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RESEARCH ARTICLE

Gle1 Regulates RNA Binding of the DEAD-Box Helicase Ded1 in Its Complex Role in Translation Initiation

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ABSTRACT DEAD-box proteins (DBPs) are required in gene expression to facilitate changes to ribonucleoprotein complexes, but the cellular mechanisms and regulation of DBPs are not fully defined. Gle1 is a multifunctional regulator of DBPs with roles in mRNA export and translation. In translation, Gle1 modulates Ded1, a DBP required for initiation. However, *DED1* overexpression causes defects, suggesting that Ded1 can promote or repress translation in different contexts. Here we show that *GLE1* expression suppresses the repressive effects of *DED1 in vivo* and Gle1 counteracts Ded1 in translation assays *in vitro*. Furthermore, both Ded1 and Gle1 affect the assembly of preinitiation complexes. Through mutation analysis and binding assays, we show that Gle1 inhibits Ded1 by reducing its affinity for RNA. Our results are consistent with a model wherein active Ded1 promotes translation but inactive or excess Ded1 leads to translation repression. Gle1 can inhibit either role of Ded1, positioning it as a gatekeeper to optimize Ded1 activity to the appropriate level for translation. This study suggests a paradigm for finely controlling the activity of DEAD-box proteins to optimize their function in RNA-based processes. It also positions the versatile regulator Gle1 as a potential node for the coordination of different steps of gene expression.

KEYWORDS DEAD box, RNA, helicase, nuclear export, translation, yeast

Gene expression is a central cellular activity, and perhaps not surprisingly, elaborate mechanisms of regulation have evolved to ensure fidelity in every aspect of this process. In eukaryotic cells, many factors interact with individual mRNAs to facilitate and regulate a variety of processes, including nuclear export, translation, and degradation, and thus the composition and structure of the resulting mRNA-protein complexes (mRNPs) are constantly changing. A major method for controlling these RNA-protein interactions is the action of RNA helicases, of which the DEAD-box proteins (DBPs) are the largest family. DEAD-box proteins play essential roles in nearly every RNA-based process in the cell through their linked activities of ATP binding, hydrolysis, and RNA binding (for a review, see reference 1). Through these activities, DEAD-box proteins are able to affect RNA secondary structure, alter RNA-protein interactions, and/or act as ATP-regulated RNA-binding proteins.

Ded1 is a DEAD-box protein in budding yeast that has been linked primarily to translation initiation (2). It has two orthologs in humans: DDX3X and DDX3Y. DDX3Y is on the Y chromosome, and deletions of the gene are associated with azoospermia and male infertility (3). On the other hand, DDX3X is widely expressed and has been strongly implicated in oncogenesis, most notably in the pediatric brain cancer medulloblastoma, in which it has been found to be the most frequently mutated gene after β-catenin (4, 5). How DDX3 contributes to cancer or male fertility is largely unknown, however, underscoring the importance of understanding the function and regulation of Ded1/DDX3.

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The traditional role ascribed to Ded1 in translation initiation is unwinding of the 5′ untranslated region (5′-UTR) during start site scanning by the 48S preinitiation complex (PIC) (2), a function supported by recent findings that the mRNA transcripts most affected by *ded1* mutation tend to have more-structured 5′-UTRs (6). Another study has suggested that Ded1 also promotes preinitiation complex formation prior to scanning (7), and our previous work suggested that Ded1 may be important for recognition of the AUG start site (8). These potential molecular activities imply that Ded1 plays a critical role in promoting translation, which is consistent with prior findings that *ded1* mutants inhibit growth and translation *in vivo* (9, 10). However, overexpression of *DED1* also inhibits growth and causes the formation of stress granules, and conflicting results have been reported for addition of exogenous Ded1 to translation assays *in vitro* (7, 11, 12). The molecular basis for these repressive effects on translation is not currently known, but it has been proposed that Ded1 serves as a "gatekeeper" for translation with the ability to promote or to repress translation (7). A fuller understanding of the molecular mechanisms of Ded1 is needed for a complete picture of this conserved protein's role in regulating translation.

Although the requirement for DEAD-box proteins in gene expression is well established, the mechanisms by which they themselves are regulated are not clear for most such proteins. Ded1 has been reported to bind eukaryotic translation initiation factor 4G (eIF4G), and it was recently shown to trimerize, which affects its enzymatic activity, but the cellular implications of these interactions remain unknown (7, 13). We have reported that Ded1 activity is also modulated by Gle1, a multifunctional regulator of DEAD-box proteins (8). Gle1 was originally identified as an essential mRNA nuclear export factor in both yeast and human cells (14, 15). Subsequently, Gle1, along with the small molecule inositol hexakisphosphate (IP_6), was shown to stimulate the activity of the DEAD-box protein Dbp5 during mRNA export (16–19). Gle1 binds to Dbp5 through a protein fold that structurally resembles those in known DEAD-box protein regulators such as eIF4G, PDCD4, and CWC22 (20–22). Our previous studies expanded the role of Gle1 by showing that it functions in translation initiation also, suggesting that Gle1 might serve to coordinate the processes of mRNA export and translation (23). In initiation, Gle1 functions independently of Dbp5 and IP_6 and instead inhibits the ATPase activity of Ded1 (8, 23). Like Ded1/DDX3, mutations in the human ortholog of *GLE1* have also been linked to pathologies, including amyotrophic lateral sclerosis (ALS) and two severe motor neuron developmental disorders, although no links to oncogenesis have been reported (24, 25).

The molecular mechanisms by which Ded1 and its regulator Gle1 affect translation appear to be complex. Both *ded1* and *gle1* mutant cells have defects in translation initiation, suggesting that both Ded1 and Gle1 promote translation (9, 23); yet Gle1 inhibits Ded1, and the initiation defects are suppressed in *ded1 gle1* double mutants (8). Further, as stated above, Ded1 also represses translation under some experimental conditions (7, 12). To begin to address this gap in understanding, here we use a combination of *in vivo* overexpression, *in vitro* biochemical assays, and mutation analysis to explore the nature of Gle1 regulation of Ded1 in translation. We propose that Ded1 plays a gatekeeper role wherein it is required for translation to proceed, but excess RNA binding by Ded1, especially in the absence of enzymatic activity, leads to translation repression. Gle1 is able to check both of these functions of Ded1 by affecting its ability to bind RNA, thus serving as a regulator of the translation gatekeeper Ded1. This work elucidates the Gle1-Ded1 interaction, which provides a paradigm for regulation of DEAD-box RNA helicases and may have implications for human disease.

RESULTS

GLE1 suppresses the effects of *DED1* overexpression *in vivo*. Previous studies have shown that while *ded1* mutations cause growth defects and inhibition of translation, overexpression of *DED1* does as well (7, 9, 12). This effect is specific to *DED1*, as similar overexpression of *DBP5* does not cause growth defects (26). These results

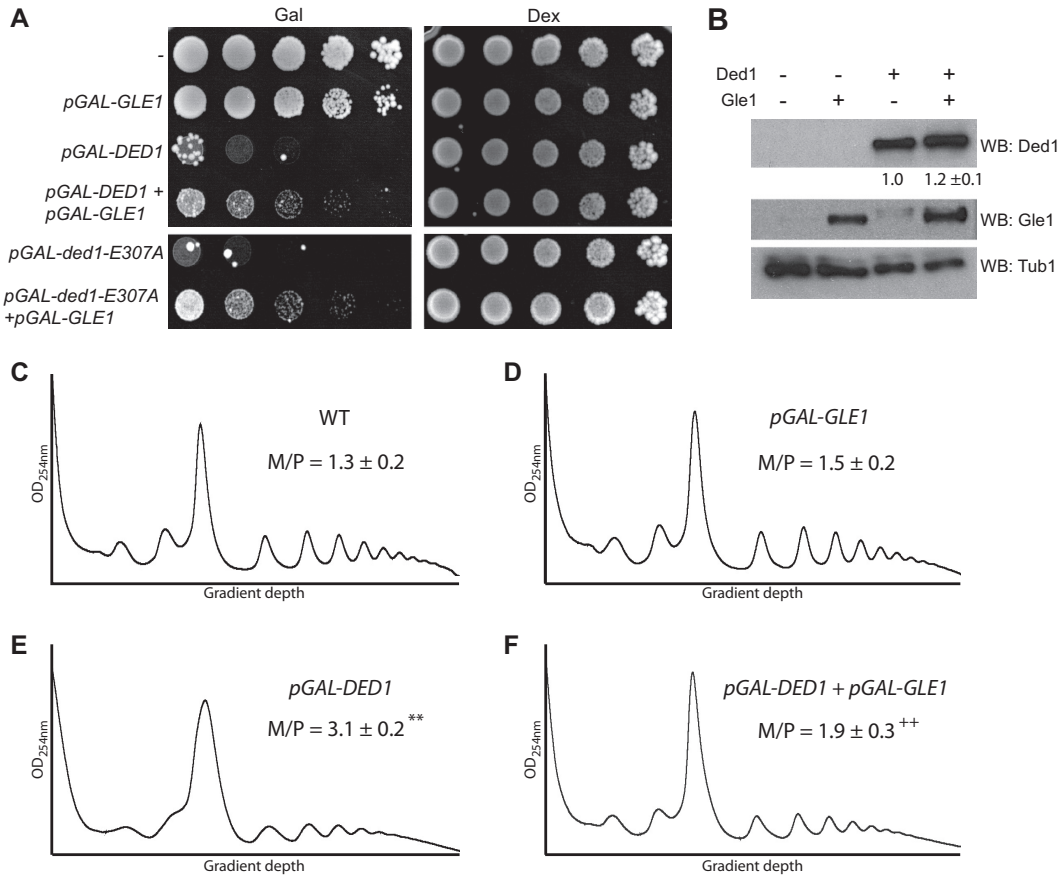


FIG 1 *GLE1* suppresses growth and translation inhibition caused by overexpression of *DED1*. (A) Wild-type (W303) cells transformed with Gal-inducible *GLE1*, *DED1*, and/or *ded1-E307A* were serially diluted, plated on selective medium containing galactose or dextrose, and incubated at 30°C. (B) Western blots (WB) of cell extracts from the strains described for panel A. Cells were grown in selective medium containing glucose and shifted to galactose-containing medium for 12 h before harvest. Samples were blotted using antibodies specific to Ded1, Gle1, and α-tubulin (Tub1). The relative intensity of the Ded1 bands is shown, normalized to Tub1, for three trials. (C to F) Cells transformed with Gal-inducible *GLE1* and/or *DED1* were shifted to galactose-containing medium for 12 h, and cell extracts were prepared. Polyribosomal profiles were generated by subjecting extracts to sucrose density centrifugation, followed by reading of the absorbance at 254 nM as the gradient was removed. Representative profiles are shown. Monosome-to-polysome (M/P) ratios were determined by calculating the area under the curve for the monosome (80S) peak versus the sum of the polysome peaks. Each M/P ratio presented is the mean from six independent trials ± standard error of the mean (SEM). **, $P < 0.01$ versus wild-type cells; ++, $P < 0.01$ versus *DED1*-overexpressing cells.

suggest that cells are sensitive to the level of Ded1 and that modulators of Ded1 activity are likely to have effects on these phenotypes. To determine whether this is the case for Gle1, we examined the phenotypes caused by *DED1* overexpression combined with manipulation of Gle1 levels. First, we overexpressed both *DED1* and *GLE1* with galactose-inducible reporters and examined growth. Overexpression of *DED1* alone severely inhibited growth, as reported previously (Fig. 1A). Interestingly, coexpression of *GLE1* with *DED1* suppressed this growth defect. Cells overexpressing *GLE1* alone also displayed moderate growth defects that were visible at early time points (see Fig. S1A in the supplemental material). Growth curves calculated from culturing the cells in liquid medium were consistent with the serial dilutions on plates (Fig. S1B). Importantly, the suppressive effect of *GLE1* on *DED1* growth inhibition was not due to a decrease in Ded1 protein levels in the cells coexpressing *DED1* and *GLE1* (Fig. 1B). These results suggest that there is an optimal level of Ded1 for growth and that Gle1 serves to check Ded1 to keep it from inhibiting growth. Additionally, an ATPase-deficient mutant, *ded1-E307A*, still inhibited growth upon overexpression, as reported previously (7). *GLE1* suppressed the growth defect from this *ded1* mutant (Fig. 1A), suggesting that the

inhibitory effect of *DED1* overexpression and its suppression by *GLE1* are not dependent on Ded1 ATPase activity *per se*.

Overexpression of *DED1* has also been reported to cause translation defects as assessed by polyribosome profiles (12). To examine whether Gle1 modulates the effect of Ded1 on translation *in vivo*, we used sucrose density sedimentation of extracts from *DED1*- and/or *GLE1*-overexpressing cells to generate polyribosome profiles. We observed defects in *DED1*-overexpressing cells with a clear increase in the ratio of monosomes to polysomes (M/P) (Fig. 1C and E), a common shift observed in translation initiation mutants (27). Consistent with the effects on growth, cooverexpression of *GLE1* with *DED1* (Fig. 1F) significantly reduced the polysome defect (M/P of 1.9, compared to 3.1 for *DED1* alone). *GLE1* overexpression alone did not have a significant effect compared to what was seen in control cells (Fig. 1D). In addition to its role in translation, Gle1 has an important role in mRNA nuclear export (14), and Ded1 has also been shown to shuttle between the nucleus and cytoplasm (28). However, we did not observe a change in the cytoplasmic localization of green fluorescent protein (GFP)-tagged Ded1 upon *GLE1* overexpression (Fig. S1C). Furthermore, we directly tested whether mRNA export was affected by *DED1* and/or *GLE1* overexpression through *in situ* assays with labeled oligo(dT). In contrast to what was seen in *mex67-5* cells, which contain a mutation in an mRNA export factor previously shown to have defects (29), neither *DED1* nor *GLE1* overexpression resulted in significant nuclear accumulation of mRNAs (see Fig. S2 in the supplemental material), suggesting that bulk mRNA export is unaffected in these cells. Taken together, these results indicate that Gle1 can serve as a counterweight to Ded1 and further suggest that the Gle1-Ded1 interaction affects translation.

Gle1 counteracts the effects of Ded1 on translation *in vitro*. *In vitro* translation assays with translation-competent extracts are a proven method for detailed examination of translation factors. Interestingly, different studies have reported different results for the addition of excess Ded1 to *in vitro* translation reactions (7, 11). To begin to elucidate this phenomenon, we added purified, recombinant Ded1 in a range of concentrations to translation reaction mixtures containing extracts from wild-type (WT) cells and *in vitro*-synthesized luciferase mRNA (Fig. 2A). We observed a clear dose-dependent effect of Ded1 in this assay. At lower concentrations, Ded1 significantly stimulated translation of the reporter, but as the concentration increased, the stimulation decreased, eventually resulting in inhibition of translation compared to controls. In contrast, addition of a control recombinant protein (MBP) did not have a significant effect at the same concentrations (see Fig. S3A in the supplemental material). We conclude that the effect of Ded1 depends, at least in part, on its concentration. This *in vitro* result is consistent with the observed effects of altering Ded1 levels *in vivo* (Fig. 1) (9, 12).

To further resolve the disagreement in the literature concerning the effects of Ded1 in translation assays *in vitro*, we prepared translation extracts using a modified protocol (described in reference 7). Interestingly, these extracts yielded a higher level of basal activity than did the extracts illustrated in Fig. 2A (data not shown). Furthermore, the addition of low concentrations of Ded1 to these high-activity extracts was sufficient to induce repression of translation (Fig. S3B). This result suggests that the modified extracts have a higher basal level of Ded1 activity or are less dependent on Ded1 than the standard preparations, thus increasing the effective concentration of Ded1. However, Ded1 abundance was in fact slightly lower in the modified, high-activity extracts (Fig. S3C, top). In contrast, Gle1 levels were more substantially reduced in the high-activity extracts (Fig. S3C, bottom). This result suggests that changes in the Ded1-to-Gle1 ratio may be at least partly responsible for the difference between extract preparations both in activity and in the effect of Ded1 addition.

We have shown previously that addition of recombinant Gle1 inhibits translation *in vitro* (8). Here we examined whether Gle1 exerts this effect through Ded1. Thus, we added Ded1 to translation reaction mixtures (using conditions under which Ded1 will

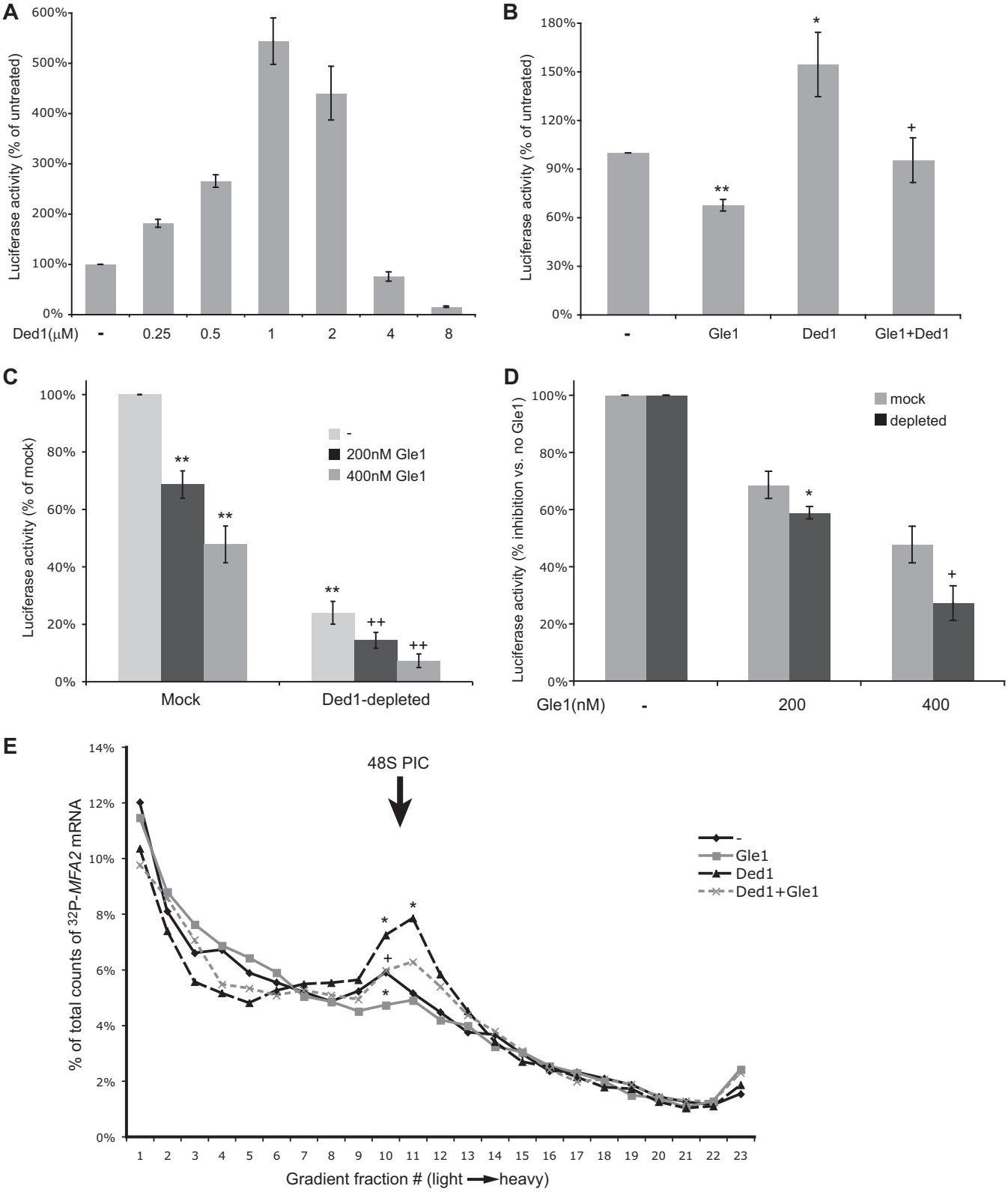


FIG 2 A balance of Ded1 and Gle1 activity is required for efficient translation and PIC assembly. (A and B) *In vitro*-transcribed luciferase mRNA was incubated with translation-competent extracts, followed by luciferase assays to determine the extent of translation. (A) Recombinant Ded1 was added to reaction mixtures with extracts from wild-type cells at 0.25, 0.5, 1, 2, 4, or 8 μM. (B) Ded1 (500 nM) and/or 250 nM recombinant Gle1 was added to translation-competent extracts from wild-type cells alone or in combination. *, $P < 0.05$ versus untreated (−); **, $P < 0.01$ versus untreated (−); +, $P < 0.05$ versus Ded1 sample. (C and D) Extracts from cells expressing protein A-tagged Ded1 were depleted of Ded1 with IgG-Sepharose resin or mock treated with glutathione-Sepharose. *In vitro* translation and luciferase assays were then performed with Gle1 added at 200 or 400 nM. (C) The activities in mock and depleted samples are directly compared.

(Continued on next page)

stimulate translation) in the presence or absence of recombinant Gle1 (Fig. 2B). When added together with Ded1, Gle1 reversed the stimulation of translation, resulting in activity levels close to the control level. This indicated that Gle1 was able to counteract the effects of Ded1 on translation. Demonstrating the specificity of this effect, addition of MBP control protein did not significantly change the effect of Ded1 on translation (Fig. S3A). To show that Gle1 works specifically through Ded1, we immunodepleted Ded1 from cell extracts containing protein A-tagged Ded1. The immunodepletion protocol substantially reduced Ded1-ProtA levels in the extract, although some Ded1 remained (Fig. S3D). Consistent with previous reports (7, 9), depletion of Ded1 caused a significant inhibition of translation (Fig. 2C). If Gle1 works primarily through inhibition of Ded1, this reduction in Ded1 levels will lower the ratio of Ded1 to Gle1 and make the translation activity in the depleted samples more sensitive to additional recombinant Gle1. Indeed, when Gle1 was added to Ded1-depleted extracts (at either 200 and 400 nM), translation was inhibited to a greater extent than for mock-treated extracts (Fig. 2D). These results indicate that Gle1 inhibits Ded1 in translation, likely to keep its activity within acceptable levels for maximal translation.

The molecular mechanism of Gle1 modulation of Ded1 in translation. Ded1 has previously been shown to play a role in the assembly of translation initiation complexes, specifically the formation of the 48S preinitiation complex (PIC) (7). To examine this step of translation, we performed sucrose density fractionation of translation-competent extracts incubated with a radiolabeled *MFA2* mRNA reporter. Addition of a nonhydrolyzable GTP analog (GMP-PNP) to these *in vitro* reaction mixtures causes accumulation of 48S PICs, observable as a peak of radioactivity in the fractions (Fig. 2E, untreated). As previously reported (7), we observed a significant decrease in the 48S PIC upon the addition of a large excess (10 μM) of Ded1 protein (data not shown). However, addition of smaller amounts of Ded1 significantly stimulated 48S PIC formation (Fig. 2E, Ded1), consistent with the increase in translation activity *in vitro* (Fig. 2A and B). Likewise, addition of recombinant Gle1 reduced 48S PIC formation, consistent with its repression of translation *in vitro*. When both Ded1 and Gle1 were added to the reaction mixtures, Gle1 largely reversed the effect of Ded1 stimulation, returning 48S PIC levels close to that of the wild type. These results suggest that Gle1 may modulate Ded1 function in a step upstream of 48S PIC formation, such as the preinitiation complex assembly itself.

Previously, we showed that *ded1* and *gle1* mutants have complementary and mutually suppressing defects in translation start site selection (8). To extend this finding, we determined whether start site defects are also present in *DED1*- and *GLE1*-overexpressing cells by utilizing two different reporter sets (see Fig. S4A in the supplemental material): one that measures "leaky scanning" defects (left), where the 40S ribosome fails to initiate at the first AUG codon encountered during start site scanning (8, 30), and a second set (right), where the start site for the coding region is mutated to a near-cognate codon (AUG to UUG), which is used to assess perturbations in start site fidelity (8, 31). We did not observe any effects from overexpression of *DED1* or *GLE1* in the leaky scanning assay (Fig. S4B), but for the second set of reporters, overexpression of *GLE1* induced a small but significant increase in translation of the mutant reporter (Fig. S4C). Consistent with Gle1 and Ded1 having opposing functions, this effect was suppressed when *DED1* was coexpressed with *GLE1*. These results are consistent with the start site defects observed previously (8), but the magnitude of the

FIG 2 Legend (Continued)
**, $P < 0.01$ versus mock; ++, $P < 0.01$ versus Ded1 depleted. (D) Activities in mock and depleted samples without Gle1 added are independently set to 100% in order to compare the responses to Gle1. *, $P < 0.05$ versus mock + 200 nM Gle1; +, $P < 0.05$ versus mock + 400 nM Gle1. For panels A to D, data represent the means from 3 to 7 independent experiments performed in duplicate. Error bars represent SEM. (E) *In vitro*-transcribed, ^{32}P-labeled *MFA2p(G)* mRNA was incubated with translation-competent extracts from wild-type cells in the presence of GMP-PNP to block initiation after 48S PIC assembly. Recombinant Gle1 (1 μM) and/or Ded1 (500 nM) was added as indicated. Samples were subjected to sucrose density fractionation, and radioactivity was quantitated by scintillation counting. The arrow marks the peak corresponding to the 48S PIC. Each data point represents the mean percentage at that fraction from three independent experiments. *, $P < 0.05$ versus untreated (−); +, $P < 0.05$ versus Ded1 sample.

defect is small, suggesting that the growth and translation defects caused by *DED1* are due primarily to other processes.

Gle1 binds to Ded1 and modulates its ATPase activity *in vitro* (8, 28), strongly suggesting that Gle1 directly regulates Ded1 activity. Two other modes of Ded1 regulation have been reported: through the translation factor eIF4G and through oligomerization of Ded1 itself (7, 13). We therefore examined whether Gle1 affected binding to eIF4G or Ded1 oligomerization through coimmunoprecipitations *in vivo*. We performed immunoprecipitations from cells with protein-A-tagged eIF4G1 (*TIF4631*) and blotted for Ded1 (Fig. S4D). We were able to specifically pull down Ded1 with eIF4G-ProtA, and this association was not significantly affected by overexpression of *GLE1*. To test Ded1 oligomerization, we immunoprecipitated GFP-tagged *DED1* from cells that also containing untagged *DED1*. We observed significant untagged Ded1 pulled down by the GFP-Ded1, and overexpression of *GLE1* did not affect the level of Ded1 self-association (Fig. S4E). In both pulldown assays, treatment of the lysates with RNase reduced the overall levels of association but did not change the effect of Gle1 (data not shown). These results suggest that the effect of Gle1 on Ded1 is not mediated through eIF4G or Ded1 oligomerization.

Mutations in *DED1* that are resistant to *GLE1* expression affect RNA-dependent ATPase activity. To further elucidate the effect of Gle1 on Ded1, we sought to generate mutations in *DED1* that make it unable to bind to Gle1. Structures of Ded1 have not been reported, but structures of a crystallized Dbp5:Gle1 complex have shown that Gle1 binds to the helicase core of Dbp5 as a "soft clamp" resembling the interaction of eIF4G with eIF4A, another member of the DEAD-box helicase family (21, 32). Reasoning that Gle1 was likely to bind Ded1 in a similar manner, we generated a deletion mutant of Ded1 lacking the auxiliary N- and C-terminal domains, leaving only the two RecA-like domains comprising the central helicase core. The ded1-ΔNΔC protein was still able to pull down MBP-tagged Gle1 *in vitro* at levels similar to that of the full-length Ded1 protein, as did mutant versions of Ded1 lacking either the N- or C-terminal domains individually (Fig. 3A and data not shown). That the Ded1 helicase core is sufficient for Gle1 binding suggests that the mode of Gle1 binding to Ded1 is similar to its binding to Dbp5.

Next, we produced a rough model of the Ded1 structure, based primarily on the partial structures of its human homolog, DDX3X, using the Phyre2 structure prediction program (33, 34). Alignment of the predicted Ded1 structure with Dbp5 from the Gle1-Dbp5 structure generated a model of the Ded1-Gle1 interaction (see Fig. S5 in the supplemental material). We then took a candidate approach to mutate amino acids in Ded1 that might be important for the interaction with Gle1. As an initial screen for whether the Ded1-Gle1 interaction was affected, we used the overexpression growth assay shown in Fig. 1A, reasoning that if the Ded1 mutant no longer interacted with Gle1, cells overexpressing the mutant would no longer be rescued by expression of *GLE1*. Additionally, this method would eliminate complete loss-of-function *ded1* mutants since these would not inhibit growth upon overexpression. Furthermore, given that the growth defect in an ATPase-deficient mutant (*ded1-E307A* mutant) is still suppressed by *GLE1* (Fig. 1A), this screening method should not simply identify Ded1 enzymatic mutants. We tested a number of different point mutants suggested by the predicted structure as well as a set of small deletion mutants generated by Hilliker et al. (7). Most of the *ded1* mutants behaved similarly to wild-type *DED1* in this assay, but in two sets of mutants, *GLE1* was unable to reverse the observed phenotype (Table 1 and Fig. 3B). These mutants included candidates from the structure model, one with a triple point mutation in the C-terminal RecA-like domain (N423A/F424A/R425A) and a derivative of that mutant (N423A/F424A mutant). We also identified one of the previously described deletion mutants in the N-terminal RecA-like domain, with deletions of amino acids (aa) 125 to 134 (Δ125–134), as a candidate.

We then undertook further testing of the candidates from the mutation analysis. When expressed at endogenous levels as the sole copy of *DED1* in the cell, the *ded1-Δ125-134* mutant has been reported to be lethal (7), and we confirmed this

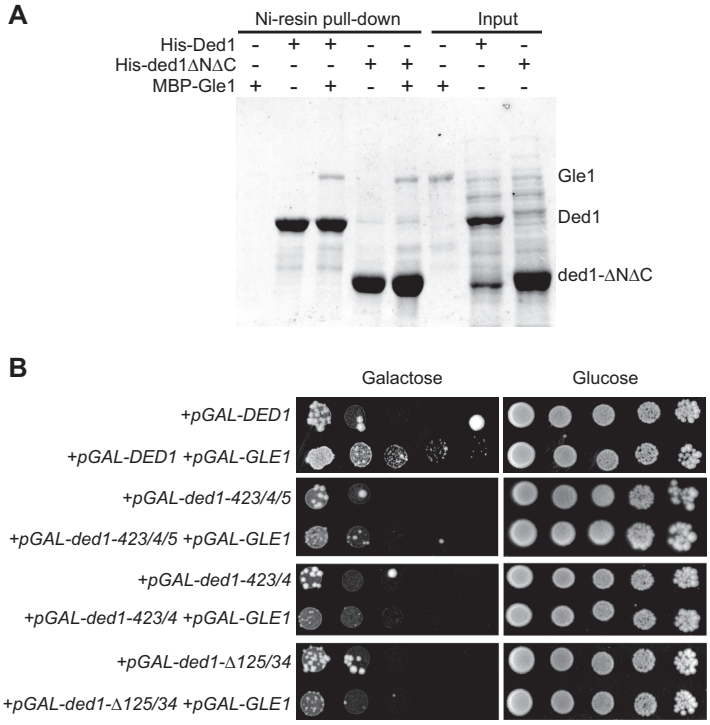


FIG 3 Targeted mutations in *ded1* affect suppression by *GLE1*. (A) *In vitro* pulldown assays were conducted with recombinant His-tagged Ded1 and a ded1-ΔNΔC deletion mutant (containing amino acids 126 to 538) from bacterial lysate. MBP-tagged Gle1 was incubated with samples as indicated. Samples were run on SDS-PAGE and Coomassie blue stained. (B) Effects on growth of the indicated mutants with or without *GLE1* overexpression. Cells containing Gal-inducible *DED1* (WT), *ded1-N423A/F424A/R425A*, *ded1-N423A/F424A*, *ded1-Δ125-134*, *GLE1*, and/or control plasmids were serially diluted on selective medium containing galactose or glucose and incubated at 30°C as described for Fig. 1A.

finding (data not shown). In contrast, both of the point mutations (*ded1-N423A/F424A/R425A* and *ded1-N423A/F424A*) were able to complement a *ded1*-null mutant at 30°C (Fig. 4A). However, the mutant cells were also temperature sensitive, growing poorly at 37°C. Furthermore, both also displayed translation defects by polysome analysis at the elevated temperature (Fig. 4B and data not shown). These defects were not the result of reduced levels of the mutant protein. Indeed, the N423A/F424A/R425A and N423A/F424A mutants remained at similar levels at 30°C and 37°C while wild-type Ded1 levels appeared to decrease somewhat (Fig. 4C and data not shown).

To determine whether the cellular defects observed in the mutant cells are indeed due to a loss of Gle1 binding, recombinant His-tagged Ded1 proteins containing the mutations were tested for *in vitro* binding to Gle1. As shown previously (Fig. 3A and reference 8), Gle1 was pulled down with His-tagged wild-type Ded1 at levels above background (Fig. 4D). Interestingly, the ded1-N423A/F424A and ded1-Δ125-134 mutant proteins were also able to pull down Gle1, indicating that the mutants do not affect Gle1 binding as was suggested by the overexpression assay (Fig. 3B). To further explore the defects in the *GLE1*-resistant mutants, we performed *in vitro* ATPase assays in the presence of total cellular RNA. As expected, wild-type Ded1 had low activity in the absence of additional RNA but was highly stimulated when RNA was added (Fig. 4E). However, the three mutants all had a severely reduced response to RNA, resulting in low ATPase activity compared to wild-type Ded1 in the presence of RNA. The mutants also displayed a severalfold increase in activity over that of the wild type in the absence of RNA. Together, these results indicate that the *GLE1*-resistant Ded1 mutants are defective in RNA-dependent ATPase activity, which may account for the *in vivo* phenotypes.

Gle1 inhibits the RNA binding affinity of Ded1. Although the Ded1 mutants that we generated were not the Gle1 binding mutants that we originally sought, the

TABLE 1 Growth phenotypes of *ded1* mutants and rescue by *GLE1*[a]

ded1 mutation(s)	*GLE1* rescue
D126/D127/D131	Yes
I128/P129/V130	Yes
L152/L153/L154	Yes
E155/K158	Yes
E155	Yes
I157/L159	Yes
K158	Yes
R161/K164	Yes
F162/T163	Yes
E307	Yes
Q338/E342	Yes
T398/D399	Yes
T398	Yes
D399	Yes
N423/F424/R425	No
N423/F424	No
N423/R425	Yes
N423	Yes
F424/R425	Yes
F424	Yes
R425	Yes
Δ21–27	Yes
Δ31–40	Yes
Δ91–100	Yes[b]
Δ101–110	Yes[b]
Δ111–118	Yes[b]
Δ125–134	No
Δ497–504	Yes[b]

[a]Cells expressing the indicated Gal-inducible *ded1* mutant with or without Gal-inducible *GLE1* were plated on selective medium as shown in Fig. 1 and then assessed for *GLE1*-mediated suppression of the growth inhibition by the *ded1* mutant. "Yes" indicates that *GLE1* rescued growth (as with wild-type *DED1*); "no" indicates that rescue was not observed.

[b]This mutant has partially compromised ability to inhibit growth, as shown in reference 7. *GLE1* still suppressed this phenotype, although less dramatically than in other "yes" cases.

inability of *GLE1* to suppress the growth defect caused by their overexpression suggested that the mutants shared properties that might give insight into the regulation of Ded1 by Gle1. The ATPase defect in the mutants further suggested that they might have alterations in RNA binding, so we tested this directly with filter binding assays and a labeled RNA oligomer. In general, the affinity of DEAD-box proteins for RNA is greatly affected by the nucleotide bound to the protein, being higher in the ATP-bound form than when ADP bound (1). Therefore, we tested the RNA binding of Ded1, the N423A/F424A mutant, and the Δ125–134 mutant in the presence of a nonhydrolyzable ATP analog (ADP-BeF_x) and ADP and in the absence of additional nucleotide. The RNA binding curves for wild-type Ded1 and the mutants were similar in the absence of additional nucleotide (Fig. 5A and D). In contrast, the affinity for RNA was 6- and 11-fold lower in the mutants in the presence of the ATP analog, and surprisingly, the affinity in the presence of ADP was significantly higher in both mutants than in wild-type Ded1 (Fig. 5B to D). These results suggest that the *ded1* mutations cause a conformational change in the protein that partially uncouples the affinity for RNA from the nucleotide bound. The differences in the presence and absence of ATP analog are also consistent with a defect in ATP binding in the mutants; however, filter-binding assays with labeled ATP show a slight increase in relative binding in the mutants compared to the wild type (see Fig. S6A in the supplemental material), suggesting that nucleotide binding *per se* is not greatly affected.

The alterations in RNA affinity in the Ded1 mutants, combined with the inability of *GLE1* expression to suppress their growth inhibition, suggested to us that Gle1 might regulate Ded1 activity by altering its RNA binding affinity. To address this question, we utilized a fluorescence anisotropy assay with recombinant Ded1, Gle1, and a fluorescein-labeled RNA oligomer, which helped overcome technical limitations in the

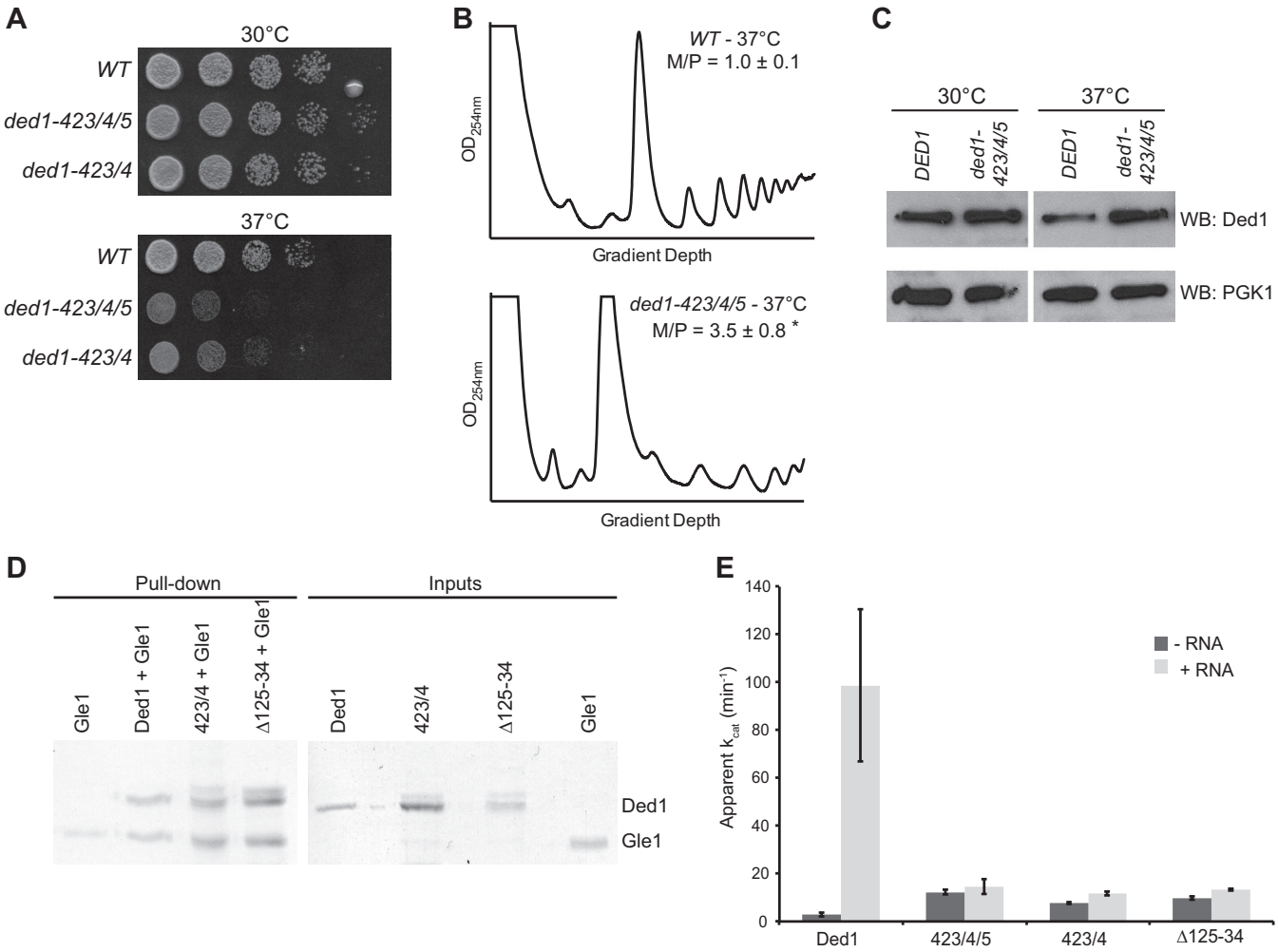
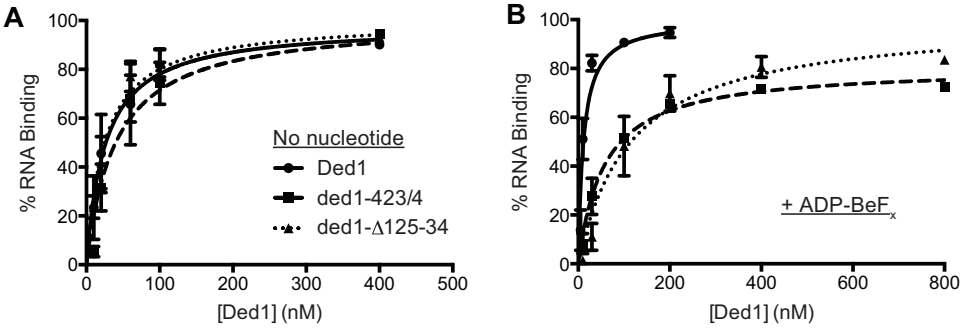


FIG 4 The *GLE1*-resistant *ded1* mutants have defects *in vivo* and *in vitro* but still bind to Gle1. (A) Low-copy-number plasmids containing wild-type (WT) *DED1*, *ded1-N423A/F424A/R425A*, or *ded1-N423A/F424A* were introduced into *ded1*-null cells, and cells were serially diluted on yeast extract-peptone-dextrose (YPD) and grown at a range of temperatures. (B) Cells containing wild-type *DED1* or *ded1-N423A/F424A/R425A* as the only copy of *DED1* were grown at 30°C before temperature was shifted to 37°C for 60 min prior to harvest. Polysome profiles were then generated as described for Fig. 1B. *, $P < 0.05$ versus WT. (C) Cell extracts from the same conditions as described for panel B were blotted with antibodies to Ded1 and PGK1 (loading control). Note that wild-type Ded1 levels decrease slightly at 37°C. (D) *In vitro* pulldown assays were performed with His-tagged, recombinant wild-type Ded1, ded1-N423A/F424A, and ded1-Δ125-134. Untagged Gle1 was incubated with samples as indicated. The proteins alone are shown in the "inputs" lanes (right). Samples were run on SDS-PAGE and Coomassie blue stained. (E) ATPase activity of purified recombinant wild-type Ded1, ded1-N423A/F424A/R425A, ded1-N423A/F424A, and ded1-Δ125-134 was determined with a colorimetric PK/LDH-coupled assay in the absence or presence of total cellular RNA (10 μg/ml). Values shown are the means from 4 independent experiments performed in duplicate.

filter-binding assay. In this assay, the apparent dissociation constant K_d with Ded1 alone in the presence of ATP was similar to that from the filter-binding assay (Fig. 5E). However, the affinity for RNA was reduced approximately 3-fold when Gle1 was added to the samples. In contrast, addition of a control protein had no effect on RNA binding by Ded1 (Fig. S6B). Interestingly, the decrease in RNA binding caused by Gle1 is independent of the nucleotide bound to Ded1, as Gle1 also reduced binding in the presence of ADP and the absence of added nucleotide (Fig. S6C and D). This result suggests that Gle1 regulates RNA binding by Ded1 directly, rather than through affecting nucleotide binding. Thus, these results indicate that Gle1 modulates the activity of Ded1 by reducing its affinity for RNA.

DISCUSSION

Our previous work showed that the DBP regulatory factor Gle1 has roles in translation in addition to its more established function in mRNA nuclear export. Specifically, Gle1 binds Ded1 and modulates its activity in translation initiation (8, 23). Here we have



D

apparent K_d (nM)

	apo:	ADP-BeF$_x$	ADP
Ded1	26 ± 13	11 ± 3	402 ± 181
423/4	40 ± 14	57 ± 11	46 ± 37
Δ125-34	25 ± 6	115 ± 37	76 ± 19

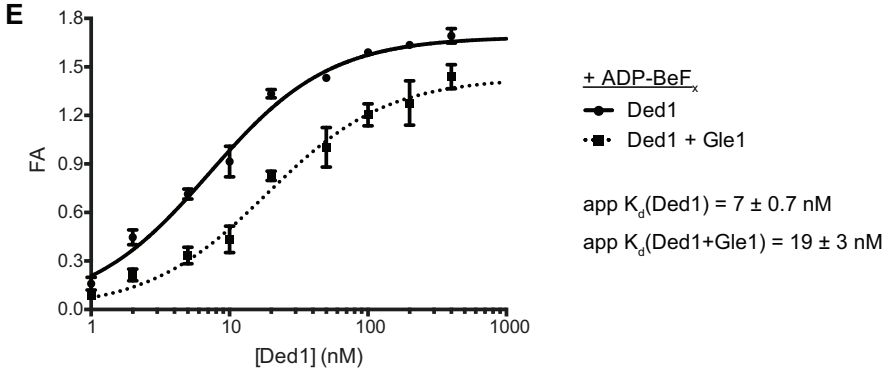


FIG 5 Gle1 inhibits the RNA binding activity of Ded1. (A to D) Equilibrium RNA binding assays were performed with wild-type Ded1, ded1-N423A/F424A, and ded1-Δ125-134. Filter binding onto nitrocellulose and nylon was assessed with radiolabeled RNA oligonucleotides incubated with recombinant protein in the presence of no additional nucleotide (apo) (A), ADP-BeF$_x$ (B), or ADP (C). (D) Apparent binding affinities under the different conditions were calculated via a single-binding-site model. (E) Fluorescence anisotropy was used to assess the RNA binding affinity of Ded1 in the presence of ADP-BeF$_x$ with or without 100 nM Gle1. Curve-fitting and apparent binding affinities were determined as described above.

made advances in understanding the mechanism by which Gle1 regulation of Ded1 controls initiation. Our data suggest a model wherein Ded1 can either promote or repress translation initiation, depending on its levels and activity, and Gle1 serves to check the effects of Ded1 by affecting its binding to RNA, thus modulating translation in a context-dependent manner. In support of this model, we show genetic and functional data indicating that Gle1 can suppress the growth and translation defects caused by *DED1* overexpression (Fig. 1). We also demonstrate that Gle1 counteracts the effects of Ded1 in translation reactions *in vitro* (Fig. 2). Further, consistent with a role for Ded1 in promoting preinitiation complex assembly (7), Gle1 also affects the formation of the 48S PIC *in vitro* (Fig. 2E). Finally, analysis of *ded1* mutations that are refractory to *GLE1* suppression *in vivo* suggested that Gle1 affects RNA binding of Ded1, which we confirmed by binding assays *in vitro* (Fig. 3 to 5).

Previous results showing the effects of Gle1 on Ded1 ATPase activity *in vitro* indicated that Gle1 targeted Ded1 activity directly (8). This conclusion is supported here by the finding that Gle1 does not appear to affect Ded1 oligomerization or interactions

with eIF4G in cells (Fig. S4) but does inhibit RNA binding by Ded1 *in vitro* (Fig. 5). Together, the data suggest that Gle1 regulation of Ded1 *in vivo* is likely due to inhibition of RNA binding (see Fig. S7A in the supplemental material). Interestingly, this model is quite similar to that proposed by Montpetit et al. for Gle1 regulation of Dbp5 (21). In that case, Gle1 binds to both the N- and C-terminal helicase domains of Dbp5, holding them in a partially open configuration that is inhibitory for RNA binding since the RNA interface spans the two domains. Since Gle1 stimulates Dbp5 activity, Montpetit et al. suggested that the conformational change induced by Gle1 aids in RNA substrate release after ATP hydrolysis, although others have interpreted these results differently, taking into account additional *in vitro* and *in vivo* studies on Gle1 and Dbp5 (21, 35). Similar modes of binding, though with distinct effects, have also been observed for other regulators of DEAD-box proteins (32, 36, 37). Given our results, it is likely that Gle1 interacts with Ded1 in a manner similar to that of Dbp5, and it decreases Ded1 activity by binding it in an open conformation that is inhibitory for RNA binding. Additionally, this mode of regulation leaves open the possibility that Gle1 stimulates Ded1 activity through an RNA release mechanism under certain conditions. Indeed, a previous study on Ded1-interacting factors reported that Gle1 moderately stimulated the ATPase activity of Ded1 *in vitro*, in contrast to our previous results (28). Significantly different conditions were used in that assay, including saturating levels of RNA and large molar excesses of Gle1, making it difficult to compare those results directly to ours, but the stimulation observed might reflect an effect on RNA substrate release. Nonetheless, the multitude of *in vivo* and *in vitro* effects that we have observed (here and as reported in reference 8) argue strongly that Gle1 generally opposes Ded1 activity.

Previous work has shown that Ded1 is critical for translation initiation, but mounting evidence suggests that its role may be more complex than simply promoting translation. Cells with hypomorphic or temperature-sensitive *ded1* mutations have severe translation defects, and depletion of Ded1 from translation extracts reduces their activity (7, 9). On the other hand, *DED1* overexpression causes defects in growth *in vivo*, and excess Ded1 represses translation *in vitro* (7, 12). At a minimum, these findings suggest that Ded1 activity needs to be within a certain range for optimal translation. Hilliker et al., taking this idea further, proposed that Ded1 serves as a "gatekeeper" for translation, either promoting translation or inducing repression (7). They further suggested that enzymatically active Ded1 is required for stimulating translation while inactive Ded1 promotes translation repression (Fig. S7B). This is supported by the persistence of growth inhibition in cells overexpressing the *ded1-E307A* ATPase-dead mutant and "hyperrepression" by ded1-E307A when added to *in vitro* translation assays (7). Interestingly, the effects of Gle1 on translation *in vivo* and *in vitro* largely mirror those of Ded1, as shown here and in our previous work (Fig. 2B and C and S1A and references 8 and 23). Therefore, we propose that Gle1 serves to control Ded1 activity during translation initiation, and perturbations to either Gle1 or Ded1 can lead to nonoptimal translation. Gle1 would thus function as a sort of gatekeeper to the gatekeeper Ded1.

Given that *GLE1* expression suppresses the effects of *DED1* overexpression (Fig. 1) and that Gle1 inhibits RNA binding by Ded1 (Fig. 5), we suggest that Ded1 mediates translation repression at least in part through binding to RNA. Inactive or excess Ded1 may also cause the sequestration of mRNAs and possibly other RNA-binding factors, preventing proper translation. Gle1 would thus suppress this effect by reducing Ded1 binding to the mRNAs, keeping them in the actively translating pool. This model is supported by the observation that *GLE1* still suppresses the growth defect from overexpression of the *ded1-E307A* mutant (Fig. 1A), which still binds RNA (38). Further, it provides an explanation for the lack of suppression of the *ded1-N423A/F424A/R425A* and *ded1-Δ125-134* mutants by *GLE1*. In wild-type Ded1, ATP binding promotes the closed conformation of the helicase domains, stimulating RNA binding; however, this coupling of RNA affinity to nucleotide status is disrupted in the mutants (Fig. 5A to D). These results suggest that mutations in the two sets of amino acids (aa 125 to 134 are

highly conserved, while N423/F424/R425 are not) cause conformational changes in Ded1 that allow it to bind RNA while still in the open configuration. Thus, the mutants are not refractory to *GLE1* suppression because of an effect on binding to Gle1, as we confirmed with the results shown in Fig. 4C. Rather, this occurs because Gle1 stabilization of an open configuration in the *ded1* mutants would have little effect on their binding to mRNAs, which would lead to translation repression and growth inhibition.

It remains somewhat unclear to what extent wild-type Ded1 can induce translation repression at physiological levels, as opposed to blocking initiation upon mutation or overexpression. However, consistent with a dual role for Ded1 in translation, even small amounts of recombinant Ded1 are sufficient to cause repression in some preparations for translation assays *in vitro* (7) (Fig. S3B). While this response differs between different extract preparations, the ratio of Ded1 to Gle1 is also altered between them. It is not known why this occurs, although it is presumably related to the proteins' biophysical characteristics during the grinding and centrifugation of the extracts. Since these are cellular extracts containing many different factors, it is not possible to state with certainty whether one is more reflective of the translation process *in vivo*. Further supporting the gatekeeper model and dual role for Ded1 in translation, Ded1 affects the assembly and disassembly of stress granules, concentrated foci of mRNPs that occur under various cellular stress conditions (7, 39). Consistent with the regulation of Ded1 by Gle1, knockdown of the human ortholog of Gle1 was also recently reported to cause defects in stress granule assembly (40), and we have observed that *GLE1* partially suppresses the formation of stress granule-like bodies induced by *DED1* overexpression (T. M. Mittelmeier, unpublished observation). However, because stress granule dynamics and translation are highly linked processes, it is difficult to disentangle whether the role of Ded1 (or Gle1) in stress granule regulation is a direct effect or a downstream one resulting from changes in translation.

The molecular mechanism for Ded1's positive role in promoting initiation also appears complex, with proposed functions for Ded1 in several different substeps, including start site scanning, PIC assembly, and possibly start site recognition (7, 8, 41). It is possible that each reported function of Ded1 is distinct, or it may be that one or more of the observed phenotypes are indirect consequences of a primary Ded1 function. For instance, it is possible that the defects in start site recognition in *ded1* mutants (8) are a result of a reduced scanning rate rather than a recognition defect *per se*. An indirect effect on start site recognition is also consistent with the very moderate phenotype that we observed with *GLE1* and *DED1* overexpression (Fig. S4A to C). There may also be some specificity for Gle1 regulation of Ded1, supported by the lack of effects in *gle1* mutant cells in a reporter assay for scanning defects (8). This specificity may thus reflect a requirement for Gle1 only during specific substeps when Ded1 activity needs to be more precisely controlled. Indeed, proteome-wide cellular abundances indicate that Ded1 levels are likely severalfold higher than Gle1 levels (42), suggesting that Gle1 might interact only transiently with Ded1 *in vivo* or affect only a subset of Ded1 mRNA targets. Furthermore, in its gatekeeper role, Gle1 is in a position to promote or repress translation depending on the state of Ded1 (Fig. S7B). This suggests that upstream regulation of Gle1 is also needed, although such regulation has not been identified to date. Ded1 also has additional modes of regulation, including binding to the translation factor eIF4G and homotrimerizing, both of which also affect its activity (7, 13). Both of these interactions are mediated through the C-terminal tail of Ded1, rather than the helicase core, and we did not observe any changes in Ded1 association with eIF4G or oligomerization upon overexpression of *GLE1* (Fig. S4D and E). A complete model of Ded1 function and regulation in the cell would need to account for all these interactions, requiring additional studies of Ded1 regulatory factors.

Gle1 has been identified as playing roles in mRNA nuclear export, translation initiation, and translation termination (14, 23). In general, Gle1 serves to promote gene expression through regulation of a DEAD-box protein, Dbp5, for export and termination, and Ded1 for initiation (8, 17, 23). Consequently, alterations in Gle1 activity would be expected to result in coordinated regulation of gene expression, suggesting that

Gle1 may be an important node in the control of gene expression. It is currently unknown, however, whether Gle1 activity is specific to particular mRNAs or mRNA subsets. While both Ded1 and Gle1 perturbations have a general effect on translation, certain mRNAs, such as those with more-structured 5′-UTRs, have been shown to be more sensitive to *ded1* mutations (6). Since the only known function of Gle1 in initiation is regulating Ded1, Gle1 would likely be limited to these same targets (or a smaller subset of them) in its initiation role. Although *gle1* mutations cause general defects in mRNA export (14), certain sets of target mRNAs may likewise be sensitive to Gle1 in export, which may or may not be the same set as in translation. The ability of Gle1 to regulate multiple steps of gene expression also make it a likely target to be perturbed in disease. Indeed, the mutations in human Gle1 identified in ALS and lethal congenital contracture syndrome 1 and lethal arthrogryposis with anterior horn cell disease (LCCS1/LAAHD) are consistent with this, although thus far the mutations appear to cause defects primarily in mRNA export rather than in translation (24, 25, 43). Interestingly, while the Ded1 ortholog DDX3 has been heavily implicated in both cancer and viral infection (2, 4, 44), human Gle1 has not, despite at least some evidence that the interaction with Ded1/DDX3 is conserved (40). Future studies will be needed to reveal whether there is any overlap in disease pathology between Ded1/DDX3 and Gle1.

MATERIALS AND METHODS

Strain and plasmid construction. Yeast strains and plasmids used are listed in Tables S1 and S2 in the supplemental material. SWY4274 and TBY2 were constructed by integration of sequences encoding a protein A tag marked with *spHIS5* at the *DED1* and TIF4631 loci, respectively, in a strain W303 background, followed by selection on His$^-$ medium. The *ded1* mutant strains (TBY5, -40, and -89) were constructed by plasmid shuffle of the relevant *CEN/HIS3* plasmid into SWY4093, a *ded1*-null strain covered by *DED1/CEN/URA3* (8). The galactose-inducible *GLE1* plasmid (pTB4) was generated by standard methods and contains the *GLE1* coding region plus 3′ sequences inserted into the BamHI and NotI sites in pBJ245, a derivative of pRS425 containing the *GAL1/10* promoter (provided by C. Hug). The Gal-inducible *ded1* mutant plasmids (Table 1) were generated via standard site-directed mutagenesis of pRP2086. The plasmids for recombinant His-tagged Ded1 mutants (pTB7, -14, -20, and -130) were cloned into the NdeI and EcoRI sites of the pET28a expression vector. The *DED1-GFP* plasmid (pSW2621) contains the *DED1* promoter and coding region cloned into XhoI and HindIII in pRS316 with *GFP* cloned in frame immediately downstream. For yeast growth assays, the indicated strains were initially cultured in selective medium with 2% glucose and then plated on medium containing glucose or 2% galactose to induce overexpression by successive 5-fold serial dilutions as previously described (45).

Ded1 and Gle1 protein levels. For checking expression of galactose-inducible Ded1 and Gle1, cells containing the indicated overexpression plasmids were cultured in selective medium containing 2% galactose for 12 h. Samples were harvested and normalized by pellet wet weight; then, crude cell extracts were prepared as described in reference 46. Briefly, cells were lysed in 1.85 M NaOH and 7.4% β-mercaptoethanol, proteins were precipitated via trichloroacetic acid, and samples were resuspended in SDS sample buffer. Samples were then separated by SDS-PAGE and blotted with specific antibodies to Ded1, Gle1, and α-tubulin (Novus) (8, 23). Band intensity was quantitated via ImageJ. For checking for depletion of Ded1, SDS sample buffer was added to untreated, mock-treated, and depleted extracts, and following SDS-PAGE, membranes were blotted with anti-Ded1. For analysis of Ded1 levels in mutant cells, the relevant strains were grown at 30°C in rich medium and then shifted to 37°C for 1 h before harvest. Blotting was performed as described above with antibodies to Ded1 and Pgk1 (Novex/ThermoFisher) as loading controls.

Polysome profiles. For polyribosome analysis of *DED1* and/or *GLE1* overexpression, cells containing the indicated plasmids were initially grown in selective medium containing 2% sucrose and then shifted to 2% galactose-containing medium for 12 h. For polyribosomes of *ded1-N423A/F424A/R425A* and *-N423A/F424A*, cells were grown at 30°C and then shifted to 37°C for 1 h. Cell extracts were prepared and subjected to sucrose density fractionation essentially as described in reference 23. Briefly, mid-log cells were incubated on ice with 100 μg/ml cycloheximide, spun down, and resuspended in buffer (20 mM HEPES-KOH [pH 7.5], 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol [DTT], 100 μg/ml cycloheximide, and protease inhibitors). Cells were lysed by vortexing with glass beads, supernatants were loaded on 7 to 47% sucrose gradients, and samples were centrifuged at 38,000 rpm for 2.5 h in a SW41-Ti rotor. Fractions from the gradients were then removed with continuous monitoring of absorbance at 254 nm with a Brandel density fractionator. Monosome-to-polysome ratios were determined by comparing the area under the curve for the 80S peak to the sum of the polyribosome peaks in ImageJ (National Institutes of Health). For the polysome profiles of the *ded1-N423A/F424A/R425A* mutant, the absorbance reading of the top portion of the monosome peak exceeded the maximum measurable by the fractionator. Thus, the true monosome area is slightly larger than the measured one, and the M/P ratio for the mutant is a minimum value. Note that the difference between the wild type and the mutant is still significant despite this limitation.

Protein purification. Recombinant His-Ded1 protein and mutant derivatives were generated essentially as described in reference 8. Rosetta cells (Novagen) containing the *DED1* plasmid were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) overnight at 23°C. Cells were harvested and lysed via sonication. His-Ded1 was then purified on Ni-nitrilotriacetic acid (Ni-NTA)–agarose (Qiagen) by conventional methods and dialyzed into buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, and 40% glycerol. Recombinant Gle1 was purified as described in reference 45. Briefly, Rosetta cells containing pMal-TEV-GLE1 were induced with 0.2 mM IPTG overnight at 16°C. Cells were lysed via sonication, and the sample was purified on an amylose column. After elution, MBP-Gle1 was cleaved by tobacco etch virus (TEV), and MBP and Gle1 were separated on an SP-Sepharose column (Sigma Fast Flow). Gle1 was then dialyzed into buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, and 20% glycerol. For the pulldown experiment shown in Fig. 3A, the MBP tag was not cleaved. For purification of MBP (used as the control protein), cells containing pMAL-cRI (NEB) were used with the same purification procedure as Gle1.

Preparation of translation extracts and RNA. For *in vitro* translation assays, the generation of translation-competent extracts was done in a fashion similar to that described in reference 47 for most assays. Briefly, wild-type cells or cells with protein-A-tagged Ded1 were grown to late log phase. Cells were spun down, washed, resuspended in Sarnow A buffer (30 mM HEPES, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT), and flash-frozen in liquid nitrogen. Extracts were finely ground by mortar and pestle while still frozen and then thawed and centrifuged twice, at 4,000 × *g* for 5 min and 38,000 × *g* for 15 min, to remove cell debris. Extracts were then run through a Zeba desalting column (molecular weight cutoff [MWCO], 7,000; Thermo Scientific) and aliquoted for use.

For Ded1 depletion experiments, extracts made from cells expressing protein-A-tagged Ded1 were incubated with 40% by volume of IgG-Sepharose beads (or glutathione-Sepharose for mock controls) at 4°C for 1 h, and then supernatants were transferred to new tubes and the depletion was repeated. Depleted and mock extracts were then used immediately for translation reactions.

RNA for *in vitro* assays was prepared using the mMessage mMachine Ultra kit (Ambion/Life Technologies). For translation assays, *pLucA50* was linearized with DraI, treated with proteinase K and SDS, and then gel purified. Luciferase reporter RNA was transcribed, capped with ARCA, and poly(A) tailed according to the kit protocol, and RNA was purified by the MegaClear kit (Ambion/Life Technologies). For PIC assembly assays, XbaI-linearized pRP803 was processed similarly, except that [α-^{32}P]UTP was added to the reaction mixture as a tracer.

***In vitro* translation assays.** *In vitro* translation assays were performed essentially as described previously (48). Extracts (one-half of total volume), and RNA (5 to 10 ng/μl) were mixed in the presence of 1 mM ATP, 250 μM GTP, 10 μM amino acids, 25 mM phosphocreatine, and 63 μg/ml creatine phosphokinase in buffer containing 10 mM HEPES (pH 7.4), 100 mM potassium acetate, 2.25 mM magnesium acetate, 1 mM DTT, and RNase inhibitor. Recombinant proteins were added as indicated. Reaction mixtures were incubated for 50 min at room temperature. Translation was assessed by luciferase assay using 100 μl of standard luciferin reagent (Promega) either on a Synergy 2 plate reader (BioTek) or on a Glomax 20/20 luminometer (Promega).

48S PIC assembly assays were performed essentially as described in reference 7. Translation reactions were performed as described above except that 5 ng/μl labeled *MFA2pG* RNA was used and 3.5 mM GMP-PNP was added instead of GTP to stop reactions after 48S PIC assembly. After incubation at room temperature for 20 min, reaction mixtures were separated on sucrose gradients as above. Fractions (500 μl) were collected by pipette, and the amount of radioactivity was assayed by Cerenkov counting.

***In vitro* pulldown assays.** Pulldown assays of recombinant Ded1 and Gle1 were performed as described in reference 8. The indicated proteins were incubated in modified His lysis (MHL) buffer (50 mM $NaHPO_4$ [pH 7.4], 150 mM NaCl, 10% glycerol, 10 mM imidazole) at room temperature for 1 to 1.5 h and then transferred to new tubes, washed with MHL buffer, and resuspended in SDS sample buffer for SDS-PAGE and Coomassie blue staining.

ATPase assays. ATPase assays were performed using a pyruvate kinase and lactate dehydrogenase (PK/LDH)-coupled colorimetric system as described in references 8 and 19. His-Ded1 or mutants (150 nM) were used in the presence or absence of 10 μg/ml total yeast RNA. Absorbance at 340 nm was read on a Versamax microplate reader (Molecular Devices), and apparent K_{cat} was calculated from the optical density at 340 nm (OD_{340}) signal decline to measure steady-state ATPase activity.

RNA binding assays. RNA filter-binding experiments were carried out similarly to what is described in reference 45. Briefly, a 41-bp, single-stranded RNA [5′-GCGUCUUUACGGUGCUUAAAA$(CA_4)_4$-3′](49) was end labeled with [^{32}P]ATP, and 2 nM was incubated with different concentrations of wild-type Ded1 or the indicated mutant for 20 min in buffer containing 20 mM HEPES (pH 7.5), 50 mM NaCl, and 20% glycerol. ADP-BeF_x (2 mM, prepared as described in reference 49) or ADP (8 mM) and equivalent concentrations of $MgCl_2$ were also added as indicated. Samples were then added to a slot blotter with a nitrocellulose membrane above a nylon membrane. The membranes were exposed to a phosphor screen and then visualized on a Typhoon FLA 7000 instrument (GE). The fraction of RNA bound was then determined via densitometry. Curve fitting was done via Graphpad Prism. Each data point represents the mean from 3 to 5 independent trials performed in duplicate.

For RNA binding by fluorescence anisotropy, a 16-bp RNA oligomer (5′-AGCACCGUAAAGACGC-3′) labeled at the 5′ end with 6-carboxyfluorescein (IDT) was used. Ded1 was preincubated in the presence or absence of 100 nM Gle1 and the indicated nucleotide in buffer containing 20 mM HEPES (pH 7.5), 50 mM NaCl, and 20% glycerol, and then 2 nM the labeled RNA was added to the samples for 30 min. The polarized fluorescence was then read on a Synergy 2 plate reader (BioTek), and anisotropy values were calculated. Data are plotted according to the formula $(A - A_0)/A_0$, where A_0 is the anisotropy value in the

absence of Ded1 for each set of samples. Curve fitting was done as described above via Prism. Data represent the means from three independent experiments performed in triplicate. For Fig. S6C and D in the supplemental material, graphs shown are from representative assays.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MCB.00139-17.

SUPPLEMENTAL FILE 1, PDF file, 11.5 MB.

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