JBC Papers in Press. Published on June 8, 2012 as Manuscript M112.372672
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M112.372672

Elongation factor 1A is the target of growth inhibition in yeast caused by *Legionella pneumophila* glucosyltransferase Lgt1

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Running title: *Glucosylation of yeast eEF1A by Lgt1*

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Keywords: Glucosyltransferase, *Legionella pneumophila*, *Saccharomyces cerevisiae*, protein synthesis, eukaryotic elongation factor eEF1A, Hbs1

Background: *Legionella pneumophila* glucosyltransferase Lgt1 modifies elongation factor 1A and Hbs1 (Hsp70 subfamily B suppressor 1).
Results: In *Saccharomyces cerevisiae* deleted of endogenous eEF1A and Hbs1, Lgt1 inhibits growth by glucosylation of ectopically expressed eEF1A at serine-53.
Conclusion: Glucosylation of eEF1A but not of Hbs1 is essential for Lgt1-induced toxicity.
Significance: Yeast as a model to study the action of *Legionella* glucosyltransferases.

SUMMARY

Legionella **is a pathogenic gram-negative bacterium able to multiply inside eukaryotic cells. It translocates numerous bacterial effector proteins into target cells to transform host phagocytes into a niche for replication. One effector of *L. pneumophila* is the glucosyltransferase Lgt1, which modifies serine-53 in mammalian elongation factor 1A (eEF1A), resulting in inhibition of protein synthesis and cell death. Here, we demonstrate that similar to mammalian cells, Lgt1 was severely toxic when produced in yeast and effectively inhibited *in vitro* protein synthesis. *S. cerevisiae* strains, which were deleted of endogenous eEF1A, but harbored a mutant eEF1A not glucosylated by Lgt1, were resistant towards the bacterial effector. In contrast, deletion of Hbs1, which is also an *in vitro* substrate of the glucosyltransferase, did not influence the toxic effects of Lgt1. Serial mutagenesis in yeast showed that F^{54}, Y^{56} and W^{58}, located immediately downstream of serine-53 of eEF1A, are essential for the function of the elongation factor. Replacement of serine-53 by glutamic acid, mimicking phosphorylation, produced a non-functional eEF1A, which failed to support growth of *S. cerevisiae*. Our data indicate that Lgt1-induced lethal effect in yeast depends solely on eEF1A. The region of eEF1A encompassing serine-53 plays a critical role in functioning of the elongation factor.**

Legionella pneumophila is a gram-negative bacterium, which causes severe pneumonia in humans known as Legionnaires' disease (1,2). The pathogen is able to multiply inside eukaryotic cells, including free-living protozoa or mammalian cells (3). A type 4B secretion system, encoded by *dot/icm* gene clusters, translocates hundreds of *Legionella* proteins (effectors) into target cells and, thereby, changes the hostile intracellular environment of phagocytes into a niche for *Legionella* replication

(4,5).

Among *L. pneumophila* effectors are cytotoxic glucosyltransferases of the Lgt family (6). These enzymes use UDP-glucose as a cofactor and target eukaryotic substrates by covalent attachment of a glucosyl moiety (7). The crystal structure of Lgt1 has been solved, allowing the characterization of the *Legionella* enzyme as a GT-A glucosyltransferase structurally related to clostridial glucosylating toxins (8,9). Known substrates of Lgts are $eEF1A^4$ (eukaryotic elongation factor 1A) and eRF3 (eukaryotic release factor 3) -related protein Hbs1 (Hsp70 subfamily B suppressor 1) (10).

Lgt1 mono-glucosylates eEF1A on serine-53 and yeast Hbs1 – on serine-213 (10). These target serine residues are located within conserved regions, exhibiting significant homology between the two proteins. Recently, it has been shown that the preferred substrate for modification of eEF1A is its complex with charged tRNA and GTP (11). In relation to Hbs1 it is not known if any cofactors are able to increase the level of its modification by Lgt1.

Glucosylation by Lgt1 parallels inhibition of protein synthesis both *in vitro* and *in vivo* and leads to death of target mammalian cells (12,13). So far it is not clear whether the lethal effect of Lgt1 is linked solely to modification of eEF1A or glucosylation of Hbs1 also contributes to toxic effects caused by the *Legionella* enzyme. Moreover, it was demonstrated recently that a short peptide encompassing region of the elongation factor from glycine-50 to valine-59 was effectively recognized and glucosylated by Lgt1 (10). This fact suggested that other not yet identified eukaryotic protein(s), possessing homologous sequences can be also substrate(s) of *Legionella* glucosyltransferases and participate in intoxication mechanisms. On the other hand, natural target selection of a region encompassing serine-53 in eEF1A for glucosylation by a microbial effector toxin suggests importance of this area for biological functions of the elongation factor.

To address these issues the budding yeast *Saccharomyces cerevisiae* was used as a model. This is appropriate, because mammalian eEF1A, which is a target of *L. pneumophila* glucosyltransferase Lgt1, shares more than 80% of identical amino acid residues with the yeast analogs elongation factors Tef1 and Tef2. Here we report that Lgt1 is toxic for yeast. This effect depends solely on yeast eEF1A, containing the glucose acceptor serine-53, but not on Hbs1. Moreover, we show that the narrow region of eEF1A, which is recognized by Lgt1, is characterized by several functionally essential amino acid residues.

EXPERIMENTAL PROCEDURES

Strains, vectors, and culture conditions - Cloning and recombinant protein production was performed in *E. coli* DH10B and BL21 (DE3) (Invitrogen, Grand Island, NY, USA). Genomic DNA from strain D273-10B was used as a source for the amplification of *TRP1*, *HIS3* and *LEU2* marker genes. MH272-3fα (*ura3, leu2, his3, trp1, ade2*) or the diploid MH272-3fα/a (*ura3/ura3, leu2/leu2, his3/his3, trp1/trp1, ade2/ade2*) (14) are the parental strains of the mutant *S. cerevisiae* employed in this study (a summary of all strains is given in Table S1). Plasmids for cloning and recombinant protein expression in *E. coli* are based on pUC19 (NEB, Frankfurt am Main, Germany), pBC KS (+), pBluescript KS (+) (Stratagene, Waldbronn, Germany) and pET28a (Novagen, Madison, WI, USA). Yeast expression vectors were constructed in pRS313 (15), pESC-Ura (Stratagene), YEPLac195 (16), YCPLac444 and YEPLac555 (17). A summary of all plasmids is given in Table S2; primers employed for cloning are listed in Table S3.

Yeast strains were grown on rich medium containing glucose (YPD: 1% yeast extract, 2% peptone, 2% glucose) or on minimal medium (SD: 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose; SGal: 0.67% yeast nitrogen base without amino acids , 2% galactose). SD and SGal media were supplemented with the appropriate supplements. Yeast transformations were performed by the lithium acetate method (18).

Purification of Lgt1, Hbs1 and Tef1 - The coding sequence of glucosyltransferase Lgt1 was amplified from genomic DNA of *L. pneumophila* Philadelphia-1 strain (19) and was cloned into the EcoRI/SacI restriction sites of pET28a. The resulting plasmid p558 (Table S2) encodes His-tagged Lgt1 and served as the template for the construction of mutated *lgt1* versions. Lgt1

containing $D^{246}N$ (plasmid p562), $D^{246}N/W^{520}A$ (p563), or $D^{246}N/W^{520}A/N^{293}A$ (p578) were prepared step-wise by QuikChange mutagenesis on p558 (Promega, Mannheim, Germany) using primers #183-#184, #923-#924, or #548-#549 (Table S3) respectively. Yeast expression vectors were constructed by transferring wild type and mutated versions of *lgt1* into pESC-Ura under the control of *GAL* promoter. The resulting pESC-Ura series plasmids p569, p590, p570 and p591 (Table S2) encode for wild type Lgt1, Lgt1-$D^{246}N$, Lgt1-$D^{246}N/W^{520}A$ and Lgt1-$D^{246}N/W^{520}A/N^{293}A$.

Purification of His-tagged Hbs1 from *E. coli* and His-tagged Tef1 (eEF1A) from *S. cerevisiae* was described previously (10,11). Wild type and mutant versions of His-tagged Lgt1 were expressed from plasmids p558, p562, p563, and p578 (Table S2) in *E. coli* BL21 (DE3). Induction was performed overnight with 0.2 mM IPTG at 22ºC. Proteins were subsequently purified via nickel-affinity chromatography using HisTrap columns (GE Healthcare, Moscow, Russia) connected to an ÄKTA Purifier (GE Healthcare) according to the instructions of the manufacturer and stored in 10% glycerol/TBS (Tris-buffered saline – 20 mM Tris-HCl, pH=7.4, 150 mM NaCl) at -20ºC.

Cloning and mutagenesis of TEF1 - *TEF1* (yeast eEF1A) plus 500 bp regions up- and downstream of the *orf* was amplified using primers #518-#492 (Table S3) and yeast genomic DNA as a template. The PCR product was cloned into the BamHI/SalI sites of YEPlac195, YCPlac444 and pRS313. The resulting plasmids are termed p561 (pYE-*TEF1*), p815 (pYC-*TEF1*) and p572 (pRS-*TEF1*) (Table S2). For mutagenesis experiments a portion of *TEF1*, containing the coding sequence of the recognized by Lgt1 decapeptide G^{50}-V^{59} (10), was cut from p572 (pRS-*TEF1*) with SacI/ClaI and was ligated into pBC, resulting in plasmid p574 (Table S2). QuikChange mutagenesis of p574 was employed to generate the following substitutions in Tef1: $G^{50}A$ (p644), $S^{53}A$ (p575), $S^{53}E$ (p688), $S^{53}C$ (p686), $S^{53}K$ (p687), $F^{54}A$ (p579), $K^{55}A$ (p645), $Y^{56}A$ (p580), $W^{58}A$ (p581) and $V^{59}A$ (p668) (Table S2, for primers used see Table S3). SacI/ClaI fragments from these pBC-based mutagenised plasmids were then transferred back to p572, substituting the wild type portion of the gene. The resulting pRS313-based yeast expression plasmids encode Tef1-$G^{50}A$ (p664), Tef1-$S^{53}A$ (p577), Tef1-$S^{53}E$ (p697), Tef1-$S^{53}C$ (p701), Tef1-$S^{53}K$ (p696), Tef1-$F^{54}A$ (p584), Tef1-$K^{55}A$ (p665), Tef1-$Y^{56}A$ (p585), Tef1-$W^{58}A$ (p586) and Tef1-$V^{59}A$ (p670) under the control of *TEF1* promoter. To construct $S^{53}A$ mutated variant of p815 (pYC-*TEF1*), BamHI/SalI fragment from p577 (pRS-*TEF-*$S^{53}A$) was cloned into YCPLac444 to give rise p828 (pYC-*TEF*-$S^{53}A$). Plasmids p815 and p828 were used in co-transformation studies (see Results section and Figure 5).

Generation of Δ*tef1 and* Δ*tef2 deletion strains - tef1::TRP1 (*Δ*tef1*) was constructed by replacing nucleotides 363 to 967 within the *TEF1 orf* with the *TRP1* marker gene. *tef2::LEU2* (Δ*tef2*) was constructed by replacing nucleotides 10 to 1368 within the *TEF2 orf* with the *LEU2* marker gene. The deletion constructs were integrated into the genome of the diploid *S. cerevisiae* MH272a/α strain using standard yeast genetics methods (18). Disruptions were confirmed via PCR using genomic DNA of grown transformants. Due to the fact that the Δ*tef1*Δ*tef2* double mutation is lethal (20), a diploid strain containing one copy of each *tef1::TRP1* and *tef2::LEU2* disrupted genes was transformed with p561 (pYE-*TEF1*) before been sporulated and dissected (Singer Instruments, Somerset, UK). After tetrad analysis a haploid Δ*tef1*Δ*tef2* strain rescued by pYE-*TEF1* was selected (*S. cerevisiae* SC33) and was used for further 5-FOA experiments. pYE-*TEF1* was replaced with pRS313-based plasmids encoding for the different *TEF1* mutants via plasmid shuffling using the 5-FOA method (21). The resulting yeast strains are summarized in Table S1. Description of Δ*tef1* and Δ*tef2* deletion procedure is given in Supplementary results section. Analysis of *tef* deletion strains is presented on Supplementary Figures S1-S3.

Generation of Δ*hbs1 deletion strains - hbs1::HIS3 (*Δ*hbs1*) was constructed by replacing nucleotides 436 to 1212 within the *HBS1 orf* with the *HIS3* marker gene. The construct was transformed into the haploid *S. cerevisiae* MH272α strain. The *hbs1::HIS3* deletion was confirmed by PCR and western blotting with anti-Hbs1 serum and the corresponding strain (*S.*

cerevisiae SC31) was used in subsequent experiments.

Δ*tef1*Δ*tef2*Δ*hbs1* triple deletion strains were constructed in *S. cerevisiae* SC39 (Δ*tef1*Δ*tef2* + pRS-*TEF1*) or *S. cerevisiae* SC40 (Δ*tef1*Δ*tef2* + pRS-*TEF1*-$S^{53}A$) (Table S1). To that end, *HBS1* was replaced with the *hbs1*::*kanMX4* deletion cassette from strain Y16000 (Euroscarf). Recombinant clones were selected on YPD supplemented with 0.4 mg/ml G418 (Invitrogen). The *HBS1* deletion was confirmed by PCR and western blotting with anti-Hbs1 serum and the resulting strains, termed *S. cerevisiae* SC200 (Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1*) and *S. cerevisiae* SC201 (Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1*-$S^{53}A$), were used in subsequent experiments. Analysis of *hbs1* deletion strains is presented on Supplementary Figures S4 and S5.

Yeast in vitro *translation system* - Yeast translation extracts were prepared as described (22). Transcription was performed using SP6 polymerase (22) and pSP-luc+ (Promega) as a template to generate firefly luciferase mRNA. Translation reactions were performed as described (22-24). To test for the ability of Lgt1 to inhibit translation in yeast system, purified His-tagged Lgt1 at 9, 90, or 280 nM final concentrations and 10 µM UDP-glucose as a Lgt1 co-substrate were added to the translational mixes lacking luciferase mRNA. The reactions were pre-incubated for 10 min at 20°C. Translation was started after this preincubation step by adding luciferase mRNA and was allowed to proceed for 50 min at 20ºC. After that, 10 µl of the reaction mix was diluted into 100 µl of luciferase assay buffer (100 mM KH_2PO_4, 1 mM EDTA, 1 mM DTT, pH adjusted to 7.8 with KOH). The diluted reaction mixes (100 µl) were mixed with 100 µl of luciferase reagent (20 mM Tricine, 5 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 270 µM coenzyme A, 500 µM D-luciferin, 500 µM ATP) and luminescence was determined using a Lumat LB 9507 device (Berthold Technologies GmbH, Wildbad, Germany) (25). Luciferase activity obtained in the absence of Lgt1 was set to 100%.

Spot-test assay - The toxicity of Lgt1 in different yeast strains was analyzed on SD or SGal plates. To that end, 5-fold serial dilutions of cultures were spotted onto agar plates with the required supplements and either glucose or galactose as indicated on the corresponding figures. Plates were incubated for the times and at temperatures indicated in the figure legends. For liquid culture experiments 3 ml SGal medium in plastic tubes (cat. number 62.515.006, Sarstedt) was inoculated with yeast cells to a starting OD_{600} of 0.1. Cultures were then incubated at 30ºC for up to 48 h under vigorous shaking.

Production of polyclonal antibodies - Monospecific sera against Lgt1 and Hbs1 were produced by injecting 10 µg of purified recombinant proteins into mice intraperitoneally three times with 4-days intervals. Sera were collected 5 days after the last injection. Monospecific anti-Tef1 serum was produced by GeneScript (Piscataway, NJ, USA) in rabbits by injecting synthesized Tef1-derived peptide (C^{31}GGIDKRTIEKFEK^{44}) conjugated to keyhole limpet hemocyanin (KLH) via the first cysteine residue of the peptides.

Electrophoresis and western blotting - Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and western blotting were performed according to published protocols (26,27). Preparation of yeast cell extracts was accomplished by NaOH/trichloroacetic acid procedure or by glass beads disruption (28).

Glucosyltransferase assay - Glucosylation was performed with 167 pmol of recombinant His-tagged wild type or mutated Lgt1 and 67 pmol of GST-tagged target decapeptide in a total volume of 20 µl (10). The standard reaction was allowed to proceed at 37°C for up to 60 min in 20 mM Tris-HCl, pH=7.5, 150 mM NaCl, 1 mM $MnCl_2$ and 10 µM UDP-[^{14}C]glucose (American Radiolabeled Chemicals, St. Louis, MO, USA). The reaction was stopped at various time points by the addition of SDS-sample buffer and incubation at 95°C for 5 min. Subsequently, samples were subjected to SDS-PAGE and autoradiography. Radiolabeled bands were analyzed by PhosphorImager and quantified with ImageQuant 5.2 (GE Healthcare).

RESULTS

Toxicity of Lgt1 in a wild type S. cerevisiae *background* - At first, we studied whether expression of *lgt1* in the cytosol of *S. cerevisiae* produces toxic effects. To this end, we generated mutated variants of the

glucosyltransferase with gradually decreased enzymatic activity. Such Lgt1 mutations included the single replacement $D^{246}N$, the double substitution $D^{246}N/W^{520}A$ and the triple mutant $D^{246}N/W^{520}A/N^{293}A$. Aspartate-246 represents an important element of the DxD motif and has been shown to participate in orienting the distal part of glucose moiety of the co-factor UDP-glucose. Tryptophan-520 represents a critical residue of a flexible C-terminal loop, which is suggested to fix the co-substrate into the correct position for catalysis. Finally, asparagine-293 appears to be involved in the guidance and/or binding of the protein substrate eEF1A (8,9). The proteins with described mutations were initially purified from recombinant *E. coli* cultures and tested in the ^{14}C-glucosylation assay. As shown in Fig. 1A, substitution of $D^{246}N$ resulted in a drastic decrease of glucosylation activity of Lgt1. The double $D^{246}N/W^{520}A$ mutant demonstrated further diminished enzymatic activity, while the triple mutation produced a protein with catalytic activity below the detection limit of the method.

Next, coding sequences of the wild type and mutated Lgt1 variants were cloned into a galactose inducible yeast expression vector and were transferred into wild type *S. cerevisiae*. Wild type Lgt1 was severely toxic and resulted in cell death upon induction in the presence of galactose (Fig. 1B). Yeast cells demonstrated a slow growth even under glucose-repressing conditions, possibly due to "promoter leakage". Similar to wild type Lgt1, the enzyme with the $D^{246}N$ substitution also inhibited *S. cerevisiae* plated on galactose-containing medium, while growth on glucose-supplemented agar was almost completely restored. Decreasing enzymatic activity in the double $D^{246}N/W^{520}A$ and the triple $D^{246}N/W^{520}A/N^{293}A$ mutants paralleled with a diminished toxic phenotype (Fig. 1B). However, even the triple Lgt1 mutant exhibited reduced growth on galactose-containing media as compared with control yeast transformed with the empty pESC-Ura vector (compare the upper and lower rows on Fig. 1B).

Next, we tested Lgt1 production by western blotting, using anti-Lgt1 serum. As expected no protein production was observed when yeast strains were cultivated in the presence of glucose (Fig. 1C, lanes 1-5). Wild-type Lgt1 and Lgt1-$D^{246}N$ were also not detected when the respective strains were cultivated in the presence of galactose (Fig. 1C, lanes 7, 8). Low toxicity of the double and triple mutants allowed production of Lgt1, detected by western blotting (Fig. 1C, lanes 9, 10).

Using a reticulocyte-based translation system, we recently showed that Lgt1 effectively blocks mammalian protein synthesis *in vitro* (12,13). Similar results were obtained, when translation extracts from *S. cerevisiae* were programmed with mRNA coding for firefly luciferase. In the presence of UDP-glucose, increasing concentrations of purified Lgt1 resulted in a dose-dependent inhibition of luciferase mRNA translation, observed already at 9 nM Lgt1 (Fig. 2A, column 2). Inhibition of translation was paralleled by glucosylation of Tef1/2 (the homologs of elongation factor 1A in yeast) as shown in ^{14}C-glucosylation assay (Fig. 2B).

Engineering of S. cerevisiae *strains with mutations in elongation factor 1A gene* - Because Lgt1 has been reported to use eEF1A as protein substrate both *in vitro* and *in vivo* through the recognition of a 10-aa peptide (10) we wanted to know whether amino acid residues, comprising this recognition sequence, were essential for eEF1A function. Therefore, we engineered *S. cerevisiae* strains by disrupting both chromosomal copies of eEF1A (*TEF1* and *TEF2*). The lethal phenotype of the Δ*tef1*Δ*tef2* mutation was rescued by plasmid encoded *TEF1* or mutated versions of it (see *Experimental Procedures* and *Supplementary results* section).

As shown in our previous investigations with mammalian eEF1A, substitution of the glucose-accepting serine-53 of the elongation factor by an alanine residue resulted in a variant protein, which is not glucosylated by Lgt1 *in vitro* (12). Moreover, $F^{54}A$, $Y^{56}A$ and $W^{58}A$ substitutions severely affected glucosylation efficiency of mutated eEF1A-derived peptides, whereas $G^{50}A$, $K^{51}A$, $G^{52}A/K^{55}A$ and $V^{59}A$ produced only minor effects (10).

Based on this information, we developed a panel of pRS313-based plasmids, containing as an insert *TEF1-WT*, *TEF1-*$G^{50}A$, *TEF1-*$S^{53}A$, *TEF1-*$S^{53}E$, *TEF1-*$S^{53}C$, *TEF1-*$S^{53}K$, *TEF1-*$F^{54}A$, *TEF1-*$K^{55}A$, *TEF1-*$Y^{56}A$, *TEF1-*$W^{58}A$ and *TEF1-*$V^{59}A$ under the control of *TEF1* promoter. These constructs were transformed into *S. cerevisiae* (Δ*tef1*Δ*tef2* + pYE-*TEF1*). After cultivation of

yeast on 5-FOA-supplemented medium, causing elimination of *URA3*-containing pYE-*TEF1*, plates were inspected for growth of the resulting strains. As expected, *S. cerevisiae*, containing pRS313 vector without the *TEF1* insert, failed to grow (Fig 3A), while the strain with the wild-type *TEF1* produced numerous colonies on the plate. Surprisingly, yeast strains, containing eEF1A with $S^{53}A$, $S^{53}C$ and $S^{53}K$ mutations were viable, while substitution of $S^{53}E$ was lethal for *S. cerevisiae*. Remarkably, analysis of the growth phenotype in the other mutated strains displayed critical importance for functional activity of eEF1A amino acid residues Y^{56} and W^{58}, substitutions which by alanine residues precluded growth of yeast. In addition, a strain with $F^{54}A$ substitution demonstrated growth on agar plates under favorable conditions, but displayed a growth-limiting phenotype under conditions of cold- and heat stress (Fig. 3B). By contrast, alanine substitutions of G^{50}, K^{55} and V^{59} in Tef1 failed to produce growth defects under the cultivation conditions tested.

Toxicity of Lgt1 in S. cerevisiae *Tef1-mutants* – As demonstrated in the above experiments yeast expressing exclusively *TEF1-$S^{53}A$* was viable. Therefore, we were able to test the toxic activity of Lgt1 in this yeast mutant, in which eEF1A could not be modified by Lgt1. To this end, *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1*) and (Δ*tef1*Δ*tef2* + pRS-*TEF1-$S^{53}A$*) strains were transformed with galactose-inducible *lgt1*-containing plasmids. In line with our previous findings with wild-type *S. cerevisiae*, transformation of *lgt1* into yeast strain (Δ*tef1*Δ*tef2* + pRS-*TEF1*) produced a lethal effect on galactose-containing medium and a partial defect on glucose-containing agar. *lgt1-$D^{246}A/W^{520}A$* was less toxic when expressed in this yeast variant (Fig. 4A). In contrast, *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1-$S^{53}A$*) was protected against the toxic activity of Lgt1 and demonstrated an improved survival rate (Fig. 4A).

Western blotting analysis with anti-Lgt1 serum demonstrated that good growth of *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1-$S^{53}A$*) was accompanied by high level of Lgt1 production, while even undetectable amounts of the wild type glucosyltransferase efficiently killed yeast strain (Δ*tef1*Δ*tef2* + pRS-*TEF1*) (Fig. 4B, compare lanes 2 and 6).

To confirm that Lgt1 was not only produced but was fully functional in *S. cerevisiae* with plasmid-born Tef1-$S^{53}A$, we prepared extracts of yeast cells and tested them in the ^{14}C-glucosylation assay. As shown in Fig. 4C, extracts obtained from *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1-$S^{53}A$*) displayed no endogenous glucosylation of eEF1A in line with the fact that the major substrate Tef1 did not contain the acceptor serine-53 in this strain. However, addition of purified wild type Tef1 to the reaction mix resulted in ^{14}C-glucosylation of the substrate.

Next we investigated whether expression of *TEF1-$S^{53}A$* would rescue the lethal effect of *lgt1* expression in the strain (Δ*tef1*Δ*tef2* + pRS-*TEF1*). To that end, we transformed *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1*) with pYC-*TEF1-$S^{53}A$*, or as control with pYC-*TEF1*. Then, these strains were transformed with the plasmid, containing wild type *lgt1* under the control of galactose promoter or, as a control, with pESC-Ura vector. All four engineered *S. cerevisiae* strains were tested for growth on galactose-containing liquid and solid media.

As illustrated on Fig. 5, additional copies of Tef1-$S^{53}A$ reduced the lethal phenotype in comparison to the strain expressing an additional copy of wild type Tef1. The rescued phenotype was observed even in spite of high production of Lgt1, detected in western blotting with anti-Lgt1 serum (Fig. 5C). Semi-quantitative investigation of Lgt1-positive yeast extract in western blotting suggested that single *S. cerevisiae* cell may contain up to 160 000 molecules of the glucosyltransferase (Fig. S1).

Toxicity of Lgt1 in HBS1-*deleted* S. cerevisiae - The experiments described above indicated that glucosylation of eEF1A causes a toxic effect in the *S. cerevisiae* model. However, to clarify whether modification of the other Lgt1 substrate, protein Hbs1, also influences yeast cell growth, we generated Δ*hbs1 S. cerevisiae* strains by disrupting the gene either with the *HIS3* marker or with geneticin antibiotic cassette. Next, we transformed the wild type or mutated *lgt1* genes into the engineered *S. cerevisiae* strains and compared the toxic effects observed in Δ*hbs1*

strains with that of the yeast strains containing an intact copy of chromosomal *HBS1*.

Expression of galactose-inducible *lgt1-D^{246}N* resulted in a lethal phenotype in the wild type as well as in the Δ*hbs1* strain on galactose- but not on glucose-containing media (Fig. 6). Lgt1-D^{246}N/W^{520}A, the enzymatically less active glucosyltransferase mutant, produced moderate toxic effects in *S. cerevisiae* also irrespective of the presence or absence of *HBS1*. These results indicate that deletion of *HBS1* in a wild type yeast background, i.e. in *S. cerevisiae* containing intact chromosomal *TEF1*/*TEF2* genes, did not influence toxic effects caused by Lgt1.

Next we studied the impact of Hbs1 upon Lgt1-caused toxicity in *S. cerevisiae* with plasmid-born Tef1-S^{53}A or Tef1-WT as sole elongation factors, i.e. in the yeast strains (Δ*tef1*Δ*tef2* + pRS-*TEF1*) and (Δ*tef1*Δ*tef2* + pRS-*TEF1-S^{53}A*). As shown in Fig. 7, deletion of *HBS1* did not alter the growth phenotypes of and Lgt1 production by the corresponding Hbs1-engineered *S. cerevisiae*. Strains without *HBS1* but containing wild type *TEF1* on a plasmid were efficiently killed by Lgt1-WT. In contrast, *HBS1*-deleted strains containing Tef1-S^{53}A were protected against lethal effect of the highly produced wild type glucosyltransferase (Fig. 7C, lane 4) similar to that of *HBS1*-containing *S. cerevisiae* (Fig. 4B, lane 6). Interestingly, the *S. cerevisiae* strain lacking Hbs1 and containing non-modifiable eEF1A (i.e. lacking all known catalytic substrates for glucosyltransferases of *L. pneumophila*), still demonstrated a moderate growth-limiting phenotype even in the presence of triple-mutated Lgt1-D^{246}N/W^{520}A/N^{293}A (Fig. 7B, *lgt1-mut*, lower row).

DISCUSSION

As a first step towards elucidation of *S. cerevisiae* as a model to study the toxic activity of *Legionella* glucosylating enzymes, we checked the effects of Lgt1 in *in vitro* translation using yeast extract. Addition of purified glucosyltransferase to *S. cerevisiae* translational extract inhibited protein synthesis in a dose-dependent manner. These results were similar to those obtained with mammalian reticulocyte lysates recently (12,13). Also similar to experiments with mammalian cells, expression of *lgt1* in the cytosol of *S. cerevisiae* resulted in severe toxic effects. A slight inhibitory growth phenotype was observed already in the presence of glucose in the medium (i.e. under non-inducing conditions) precluding over-expression of the recombinant protein. Induction of *lgt1* gene with galactose completely stopped growth of yeast. Such inhibition was observed in spite of very low synthesis of the toxic protein, because Lgt1 was not detected by western blotting with the monospecific anti-Lgt1 serum. Transformation of yeast with mutated *lgt1* genes produced toxic effects proportional to glucosylation activity of the proteins. These results demonstrated that *S. cerevisiae* is an adequate model to study Lgt activity in intact cells.

As shown in our previous studies, substitution of serine-53 by alanine turned elongation factor into a protein not modifiable *in vitro* by *L. pneumophila* glucosyltransferases Lgt (12,13). In the current study we engineered *S. cerevisiae* strains, which contained such non-modifiable Tef1-S^{53}A as the only elongation factor present. In contrast to *S. cerevisiae* with modifiable wild type Tef1, Tef1-S^{53}A-containing yeast cells were protected towards toxic action of Lgt1. The positive impact of Tef1-S^{53}A upon survival of *S. cerevisiae* is even more evident when the high level of Lgt1 production in the Tef1-S^{53}A-possessing yeast strains is compared to the very low amount of toxic protein that produced lethal effect in yeast with wild type eEF1A. These results demonstrated that eEF1A is a major target responsible for toxic effect after its glucosylation by Lgt1.

Another substrate of *L. pneumophila* glucosyltransferases, Hbs1 (Hsp70 subfamily B suppressor 1 (29)), was shown to be modified by *in vitro* glucosylation. For Hbs1 the target amino acid residue was identified as serine-314 (Hbs1-like protein of human origin, GenBank accession number AK292656) or the corresponding serine-213 of Hbs1 in *S.cerevisiae* (10).

Both substrates of Lgt, eEF1A and Hbs1, represent important players in translational processes in eukaryotes. eEF1A is necessary for active translation and represents an essential protein. In contrast, disruption of *HBS1* does not cause an obvious phenotype, but the protein has been shown to play an important role during situations of translational stalls due to inhibitory structures (e.g. strong hairpin loops), premature or lacking stop codons and other defects of mRNA or

deleterious mutations in rRNA (30,31). So far a role of Hbs1 in toxic effects of Lgt1 was not clarified. To address this issue, we deleted the *HBS1* gene in wild type *S. cerevisiae* or in *S. cerevisiae* containing Tef1-S^{53}A. However, when such deletion strains were transformed with the *lgt1* gene, toxic effects produced by Lgt1 did not differ considerably from that of control background strains, containing Hbs1. Thus, these findings indicate that modification ofHbs1 is not essential for toxicity.

Of interest is our observation that triple mutant of Lgt1, possessing undetectable level of glucosyltransferase activity, still demonstrated noticeable growth-limiting effect towards *S. cerevisiae* (*TEF1-S^{53}A*, *Δhbs1*) variant. Such effect cannot be explained simply by non-specific action of overproduced foreign protein upon cell metabolism since control yeast strain, transformed with the similarly highly expressed luciferase gene-containing plasmid (Fig. 7B) or hyper-producing fragments of Lgt1 (data not shown), did not demonstrate decrease in growth. One possible explanation for the mechanism of the observed phenomenon can be non-productive interaction of catalytically inactive glucosyltransferase with its enzymatically incompetent Tef1-S^{53}A substrate leading to restraint of eEF1A functions in yeast cells. However, so far we did not observe a strong non-productive interaction of Lgts with eEF1A in mammalian systems.

Elongation factor 1A is necessary for delivery of aminoacylated tRNA molecules to the A-site of a translating ribosome (32). In spite of thorough and long lasting investigations many aspects of mechanisms of its participation in protein synthesis and in other processes in eukaryotic cells remain unanswered. eEF1A has undergone thorough mutational analysis to discover amino acid residues important for its functioning in yeast (33-38). These studies were successful in identifying mutations that affected fidelity of translation, dependence upon nucleotide exchange factor, rate of GTP binding and GTP hydrolysis etc. Few of the obtained mutations resulted in non-functional eEF1A, producing non-viable phenotype of *S. cerevisiae*. Although mechanisms of lethality remain unexplained, the obtained results allow identification of vitally important regions of the protein.

We studied the decapeptide G^{50}-V^{59} of Tef1, which includes Lgt1-modifiable serine-53. So far this region remained a largely unexplored segment of eEF1A. By using yeast strains with mutated Tef1 as the only elongation factor 1A, we identified various amino acid residues within this area, which appear to be essential, as their mutation to alanine resulted in severely affected or even non-viable yeast. Such essential amino acids include F^{54}, Y^{56} and W^{58}.

Currently we are not able to explain the mechanisms of the functional incompetence of the mutated forms of elongation factor 1A. Strikingly, in previous studies we identified the very similar aromatic residues as absolutely important for substrate recognition of eEF1A by Lgt1 (10). Thus, it is likely that the mutations in F^{54}, Y^{56} and W^{58} result in structural changes or alterations, which similarly affect cellular function of eEF1A in translation and in recognition by Lgt1. Thus, this region around serine-53 appears to be a hot spot for functional efficiency of eEF1A.

Available genome sequencing data allow computer analysis of this area (Table S5). Interestingly, representatives of more than 40 species of mammals, fish, insects, nematodes demonstrated strict sequence conservation in this region and contained the typical decapeptide G^{50}KGSFKYAWV^{59}. Sequences of eEF1A of plant origin also demonstrated considerable homology. However structure of this region contained G^{50}N and G^{52}R substitutions. Archaeal phyla displayed considerable variations in elongation factor 1A sequences. Whereas in few examples F^{54} was substituted by non-aromatic amino acid residues, positions 56 and 58 of the protein were occupied exclusively by phenylalanine/tyrosine/tryptophan residues. Thus, *in silico* data demonstrated evolutionary conservation of the decapeptide structure and supported in general our yeast mutational experiments on essential roles of aromatic amino acid residues at positions 54, 56 and 58 in elongation factor 1A.

Of interest is also the finding that substitution of serine-53 with alanine, cysteine or lysine residues did not considerably affect the growth phenotype of engineered yeast. However substitution S^{53}E resulted in non-functional Tef1 failing to support growth of *S. cerevisiae*.

In numerous published papers substitution of serine in a protein by negatively charged glutamate residue has been attributed to as "pseudophosphorylation" (39-41). Thus, our data leads to the assumption that the modification of serine-53 residue by phosphorylation can shut-down protein synthesis. Interestingly, in a recent report phosphorylation of serine-53 has been demonstrated by mass-spectrometry approach although physiological consequences of such modification remain to be determined (42).

REFERENCES

1. McDade, J. E., Shepard, C. C., Fraser, D. W., Tsai, T. R., Redus, M. A., and Dowdle, W. R. (1977) *N. Engl. J. Med.* **297,** 1197-1203
2. Fraser, D. W., Tsai, T. R., Orenstein, W., Parkin, W. E., Beecham, H. J., Sharrar, R. G., Harris, J., Mallison, G. F., Martin, S. M., McDade, J. E., Shepard, C. C., and Brachman, P. S. (1977) *N. Engl. J. Med.* **297,** 1189-1197
3. Jules, M. and Buchrieser, C. (2007) *FEBS Lett.* **581,** 2829-2838
4. Ensminger, A. W. and Isberg, R. R. (2009) *Curr. Opin. Microbiol.* **12,** 67-73
5. Hubber, A. and Roy, C. R. (2010) *Annu. Rev. Cell Dev. Biol.* **26,** 261-283
6. Belyi, Y., Jank, T., and Aktories, K. (2011) *Front Microbiol.* **2,** 76
7. Belyi, I., Popoff, M. R., and Cianciotto, N. P. (2003) *Infect. Immun.* **71,** 181-186
8. Hurtado-Guerrero, R., Zusman, T., Pathak, S., Ibrahim, A. F., Shepherd, S., Prescott, A., Segal, G., and van Aalten, D. M. (2010) *Biochem. J.* **426,** 281-292
9. Lu, W., Du, J., Stahl, M., Tzivelekidis, T., Belyi, Y., Gerhardt, S., Aktories, K., and Einsle, O. (2010) *J. Mol. Biol.* **396,** 321-331
10. Belyi, Y., Stahl, M., Sovkova, I., Kaden, P., Luy, B., and Aktories, K. (2009) *JBC* **284,** 20167-20174
11. Tzivelekidis, T., Jank, T., Pohl, C., Schlosser, A., Rospert, S., Knudsen, C. R., Rodnina, M. V., Belyi, Y., and Aktories, K. (2011) *PLoS. One.* **6,** e29525
12. Belyi, Y., Niggeweg, R., Opitz, B., Vogelsgesang, M., Hippenstiel, S., Wilm, M., and Aktories, K. (2006) *Proc. Natl. Acad. Sci. U. S. A* **103,** 16953-16958
13. Belyi, Y., Tabakova, I., Stahl, M., and Aktories, K. (2008) *J. Bacteriol.* **190,** 3026-3035
14. Heitman, J., Movva, N. R., Hiestand, P. C., and Hall, M. N. (1991) *Proc. Natl. Acad. Sci. U. S. A* **88,** 1948-1952
15. Sikorski, R. S. and Hieter, P. (1989) *Genetics* **122,** 19-27
16. Gietz, R. D. and Sugino, A. (1988) *Gene* **74,** 527-534
17. Conz, C., Otto, H., Peisker, K., Gautschi, M., Wolfle, T., Mayer, M. P., and Rospert, S. (2007) *JBC* **282,** 33977-33984
18. Sherman, F. (2002) *Methods Enzymol.* **350,** 3-41
19. Brenner, D. J., Steigerwalt, A. G., and McDade, J. E. (1979) *Ann. Intern. Med.* **90,** 656-658
20. Cavallius, J. and Merrick, W. C. (1998) *JBC* **273,** 28752-28758
21. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) *Methods Enzymol.* **154,** 164-175
22. Garcia, P. D., Hansen, W., and Walter, P. (1991) *Methods Enzymol.* **194,** 675-682
23. Funfschilling, U. and Rospert, S. (1999) *Mol. Biol. Cell* **10,** 3289-3299
24. Berndt, U., Oellerer, S., Zhang, Y., Johnson, A. E., and Rospert, S. (2009) *Proc. Natl. Acad. Sci. U. S. A* **106,** 1398-1403
25. Rakwalska, M. and Rospert, S. (2004) *Mol. Cell Biol.* **24,** 9186-9197
26. Laemmli, U. K. (1970) *Nature* **227,** 680-685
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A* **76,** 4350-4354
28. Yaffe, M. P. and Schatz, G. (1984) *Proc. Natl. Acad. Sci. U. S. A* **81,** 4819-4823
29. Nelson, R. J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., and Craig, E. A. (1992) *Cell* **71,** 97-105
30. Chen, L., Muhlrad, D., Hauryliuk, V., Cheng, Z., Lim, M. K., Shyp, V., Parker, R., and Song, H. (2010) *Nat. Struct. Mol. Biol.* **17,** 1233-1240
31. Cole, S. E., LaRiviere, F. J., Merrikh, C. N., and Moore, M. J. (2009) *Mol. Cell* **34,** 440-450
32. Ramakrishnan, V. (2002) *Cell* **108,** 557-572
33. Carr-Schmid, A., Pfund, C., Craig, E. A., and Kinzy, T. G. (2002) *Mol. Cell Biol.* **22,** 2564-2574
34. Cavallius, J. and Merrick, W. C. (1998) *JBC* **273,** 28752-28758
35. Dinman, J. D. and Kinzy, T. G. (1997) *RNA.* **3,** 870-881

36. Magazinnik, T., Anand, M., Sattlegger, E., Hinnebusch, A. G., and Kinzy, T. G. (2005) *Nucleic Acids Res.* **33,** 4584-4592
37. Ozturk, S. B., Vishnu, M. R., Olarewaju, O., Starita, L. M., Masison, D. C., and Kinzy, T. G. (2006) *Genetics* **174,** 651-663
38. Ozturk, S. B. and Kinzy, T. G. (2008) *JBC* **283,** 23244-23253
39. Yang, Y., Craig, T. J., Chen, X., Ciufo, L. F., Takahashi, M., Morgan, A., and Gillis, K. D. (2007) *J. Gen. Physiol* **129,** 233-244
40. Rankin, C. A., Sun, Q., and Gamblin, T. C. (2005) *Brain Res. Mol. Brain Res.* **138,** 84-93
41. Eidenmuller, J., Fath, T., Maas, T., Pool, M., Sontag, E., and Brandt, R. (2001) *Biochem. J.* **357,** 759-767
42. Piazzi, M., Bavelloni, A., Faenza, I., Blalock, W., Urbani, A., D'Aguanno, S., Fiume, R., Ramazzotti, G., Maraldi, N. M., and Cocco, L. (2010) *Mol. Cell Proteomics.* **9,** 2719-2728
43. Gietz, R. D. and Schiestl, R. H. (2007) *Nat. Protoc.* **2,** 1-4

ACKNOWLEDGMENTS
This work was supported by the Deutsche Forschungsgemeinschaft and the Center for Biological Signalling Studies (BIOSS) in Freiburg (Germany).

FOOTNOTES
[#] Authors contributed equally to this work.

[4]The abbreviations used are:
eEF1A, eukaryotic elongation factor 1A
Hbs1, Hsp70 subfamily B suppressor 1
UDP-glucose, uridine diphospho-glucose
IPTG, Isopropyl-β-D-thiogalactopyranoside
KLH, keyhole limpet hemocyanin
GST, glutathione- S-transferase
orf, open reading frame
SD, yeast synthetic medium with glucose
SGal, yeast synthetic medium with galactose

FIGURE LEGENDS

FIGURE 1. Toxic effects of Lgt1 in wild type yeast.
(A). ^{14}C-glucosylation of GST-tagged target decapeptide G^{50}KGSFKYAWV^{59} of eEF1A by wild type Lgt1 (circles), Lgt1 with D^{246}N substitution (diamonds), Lgt1 with D^{246}N/W^{520}A substitutions (squares) and Lgt1 with D^{246}N/W^{520}A/N^{293}A substitutions (triangles). The amount of the ^{14}C-glucosylated eEF1A-derived peptide at indicated time periods is given. Left and right panels represent graphs with different Y-scales to accommodate different glucosylation activities of wild type Lgt1 and its mutated forms. Reaction with the wild type Lgt1 is not shown on the right panel. (B). Spot-test assay of Lgt1-accomplished yeast toxicity. *S. cerevisiae* MH272α was transformed with the vector pESC-Ura or pESC-Ura-based plasmids, coding for different variants of Lgt1 (wild type and mutated), titrated 5-fold and spotted onto supplemented SD agar (with glucose, Glc) or SGal agar (with galactose, Gal). Variants of proteins, coded by the plasmids, are indicated on the left. (C). Western blotting analysis of Lgt1 production in yeast, transformed with vector pESC-Ura or pESC-Ura-based plasmids, coding for different variants of Lgt1 (wild type and mutated). *S. cerevisiae* were cultivated in liquid SD (i.e. with glucose, lanes 1-5) or SGal (i.e. with galactose, lanes 6-10). Lanes 1 and 6, pESC-Ura; lanes 2 and 7, *lgt1-WT*; lanes 3 and 8, *lgt1-D^{246}N*; lanes 4 and 9, *lgt1-D^{246}N/W^{520}A*; lanes 5 and 10, *lgt1-D^{246}N/W^{520}A/N^{293}A*. Nitrocellulose membranes were probed with anti-Lgt1 serum overnight at 4ºC and anti-mouse horseradish peroxidase conjugate for 1h at 22ºC. The position of reacting protein bands is marked by an asterisk.

FIGURE 2. Influence of Lgt1 upon protein synthesis in *S. cerevisiae* translation extracts. (A). Synthesis of luciferase in yeast translation extracts in the absence (bars 1 and 5) or presence of 9 nM (bar 2), 90 nM (bar 3) and 280 nM (bar 4) Lgt1. Bar 5 – experiment without luciferase-coding mRNA (i.e. no translation). Each bar represents means of two experiments +/- SD. Data are shown as percentage of maximal translation without added Lgt1. (B). Modification of eEF1A in yeast translational extracts by Lgt1. The reaction conditions were exactly the same as for *in vitro* translation but with 10 μM UDP-^{14}C-glucose instead of unlabeled UDP-glucose. Subsequently, the reaction mix was subjected to SDS-PAGE and autoradiography. Lanes 1 and 2, 9 nM Lgt1; lanes 3 and 4, 90 nM Lgt1; lanes 5 and 6, 280 nM Lgt1. Lanes 1, 3 and 5, incubation time 10 min; lane 2, 4 and 6, incubation time 60 min.

FIGURE 3. Growth phenotypes of yeast strains expressing wild type (WT) and mutated *TEF1*. (A). Δ*tef1*Δ*tef2* yeast strain expressing *TEF1* on a *URA3*-containing plasmid p561 (pYE-*TEF1*) was transformed with different variants of *TEF1* cloned into pRS313. Strains harboring both plasmids were cultivated on 5-FOA-containing plates for 1 week at 30°C. Only strains which had lost the *URA3*-containing plasmid and in addition harbored a functional variant of *TEF1* as an insert in pRS313 vector were able to form colonies. Constructions resulting in non-functional Tef1 are shown in bold letters. The experiment was repeated twice with identical results. Representative plates are shown. (B). Spot-test assay of growth phenotypes of selected *TEF1* mutants grown on YPD at 30ºC, 10ºC, or 40ºC and in the presence of 0.9 M NaCl. *TEF1* mutants, encoded on pRS313-based plasmids, are shown on the left.

FIGURE 4. Toxic effect of Lgt1 in *S. cerevisiae* variants expressing wild type and mutated *TEF1* genes. (A). Spot-test assay of growth phenotypes of *S. cerevisiae* containing plasmid-born wild type Tef1 and Tef1-S^{53}A as the only eEF1A present in yeast cells. Yeast variants were transformed with pESC-Ura or pESC-Ura-based plasmids coding for wild type *lgt1* and *lgt1*-D^{246}A/W^{520}A. Strains were analyzed as described in Experimental Procedures on SD (left panel, glucose, Glc) and SGal (right panel, galactose, Gal). The transformed variants of *lgt1* and types of Tef1 in recipient yeast strains are indicated on the left. (B). Western blotting, demonstrating Lgt1 production in *S. cerevisiae* strains transformed by pESC-Ura-based plasmids coding for wild type *lgt1* and *lgt1*-D^{246}A/W^{520}A. Yeast cultures were grown in SD liquid medium (i.e. with glucose, lanes 1, 3, 5, 7) or SGal (i.e. with galactose, lanes 2, 4, 6, 8). Lanes 1 and 2, *lgt1-WT* in *S. cerevisiae* expressing wild type *TEF1*; lane 3 and 4, *lgt1-D^{246}A/W^{520}A* in *S. cerevisiae*

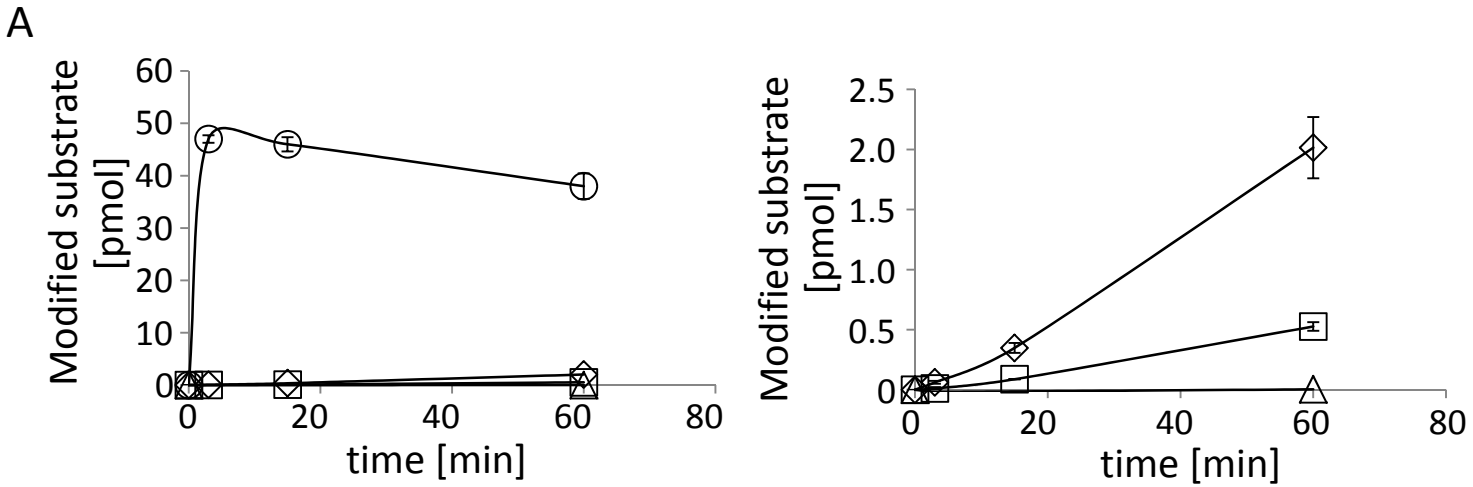
expressing wild type *TEF1*; lanes 5 and 6, *lgt1* in *S. cerevisiae* expressing *TEF1-S^{53}A*; lane 7 and 8, *lgt1-D^{246}A/W^{520}A* in *S. cerevisiae* expressing *Tef1-S^{53}A*. Western blotting was probed with anti-Lgt1 serum. The position of the glucosyltransferase is labeled with an asterisk. (C). *In vitro* glucosylation of yeast extract prepared from *S. cerevisiae* containing Tef1-S^{53}A and transformed with wild type *lgt1*. After cultivation in SGal, yeast cells were collected, disrupted by glass beads and tested for Lgt1-dependent glucosylation in the ^{14}C-glucosylation assay without (lane 1) and with (lane 2) addition of purified His-tagged Tef1. Coomassie stained purified His-tagged Tef1 (lane 3) and molecular mass markers (lane 4) are shown. Molecular masses of used markers are shown on the right. The position of a protein band representing glucosylated Tef1 is labeled with an asterisk.

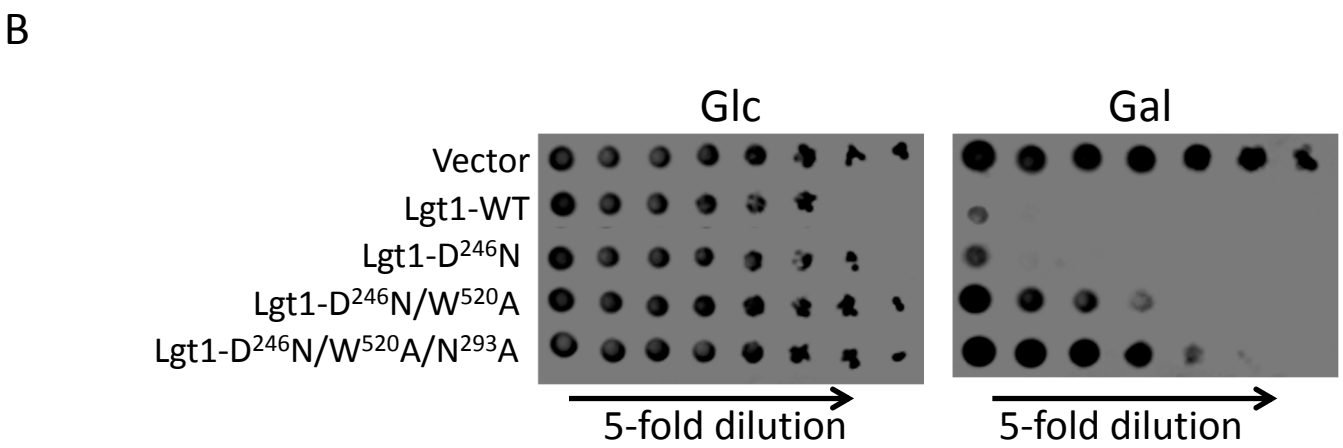
FIGURE 5. Rescue of toxic effects of Lgt1 in the *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1-WT*) strain by pYC-*TEF1-S^{53}A*. (A). Growth of yeast strains in liquid SGal. *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1-WT*) co-transformed by pESC-Ura and pYC-*TEF-WT* (squares), by pESC-Ura and pYC-*TEF1- S^{53}A* (diamonds), by pESC-*lgt1* and pYC-*TEF1-WT* (triangles), or by pESC-*lgt1* and pYC-*TEF1- S^{53}A* (circles). (B). Spot-test assay of strains as in (A) on SD (left panel, glucose, Glc) or SGal (right panel, galactose, Gal). (C). Lgt1 production in *S. cerevisiae* strains grown in SGal. *S. cerevisiae* (Δ*tef1*Δ*tef2* + pRS-*TEF1-WT*) co-transformed by pESC-Ura and pYC-*TEF-WT* (lane 1), by pESC-Ura and pYC-*TEF1-S^{53}A* (lane 2), by pESC-*lgt1* and pYC-*TEF1-WT* (lane 3), and by pESC-*lgt1* and pYC-*TEF1- S^{53}A* (lane 4). Western blottings were probed with anti-Lgt1 serum (upper panel) or anti-eEF1A antibody (lower panel).

FIGURE 6. Toxicity of Lgt1 in wild type and Δ*hbs1* strains. Wild type and Δ*hbs1 S. cerevisiae*, containing pESC-Ura or *lgt1-D^{246}N* or *lgt1-D^{246}N/W^{520}A* within pESC-Ura-based plasmids, were analyzed on SD (left panel, glucose, Glc) or SGal (right panel, galactose, Gal). Description of strains is indicated on the left.

FIGURE 7. Toxicity of Lgt1 in Δ*hbs1*, expressing exclusively plasmid-born *TEF1-WT* or *TEF1-S^{53}A*. Growth of *S. cerevisiae* (Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1-S^{53}A*) harboring pESC-Ura, pESC-Ura-coded *lgt1*, or pESC-Ura-coded *lgt1-D^{246}N/W^{520}A/N^{293}A* was compared to growth of the corresponding strain expressing wild type *HBS1*. (A). Growth of engineered yeast strains in liquid SGal. Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1*+ *lgt1* (closed triangles), Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1-S^{53}A* + pESC-Ura (circles), Δ*tef1*Δ*tef2* + pRS-*TEF1-S^{53}A* + *lgt1* (squares) and Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1-S^{53}A* + *lgt1* (open triangles). (B). Growth phenotypes of yeast strains on SD and SGal plates. Description of strains is indicated on the left. *lgt1-mut* stands for *lgt1-D^{246}N/W^{520}A/N^{293}A*. A strain expressing firefly luciferase (*lucifer*) served as an additional control. (C). Expression of *lgt1* in SGal by engineered *S. cerevisiae* strains: Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1*+ *lgt1* (lane 1), Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1* + *lgt1- D^{246}N/W^{520}A/N^{293}A* (lane 2), Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1-S^{53}A* + pESC-Ura (lane 3), Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1-S^{53}A* + *lgt1* (lane 4), and Δ*tef1*Δ*tef2*Δ*hbs1* + pRS-*TEF1-S^{53}A* + *lgt1-D^{246}N/W^{520}A/N^{293}A* (lane 5). Western blottings were probed with anti-Lgt1 serum (upper panel) or anti-eEF1A antibody (lower panel).

Fig. 1





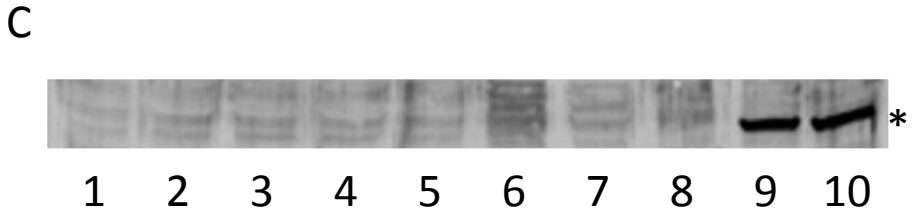


Fig. 2

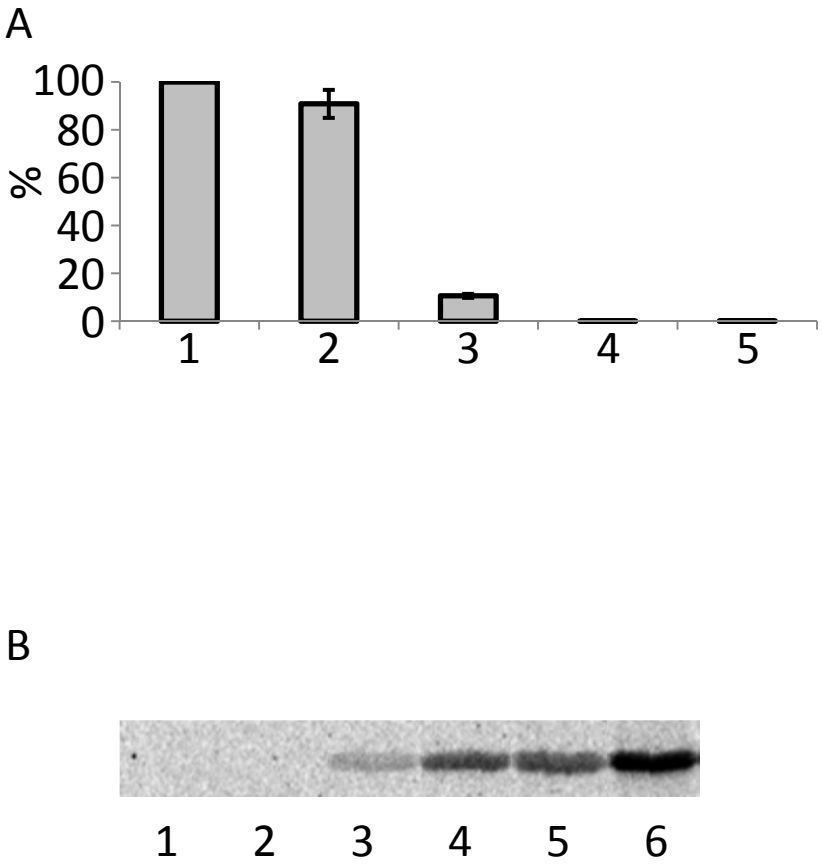


Fig. 3

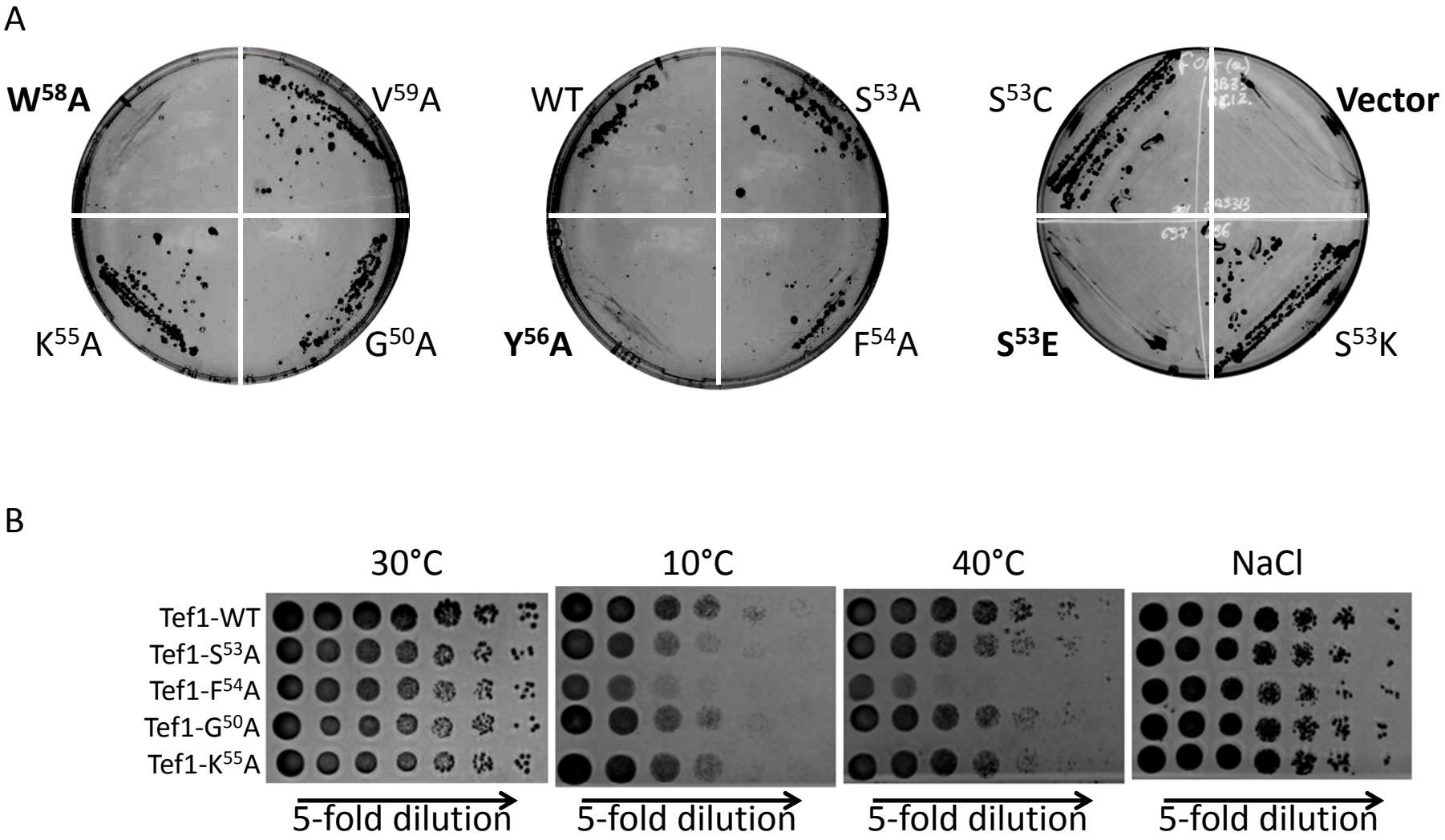


Fig. 4

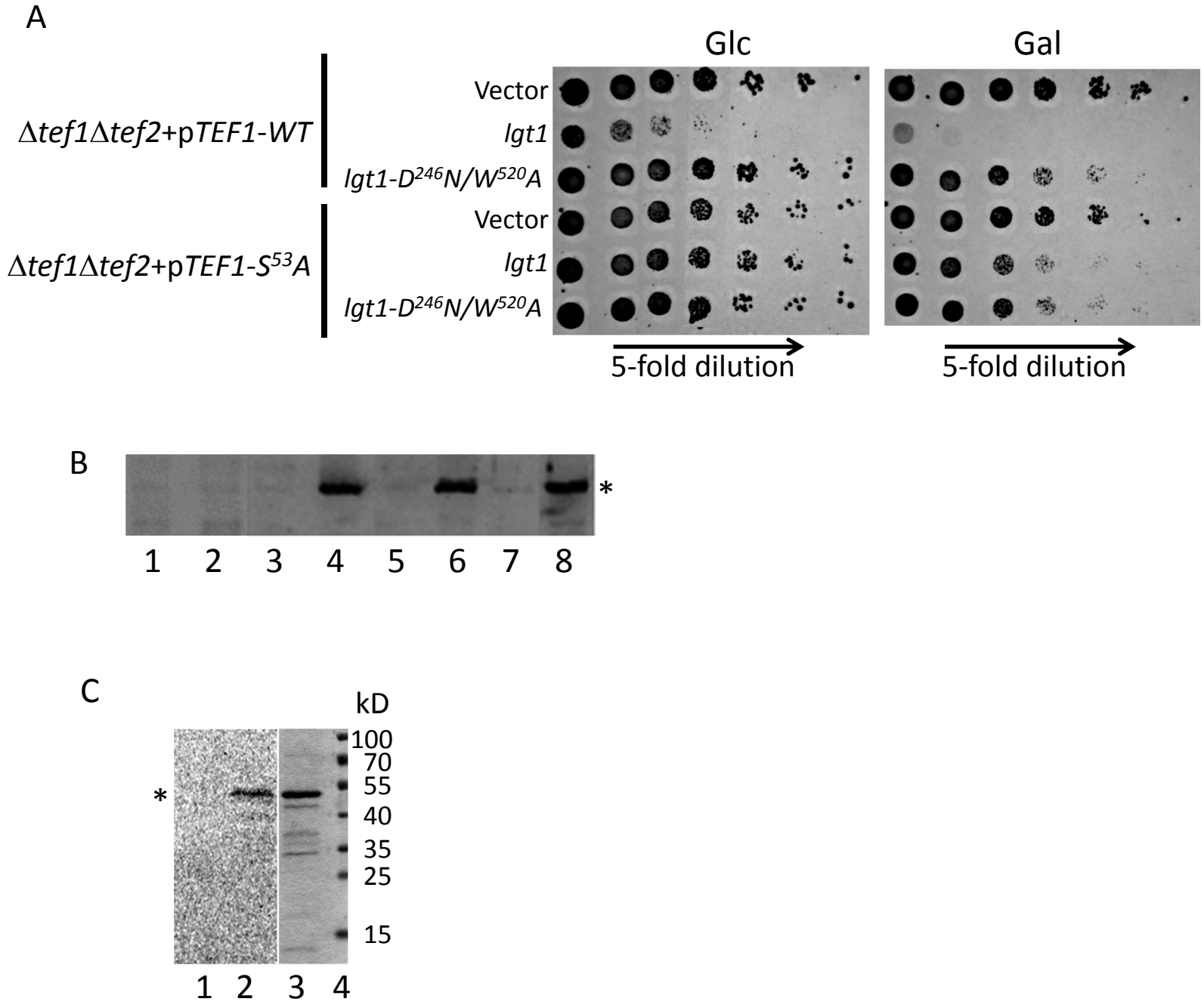


Fig. 5

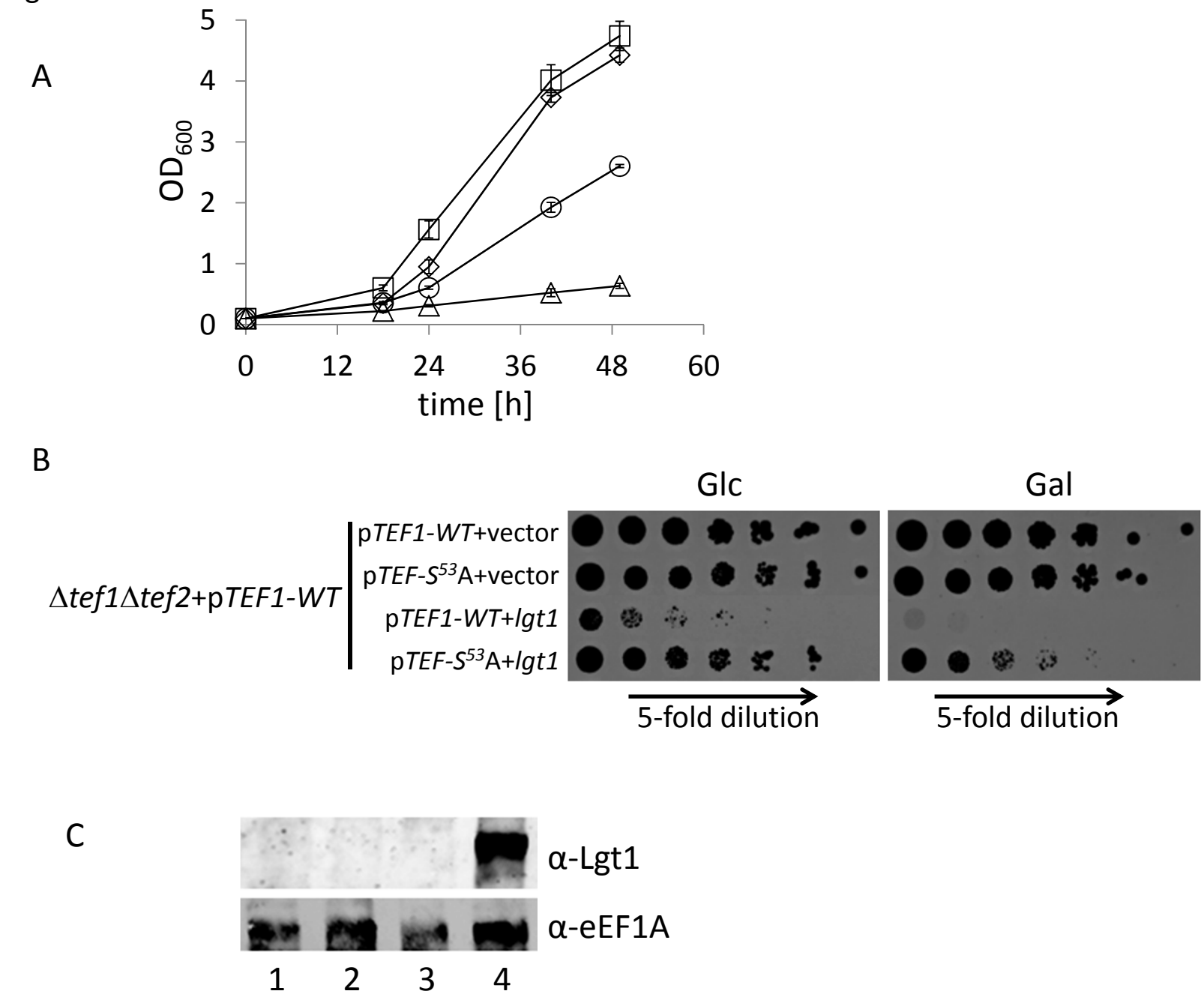


Fig. 6

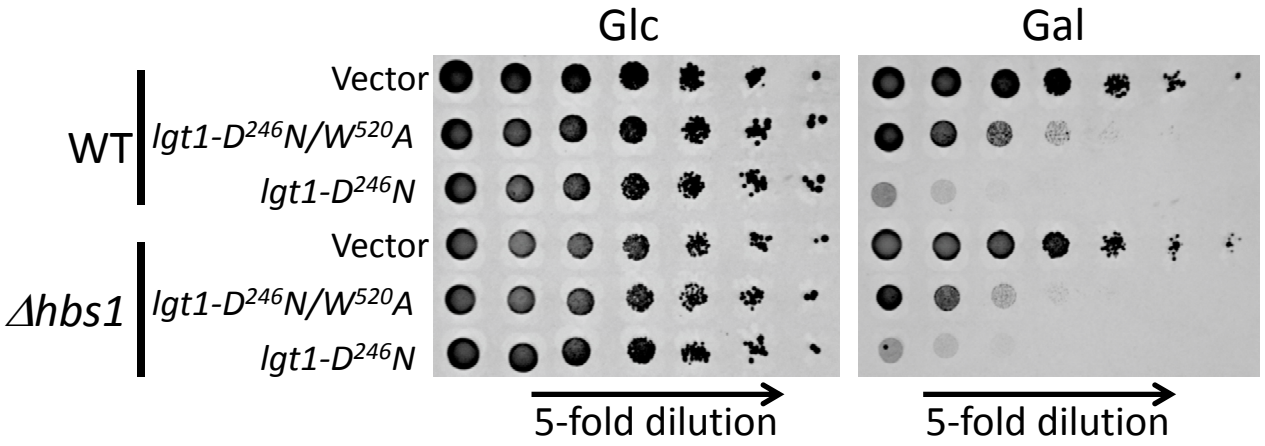


Fig. 7