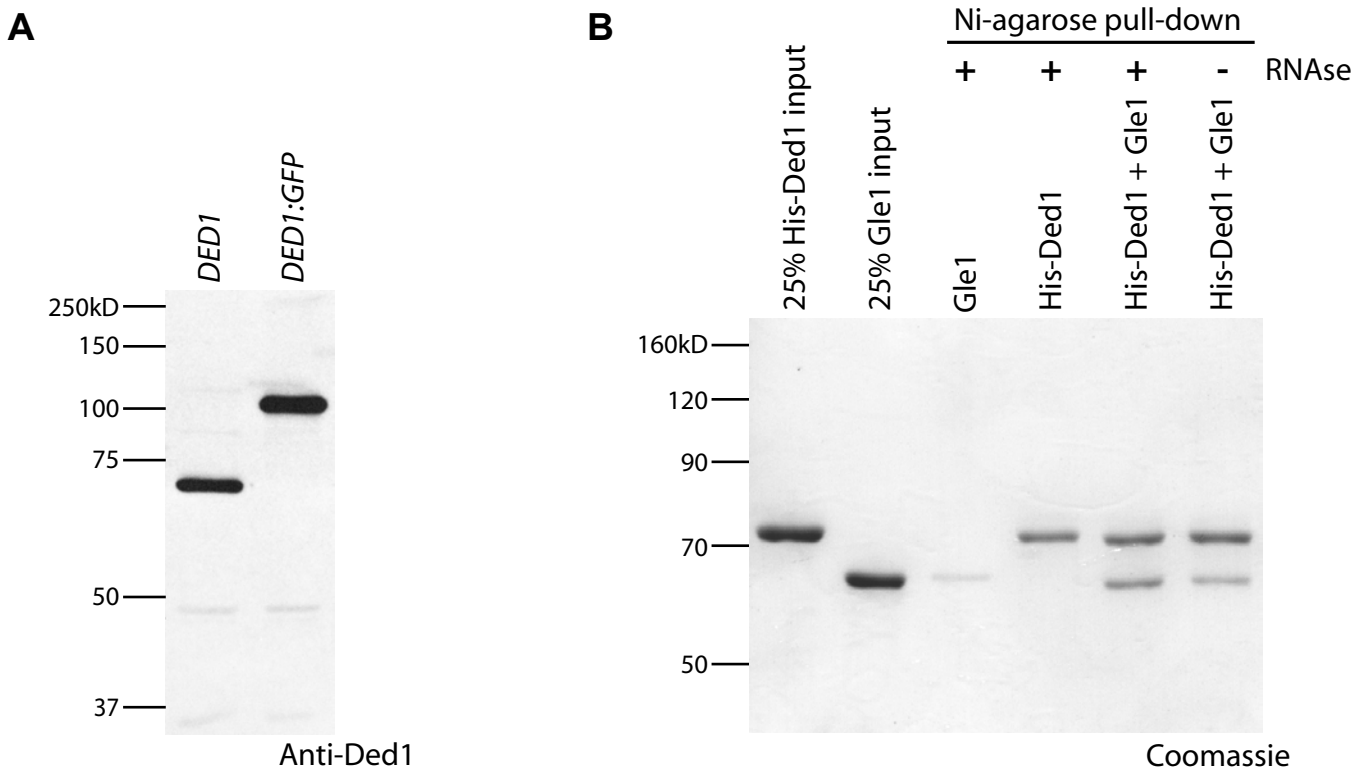
Supplemental Data:

Supplemental Table S1: Yeast strains used in this study

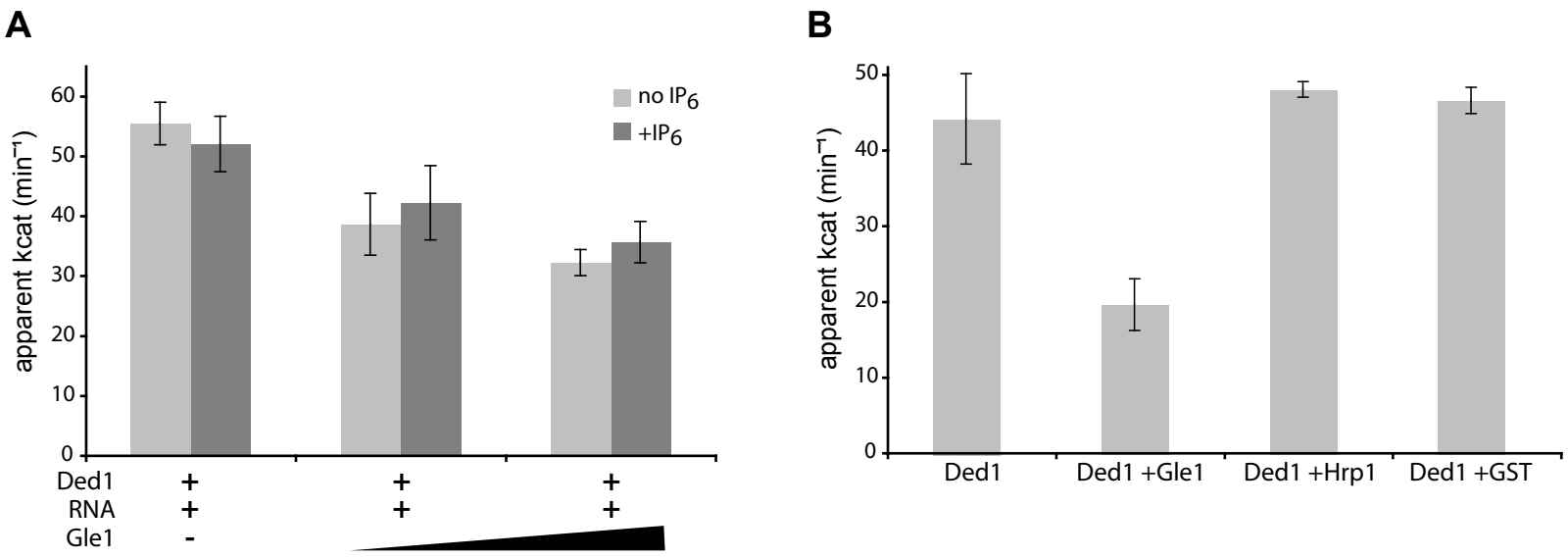
Strain Name	Genotype	Source
SWY4276	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/DED1*	This study
SWY4277	*MATα ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/ded1-120*	This study
SWY4264	*MATα ded1::KAN gle1-4 ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/DED1*	This study
SWY4265	*MATα ded1::KAN gle1-4 ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/ded1-120*	This study
SWY4322	*MATα ded1::KAN ipk1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/DED1*	This study
SWY4323	*MATα ded1::KAN ipk1::KAN gle1-4 ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/ded1-120*	This study
SWY4324	*MATα ded1::KAN rat7-1 (nup159) ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/DED1*	This study
SWY4325	*MATα ded1::KAN rat7-1 (nup159) ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/ded1-120*	This study
SWY4329	*MATα ded1::KAN rat8-2 (dbp5) ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/LEU2/DED1*	This study
SWY3826	*MATα gle1::HIS3 ade2 ura3 his3 leu2 trp1 can1-100 +pCEN/LEU2/GLE1*	(1)
SWY4414	*MATa gle1::HIS3 ade2 ura3 his3 leu2 trp1 +pCEN/LEU2/GLE1-PROTA*	This study
SWY4092	*MATa ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/URA3/DED1*	This study
SWY4585	*MATa ded1::KAN ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 +pCEN/TRP1/DED1-GFP*	This study

Supplemental Table S2: Plasmids used in this study

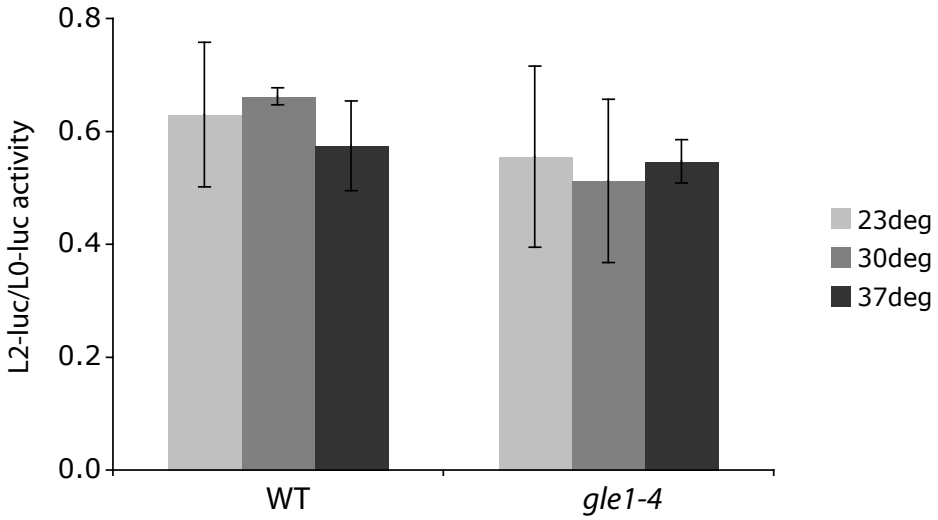
Plasmid Name	Description	Source
DED1	*CEN/URA3/DED1*	(2)
pSW3619	*CEN/LEU2/DED1*	This study
ded1-120	*CEN/LEU2/ded1-120*	(2)
pSW3622	*CEN/TRP1/DED1-GFP*	This study
pSW399	*CEN/LEU2/GLE1*	(3)
pSW3620	*CEN/LEU2/GLE1-PROTEIN-A*	This study
pSW3576	*pET28a-DED1*	This study
pSW3738	*pET28a-ded1-R438G*	This study
pSW3242	*pMAL-TEV-GLE1*	(4)
pSW3734	*pMAL-TEV-gle1-VAI/DDD*	This study
pSW3739	*pRS424-Gal1/10-DED1*	This study
pSW3740	*pRS424-Gal1/10-ded1-R438G*	This study
pLucA50	*pLuc-A50*	(5)
L0-luciferase	*YCp33Supex2-L0-LUC*	(6)
L2-luciferase	*YCp33Supex2-L2-LUC*	(6)
pM199Z	uORF1, *GCN4-lacZ* with uORF1 at uORF4 position	(7)
pM226Z	uORF1x, derivation of 199, uORF1 extended into GCN4 cds	(7)
p367	HIS4-AUG, *HIS4-lacZ* with AUG start codon	(8)
p391	HIS4-UUG, *HIS4-lacZ* with UUG at 1st and 3rd codons	(8)



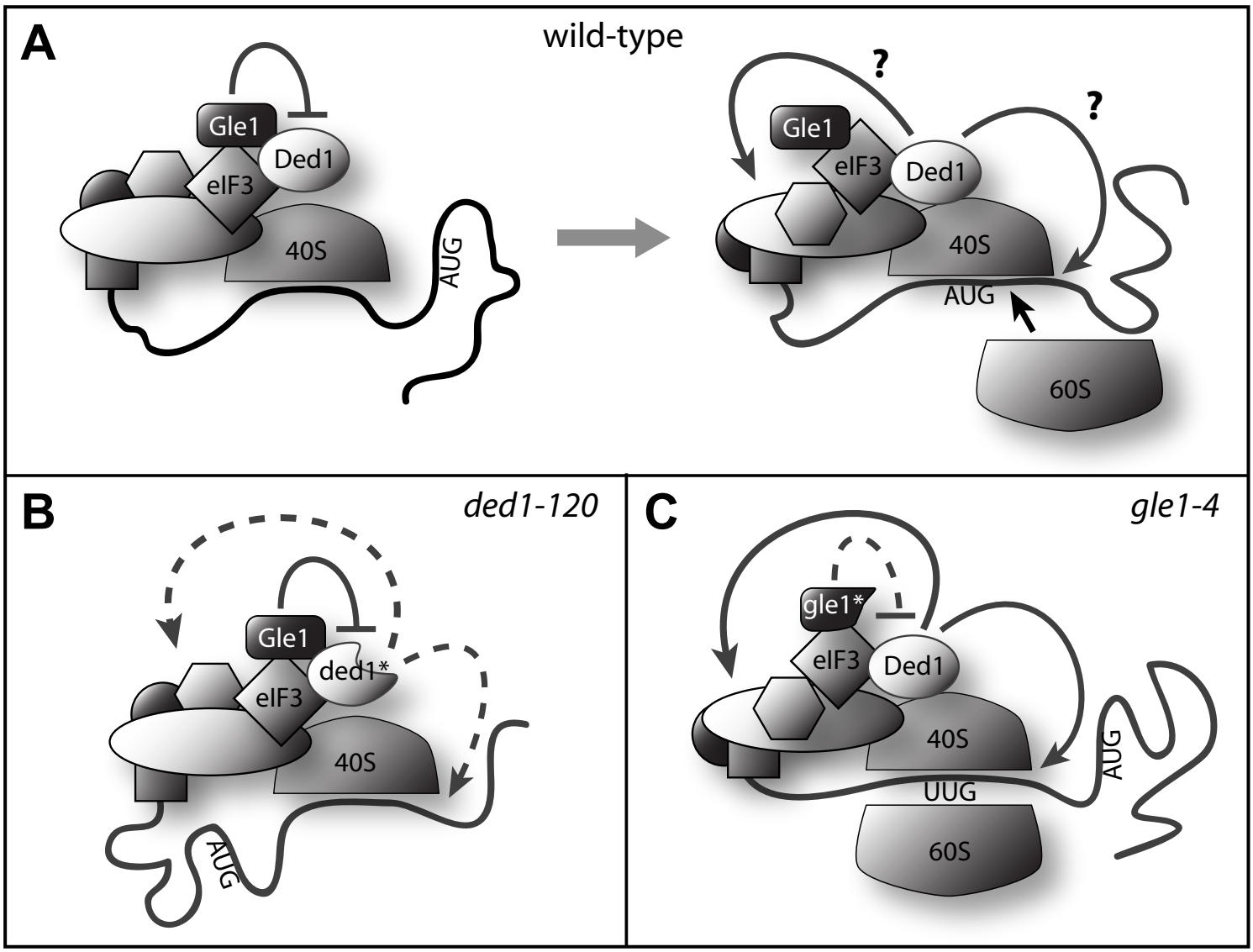
Supplemental Figure S1: The interaction of Gle1 and Ded1 is specific. (A) Cultures of wild-type (*DED1*) and GFP-tagged Ded1 (*DED1:GFP*) strains were harvested and lysed. Samples were subjected to SDS-PAGE followed by immunblotting using Ded1 antisera. Note the shift in mobility of the major band due to GFP tagging.(A) Purified His-Ded1 was incubated with purified Gle1 in the presence of RNase A and then isolated via Ni-agarose resin as in Figure 1C. Samples were subjected to SDS-PAGE and Coomassie-stained.



Supplemental Figure S2: Gle1 inhibition of Ded1 ATPase activity is specific. (A) ATPase assays were performed with 100nM Ded1, 0.3 µg/ml RNA, 1mM ATP, and 200 or 400nM Gle1 in the presence or absence of 200nM IP6. Mean and standard error of means were calculated from three to five independent experiments. (B) ATPase assays were performed as above with 300nM Gle1, Hrp1, or GST. Mean and standard error of means were calculated from three independent experiments.



Supplemental Figure S3: Mutants of *gle1* do not have defects in scanning. Luciferase assays were performed with wild-type and *gle1-4* strains transformed with the L0-Luc and L2-Luc plasmids after incubation at the indicated temperatures for 4 hours, and scanning efficiency was calculated as in Figure 5A. Mean and standard error of means are shown.



Supplemental Figure S4: Speculative model for Gle1 regulation of Ded1 in translation. (A) In wild-type cells, Gle1 inhibits Ded1, which prevents premature AUG recognition (left). However, upon start site recognition, conformational changes in the PIC may cause rearrangement or dissociation of Gle1 such that it no longer inhibits Ded1. Ded1 is required for efficient initiation at the start codon, perhaps through an unknown remodeling target (right). (B) In a *ded1-120* strain, altered ded1 protein results in defective start site recognition and/or subsequent steps, leading to leaky scanning past the first AUG codon. (C) In a *gle1-4* strain, altered gle1 protein is impaired in its ability to restrain Ded1 activity, leading to inappropriate initiation at near-cognate (UUG) start codons.

Supplemental References:

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