative genetic *SSD1* interaction with *ELP3* [30].

In this study, we demonstrated that this is the case, as growth phenotypes of both *elp3* and *deg1* were found to be more pronounced in the W303 *ssd1-d* strain compared to the BY4741 *SSD1-v* strain, including sensitivity to elevated temperatures and the TORC1 inhibitor drug rapamycin. Additionally, the genetic interactions between *ELP3*, *DEG1*, and *URM1* were found to be enhanced from synthetic sickness in the *SSD1-v* strain [7] to synthetic lethality in the *ssd1-d* strain (Figure 3). To exclude the option that the observed variation in stress phenotypes was due to differences other than the *SSD1* locus, we verified that (i) growth phenotypes in the *ssd1-d* strain were rescued by the ectopic expression of *SSD1-v* (Figure 1B) and that (ii) growth phenotypes in the *SSD1-v* strain were similarly enhanced by the complete loss of *SSD1* (Figure S3). Phenotype assessment, however, clearly demonstrated that *ssd1* mutation itself is linked to the same phenotypes as for *elp3* and *deg1* (Figure 1A and Figure S3). Hence, it appears that the observed phenotypic variation of *elp3* and *deg1* mutants by *ssd1* is caused by related cellular effects of either mutation alone rather than a tRNA-modification-specific effect of *ssd1*. Our observation that *deg1* phenotypes are enhanced by *ssd1-d* (Figure 1) but not the expression defect of gene encoding a Gln-rich protein (Figure 4) further supports our assumption that *ssd1-d* does not specifically aggravate the effect of modification loss at the level of translation. It currently remains unknown why the relative reduction of Rnq1-GFP signal strength is actually reduced in the *ssd1-d deg1* mutant. An impact of the strain background on the amyloid aggregate formation potential of the protein might contribute to the observed differences.

Both Deg1 and Elongator have been implicated in the maintenance of protein homeostasis [7,28,29,53,54]. A deficiency in protein homeostasis is thought to account for stress phenotypes of certain combined mutants involving Elongator, tRNA thiolation, and/or Deg1 defects (e.g., temperature sensitivity) [7,28]. Since Ssd1 also has a documented role in protein disaggregation by influencing the ability of heat shock protein Hsp104 to bind protein aggregates [55], the phenotypic variation of *deg1* and *elp3* could be at least partly mediated by effects on protein aggregation. Indeed, protein homeostasis defects of a combined *elp3 ncs2* mutant [30,56] and of a *deg1* single mutant (Figure 5) were moderately elevated by the presence of the *ssd1-d* allele. It is worth noting that the presence of the *ssd1-d* allele alone already increased the amount of detectable cellular protein aggregates (Figure 5A). Thus, Ssd1 could prevent protein aggregation in a mechanistically distinct way compared to the tRNA modifications, potentially involving the described effect on Hsp104 function. Such an independent effect on protein homeostasis could explain the observed additive phenotypes of *ssd1-d* in conjunction with *elp3* or *deg1* mutations.
In addition to the effect on protein homeostasis, a phenotypic similarity between elp3, deg1, and ssd1 could also be related to similar effects of either mutation on cell wall integrity. Like ssd1 mutant cells, elp3 mutants display an increased sensitivity to cell wall stressor CFW [38], and combined mutants involving elp3, uba4, urm1, and deg1 mutations in the SSD1-v background were shown to exhibit hyperpolarized growth and cell lysis phenotypes that likely imply cell wall defects [7,27]. Along these lines, it was further demonstrated that elp3 mutant phenotypes are partially suppressed by genetically upregulating the cell wall integrity (CWI) pathway [30] and by osmotic stabilization [38]. Similar to ssd1-d elp3, we found that ssd1-d deg1 mutants are also in part phenotypically suppressed by osmotic stabilization (Figure S1). Hence, if ssd1 and elp3(deg1) individually induced cell wall defects by distinct mechanisms, these could be elevated in their respective double mutants with a single tRNA modification defect and a null or truncated allele of SSD1. In support of this idea, we found that like elp3, the deg1 mutation also increased CFW sensitivity and a double ssd1 deg1 mutant showed additivity, in not only rapamycin and temperature sensitivity but also CFW sensitivity (Figure S3). While the molecular basis for rapamycin sensitivity of different tRNA modification mutants is not fully understood, it is noteworthy that modification mutants involving elp3 and/or deg1 mutations display hallmarks of reduced TORC1 activity [29,57]. Since TORC1 has, amongst others, a role in the maintenance of cell wall integrity [58,59], it could be hypothesized that rapamycin increases the chronic cell wall integrity defects of the tRNA modification mutants, which are further elevated upon the loss of Ssd1 and result in additive phenotypes in combination with ssd1-d.

We additionally observed that the deg1 mutation is linked to reduced chronological lifespan, which was specifically obvious in the ssd1-d strain background (Figure 6). It was previously demonstrated that SSD1 mutations affect the transcript levels of longevity genes and thereby drastically reduce chronological lifespan [36] Additionally, proper CWI signaling positively contributes to long-term survival of the stationary phase [60]. Hence, additive effects of deg1 and ssd1 mutations on the CWI and mRNA levels of longevity genes might explain the ssd1-d-specific aging phenotype of deg1. Additionally, the clearance of protein aggregates that accumulate during chronological aging [61] might influence long-term stationary phase survival. Therefore, the shortened lifespan of the ssd1-d deg1 mutant might partly be related to the elevated protein aggregation that we detected (Figure 5). However, since a W303 deg1 ssd1-d [SSD1-v] mutant showed a reduced chronological lifespan compared to the ssd1-d [SSD1-v] control (Figure 6B), other genetic differences and factors between BY4741 and W303 probably contribute to the short-lived phenotype observed in W303 deg1 rather than BY4741 deg1 cells.

Unexpectedly, other tRNA modification defects that share the temperature-sensitive phenotype with elp3 and deg1 did not generally exhibit enhanced phenotypes in the ssd1-d W303 strain compared to SSD1-v BY4741. On the contrary, we observed ameliorated effects for pus1, trm1, and ncl1 in W303. This was not limited to temperature sensitivity, as it also extended to sensitivity against the anticancer drug 5-FU, which was weakened rather than enhanced in W303 pus1 and trm1 mutants compared to the BY4741 counterparts (Figure S6). Intriguingly, phenotypic enhancement by ssd1-d appears to be refined to two specific modification defects that induce a pleiotropic set of phenotypes overlapping with ssd1. Of particular relevance could be the CFW sensitivity indicative of cell wall defects and shared between ssd1, elp3, and deg1 but not, for instance, with other pseudouridine synthase defects (Figure S3B). Hence, the phenotypic variation of tRNA modification defects by ssd1 could be restricted to those tRNA modifications that play roles in cell wall integrity and might therefore reflect additive cell wall damage.

4. Conclusions

We demonstrated that in *S. cerevisiae*, the RNA-binding Ssd1 protein influences the cellular consequences of tRNA modification loss. In addition to the previously described SSD1-dependent phenotypic modulation of elp3 mutants lacking specific wobble uridine modifications, a similar effect was observable in deg1 mutants defective in U38 and U39.
pseudouridylation. However, phenotypes associated with other tRNA modification defects were not similarly affected. Genetic evidence suggests that *elp3*, *deg1*, and *ssd1* mutants share cell wall integrity defects that might be responsible for additive negative phenotypes.

5. Materials and Methods

5.1. Strains and General Methods

The strains of *Saccharomyces cerevisiae* used in this study are listed in Supplementary Table S1, and standard methods were used for yeast growth and maintenance [62]. When plasmid maintenance was required, a synthetic complete medium lacking either leucine or uracil (depending on the selectable marker of the plasmid) was used. Genomic deletions were generated with the help of PCR [63] and oligonucleotides targeting *ELP3*, *URM1*, *DEG1*, *PU51*, *TRM1*, *TRM8*, or *NCL1* (Supplementary Table S2). Replacements were confirmed by PCR using primers located outside of the target genes (Supplementary Table S2). For temperature and drug sensitivity assays, freshly grown yeast cells were resuspended in sterile water and adjusted to an initial optical density (OD$_{600nm}$) of 1. These suspensions were used for 10-fold serial dilutions and spotted on YPD plates with or without the presence of indicated drugs. For temperature assays, identical replicate plates were prepared from the same cell dilutions and incubated at different temperatures.

5.2. Plasmid Construction and Shuffling

For the deletion of *DEG1* in *urm1:SpHIS3* or *elp3::SpHIS3* strains, they were first transformed with pFF8 (*ELP3*; [27]) or pHA-URM1 (*URM1*; [64]) to genetically complement the genomic deletions. Subsequently, *DEG1* was deleted by using a PCR-generated deletion cassette (*deg1:SpHIS5*), and the *URA3* plasmids pFF8 or pHA-URM1 were eliminated by growth on minimal media containing uracil and 5-fluoro-orotate (1 mg/mL). For the plasmid-based complementation of *ssd1-d*, the *SSD1-v* plasmids pPL091 (*LEU2*) and pPL092 (*URA3*) [58] were used. For control purposes, the *ssd1-d* plasmid pPL093 (*URA3*) [58] was utilized. tRNA$_{Gln}$$_{UUG}$ overexpression used pRK55 [7]. Rnq1 was expressed as a GFP fusion from plasmid p1332 [65].

5.3. Protein Isolation and Western Blotting

Protein extracts were obtained from cells grown to OD$_{600nm}$ = 1 using disruption with glass beads [66]. Protein yield was quantified according to [67], and equal amounts of total protein (50 µg) from different strains were used for loading the gels. Transfer and detection were done as described previously [38] and involved anti-GFP (Santa Cruz Biotechnology, Dallas, TX, USA) and anti-Cdc19 antibodies.

5.4. Isolation of Protein Aggregates

Aggregated proteins were obtained from 50 mL YPD cultures grown to OD$_{600nm}$ = 1, as previously described [28,68]. This method is based on the isolation of aggregated proteins from total protein extracts by centrifugation. Identical amounts of total protein extract were subjected to centrifugation and washing steps as previously described [27,65]. The obtained aggregate pellet was dissolved and analyzed by denaturing SDS acrylamide gel electrophoresis and Coomassie staining. To control for the identical input of total protein, a portion of each extract (25 µg) was analyzed along with the aggregate samples. After breaking cells by sonication, a Bradford assay was used to determine the obtained amount of protein for each strain; 4 mg of total protein were subjected to centrifugation and washing [68]. Aggregates from the pellet were boiled in a Laemmli buffer and were separated on NuPAGE Bis–Tris 4–12% gradient gels. As a control, 25 µg of the total protein extract used for aggregate isolation were run on the same gel.

5.5. Chronological Aging Assay

Yeast chronological lifespan (CLS) was determined according to [69]. Freshly streaked colonies were inoculated into a preculture consisting of a 2 mL synthetic complete dextrose
Int. J. Mol. Sci. 2021, 22, 8753

13 of 16

(SDC) medium and incubated at 30 °C overnight. Main cultures were inoculated at OD\textsubscript{600nm} = 0.1 in a 10 mL SDC medium in Erlenmeyer flasks covered with aluminum foil. The optical density of the main culture was measured until the stationary phase, considered as time point day zero with an initial survival of 100%, was reached [69]. To determine viability, cells from each flask were diluted and plated on two YPD plates that were incubated at 30 °C until colonies appeared. Colony counts were used to calculate colony forming units per ml (CFU/mL) for each culture and time point (0–17 days). Relative viability represents the CFU/mL value normalized to the value obtained for day zero. Each strain was analyzed using three independent cultures that were cultivated in parallel.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22168753/s1, Table S1: Strains used or generated in this study. Table S2: Oligonucleotides used in this study. Figure S1: Temperature and drug sensitivity of elp3 and deg1 modification mutants in both SSD1 backgrounds. Figure S2: Rapamycin sensitivity in the presence and absence of SSD1. Figure S3: Drug sensitivities of gene deletion mutants in the SSD1-v background. Figure S4: Rescue of the lethality of deg1 urm1 and deg1 elp3 strains. Figure S5: Densiometric analysis of protein aggregation shown in Figure 5. Figure S6: 5-fluorouracil (5-FU) phenotype of tRNA modification mutants in ssd1-d and SSD1-v backgrounds.

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