THE RNA HELICASE DED1 INTERACTS WITH EIF4G1 TO CONTROL TRANSLATION INITIATION

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

The DEAD-box RNA helicase Ded1 has an essential role in translation initiation in *Saccharomyces cerevisiae*. Mutations in the human homolog DDX3 have been implicated in several human diseases, particularly medulloblastoma, a pediatric brain cancer. Previous *in vitro* data and immunoprecipitation assays have shown that Ded1 can both bind to the initiation factor eIF4G1 and homo-oligomerize through its C-terminal domain. The interaction with eIF4G1 is essential to effectively synthesize proteins. Studies have also shown that *ded1* mutants lacking a C-terminus (*ded1*-Δ*CT*) do not efficiently regulate translation, and a similar effect is also observed when only the last 14 amino acids of the C-terminus are deleted (*ded1*-Δ*591-604*). Here we characterized the *in vivo* effects of these interactions with the Ded1 C-terminus. In serial dilution growth assays, we observed growth defects in both *ded1*-Δ*CT* mutants and in strains lacking eIF4G1 (*tif4631Δ)*, along with a synthetic phenotype in *ded1*-Δ*CT tif4631*Δ double mutant strains. Similarly, *ded1* double mutants lacking both eIF4G1 and the last 14 amino acids of the Ded1 C-terminus partially phenocopied the full *ded1*-Δ*CT* mutant. These growth defects suggest a decreased binding affinity by *ded1* with eIF4G1 due to the lack of the C terminus that leads to diminished translation efficiency. Furthermore, we show that *ded1* mutants show an increased resistance to rapamycin, a known target of the TOR pathway. This could suggest an important role for Ded1 C-terminus in regulating the TOR pathway in response to rapamycin stress. We also show that altering eIF4G1 levels in the presence of heat significantly affects cell viability. This might suggest a tight regulation of eIF4G1 levels during the cell's response to heat stress.

Introduction

Translation is a fundamental process in gene expression that enables the conversion of genetic information encoded in mRNA into functional proteins. It involves the coordinated actions of multiple cellular components including ribosomes, translation factors, and RNA helicases, among others. The yeast protein Ded1, a DEAD-box RNA helicase, has an essential role in translation by mediating protein-protein and protein-RNA interactions which lead to translation initiation, among other gene expression processes[1]. Dead-box proteins are a family of RNA helicases that are involved in many aspects of RNA metabolism, including splicing, translation, and degradation. They are characterized by the presence of a DEAD motif, named according to their amino acid composition, which is essential for their helicase activity. DEAD-box proteins usually form part of larger multi-subunit complexes[2], such as the translation initiation machinery, and mutations in them have been linked to diseases such as cancer[3] which is the case with the Ded1 human homolog DDX3. Similar to its DEAD-box family members, Ded1 consists of a central helicase core which promotes unwinding of RNA secondary structures. Flanking the helicase core are N- and C-terminal disordered domains which mediate binding to the eIF4F translation complex and oligomerization to other Ded1 proteins[1, 2].

While RNAs are primarily imagined as being single stranded, they can form secondary structures that physically impede translation machinery progression and access to the start site. This is where RNA helicases like Ded1 demonstrate their importance. Sen et al. (2015) showed that there is a group of genes that are particularly dependent on efficient Ded1 activity. Upon further investigation of these genes, it was shown that their transcripts share common patterns in the form of atypically long 5’ untranslated regions (UTRs) and a greater propensity to form secondary structures than the average messenger RNA[10]. The group found that RNAs with a

decreased translational efficiency in a *ded1* strain, that is, RNAs that are more dependent on Ded1, had a 5' UTR that was close to double the length of the average yeast 5' UTR[10]. Additionally, the group measured the propensity of this same group of RNAs in forming secondary structures in their 5' UTRs, finding that they were double stranded far more often than the genome average[10], again indicating that these genes have developed a dependency on Ded1 due to its ability of unwinding the secondary structures in the 5' UTR. The unwinding of these structures allows the translation machinery to scan the mRNA unimpeded and efficiently reach the start site where the protein can begin to be synthesized. To explore this phenomenon further, Guenther et al. (2018) showed that defects in Ded1 can promote translation initiation at a near cognate start site, a start site resembling the canonical AUG but differing in a single nucleotide. The group showed that Ded1 was crucial in consistent mRNA translation in the correct reading frame, avoiding near cognate start codons and translation initiation at an Alternate Translation Initiation Site (ATIS), which could lead to unwanted consequences. They were able to demonstrate that the mechanism by which Ded1 accomplishes this function is by allowing quick scanning through the 5' UTRs and avoiding any stalling at near cognate start codons where translation could begin[12]. These findings implicate Ded1 as an initiator of translation by efficiently unwinding RNA structures that could physically block the translation initiation machinery and by promoting read-through of near cognate start codons which would alter the expression of a particular mRNA.

Accompanying Ded1 in translation initiation is the eukaryotic initiation factor 4G1 (eIF4G1), which serves as a scaffold in the larger eIF4F complex[5]. Ded1 is believed to interact with the eIF4F complex, primarily through eIF4G1, which functions in the early stages of translation initiation. Interestingly, the interaction between Ded1 mutants lacking a C-terminus

(ded1ΔCT) and eIF4G1 is greatly reduced *in vivo*[5] and *in vitro*[5, 8], while the interaction remains unaffected following deletions in the Ded1 helicase core or N-terminal domain. This confirms the idea that Ded1 interacts with eIF4G1 through its C-terminus in order to form a Ded1-mRNA-eIF4F complex. It was also shown that the portion of eIF4G1 that interacts with Ded1 is one of its RNA binding domains[5]. This was shown by using a fragment of eIF4G1 that only included its RNA3 domain and proving that it could interact with the Ded1 protein. It is worth noting that the "RNA binding domain" in eIF4G1 has not been conclusively associated with RNA binding, meaning that it has potential for other regulatory functions. Furthermore, the Ded1 N-terminus has been implicated in interactions with the cap-binding protein eIF4E and eIF4A, yet another helicase that forms part of the eIF4F complex[6]. The interactions between the small ribosomal subunit, the eIF4F complex and other initiation factors form what is known as the PreInitiation Complex (PIC), a major protein complex to bind the mRNA in preparation for translation. Hilliker et al. (2011) demonstrated that Ded1 plays a fundamental role in the assembly of the PIC by mediating binding between itself, the eIF4F complex, and the mRNA. The group showed that the assembly of this complex is a prerequisite for translation initiation, as strains that were unable to form the assembly had a significantly reduced translation activity[5]. In another study, Gulay et al. (2020) demonstrated that the interactions between Ded1 and two components of the eIF4F complex, eIF4A and eIF4E, are important for efficient PIC assembly, especially on mRNAs that contain secondary structures. The group selectively disrupted Ded1 binding to each protein and revealed a dependence on these interactions for PIC assembly to progress as usual[11].

In addition to its interaction with the eIF4F complex, Ded1 has the ability to oligomerize with itself in order to optimize its helicase activity[2]. This intrinsic ability to oligomerize without the need for RNA or other cofactors was demonstrated by Putnam et al. (2015), who found that

Ded1 could form dimers and, albeit less often, trimers through direct protein-protein interactions. The same group examined the role of the C-terminus in oligomerization, finding that a ded1ΔCT mutant could form dimers *in vitro* but it was unable to form trimers[2]. This indicates that the C-terminus has an essential role in forming the Ded1 trimer, although not as important for direct Ded1-Ded1 interactions. The group then examined the effect of Ded1 oligomerization on its helicase activity, finding that the protein's helicase activity was related to its concentration in a sigmoidal fashion, indicating that it functioned through cooperative oligomerization[2]. The optimal form for Ded1 helicase activity was found to be as a trimer, although it could bind RNA as a monomer, dimer, or trimer[2]. To investigate the effect of eIF4G1 on Ded1 oligomerization, Putnam et al. (2015) examined the relationship between eIF4G1 presence and Ded1 helicase activity. They found that if eIF4G1 was present, Ded1 helicase activity was significantly hindered and cooperative oligomerization was no longer observed, suggesting that the Ded1-eIF4G1 interaction disrupts the Ded1 trimer. However, the implications of this competitive binding *in vivo* remain unclear.

More recently, a novel Ded1 function was characterized in the context of stress responses. In order to efficiently manage and prioritize cellular resources in stressful conditions, cells must be able to alter protein synthesis appropriately. The target of rapamycin (TOR) pathway is a major pathway involved in this regulation, named so because mutations in TOR conferred rapamycin resistance[7]. The role of Ded1 as a translation initiator along with the aforementioned initiation factors eIF4G1 and eIF4A has long been known; however, Aryanpur et al. (2019) demonstrated that Ded1 had an additional role as a translation repressor when exposed to rapamycin, an inhibitor of the growth regulator TOR and a mimic of stress for the cell. The group also identified that the Ded1 C-terminus played an important role in the response to TOR

inhibition, as deleting it conferred rapamycin resistance. Rapamycin resistance was accompanied by an inhibited ability to downregulate translation, an essential process during the stress response to limit the expenditure of valuable resources for the synthesis of non-essential proteins. The ded1ΔCT protein was unable to attenuate new protein synthesis as efficiently as its WT counterpart, indicating a novel role of the C-terminus in translation repression following rapamycin treatment[9]. Another indicator of stress response is the ability of the cell to form phase separated aggregates of mRNAs and proteins termed stress granules. Hilliker et al. (2011) was the first to show that Ded1had an important role in the formation and resolution of stress granules. By doing this, Ded1 is able to both repress translation by forming mRNP condensates stalled in translation initiation and promote translation by resolving these condensates. Building on this research, Iserman et al. (2020) showed that Ded1 forms condensates *in vitro* in response to stresses such as heat and pH, and these condensates colocalize with stress granule markers *in vivo*, indicating the likely possibility that Ded1 is part of stress granules. Additionally, the group demonstrated that the condensates of Ded1 impaired its ability to aid in the translation of mRNAs with highly structured 5' UTRs, all the while leaving translation of less structured mRNAs relatively unchanged[12]. This illustrates a role for Ded1 condensation in selectively translating mRNAs during times of stress and nutrient deprivation. Another study by Hondele et al. (2019) argued that a human subtype of DEAD-box proteins, DDXs, were regulators of mRNA translation by sequestering them into stress granules to control their fate more closely. The group also demonstrates that RNA release is mediated by Ded1 ATP hydrolysis, as inhibition of the ATPase domain greatly reduced the rate of disassembly of stress granules among other cellular condensates[13]. Notably, Aryanpur et al. (2022) showed that *ded1ΔCT* mutants formed stress granules more readily than wild-type cells when exposed to oxidative

stress, possibly indicating that the Ded1 C-terminus has an important role in regulating stress granule formation dynamics under stress[9]. We are currently in the process of further examining the role of stress granule formation after exposing cells to rapamycin stress to test whether the same phenotype is observed.

In this thesis, we will further examine the genetic interactions between Ded1 and eIF4G1, assessing which sections of the Ded1 C-terminus are important for the interactions. We will also attempt to define which sections of the C-terminus are important for Ded1 activity in unwinding 5' untranslated region (UTR) structures in mRNAs.

Materials and Methods

Yeast strains and plasmids

Yeast strains and plasmids used are listed in Tables 1 and 2. Strains containing different plasmid-based *ded1* mutants were created by plasmid shuffle starting with the strain SWY4093 (*ded1::KAN +pDED1/CEN/URA3*). Strains carrying genomic *ded1* mutant genes were generated by integrating PCR products synthesized using pairs of primers listed in Table 3. Integration of the PCR products at the *DED1* locus of strain OLY3 resulted in the replacement of the wild-type *DED1* coding sequence with codons for the noted amino acids deleted, followed by a gene encoding the selectable marker noted in Table 1. Strains TBY134-137 were created by integrating a sequence encoding a protein conferring resistance to hygromycin B into the *TIF4631* locus of SWY4093. Plasmid shuffle was then used to create the different *ded1 tif4631* double mutant strains. *DED1* mutant plasmids were created by site-directed mutagenesis of either *DED1* or pSW3619. pTB111-115 were created by amplifying pSW3619 using PCR primers designed to exclude the targeted 14 amino acid region, followed by blunt end ligation.

Media

YPD (2% peptone, 1% yeast extract, 2% dextrose) was used as primary nutrient-rich media for growth of yeast strains. Solid medium contained 2% agar. To retain plasmids during growth, minimal medium was used (0.17% yeast nitrogen base, 0.5% NH_4SO_4, 2% dextrose + required amino acids). When noted, hygromycin B was used at a concentration of 0.4 mg/ml and rapamycin was used at 200 ng/ml. Unless otherwise noted, strains were grown at 30°C.

Strain analysis with tetrad dissections

To analyze phenotype segregation in individual genomic integrants of *ded1Δ536-590*, individual integrants/clones were mated with strain OLY2 on solid YPD medium. After overnight incubation at 30°C, zygotes were picked from the mating mixture using a Singer Sporeplay dissection microscope and allowed to sporulate. Tetrads were dissected and tested for resistance to hygromycin B, indicating the presence of the *ded1Δ536-590* allele. The growth phenotypes of individual *ded1Δ536-590* strains resulting from these backcrosses were then analyzed to ensure that the mutant phenotype bred true.

Cell lysate preparation for Western Blot

Approximately 30mg of cells from an overnight culture were pelleted, washed once with H_2O, and resuspended in 160µL of 1.85M NaOH, 7.4% β-mercaptoethanol. After incubating on ice for 10 minutes, 160µL of 50% TCA was added and the mixture was again incubated on ice for 10 minutes. Precipitated protein was pelleted, washed in 0.5mL 1M Tris Base, and resuspended in 150 µl/1 OD_{600} of protein loading buffer (28.5% glycerol, 4.4% SDS, 55 mM Tris, pH=6.5, 0.1 M DTT + bromophenol blue).

Western blotting of cell lysates

Cell lysates were heated to 95°C prior to loading onto a 10% polyacrylamide gel, separated by SDS–PAGE, and transferred to PVDF membrane. Membranes were probed with antibodies directed against Ded1p (VU318) diluted at 1:3,000 or eIF4G1p (gift from R. Parker, University of Colorado Boulder) diluted at 1:10,000. Anti-Ded1p and anti-eIF4G1p were detected with HRP-conjugated goat, anti-rabbit IgG (Invitrogen), diluted at 1:10,000. HRP-conjugated secondary antibodies were detected using the SuperSignal West Durachemiluminescent substrate (ThermoFisher) and Sapphire imager (Azure Biosystems). Band intensity was measured using ImageJ/Fiji software.

Serial Dilution Growth Assay

Yeast growth assays were performed by serial dilution. Cells were diluted to 2 x 10^7 cells/ml, then serially diluted 1:5 fold. 5 μl of the original dilution and of 4 serial dilutions were spotted onto solid medium. Images of growth on YPD plates were taken 24 hours after plating. Images of growth on rapamycin plates were taken after 5 days. Rapamycin (Santa Cruz Biotechnology) was dissolved in dimethylsulfoxide (DMSO) and was used at concentrations of 200 ng/ml in YPD plates and 100 ng/ml in selective auxotrophy plates.

Table 1 Yeast strains used in this thesis

Strain Name	**Genotype**	**Backbone**	**Source**
OLY2	*MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	W303	SR Wente
OLY3	*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	W303	SR Wente
SW4093	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/URA3/DED1)*	W303	Bolger and Wente, JBC, 2011
OLY14	*MATa his1*	W303	GR Fink via R. Parker
TBY152	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/DED1)*	W303	This Thesis
TBY153	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ535-548)*	W303	This Thesis
TBY154	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ549-562)*	W303	This Thesis

TBY155	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ563-576)*	W303	This Thesis
TBY156	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ577-590)*	W303	This Thesis
TBY157	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ591-604)*	W303	This Thesis
TBY158	*MATαded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ536-604)*	W303	This Thesis
TBY159	*MATαded1::KAN tif4631::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/DED1)*	W303	This Thesis
TBY160	*MATαded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ535-548)*	W303	This Thesis
TBY161	*MATαded1::KAN tif4631::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ549-562)*	W303	This Thesis

TBY162	*MATαded1::KAN tif4631::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ563-576)*	W303	This Thesis
TBY163	*MATαded1::KAN tif4631::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ577-590)*	W303	This Thesis
TBY164	*MATαded1::KAN tif4631::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ591-604)*	W303	This Thesis
TBY165	*MATαded1::KAN tif4631::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 + (pCEN/LEU2/ded1Δ536-604)*	W303	This Thesis
TBY183	*MATαded1Δ536-604::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	W303	Bolger Lab, University of Arizona
TBY198	*MATαded1Δ536-590::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	W303	Bolger Lab, University of Arizona
TBY208	*MATαtif4631::HYG ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	W303	Bolger Lab, University of Arizona

Table 2 Plasmids used in this thesis

Plasmid Name	**Description**	**Source**
pRS315	*CEN/LEU2*	Sikorski and Heiter, Genetics, 1989
pRS316	*CEN/URA3*	Sikorski and Heiter, Genetics, 1989
pOL76	*CEN/LEU2/DED1*	Bolger and Wente, JBC, 2011
pTB111	*CEN/LEU2/ded1Δ535-548*	Aryanpur et al., MBoC, 2019
pTB112	*CEN/LEU2/ded1Δ549-562*	Aryanpur et al., MBoC, 2019
pTB113	*CEN/LEU2/ded1Δ563-576*	Aryanpur et al., MBoC, 2019
pTB114	*CEN/LEU2/ded1Δ577-590*	Aryanpur et al., MBoC, 2019
pTB115	*CEN/LEU2/ded1Δ591-604*	Aryanpur et al., MBoC, 2019
pTB136	*CEN/LEU2/ded1Δ536-604*	Aryanpur et al., MBoC, 2019

Table 3 Primers used in this thesis

Primer Name	**Sequence**	**Final Construct**	**Source**
DED1-1004-seqF	GACTCCTGTTGGTGAAAG	Forward primer to check for correct integration of Hygromycin resistance cassette.	Integrated DNA Technologies

Hygrocheck_2	GGATGTATGGGCTAAATG	Reverse primer to check for correct integration of Hygromycin resistance cassette.	Integrated DNA Technologies
Ded1_CTKO_F	GCTAACCAAGAAGTCCCATCATTCTTGAAGGACGCTATGATGAGTTGACAGCTGAAGCTTCGTACGC	Forward primer used to generate *ded1*Δ*CT::HYG* and *ded1::HYG* mutants.	Integrated DNA Technologies
Ded1_DelRVS_SC	CATGCTAGAGCAGAAAACGAAGAATCCTCACCCTAGTTTGTCTGCATAGGCCACTAGTGGATCTG	Reverse primer used to generate *ded1*Δ*CT::HYG* and *ded1::HYG* mutants.	Integrated DNA Technologies

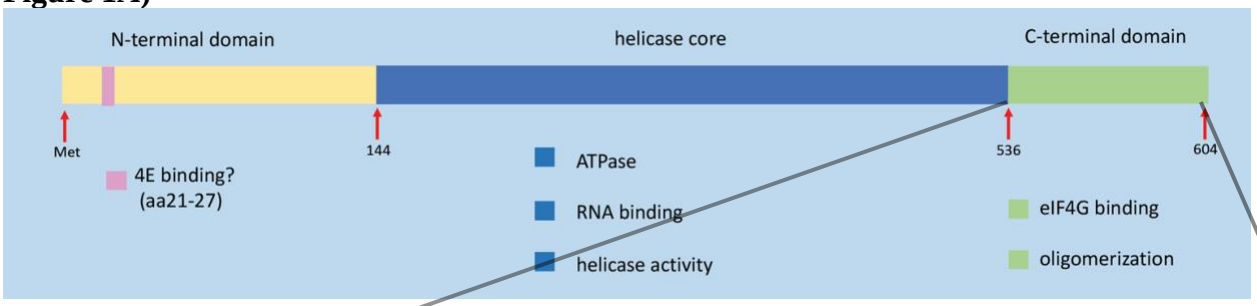
Results

Ded1 C-Terminus Mutants Affect Cell Viability

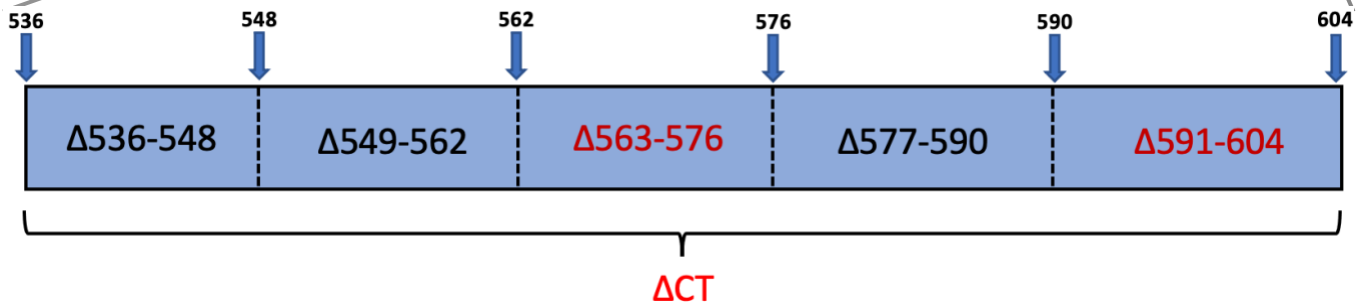
The low complexity Ded1 C-terminus is important for interactions with itself during oligomerization and with the eIF4F complex[1, 2]. To explore the role of the C-terminal domain in cell viability, we generated strains in which the endogenous *DED1* gene is deleted with low-copy plasmids expressing either wildtype *DED1* or *ded1* mutants that delete the C-terminus in 14 amino acid segments (Fig. 1B). We then measured cell viability and growth for each mutant in serial dilution assays (Fig. 1C). This experiment revealed that there is little to no effect on cell

viability when deleting the C-terminal region of Ded1 from residues 536 to 590 (Fig. 1C). However, we saw a significant effect on cell viability when mutating the final 14 amino acids in the C-terminus (*ded1-Δ591-604*, Fig. 1C). This effect was an intermediate phenotype between wild-type *DED1* and the full C-terminus deletion, suggesting that these 14 amino acids play a significant role in the full C-terminal deletion phenotype.

Figure 1A)



B)



C)

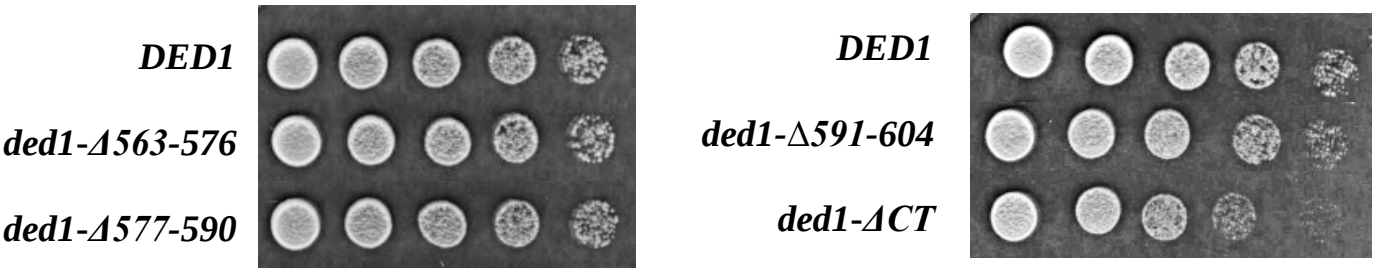
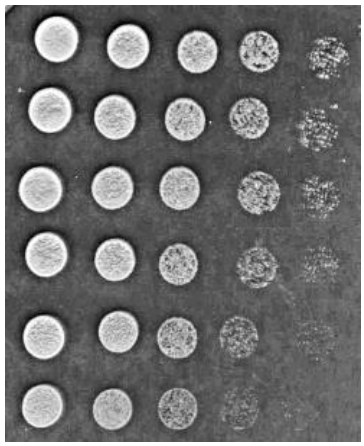


Fig. 1: Establishing the role of different C-terminal mutants in cell viability. **A)** Map of the Ded1 protein and its studied domains with their functions. **B)** Zoomed in map of Ded1 C-terminus with amino acid sections studied, with shown deletions highlighted in red. **C)** Serial dilution scans of different *ded1* C-terminal mutants grown on minimal media at 30°C.

eIF4G1 is Important to Cell Viability

The early stages of translation are believed to be modulated, in part, by the interactions between Ded1 and the eIF4F complex. This complex and Ded1 are involved in the recruitment of the rest of the proteins that make up the larger translation pre-initiation complex (PIC)[5]. As first shown by Hilliker et al. (2011), the Ded1-eIF4F interaction happens between the Ded1 C-terminus and the eIF4G1 component[6]. While this interaction has been explored biochemically, we aimed to characterize its impact on cell viability through serial dilution assays. To begin exploring this effect, we generated *ded1*Δ*591-604* and *ded1*-Δ*CT* mutants as above in strains that either contained or were missing the eIF4G1 gene (*tif4631Δ*) (Fig. 2A). We observed a mild synthetic growth defect in the *ded1*Δ*591-604 tif4631Δ* strain compared to the single mutant controls. However, this defect was exacerbated in the *ded1*-Δ*CT tif4631Δ* double mutant. Quantification of these growth assays revealed clear synthetic effects between these mutations (Fig. 2B). We can thus infer that removal of the Ded1 C-terminus weakens the interaction between Ded1 and eIF4G1 and Ded1's efficiency in undergoing its canonical role in translation initiation. Interestingly, the synthetic effect also suggests that removal of the C-terminal region may not entirely eliminate Ded1 association with eIF4G1, which might be mediated indirectly through Ded1 binding to other members of the eIF4F complex. Removal of eIF4G1 in a wild-type Ded1 strain also impacts cell also impacted cell growth, likely due to an impaired ability to regulate PIC assembly.

Figure 2A)



B)

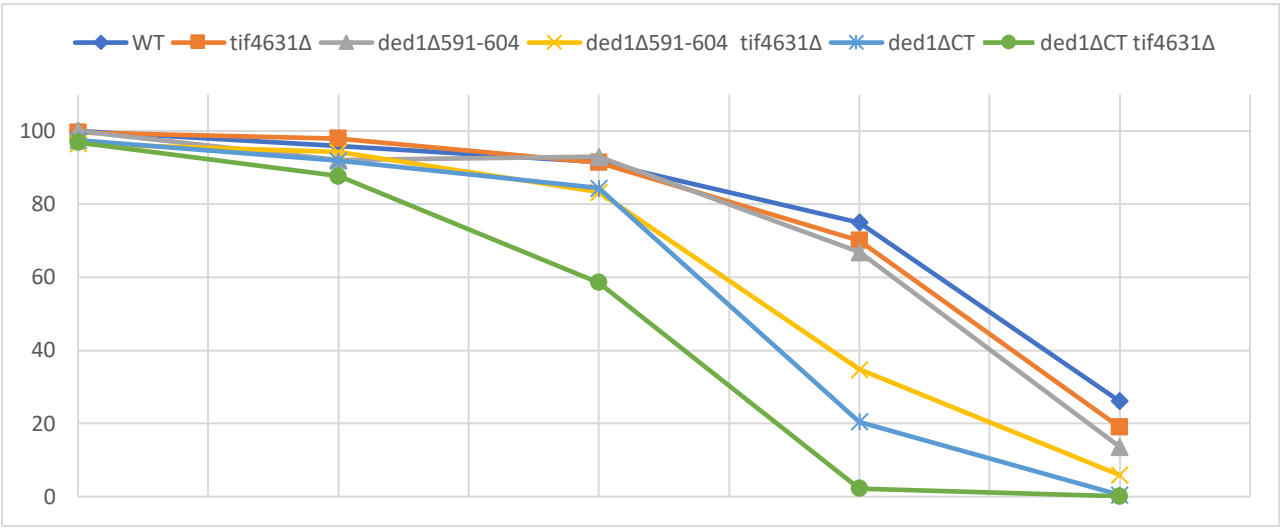


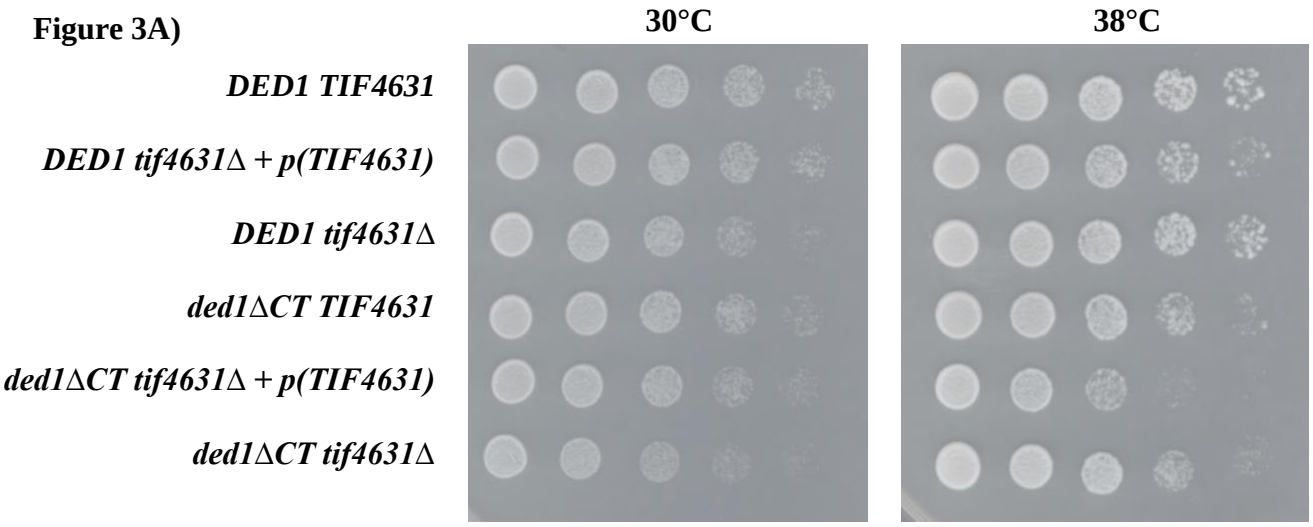
Fig. 2: Establishing additive effect of C-terminal mutants in ΔeIF4G1 strains. **A)** Serial dilution of previously established *ded*1 C-terminal mutant strains with strong growth defects with eIF4G1 deletions, grown at 30°C on minimal media. **B)** Quantified growth of strains by percent area covered in each consecutive dilution, first spot of WT *DED1* set to 100.

eIF4G1 Protein Has a Role in Heat Shock Response

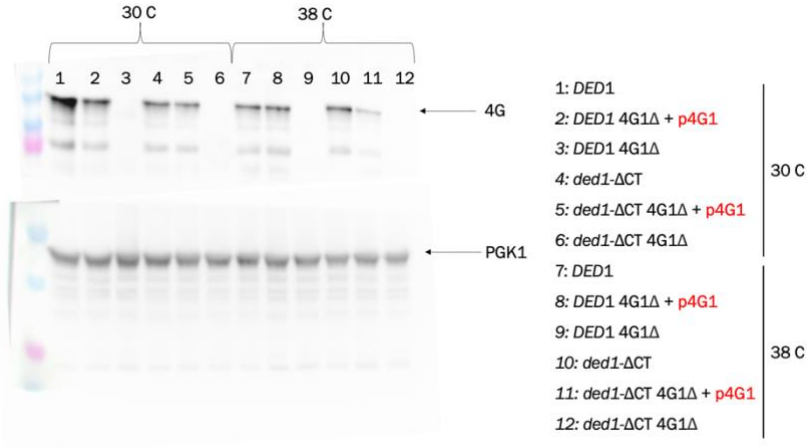
The yeast heat shock response gets activated at around 38°C, which induces cells to completely change their transcriptome and alter RNA stability[15]. The process of translation also gets significantly altered to prioritize certain mRNAs over others involved in the response. Therefore, we tested whether eIF4G1 has an important role in this response. Upon introduction of wild-type eIF4G1 on a plasmid in a strain lacking the genomic version, we compared it to

wild-type cells in order to make sure that the plasmid would rescue the growth defect seen in *tif4631*Δ cells. There was no effect on cells grown at 23°C (data not shown) or 30°C; however, when the plasmid was introduced into cells growing at 38°C, there was a significant impact on cell viability (Fig. 3A). Intriguingly, these cells grew worse than cells lacking the eIF4G1 protein altogether. Western blotting did not reveal an evident connection between eIF4G1 protein levels and cell viability (Fig. 3B/C). Further studies will be required to understand the role of eIF4G1 in heat shock.

Figure 3A)



B)



C)

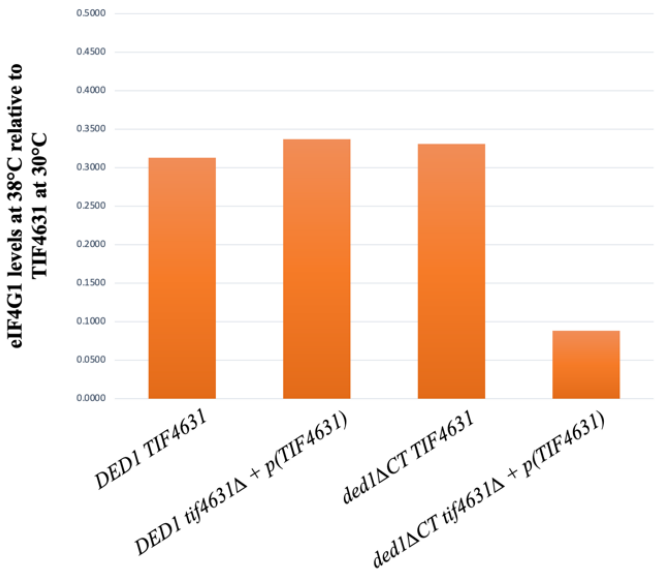


Fig. 3: Establishing role of eIF4G1 in heat shock response. **A)** Serial dilutions comparing genomic and plasmid based eIF4G1 (*TIF4631*) in *DED1* and *ded1-ΔCT* strains grown in selective media. **B)** Western Blot of eIF4G1 levels in different mutant strains at 30 and 38°C. **C)** Quantification of eIF4G1 levels in genomic and plasmid-based strains at 38°C compared to wild-type levels at 30°C.

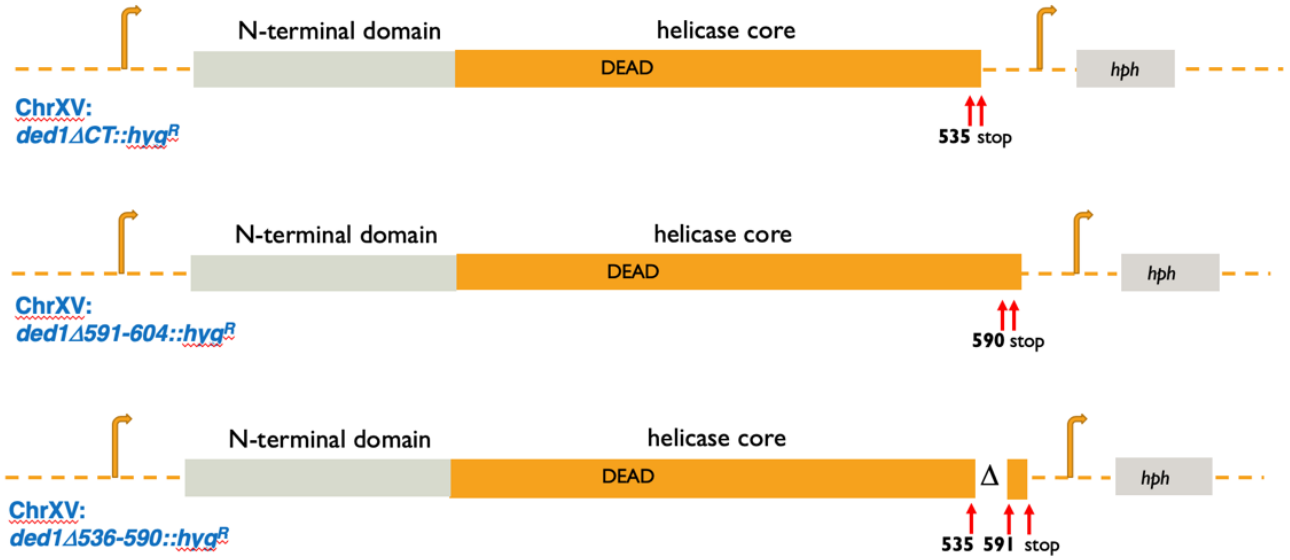
Genomic CT Mutants Have a Stronger Effect on Cell Viability

All our previous work has involved deleting the genomic version of *DED1* and introducing a plasmid expressing *ded1* with our desired mutations. While this is a common practice in yeast genetics, we were able to see minor inconsistencies in protein abundance when introducing the protein on a plasmid. Notably, the ded1-ΔCT protein was about 2-fold more abundant compared to the WT Ded1 protein (see Fig. 4C below). This led us to ask the question of whether the Ded1 protein becomes more stable when missing the C-terminus, or if this was an artifact of the fact that the protein was being expressed from a plasmid. We set out to make genomic versions of the same mutants we had been experimenting with on plasmids (Fig. 4A) and tested these strains with serial dilution assays to assess cell viability (Fig. 4B). Surprisingly, we observed that the genomic C-terminal deletion has a stronger effect on general cell viability

than the plasmid version (Fig. 4B). This strain also behaved similarly when grown on rapamycin, growing a bit slower than its plasmid-based counterpart (Fig. 5A). We then tested whether protein abundance in a plasmid *ded1*Δ*CT* strain was the same as a genomic *ded1*Δ*CT* strain and found that the mutant protein was more abundant when introduced on a plasmid (Fig. 4C). The genomic *ded1*-Δ*CT* showed Ded1 levels only slightly higher than that of the wild-type protein. Our findings preliminarily show that our work with plasmids has potentially masked phenotypes since protein abundance can compensate for a functional deficiency of the mutated protein.

Following testing of the genomic *ded1*-Δ*CT* mutant, we began testing two other genomic mutants, *ded1*-Δ*591-604* and -*Δ536-590*. On nutrient-rich media, there was no significant difference in growth as compared to the plasmid version of the *ded1*-Δ*591-604* mutants (data not shown). However, when grown on rapamycin, the genomic version grew somewhat better (Fig. 5A). Of more significance, however, was the fact that the *ded1*-Δ*591-604* mutant grew even better than *ded1*-Δ*CT* on rapamycin (Fig. 5B). Consistent with our model for Ded1 function in stress and pro-growth conditions, we speculate that the *ded1-Δ591-604* can better stimulate translation than the *ded1-ΔCT* in both conditions. Serial dilutions of the *ded1-Δ536-590* mutant, however, showed that the removal of all amino acids except for the last 14 resulted in cell viability inferior to that of the full C-Terminus deletion on rapamycin (Fig. 5B). Taken together, this means that the C-terminus has a complex role in its regulation of translation in multiple conditions. Our findings led us to hypothesize that the final 14 amino acids of the Ded1 C-terminus play an essential role in conferring rapamycin sensitivity, and their loss leads to proliferative growth in the presence of this drug.

Figure 4A)



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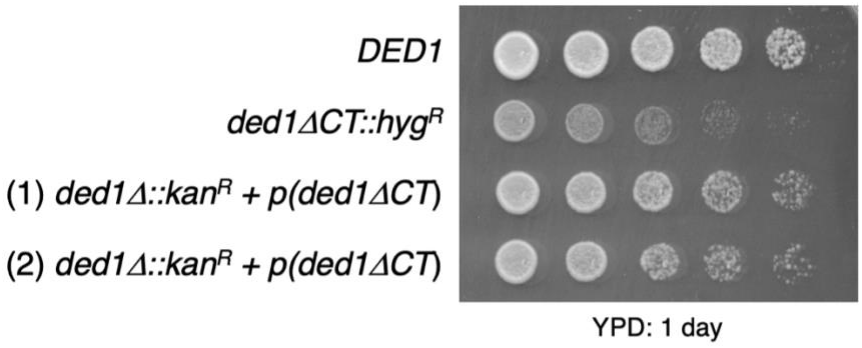

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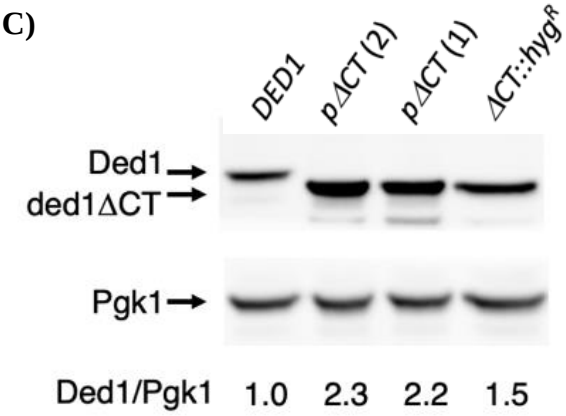
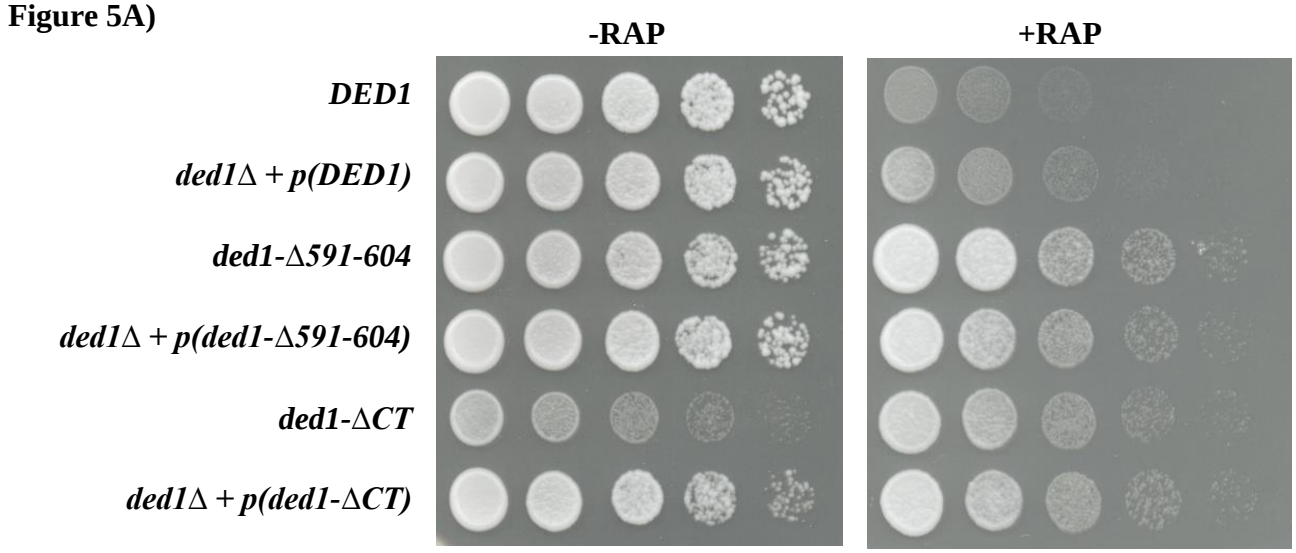


Fig. 4: Assessing relationship between plasmid Ded1 and genomic Ded1 mutants. **A)** Genomic map of mutants made. **B)** Serial dilution comparing genomic *ded1ΔCT* with plasmid versions of the same mutant. **C)** Western Blot of protein levels comparing Wild-Type Ded1 to two different plasmid versions (1 and 2) and genomic versions of *ded1ΔCT.*

Figure 5A)



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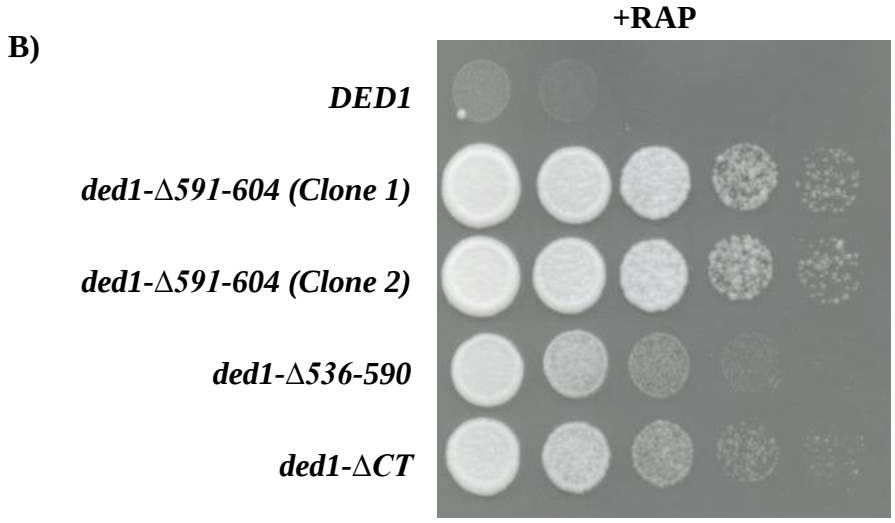

Fig. 5: Understanding role of genomic C-terminal mutants in rapamycin response. **A)** Serial dilutions comparing genomic versions to plasmid versions of C-terminal mutants in rapamycin. **B)** Serial dilution comparing different C-terminal mutant growth in rapamycin

Discussion

In this study, we investigated the role of the Ded1 C-terminus in cellular growth and response to rapamycin stress. Our findings demonstrate that the final 14 amino acids of the C-terminus play a crucial role in regulating cell viability, particularly in the presence of rapamycin.

We also discovered that using genomic mutants more accurately reflects protein levels observed in wild-type cells, compared to plasmid-mediated introduction of the same mutations. Additionally, we identified important genetic interactions between Ded1 and eIF4G1 in cell viability assays, leading us to propose a model for Ded1-eIF4G1 function. Our model is depicted in Figure 6 below. These results shed new light on the functions of Ded1 and provide insights into its regulatory mechanisms, which may have important implications for understanding cellular growth and response to stress.

A key outcome of this thesis is the elucidation of the role of the Ded1 C-terminus in various areas of cell function. One of these is general cell viability. While the specific mechanism through which the C-terminus influences Ded1 function cannot be determined precisely from this data, we can speculate that one of its roles is mediating the interaction between Ded1 and eIF4G1. Hilliker *et al.* (2011) showed that Ded1 interaction with the eIF4F complex is essential for efficient initiation of translation, so disruptions in this interaction may lead to major stalls in translation and consequently influence cell viability. As shown in Figure 4, in cells with wild-type Ded1, the interaction between eIF4G1 and Ded1 is stable and leads to efficient translation of mRNAs and protein synthesis. Conversely, in cells with *ded1-Δ591-604*, the interaction between eIF4G1 and Ded1 is destabilized, leading to inefficient translation of mRNAs and lower protein synthesis. Finally, in

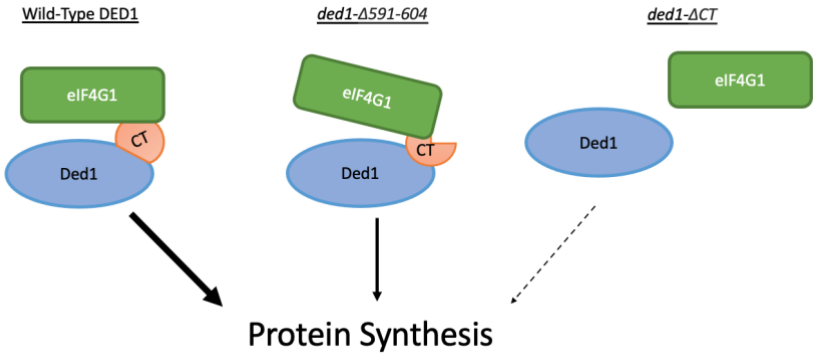

Fig. 6: Speculative model for Ded1 mutants in translation

cells with *ded1-ΔCT*, the interaction between Ded1 and eIF4G1 is further destabilized, leading to even lower translation of mRNAs and extremely inefficient protein synthesis. In support of this model, we show growth assays which correlate cell viability to interactions between the RNA helicase Ded1 and the scaffolding factor eIF4G1.

Our work also strongly suggests an important role for the final 14 amino acids in the response to rapamycin stress in *S. cerevisiae.* Aryanpur *et al.* (2019) showed that *ded1-ΔCT* cells conferred rapamycin resistance far superior to that seen in wild-type cells. Furthermore, the *ded1-Δ536-590* mutant, which lacks every amino acid except for the last 14, grew somewhat worse than the *ded1-ΔCT* cells, although better than wild-type cells on rapamycin. This result indicates that regulation of the rapamycin response is encoded throughout the C-terminus, but a critical part lies in the last 14 amino acids. Building on this, our data shows that *ded1-Δ591-604* cells grow even more readily in rapamycin media than do *ded1-ΔCT* cells. While these results seem puzzling, we believe this can be attributed to a combination of effects from *ded1-ΔCT* and *DED1* in their response to rapamycin induced stress. We conclude that the final 14 amino acids hold the key to rapamycin sensitivity and losing them as we do in both *ded1-Δ591-604* and *ded1-ΔCT* cells confers rapamycin resistance. However, *ded1-ΔCT*

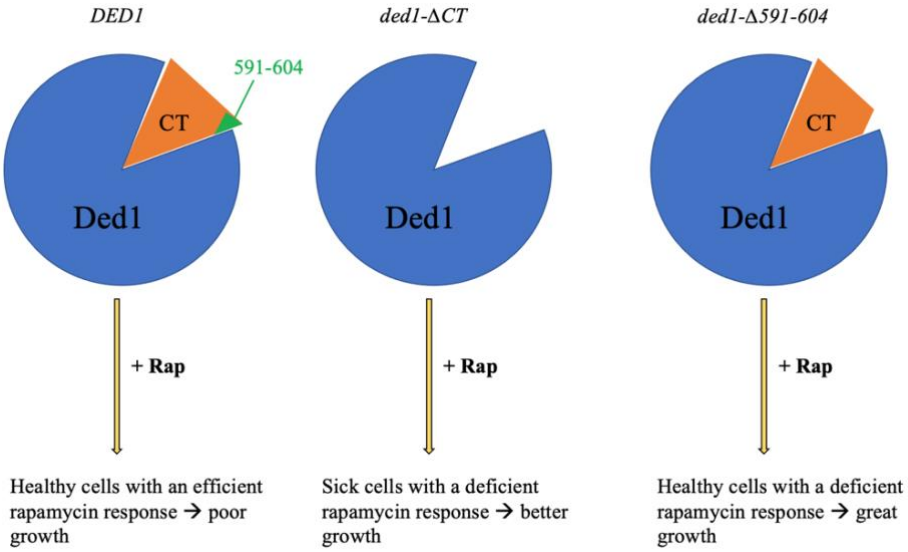

Fig. 7: Speculative model for Ded1 mutants in rapamycin response

cells also lose their interaction with eIF4G1, which decreases their ability to efficiently promote translation, causing poor cell growth. This is summarized in the model relating Ded1 to the rapamycin response (Figure 7). The Target of Rapamycin Complex 1 (TORC1) is a master regulator of cell growth and metabolism, so it is unsurprising that mutations in proteins involved with this complex may lead to cancer[14]. Our understanding of the role of DDX3 in cancer is limited; however, we present a mechanism to explain the potential role of DDX3 mutations in losing the ability to efficiently regulate the stress response.

Previously, our research on *ded1* mutants involved the use of plasmid-mediated introduction. However, we have recently shifted towards generating genomic versions of the mutants. This change has allowed us to more accurately replicate wild-type protein levels within the cell, as plasmid introduction can result in slight overexpression of the Ded1 protein, leading to unintended consequences. Studies by Aryanpur et al. (2022) have demonstrated that overexpression of Ded1 can inhibit growth and promote stress granule formation, highlighting the tight regulation of cellular stress response by Ded1 protein levels. By generating strains with protein levels that remain faithful to those observed in a wild-type strain, we can improve the accuracy and reliability of our results.

Future work will be involved in understanding the mechanism through which the Ded1 C-terminus amino acids 591-604 are involved in regulating the response to stress. In order to claim that these amino acids are truly central in regulation of the rapamycin response, we aim to replace the endogenous Ded1 C-terminus with an exogenous low-complexity domain that includes the final 14 amino acids of the native Ded1 C-terminus. If we were to observe results similar to what we see in *ded1-*Δ*591-604* mutants, then we can be confident that the final 14 amino acids are truly central to this regulatory pathway. Additionally, we will further

characterize genomic *ded1* proteins' ability to translate structured mRNAs to understand how differences in protein abundance and C-terminus mutations influence mRNA translation, particularly focusing on the *ded1*-Δ*536-590* mutant. Further investigation of this mutant would give us insight as to what the role of the rest of the C-terminus could be. Since prior studies have shown that the C-terminus is important for Ded1 oligomerization[2], it might be worth determining what parts of the C-terminus are most important for this function as well.

References

1. Linder, P., & Jankowsky, E. (2011). From unwinding to clamping — the dead box RNA helicase family. *Nature Reviews Molecular Cell Biology*, *12*(8), 505–516. https://doi.org/10.1038/nrm3154
2. Putnam, A. A., Gao, Z., Liu, F., Jia, H., Yang, Q., & Jankowsky, E. (2015). Division of Labor in an oligomer of the dead-box RNA helicase ded1p. *Molecular Cell*, *59*(4), 541–552. https://doi.org/10.1016/j.molcel.2015.06.030
3. Gupta, N., Lorsch, J. R., & Hinnebusch, A. G. (2018). Yeast ded1 promotes 48s translation pre-initiation complex assembly in an mrna-specific and EIF4F-dependent manner. *ELife*, *7*. https://doi.org/10.7554/elife.38892
4. Hilliker, A., Gao, Z., Jankowsky, E., & Parker, R. (2011). The dead-box protein Ded1 modulates translation by the formation and resolution of an EIF4F-mrna complex. *Molecular Cell*, *43*(6), 962–972. https://doi.org/10.1016/j.molcel.2011.08.008
5. Abdelhaleem, M. (2004). Do human RNA helicases have a role in cancer? *Biochimica Et Biophysica Acta (BBA) - Reviews on Cancer*, *1704*(1), 37–46. https://doi.org/10.1016/j.bbcan.2004.05.001
6. Gao, Z., Putnam, A. A., Bowers, H. A., Guenther, U.-P., Ye, X., Kindsfather, A., Hilliker, A. K., & Jankowsky, E. (2016). Coupling between the dead-box RNA helicases ded1p and eIF4A. *ELife*, *5*. https://doi.org/10.7554/elife.16408
7. González, A., & Hall, M. N. (2017). Nutrient sensing andtorsignaling in yeast and mammals. *The EMBO Journal*, *36*(4), 397–408. https://doi.org/10.15252/embj.201696010

8. Aryanpur, P. P., Renner, D. M., Rodela, E., Mittelmeier, T. M., Byrd, A., & Bolger, T. A. (2019). The dead-box RNA helicase DED1 has a role in the translational response to torc1 inhibition. *Molecular Biology of the Cell*, *30*(17), 2171–2184. https://doi.org/10.1091/mbc.e18-11-0702
9. Aryanpur, P. P., Mittelmeier, T. M., & Bolger, T. A. (2022). The RNA helicase Ded1 regulates translation and granule formation during multiple phases of cellular stress responses. *Molecular and Cellular Biology*, *42*(1). https://doi.org/10.1128/mcb.00244-21
10. Sen, N. D., Zhou, F., Ingolia, N. T., & Hinnebusch, A. G. (2015). Genome-wide analysis of translational efficiency reveals distinct but overlapping functions of yeast dead-box RNA helicases DED1 and EIF4A. *Genome Research*, *25*(8), 1196–1205. https://doi.org/10.1101/gr.191601.115
11. Gulay, S., Gupta, N., Lorsch, J. R., & Hinnebusch, A. G. (2020). Distinct interactions of eIF4A and eIF4E with RNA helicase DED1 stimulate translation in vivo. *ELife*, *9*. https://doi.org/10.7554/elife.58243
12. Iserman, C., Desroches Altamirano, C., Jegers, C., Friedrich, U., Zarin, T., Fritsch, A. W., Mittasch, M., Domingues, A., Hersemann, L., Jahnel, M., Richter, D., Guenther, U.-P., Hentze, M. W., Moses, A. M., Hyman, A. A., Kramer, G., Kreysing, M., Franzmann, T. M., & Alberti, S. (2020). Condensation of ded1p promotes a translational switch from housekeeping to stress protein production. *Cell*, *181*(4). https://doi.org/10.1016/j.cell.2020.04.009
13. Hondele, M., Sachdev, R., Heinrich, S., Wang, J., Vallotton, P., Fontoura, B. M., & Weis, K. (2019). Dead-box ATPases are global regulators of phase-separated organelles. *Nature*, *573*(7772), 144–148. https://doi.org/10.1038/s41586-019-1502-y

14. Murugan, A. K. (2019). MTOR: Role in cancer, metastasis and drug resistance. *Seminars in Cancer Biology, 59,* 92–111. https://doi.org/10.1016/j.semcancer.2019.07.003

15. Castells-Roca, L., García-Martínez, J., Moreno, J., Herrero, E., Bellí, G., & Pérez-Ortín, J. E. (2011). Heat shock response in yeast involves changes in both transcription rates and mrna stabilities. *PLoS ONE, 6*(2). https://doi.org/10.1371/journal.pone.0017272