Supplementary Material For:

eIF5 is a GDP dissociation inhibitor necessary for translational control by eIF2 phosphorylation

Martin D. Jennings and Graham D. Pavitt

Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, United Kingdom

Correspondence and requests for materials should be addressed to graham.pavitt@manchester.ac.uk.

This supplementary material file includes three tables, six figures and associated additional references.

Table S1. Plasmids used in this study

Name	Description	Construction[a]/ Reference
pRS316	CEN6 *URA*3	Ref [30]
p722	pRS316 CEN6 *URA*3 GCN2	Ref [22]
p1055	pRS316 CEN6 *URA*3 GCN2-*M788V,E1591K*	Ref [22]
p1056	pRS316 CEN6 *URA*3 GCN2-*E601K,E1591K*	Ref [22]
pGEX-4T-1	IPTG inducible GST fusion vector	GE Healthcare
YEpTIF5-FL	YEplac181 2µ *TIF5-FLAG LEU2*	Ref [16]
pGEX-TIF5	pGEX-4T-1 *GST-TIF5*	Ref [28]
pAV1915	pGEX-4T-1 *GST-TIF5 (R15M)*	Site-directed mutagenesis of pGEX-TIF5 using oligonucleotides eIF5(R15M)1 and eIF5(R15M)2
pAV1919	pGEX-4T-*1 GST-TIF5 (W391F)*	Site-directed mutagenesis of pGEX-TIF5 using oligos eIF5(W391F)1 and eIF5(W391F)2
pAV1936	YEplac181 2µ *TIF5-FLAG (W391F) LEU2*	Site-directed mutagenesis of YEpTIF5-FL using oligos eIF5(W391F)1 and eIF5(W391F)2
pAV1937	YEplac181 2µ *TIF5-FLAG (R15M) LEU2*	Site-directed mutagenesis of YEpTIF5-FL using oligos eIF5(R15M)1 and eIF5(R15M)2
YEpTIF5-FL-7A	YEplac181 2µ *TIF5-FLAG-7A LEU2*	Ref [16]
pAV2010	pGEX-4T-1 *GST-TIF5-NTD*	PCR amplification of 1-456bp of TIF5 from pGEX-TIF5 using oligos TIF5ntdF and TIF5-152R to include 5' BamHI and 3' XhoI sites, then inserted in pGEX-4T-1
pAV2011	pGEX-4T-1 *GST-TIF5-NTD+LR*	PCR amplification of 1- 720bp of TIF5 from pGEX-TIF5 using oligos TIF5ntdF and TIF5-240R to include 5' BamHI and 3' XhoI site, then inserted in pGEX-4T-1
pAV2012	pGEX-4T-1 *GST-TIF5-CTD*	PCR amplification of 721-1215bp of TIF5 from pGEX-TIF5 using oligos TIF5ctdF2 and TIF5ctdR to include 5' BamHI and 3' XhoI site, then inserted in pGEX-4T-1
pAV2013	pGEX-4T-1 *GST-TIF5-LR+CTD*	PCR amplification of 457-1215bp of TIF5 from pGEX-TIF5 using oligos TIF5-153F and TIF5ctdR to include 5' BamHI and 3' XhoI site, then inserted in pGEX-4T-1
pAV1043	*6His-GCD11* in pALTER-1	Ref [13,31]
pAV2021	*3Myc-6His-GCD11* pALTER-1	Annealing of complementary oligos MYCtagF and MYCtagR and ligation into BspE1 site of pAV1043
pAV2034	pRS424 2µ *3Myc-6His-GCD11 TRP1*	NheI-EcoRI fragment from pAV2021 cloned into EcoRI-SpeI of pRS424
pAV2052	pRS314 CEN6 3Myc-6His-GCD11 *TRP1*	NheI-EcoRI fragment from pAV2021 cloned into EcoRI-SpeI of pRS314
pAV2053	YCplac111 *CEN4 TIF5-FLAG (R15M) LEU2*	EcoRI-HinDIII fragment from pAV1937 cloned into YCPlac111
pAV2054	YCplac111 *CEN4 TIF5-FLAG LEU2*	EcoRI-HinDIII fragment from YEpTIF5-FL cloned into YCPlac111
pAV2055	YCplac111 *CEN4 TIF5-FLAG (W391F) LEU2*	EcoRI-HinDIII fragment from pAV1936 cloned into YCPlac111
pAV2091	Fragment of *TIF5* (BsmI-KpnI) with LR7A mutation in vector pMA-AP	Custom synthesis by Mr.Gene GmbH of *TIF5* (540-1098bp) with the following mutations introduced (D220A, D221A, W223A, D226A, E230A, R235A, E238A). A BsmI-KpnI fragment.
pAV2093	pGEX-4T-1 *GST-TIF5-LR7A*	Replacement of BsmI-KpnI region of pGEX-TIF5 with BsmI-KpnI region of pAV2091
pAV2100	YEplac181 2µ *TIF5-FLAG (LR7A) LEU2*	PCR using oligos TIF5FLAGF and TIF5FLAGR and pAV2093 as a template adding NdeI at 5' and FLAG tag and SalI at 3'. Then used to replace NdeI-SalI of YEpTIF5-FL.
pAV2101	YCplac111 *CEN4 TIF5-FLAG (LR7A) LEU2*	PCR using oligos TIF5FLAGF and TIF5FLAGR and pAV2093 as a template adding NdeI at 5' and FLAG tag and SalI at 3'. Then used to replace NdeI-SalI of pAV2054.

[a]All constructed for this study unless referenced

Table S2. Oligonuclotides used in this study

Name	Sequence (5'-3')
MYCtagF	CCGGAGAACAAAAATTGATTTCTGAAGAAGATTTGA
MYCtagR	CCGGTCAAATCTTCTTCAGAAATCAATTTTTGTTCT
eIF5(R15M)1	GAGGCATTTTGTACATGTAAAATGGATCATGATTATC
eIF5(R15M)2	GATCCATTTTACATGTACAAAATGCCTCCCATCCAAG
eIF5(W391F)1	GCGGTTTCTAAGAACGTAATGAATGGCTTAGCAGCCC
eIF5(W391F)2	GCCATTCATTACGTTCTTAGAAACCGCTGAAAGTGACG
TIF5FLAGF	GCGTGGCATATGTCTATTAATATTTGTAGAGATAATC
TIF5FLAGR	GTCGTCGACCTATTTGTCATCGTCGTCCTTGTAGTCTTCGTCGTCTTCTTCATCAT
TIF5ctdF2	AGATGCGGATCCGTGAACTCTGAGCTCACTCA
TIF5ctdR	ATCGATCTCGAGTCACTATTCGTCGTCTTCTTCATCAT
TIF5ntdF	GGTCGCGGATCCATGTCTATTAATATTTGTAGAG
TIF5-152R	ATCGATCTCGAGTCACTACTTGGAACCAGAAACGGAGTC
TIF5-240R	ATCGATCTCGAGTCACTATTCTAGTTCCTTGGCACGAGC
TIF5-153F	GGTCGCGGATCCAAGAAGAAGAAAGCAGCTACC

Table S3. Yeast Strains used in this study

Name	Genotype	Construction/ reference
KAY24	*MAT***a** *leu2-3 leu2-112 gcn2Δ tif5Δ trp1Δ63 ura3-52* <pKA235 [*TIF5 CEN URA3*]>	Ref [16]
GP3511	*MATα gcn2Δ ino1 leu2-3 leu2-112 pep4::LEU2 sui2Δ ura3-52* (*HIS4-lacZ*) <pAV1089 [2μ his6-eIF2 *URA3*]>	Ref [13,31]
GP4597	*MATα trp1Δ63 ura3-52 leu2-3 leu2-112 GAL2+ gcn2Δ pep4::hisG*	Ref [32]
GP5012	*MAT***a** *gcd11::hisG leu2-3 leu2-112 trp1Δ::hisG ura3-52* (*HIS4-lacZ*) Ep517 [*LEU2 CEN GCD11*]	*This study, trp1*Δ in EY551, from E. Hannig see ref [33]
GP5134	KAY24 + pAV1937 [*TIF5-R15M LEU2 2*μ]	*This study,* Transformation of KAY24 with pAV1937
GP5137	KAY24 + pAV1948 [*TIF5-R15M LEU2 CEN*]	*This study,* Transformation of KAY24 with pAV1948
GP5423	GP4597 + YEpTIF5-FL-7A [2μ *TIF5-7A-FLAG LEU2*]	*This study,* Transformation of GP4597 with YEpTIF5-FL-7A
GP5424	GP4597 + YEpTIF5-FL [2μ *TIF5-FLAG LEU2*]	*This study,* Transformation of GP4597 with YEpTIF5-FL
GP5463	GP5012 + pAV2052 [*3myc-6His-GCD11 TRP1* CEN]	*This study,* Transformation of GP5012 with pAV2052
GP5627	KAY24 + YEpTIF5-FL [*TIF5-FLAG LEU2* 2μ] pAV1198 [*CEN6 URA3 GCN2*]	*This study,* Transformation of KAY24 with YEpTIF5-FL, plasmid shuffle to lose pKA235 then transformation of pAV1198
GP5628	KAY24 + pAV1936 [*TIF5-W391F-FLAG LEU2* 2μ] pAV1198 [*CEN6 URA3 GCN2*]	*This study,* Transformation of KAY24 with pAV1936, plasmid shuffle to lose pKA235 then transformation of pAV1198
GP5631	KAY24 + pAV2054 [*TIF5-FLAG LEU2 CEN*] pAV1198 [*CEN6 URA3 GCN2*]	*This study,* Transformation of KAY24 with pAV2054, plasmid shuffle to lose pKA235 then transformation of pAV1198
GP5632	KAY24 + pAV2055 [*TIF5-W391F-FLAG LEU2 CEN*] pAV1198 [*CEN6 URA3 GCN2*]	*This study,* Transformation of KAY24 with pAV2055, plasmid shuffle to lose pKA235 then transformation of pAV1198
GP5633	KAY24 + pAV2100 [*TIF5-LR7A LEU2* 2μ] pAV1198 [*CEN6 URA3 GCN2*]	*This study,*Transformation of KAY24 with pAV2100, plasmid shuffle to lose pKA235 then transformation of pAV1198
GP5634	KAY24 + pAV2101 [*TIF5-LR7A LEU2 CEN*] pAV1198 [CEN6 *URA*3 GCN2]	*This study,* Transformation of KAY24 with pAV2101, plasmid shuffle to lose pKA235 then transformation of pAV1198
GP5637	KAY24 + pAV2054 [*TIF5-FLAG LEU2 CEN*] pRS316 [*CEN6 URA3*]	*This study,* Transformation of KAY24 with pAV2054, plasmid shuffle to lose pKA235 then transformation of pRS316
GP5638	KAY24 + pAV2055 [*TIF5-W391F-FLAG LEU2 CEN*] pRS316 [*CEN6 URA3*]	*This study,* Transformation of KAY24 with pAV2055, plasmid shuffle to lose pKA235 then transformation of pRS316
GP5639	KAY24 + pAV2101 [*TIF5-LR7A LEU2 CEN*] pRS316 [*CEN6 URA3*]	*This study,* Transformation of KAY24 with pAV2101, plasmid shuffle to lose pKA235 then transformation of pRS316
GP5640	KAY24 + YEpTIF5-FL [*TIF5-FLAG LEU2* 2μ] pRS316 [*CEN6 URA3*]	*This study,* Transformation of KAY24 with YEpTIF5-FL, plasmid shuffle to lose pKA235 then transformation of pRS316
GP5641	KAY24 + pAV1936 [*TIF5-W391F-FLAG LEU2* 2μ] pRS316 [*CEN6 URA3*]	*This study,* Transformation of KAY24 with pAV1936, plasmid shuffle to lose pKA235 then transformation of pRS316
GP5642	KAY24 + pAV2100 [*TIF5-LR7A LEU2* 2μ] pRS316 [*CEN6 URA3*]	*This study,* Transformation of KAY24 with pAV2100, plasmid shuffle to lose pKA235 then transformation of pRS316
GP5661	GP5012 + pAV2034 [*3c-Myc-6His-GCD11 TRP1* 2μ]	*This study,* Transformation of GP5012 with pAV2034

Continued over

Name	Genotype	Construction/ reference
GP5842	KAY24 + YEpTIF5-FL [*TIF5-FLAG LEU2* 2μ] p1056 [*GCN2^c-516 URA3 CEN*]	*This study,* Transformation of KAY24 with YEpTIF5-FL, plasmid shuffle to lose pKA235 then transformation of p1056
GP5843	KAY24 + pAV1936 [*TIF5-W391F-FLAG LEU2* 2μ] p1056 [*GCN2^c-516 URA3 CEN*]	*This study,* Transformation of KAY24 with pAV1936, plasmid shuffle to lose pKA235 then transformation of p1056
GP5844	KAY24 + pAV2054 [*TIF5-FLAG LEU2* CEN] p1056 [*GCN2^c-516 URA3 CEN*]	*This study,* Transformation of KAY24 with pAV2054, plasmid shuffle to lose pKA235 then transformation of p1056
GP5845	KAY24 + pAV2055 [*TIF5-W391F-FLAG LEU2* CEN] p1056 [*GCN2^c-516 URA3 CEN*]	*This study,* Transformation of KAY24 with pAV2055, plasmid shuffle to lose pKA235 then transformation of p1056
GP5863	KAY24 + YEpTIF5-FL [*TIF5-FLAG LEU2* 2μ] p1055 [*GCN2^c-513 URA3 CEN*]	*This study,* Transformation of KAY24 with YEpTIF5-FL, plasmid shuffle to lose pKA235 then transformation of p1055
GP5864	KAY24 + pAV1936 [*TIF5-W391F-FLAG LEU2* 2μ] p1055 [*GCN2^c-513 URA3 CEN*]	*This study,* Transformation of KAY24 with pAV1936, plasmid shuffle to lose pKA235 then transformation of p1055
GP5865	KAY24 + pAV2054 [*TIF5-FLAG LEU2* CEN] p1055 [*GCN2^c-515 URA3 CEN*]	*This study,* Transformation of KAY24 with pAV2054, plasmid shuffle to lose pKA235 then transformation of p1055
GP5866	KAY24 + pAV2055 [*TIF5-W391F-FLAG LEU2* CEN] p1055 [*GCN2^c-515 URA3 CEN*]	*This study,* Transformation of KAY24 with pAV2055, plasmid shuffle to lose pKA235 then transformation of p1055

Supplementary Figures

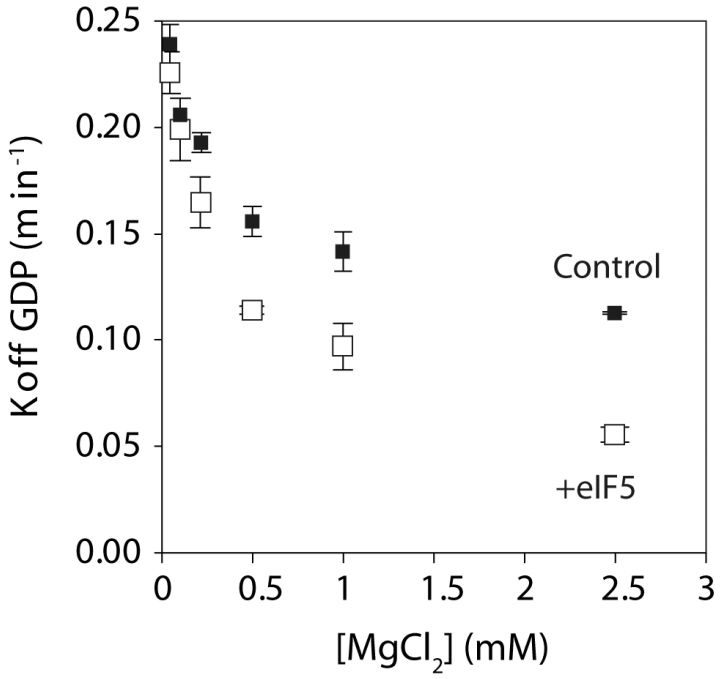

Figure S1: eIF5 GDI activity functions over a range of $MgCl_2$ concentrations. Affect of $MgCl_2$ concentration on GDP release (K_{off} GDP) from eIF2 (60 pmol, filled squares) alone or eIF2 (60 pmol) + eIF5 (120 pmol; open squares).

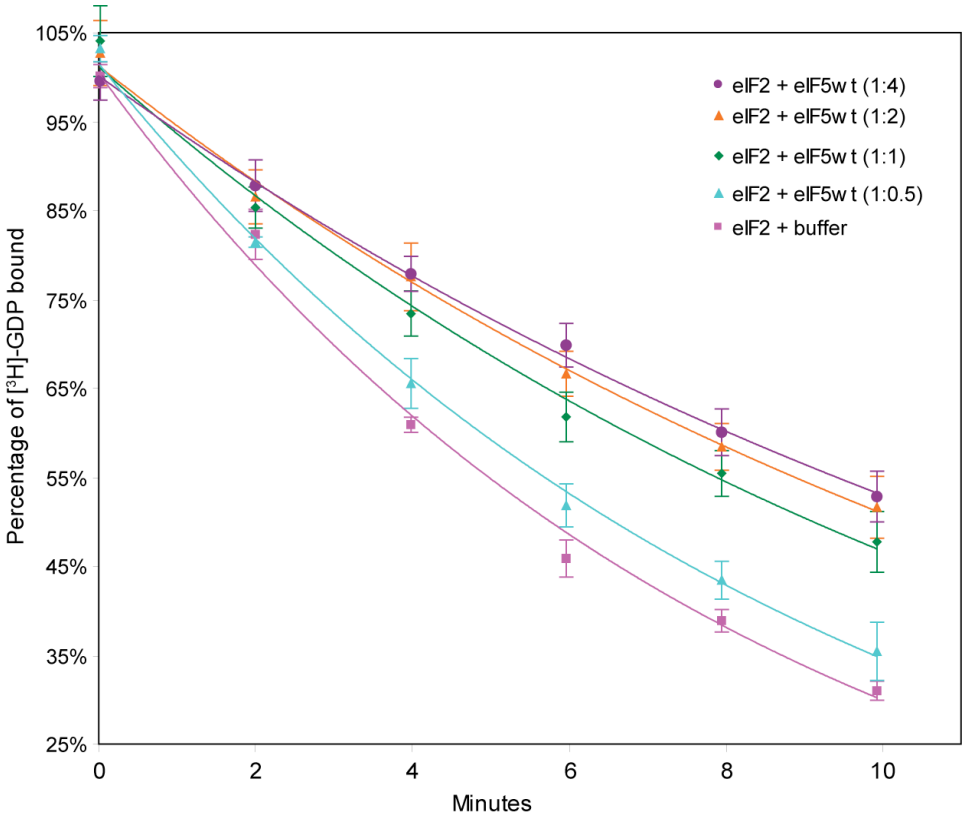

Figure S2: Example eIF2-[^{3}H]-GDP dissociation assay data. [^{3}H]-GDP dissociation from eIF2 (60 pmol) with either buffer or increasing amounts of GST-eIF5. data used to calculate K_{off} GDP values shown in Fig.1b. The molar eIF2:GST-eIF5 ratio in indicated.

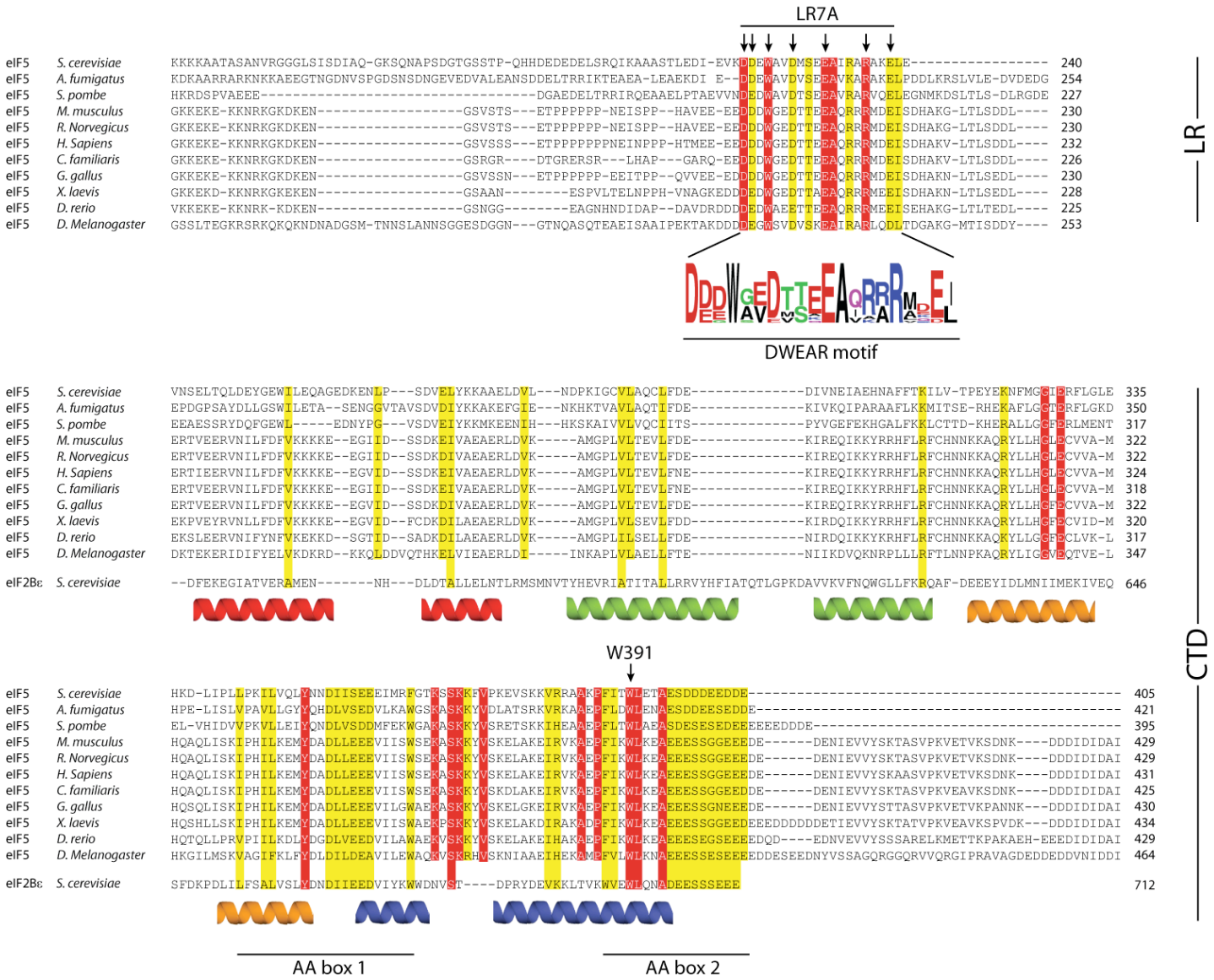

Figure S3: Multiple sequence alignment of eIF5 LR+CTD. Yeast eIF5-CTD, Human eIF5-CTD and yeast eIF2Bε-CTD were aligned structurally using DALI[34] then aligned to the other species using Clustal W[35]. Identical residues are highlighted in red and functionally similar residues are highlighted in yellow. The conserved DWEAR motif is shown using WebLogo[36] and the positions of the W391 and LR7A mutations are indicated, as are the previously defined AA box 1 and 2 motifs[15] important for eIF5 function.

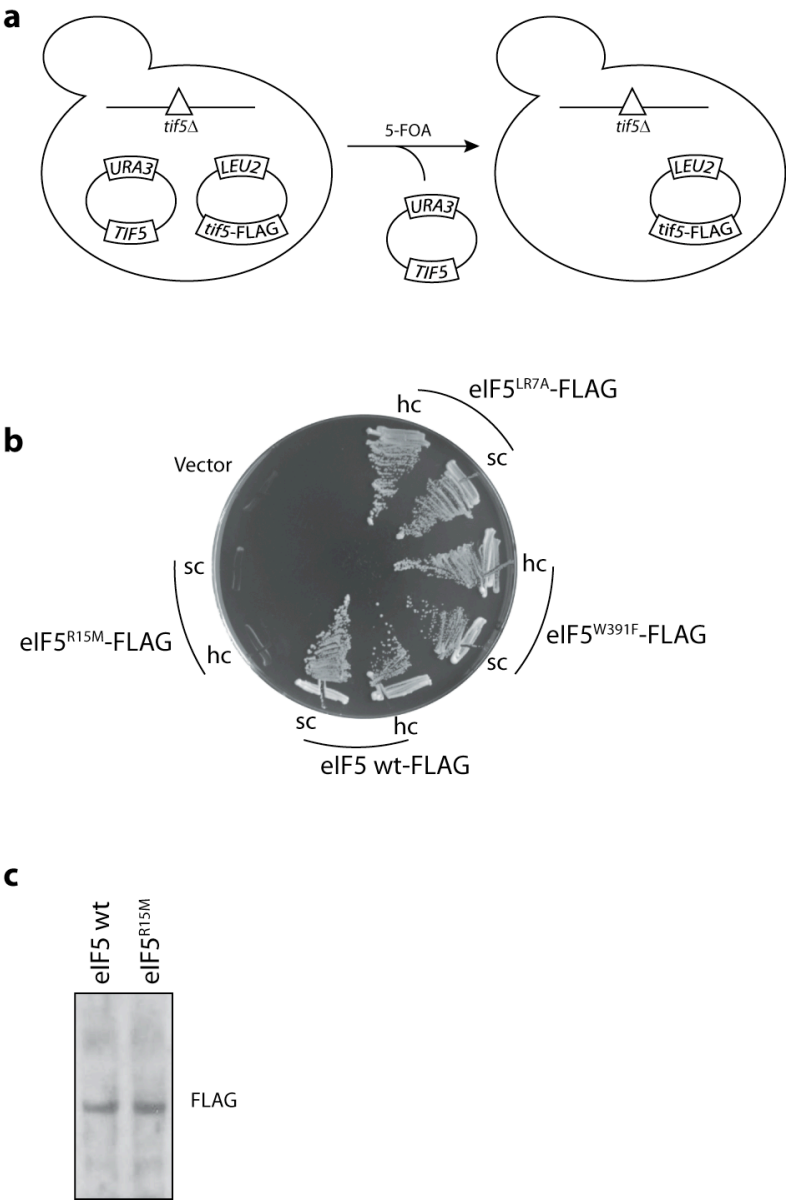

Figure S4: GAP activity is the essential function of eIF5. a) Cartoon depicting 5-FOA plasmid shuffling strategy in a *tif5*Δ (eIF5 deletion) strain. **b**) Growth of *tif5*-FLAG mutants on 5-FOA selective medium. **c**) Anti-FLAG immunoblot shows sc eIF5^{R15M} is expressed equivalent to wild-type (wt).

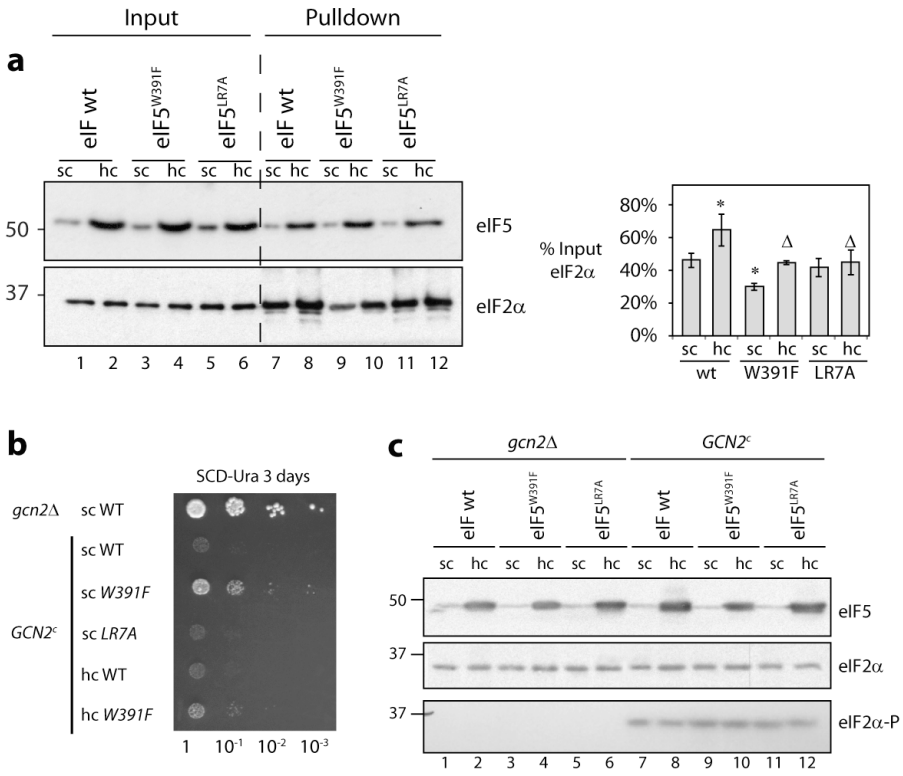

Figure S5: eIF5 W391F affects the global response to eIF2α phosphorylation. a) Left: Inputs (10 %, lanes 1-6) and co-immune precipitates of eIF2 with FLAG-eIF5 and immunoblotting with specific antibodies as indicated from cells bearing indicated sc and hc alleles of eIF5 (lane 7-12). Right: Densitometry, percentage input eIF2 in complex with eIF5 ±SD (n=3). Significant difference to wildtype (wt) sc (✻) or hc (Δ) ($p<0.05$, unpaired Student's *t*-test**)**. **b**) Yeast strains expressing indicated allele of eIF5 from either sc or hc plasmids as sole source of eIF5 were transformed with the severe constitutively active *GCN2^c-516* plasmid (p1056, *GCN2-E601K,E1591K*) and grown on selective medium. **c)** Whole cell protein extracts (10 µg) prepared from the indicated strains were subject to SDS-PAGE and immunoblotting with indicated specific antibodies.

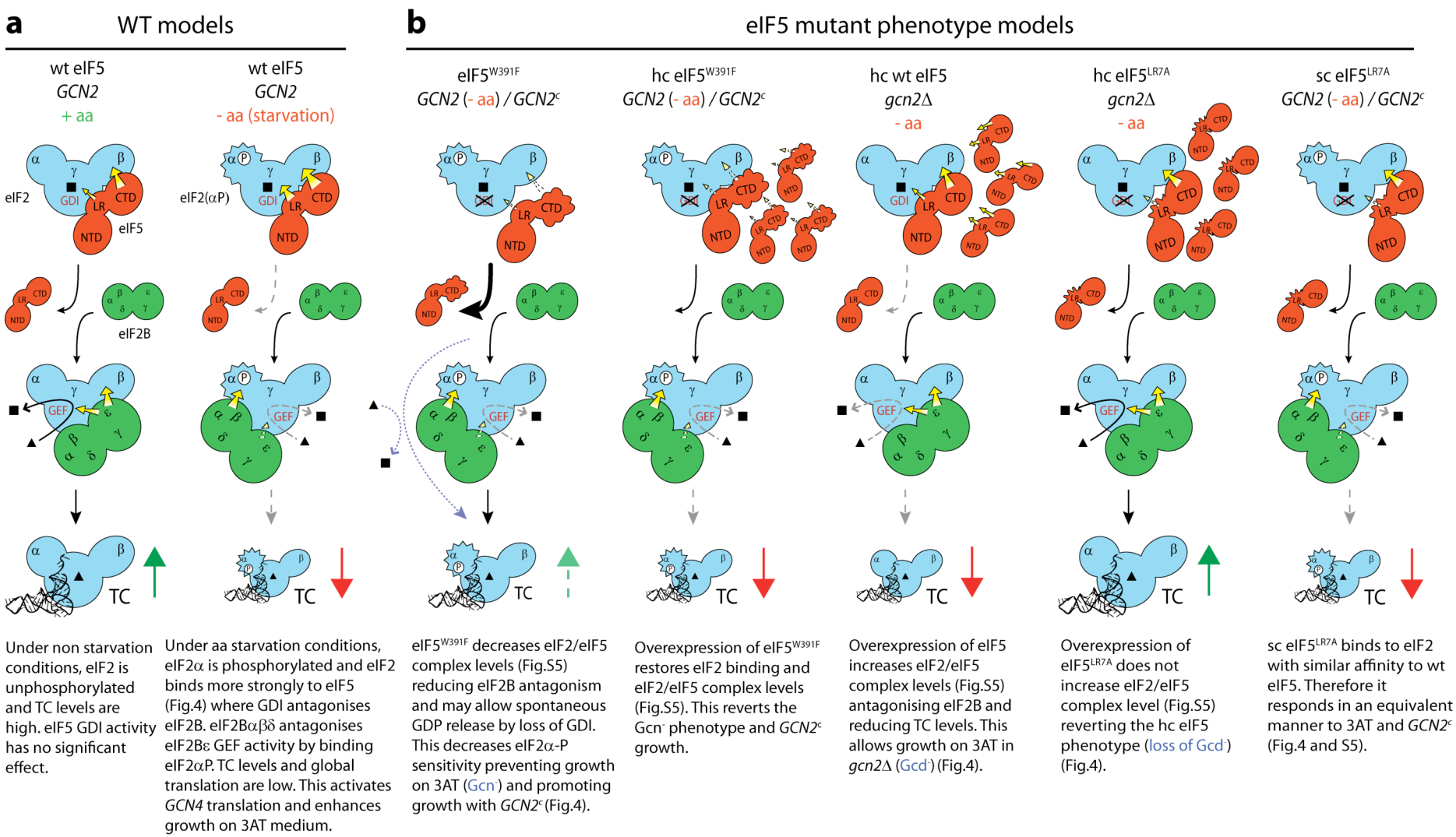

Figure S6: **Models**. Cartoons to explain domain interactions between eIF5 and eIF2 to mediate GDI activity and to account for phenotypes in Figs 4 and S5. **a**) a more detailed cartoon model than shown in Fig.4e for eIF5 GDI activity, nucleotide exchange and its inhibition by phosphorylated eIF2α showing presumptive domain interactions between factors. Here the three eIF5 domains delineated in the text are shown and their interactions with eIF2 subunits indicated by yellow arrows (the larger the arrow the tighter the binding). Solid black and dashed grey arrows show active and restricted reactions respectively, ▲ GTP, ■ GDP. Green and red arrows indicate increased and reduced ternary complex abundance (TC) **b**) changes to the wildtype interactions and reactions caused by W391F, LR7A mutations and overexpression. Altered domain shapes and diminished yellow arrows indicate the proposed altered interactions. Possible route for spontaneous nucleotide release indicated for W391F (by dotted blue arrow) is inferred from enhanced eIF2•GDP off-rate (Fig.1) and previous work showing that eIF2B independent nucleotide exchange is possible when TC is overexpressed[37,31].

Supplementary Additional References

30 Sikorski, R. S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19-27 (1989).

31 Gomez, E., Mohammad, S. S. & Pavitt, G. D. Characterization of the minimal catalytic domain within eIF2B: the guanine-nucleotide exchange factor for translation initiation. *EMBO J.* **21**, 5292-5301. (2002).

32 Mohammad-Qureshi, S. S. *et al.* Purification of FLAG-tagged eukaryotic initiation factor 2B complexes, subcomplexes, and fragments from *Saccharomyces cerevisiae*. *Methods Enzymol.* **431**, 1-13 (2007).

33 Dorris, D. R., Erickson, F. L. & Hannig, E. M. Mutations in *GCD11*, the structural gene for eIF-2 gamma in yeast, alter translational regulation of *GCN4* and the selection of the start site for protein synthesis. *EMBO J.* **14**, 2239-2249 (1995).

34 Holm, L., Kaariainen, S., Rosenstrom, P. & Schenkel, A. Searching protein structure databases with DaliLite v.3. *Bioinformatics* **24**, 2780-2781 (2008).

35 Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876-4882 (1997).

36 Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E., WebLogo: a sequence logo generator. *Genome Res.* **14**, 1188 (2004).

37 Erickson, F. L., Nika, J., Rippel, S., and Hannig, E. M., Minimum requirements for the function of eukaryotic translation initiation factor 2. *Genetics* **158**, 123 (2001).