Fungal Kti12 proteins display unusual linker regions and unique ATPase p-loops

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Abstract
Kti12 (Kluyveromyces lactis toxin insensitive 12) is an evolutionary highly conserved ATPase, crucial for the tRNA-modification activity of the eukaryotic Elongator complex. The protein consists of an N-terminal ATPase and a C-terminal tRNA-binding domain, which are connected by a flexible linker. The precise role of the linker region and its involvement in the communication between the two domains and their activities remain elusive. Here, we analyzed all available Kti12 protein sequences and report the discovery of a subset of Kti12 proteins with abnormally long linker regions. These Kti12 proteins are characterized by a co-occurring lysine to leucine substitution in their Walker A motif, previously thought to be invariable. We show that the K14L substitution lowers the affinity to ATP, but does not affect the catalytic activity of Kti12 at high ATP concentrations. We compare the activity of mutated variants of Kti12 in vitro with complementation assays in vivo in yeast. Ultimately, we compared Kti12 to other known p-loop ATPase family members known to carry a similar deviant Walker A motif. Our data establish Kti12 of Eurotiomycetes as an example of eukaryotic ATPase harboring a significantly deviating but still functional Walker A motif.

Keywords Kti12 · PSTK · Flexible linker · Active site · P-loop · ATPase

Introduction
It was noticed several years ago that yeast cells become resistant to Kluyveromyces lactis toxin, zymocin, upon removal of any of the so-called KTI genes (Butler et al. 1991b; 1994; Kishida et al. 1996). The family of Kti protein harbors various types of proteins, including Elongator core subunits (Elp1/Kti7, Elp2/Kti3, Elp3/Kti8, Elp4/Kti9, Elp6/Kti4), methyltransferases (Trm9/Kti1), zinc-binding proteins (Kti11), WD40s (Kti13), and kinases (Hrr25/Kti14) (Frohloff et al. 2001; Huang et al. 2005a; Karlsborn et al. 2014; Krutyhołowa et al. 2019b). Zymocin itself has three individual components, namely alpha-, beta- and gamma subunits (Stark and Boyd 1986). Alpha- and beta-subunits serve as auxiliary factors allowing the delivery of the gamma subunit into fungal cells (Butler et al. 1991a). The gamma subunit cleaves cellular tRNAs that specifically carry a double 5-methoxy-carbonyl-methyl-2-thio (mcm5s2U34) modification at uridines in their wobble position (Lu et al. 2005; Jablonowski et al. 2006).

Those chemically rather complicated tRNA modifications are introduced by a cascade of enzymes, including
the Elongator complex (Huang et al. 2005b; Dauden et al. 2018), methyltransferases Trm9-Trm112/ALKBH8 (Studte et al. 2008; Songe-Møller et al. 2010; van Tran et al. 2018), and the Uba4/Urm1 thiolation pathway (Leidel et al. 2009; Noma et al. 2009). Despite the creation of a toxin-target site, these tRNA modifications are genuinely required for maintaining proper translation speed and preventing from co-translational protein misfolding (Nedialkova and Leidel 2015; Ranjan and Rodnina 2016; Goffena et al. 2018). The lack of these anticodon modifications in humans causes various pathophysiologies, including severe neurodegenerative diseases (Close et al. 2006; Chaverra et al. 2017; Dauden et al. 2018; Kojic et al. 2018; Hawer et al. 2018; Shaheen et al. 2019).

Recent structural analyses of the enzymatic Elp3 subunit and the Elp123 subcomplex of Elongator show that the subcomplex specifically recognizes tRNAs (Glatt et al. 2016; Dauden et al. 2019; Lin et al. 2019). The overall complex consists of six pairs of subunits and is built up by a symmetrical dimer of two Elp123 subcomplexes, decorated with a hexameric Elp456 ring (Glatt et al. 2012) on one of the Elp123 lobes (Dauden et al. 2017; Setiaputra et al. 2017). Kti12 was demonstrated to bind to the Elongator complex in vivo and in vitro (Fichtner et al. 2002; Petراكis et al. 2005), and we recently fine-mapped its binding site to the first WD40 domain of the Elp1 subunit in vitro (Krutyholowa et al. 2019a). Kti12 harbors two domains, namely an N-terminal ATPase domain and a C-terminal tRNA-binding domain, both connected by a flexible linker region (Fig. 1a) (Schaffrath et al. 2017; Krutyholowa et al. 2019a). Kti12 shows a very high overall sequence and structural similarity to O-phosphoseryl tRNASec kinase (PSTK), a kinase responsible for phosphorylation of Ser-tRNASec (Sherrer et al. 2008). Like PSTK (Chiba et al. 2010), Kti12 binds tRNA with its C-terminus and hydrolyzes ATP, if presented with tRNASec in vitro (Krutyholowa et al. 2019a).

**Fig. 1** Identification of Kti12 proteins with elongated linker and their characterization. 

**a** Architecture of Kti12 protein. ATPase domain (grey) and tRNA-tethering domain (yellow) are connected by a flexible linker. Structural overview of *Chaetomium thermophilum* Kti12 ATPase domain (PDB ID: 6QP0) with a particular emphasis on nucleotide binding pocket and K14 located in a p-loop. 

**b** Distribution of linker length across fungal Kti12 proteins. Numbers on Ox indicate middle values for particular baskets, for instance, 18 ± 4. Black triangles indicate groups to which ScKti12 and CtKti12 belong to. 

**c** Scheme of supervised a machine-learning experiment that pinpointed coincidence of L14 with elongated linkers. Sequences with linkers of a regular length (grey) always carry lysine at position 14 (K14), whereas Kti12 proteins with elongated linkers (pink) can be distinguished by the presence of leucine in a p-loop (L14). 

**d** Violin plots represents distribution of Kti12 protein sequences in a two-dimensional feature space. Linker length is on Ox whereas presence of leucine or lysine qualifies sample to one of the groups on Oy axis. Number of sequences within each group is indicated next the Oy axis.

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Other atypical kinases, like the p-loop-containing Rio1 ATPases, are known to play key roles in ribosome biogenesis, translational control, and other cellular functions (Berto et al. 2019). However, the genuine function of Kti12 and the mechanistic consequences of its direct association with the Elongator complex remain elusive (Krustyholowa et al. 2019a).

Here, we systematically analyzed all annotated Kti12 and PSTK sequences and identified a subpopulation of Kti12 proteins in Eurotiomycetes with extremely elongated linker regions. Furthermore, we used a machine-learning approach to unbiasedly identify a co-occurring feature specific to those Kti12 proteins with elongated linkers. In detail, those aberrant Kti12 proteins share a substitution of a highly conserved p-loop lysine, which was previously described as an invariable element of the phosphate-binding motif in ATPases. We investigated this evolutionary coupling and dissected its potential implications for the active site of Kti12. Our study presents complementary approaches in vitro and in vivo that deepen our understanding of Kti12 and provide important insights into the variations of p-loop-based active sites.

Materials and methods

Sequence analyses and machine learning

Annotated and validated sequences of Kti12 proteins from different domains of life were obtained from the UniProt database and aligned using Muscle web server (Edgar 2004) and Jalview software (Waterhouse et al. 2009). Alignments were manually curated, and all proteins without both ATPase domain and CTD were removed. We utilized Python, Biopython library (Cock et al. 2009), and elements of scikit-learn platform (Pedregosa et al. 2011) to conduct a machine-learning experiment. The length of the linker between ATPase domain (NTD) and tRNA-binding domain (CTD) was used for supervised learning. After randomization of multiple sequence alignment, single amino acid positions were considered as a basis for supporting vector machine classification. The significance of a linker-length difference was considered as a basis for supporting vector machine learning experiment. The length of the linker between Kti12 and provide important insights into the variations of p-loop-based active sites.

Modeling and structure visualization

The Kti12 protein structure was visualized using PyMol software (Schrödinger 2015). We used the YRB script (Hagemans et al. 2015) to analyze hydrophobicity of the nucleotide-binding pocket. Homology-based models of mu gp28 were obtained using Phyre 2.0 and AscKti12 was modelled using SWISS-MODEL web server (Kelley and Sternberg 2009; Waterhouse et al. 2018). We employed the I-TASSER server (Roy et al. 2010; Yang et al. 2015) to predict the existence of any stable secondary structure elements within the linker of AscKti12.

Strains and genetic manipulation of Saccharomyces cerevisiae

The influence of mutations on Kti12 activity was analyzed by gene replacement technology, which allows to modify specific yeast genomic loci (Rothstein 1991). The S. cerevisiae strains used and generated are listed (see Table S1). First, the genomic KTI12 gene of strain UMY2893 (Huang et al. 2005a) was disrupted by transformation with a DNA fragment containing the URA3 gene from K. lactis flanked by KTI12 homologous regions. Selection for Ura+ colonies and screening for zymocin resistance yielded kti12::KUURA3 transformants, in which the WT gene was replaced with the disrupted allele by a double crossover event in vivo. The resulting strain CKY40 was used in the second step to introduce each of the mutations by generating the corresponding DNA fragments with the desired mutation restoring the KTI12 locus except for the desired changes. The corresponding DNA fragments were generated by PCR and transformed directly. Replacement of the KUURA3 cassette by the incoming DNA yielded ura3-auxotrophs selected by plating on 5-fluoroorotic acid-containing media. The proper recombination event was verified by screening colonies using PCR. The presence of the respective mutation was confirmed by sequencing of genomic DNA.

In vivo assay for Kti12 functionality by yeast phenotyping

To analyze the influence of the different kti12-alleles on tRNA modification by the Elongator, growth inhibition by exogenous zymocin toxin was assayed as previously described (Frohloff et al. 2001). Zymocin was obtained from the culture medium of Kluyveromyces lactis killer strain AWJ137 (Frohloff et al. 2001) by filtration (Klassen et al. 2011). Tenfold serial dilutions of the different strains were spotted on rich media plates (YPD, 0.5% yeast extract, 2% peptone, 2% glucose and 2% agar) containing 50% (v/v) zymocin or without the toxin and incubated for 2–3 days at 30°C. Functionally intact Kti12 confers growth inhibition due to the cleavage of three Elongator-modified tRNAs by zymocin. Functionally impaired Kti12 causes toxin resistance (Frohloff et al. 2001). In the second assay, the Kti12 function was tested via the nonsense suppression activity of the SUP4 tRNA, which requires the U34 modification in the anticodon. The kti12 mutations were transferred to the SUP4 background and the resulting strains, which contain the ade2-1 or the can1-100 allele containing ochre stop
codons in the coding regions, were spotted on SD plates containing adenine (YNB), lacking adenine (Ade−), or medium without arginine but containing the toxic arginine analog canavanine. Plates were incubated for 3 days at room temperature and 3 days at 30°C. In cells lacking Kti12 activity, nonsense suppression of the ade2-1 mutation and, therefore, growth without adenine supplementation is impaired. No growth on adenine plates correlates with the growth on canavanine plates since the lack of nonsense suppression of the can1-100 allele means that the CAN1-encoded arginine permease, responsible for the uptake of canavanine, is not expressed. Hence a functional KTI12 gene product is indicated by growth on Ade− and no growth on canavanine plates. (Huang et al. 2005b).

**Protein purification**

The codon optimized open reading frames (ORF), encoding Chaetomium thermophilum Kti12 (CtKti12) or Aspergillus niger (AnKti12), were individually cloned into the pET24d vector using NcoI and XhoI restriction sites and purified as previously described (Krutyholowa et al. 2019a). AnKti12 protein was additionally purified in a HEPES pH 7.2 buffer using a cation-exchange Hi-Trap SP column (GE Healthcare), followed by size-exclusion chromatography on HiLoad 16/600 Superdex 75 pg column (GE Healthcare). Proteins were stored at −80°C in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM DTT buffer.

**Thermal shift assay**

Thermostability of CtKti12 WT protein and CtKti12 mutants was assessed using thermal shift assays. In short, storage buffer with 2 mM MgCl₂ without nucleotides or with addition of 1 mM ADP or ATP was used to dissect the influence of nucleotide binding on protein stability. 10 µg of protein was heated up from 4 to 98°C at a speed of 0.6°C/min in the presence of SYPRO Orange (Sigma Aldrich) hydrophobic fluorescent dye. Data represent three independent experiments conducted in at least 2 technical replicates.

**Determination of nucleotide-binding affinity by fluorescence polarization**

Dissociation constant of MANT-labeled ATP was determined using fluorescence polarization. Experiments were performed in a storage buffer after addition of 2 mM MgCl₂. Serial twofold dilutions of CtKti12 ranging from 100 µM (highest concentration) were prepared and mixed with 1 µM MANT-ATP (final concentration). Fluorescence was elicited using 340-nm excitation light and measured at 448 nm. Fluorescence polarization was measured using FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) with a gain factor of 1000 and at a system temperature of 37°C for the whole duration of the experiment. Data originate from three independent experiments. Curve fitting and $K_d$ calculation were conducted using GraphPad Prism software (La Jolla, California, USA; www.graphpad.com) assuming a single/one-site binding mode.

**Production of tRNA**

Human tRNA Sec was obtained by run-off in vitro transcription from a linearized pUC 19 vector carrying tRNA Sec gene under the T7 promoter. After overnight transcription reaction, RNA species were separated on a HiTrap DEAE FF column (GE Healthcare). RNA was precipitated with 1:1 v/v isopropanol overnight and subsequently denatured and refolded by heating to 80°C and cooling to 40°C in annealing buffer, which contained 200 mM HEPES pH 7.5, 500 mM KCl, and 500 mM NaCl. Subsequent size-exclusion chromatography on Superdex 75 10/300 GL column yielded a homogeneous population of folded tRNA Sec molecules that were used to elicit ATPase activity of Kti12.

**ATPase activity measurements**

In vitro transcribed tRNA was used to trigger the ATPase activity of different CtKti12 protein variants. Reactions were performed in the protein storage buffer after the addition of 2 mM MgCl₂. In detail, 1 µM CtKti12 was mixed with 1 µM human tRNA Sec and either 1 mM ATP or 20 µM ATP. Reactions were incubated for 15 h or 1 h, respectively. In the case of AnKti12, the reaction was conducted in the presence of 5 µM tRNA Sec for 2 h at 37°C. Reactions were monitored using malachite green ATPase assay kit (Sigma Aldrich) as per the manufacturer’s instructions. Data derived from at least three independent experiments were performed in technical duplicates.

**Results**

**Active site mutation evolutionarily coincides with elongated linker**

We harvested and aligned all annotated Kti12 protein sequences from animals, plants, and fungi to identify conserved and variable features in its domains and motifs. Protein sequences of O-phosphoseryl tRNA Sec kinase (PSTK) from animals, which show almost identical domain organization and structural similarity to Kti12, served as references in all analyses (Fig. S1A). First, we systematically compared the length of the flexible linker region that connects the ATPase domains with the C-terminal tRNA-binding domains of Kti12 and PSTK. In animals, Kti12 and
PSTK both exhibit relatively short linker regions in the range 18–22 amino acids (Fig. S1B), whereas Kt12 proteins in plants possess longer linkers of approximately 30 amino acids (Fig. S1B). Fungal Kt12 proteins have predominantly shorter linkers of an “animal type”, but some fungi, including *Saccharomyces cerevisiae*, harbor long plant-like linkers. Unexpectedly, some fungal species evolved a third, clearly separated, population of Kt12 proteins with extremely elongated linkers from other fungal Kt12 sequences. This dataset was used to run a supervised machine-learning approach based on Support Vector Machine (SVM) (Fig. 1c), where we considered single amino acid positions in a multiple sequence alignment and tested if they led to a similar classification pattern. Surprisingly, SVM could achieve a completely equal classification pattern (\( r=1 \), accuracy=100\%) based on the identity of a single amino acid, namely a central active site lysine residue (K14, Fig. 1d). Other identified single amino acid hallmarks of long linker Kt12 proteins neither separate the classes fully nor are located within the Kt12 active site (Fig. S1C). The identified lysine residue was previously demonstrated to be of major importance for the ATPase activity of Kt12, as a K14A mutant showed almost completely abolished ATPase activity in vitro and phenocopied Kt12- and Elongator-deficient *S. cerevisiae* strains (Krutyholowa et al. 2019a). Strikingly, all sequences with an elongated linker carry leucine or isoleucine residues at the respective position. An additional analysis of the amino acid content of the linker regions revealed that long linkers, in general, harbor more threonine residues, while having noticeably decreased relative number of lysine residues (Fig. S1D). It remains to be shown, if specific residues in the linker could influence the active site of Kt12 proteins carrying the K14L substitution. Species carrying Kt12\(_{L,114}\) belong to the class of *Eurotiomycetes* in Ascomycota (Fig. 2a). Inactivating Kt12 mutations would most likely also compromise Elongator’s tRNA modification activity (Schaffrath et al. 2017) and reduce the selective pressure to preserve a functional Elongator complex. Nonetheless, no reports indicate malfunctioning of Elongator or other tRNA modification pathways in *Eurotiomycetes*. Furthermore, we found that all six Elongator genes (Elp1–6) are present in several analyzed *Eurotiomycetes* species and do not contain any genomic alterations (e.g. internal stop codons, fused gene products or frameshift mutations) that would indicate a loss-of-function. The question immediately arises whether *Eurotiomycetes* that carry both Kt12\(_{L,114}\) and an elongated linker have lost the regulatory link between Elongator and the ATPase activity of Kt12 or if the naturally occurring Kt12 variants remain active.

Both K14L and long linker ScKt12 rescue the kt12Δ phenotype

As described previously, K14 of ScKt12 is of profound importance to proper functioning of Kt12 protein (Krutyholowa et al. 2019a). Thus, we decided to experimentally verify if these variations in KT12 alleles would have any functional relevance and if the linker length is indeed functionally coupled to alterations in the ATP-binding pocket. Therefore, Kt12 function was tested in *S. cerevisiae* by established assays for functional Elongator-dependent tRNA modifications. The degree of U34 modifications in vivo can roughly be estimated by zymocin sensitivity and suppression of ochre stop codons by the SUP4 suppressor tRNA (Frohloff et al. 2001; Huang et al. 2005a). The phenotypes of the generated Sckt12–K14L mutant resemble the WT (ScKT12) contrasting with the Sckt12–K14A mutants (Fig. 2b), which phenocopy the null mutant lacking Kt12 protein (Sckt12Δ) or lacking the Elongator subunit Scelp3Δ (Krutyholowa et al. 2019a). Sensitivity to zymocin and canavanine are semi-quantitative indicators of Kt12 function. Growth on media lacking adenine is complementary but sometimes less reliable due to the appearance of revertants. The phenotypes indicate that not only the K14L mutation but also shorter deletions of the N- or C-terminal part of the linker (Δ184-196 and Δ197-213) did not substantially affect Kt12 function negatively. Replacing the short linker of ScKt12 with an elongated linker from *Aspergillus niger* Kt12 (Sckt12–AnKnoLi) resulted in a variable phenotype on media deprived of adenine suggesting weaker Kt12 activity. Complete deletion of the linker (Δ184-213) inactivates ScKt12. The entire AnKTI12 gene from *Aspergillus niger* was unable to replace the function of the ScKt12, which might occur due to specific-interaction interfaces with the Elongator complex or other binding partners. Our data illustrate that the linker region in Kt12 is as important for the tRNA-modification activity of Elongator as the N-terminal ATPase domain or the C-terminal tRNA-binding domain (Schaffrath et al. 2017).

Mutation of K14 alters nucleotide binding by Kt12

Due to the poor solubility of active site mutants from *S. cerevisiae* Kt12 (ScKt12), we expressed and purified full-length *Chaetomium thermophilum* CtKt12 and the variants CtK12\(_{K14A}\) and CtKt12\(_{K14L}\) in bacteria (Fig. S2A, B). In addition, we generated another variant, namely CtKt12\(_{K14V}\), which contains a side chain with an intermediate length, allowing us to define the minimal spatial requirements for protein activity. Next, we studied thermostability, nucleotide
binding, and tRNA-induced enzymatic ATP hydrolysis activity of CtKti12 and its variants at saturating and semi-saturating substrate concentrations. CtKti12 exhibits a melting point of ~46.0 °C (Fig. 3a), which increases to 50.0 °C upon addition of ATP. As previously shown (Krutyholowa et al. 2019a), the substitution K14A does not affect the thermostability of CtKti12 by itself, but diminishes any nucleotide-dependent stabilization effects. The Kti12K14V mutated variant displays a thermostability profile similar to the nucleotide-bound CtKti12, even in the absence of a nucleotide, suggesting a dynamic rearrangement of the K14 carbon side chain during nucleotide binding. In contrast, the Kti12K14L variant is strongly destabilized, resulting in a decreased melting temperature of 37.5 °C, 8.5 °C less than the WT. Interestingly, neither K14V nor K14L exhibited ATP-dependent increase of the melting temperature, indicating strongly decreased nucleotide-binding properties of these p-loop variants (Fig. S2C), similar to K14A.

Although the thermal shift assay provided some initial indications regarding nucleotide binding, we decided
can still bind MANT-ATP (K, decrease the affinity towards ATP (Fig. 3b). Our results demonstrate that all tested substitutions of K14 at more limiting ATP concentration close to the strain. In addition, we decided to perform the same assay the K14L variant is able to rescue the presented with physiological concentrations of ATP (1 mM; Ct (Krutyhołowa et al. 2019a). We observed comparable ATPase activity using the previously established ATPase activity assay (Waterhouse et al. 2009) and Muscle server (Edgar 2004). The phylogenetic tree was built using the neighbor-Joining method. Individual branch lengths are shown above the branches and indicate the number of amino acid substitutions per site. The fraction of replicate trees in which the associated taxa clustered together in the bootstrap test {1000 replicates (Felsenstein 1985)} are shown beneath the branching points. The evolutionary distances used to calculate the tree were calculated using the Poisson correction method with the pairwise deletion of ambiguous positions. Final dataset contained 610 positions. All evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). The tree was visualized using iTOL (Letunic and Bork 2007). b Zymocin assay assessing performance of the Elongator complex and presence of U34 modifications. WT yeast strain (ScKTI12) served as a growth control, Scelpl2A and ScKTI12Δ pose negative controls. Tenfold dilutions were spotted on YEPD media with the addition of 50% Zymocin or YNB media replacing arginine by 40 µg/ml canavanine or lacking adenine (-adenine). In the different strains KTII2 was mutated (K14A/L), parts of the whole gene were exchanged with the corresponding sequences from Aspergillus niger (An knot + linker, AnKTI12) or parts of it were deleted (marked with Δ and the deleted residues in brackets). ScKTI2-NTD codes for the first 184 amino acids to quantify nucleotide-binding affinity using fluorescence polarization. Using this method, we were able to confirm the previously measured K₄ for CTki12 WT and MANT-ATP (1.5 ± 0.2 µM). Apparently a very high evolutionary constraint conserved the lysine residue in the p-loop motif of yeast Kti12, PSTK, and other family members. Both K14A and K14V proteins have very low affinity towards MANT-ATP and the K₄ can only be estimated to values higher than 100 µM. Interestingly, the naturally occurring K14L variant can still bind MANT-ATP (K₄ 25.0 ± 2.4 µM). In summary, our results demonstrate that all tested substitutions of K14 decrease the affinity towards ATP (Fig. 3b).

Next, we examined the activity of KTI12 variants using the previously established ATPase activity assay (Krutyhołowa et al. 2019a). We observed comparable ATP hydrolysis rates in the case of CTki12 WT, K14V, and K14L presented with physiological concentrations of ATP (1 mM; Fig. 3c). This finding supports our in vivo data showing that the K14L variant is able to rescue the S. cerevisiae Δkti12 strain. In addition, we decided to perform the same assay at more limiting ATP concentration close to the K₄ of the CTki12K14Δ-MANT-ATP interaction. Upon incubation with 20 µM ATP, differences in ATP hydrolysis rates between the naturally occurring mutants become unambiguous (Fig. S2D). To further validate our findings from CTki12, we expressed and purified WT Aspergillus niger Kti12 protein and its L14K and L14A mutants (Fig. S2E). The tRNA-induced ATPase activity of WT AnKti12 naturally carrying leucine at position 14 is similar to the ATPase activity of AnKti12L14K, the common variant occurring in all other Kti12 proteins. In addition, AnKti12L14A shows strongly reduced ATPase activity which further corroborates the observed decrease in ATPase activity of CtKti12K14A and in vivo phenotyping results of the Sckt12Δ-K14A strain. Our findings indicate that residues in the long threonine-rich linker of AnKti12 cannot contribute to the active site, since the activity of AnKti12L14A remains strongly reduced in vitro.

Discussion

Three different types of linkers are present in Kti12 proteins from fungi and the reason for this divergence currently remains unclear. In the case of animal Kti12 and PSTK, a relatively short linker can be explained by the presence of tRNAsec which stimulates ATP hydrolysis by Kti12. However, apart from tRNAsec, which does not exist in S. cerevisiae, ScKti12 is important for processing of 11 different tRNAs by the Elongator complex, so the relatively short 20 amino acid linkers may be preferred for tRNA-related functions. Concomitant with this, plant phenotypes caused by mutations in the Arabidopsis thaliana deformed root and leaves (DRL1) gene encoding the Kti12 homologue are similar to the phenotypes of elongata (ELO) mutants (Chen et al. 2006; Mehlgarten et al. 2010). Most of the plant genes encoding Elongator core subunits were also identified as elo mutants (Nelissen et al. 2005). All plants and some fungi, including S. cerevisiae, harbor an approximately 30-amino acid long linker composed of a putative topological knot and a disordered linker. We tried to dissect the role of these regions in S. cerevisiae by deleting individual structural elements and respective combinations in the ScKTI12 gene, but we failed to find any phenotypical difference in the wild type strains lacking the knot or the disordered linker (Fig. 2b). Complete removal of both elements, i.e. the entire linker, inactivated Kti12, whereas replacement of the ScKti12 linker by the long one of AnKti12 did not. Hence, we conclude that cooperation of the ATPase and tRNA-binding domains as described previously (Krutyhołowa et al. 2019a) is supported by a linker, which has properties found in the long AnKti12 as well as in the shorter ScKti12 linker. Attempts to model the structure of the AnKti12 linker by I-TASSER server failed, as it was not possible to find any stable secondary or tertiary structures. Taken together, these findings suggest that the linker is indeed rather flexible, most likely unstructured and not very sensitive to sequence variations. It remains to be shown experimentally, if it additionally participates in any protein–protein interactions that could be related to potential Elongator-independent “moonlighting” functions of Kti12.
Our data show that the non-canonical K14L substitution within the Walker A motif decreases nucleotide-binding affinity of Kti12, but does not result in catalytically inactive protein. Kti12 remains active under physiological conditions, as the cytoplasmatic ATP concentration in yeast is close to 2 mM (Koç et al. 2004). We have shown that the ScKTI12–K14L allele can rescue the tRNA modification phenotype of a Kti12 deficient strain in vivo (Fig. 2b) and that the CtKti12 and AnKti12 proteins have similar in vitro activities as their K14L or L14K variants, respectively (Fig. 3c). Since cytoplasmatic ATP concentration can decrease during starvation, cellular stress or other conditions the low affinity for ATP of Kti12K14L would allow to effectively shut down tRNA modification by Elongator via Kti12. Perhaps at a low-energy state of the cell Kti12 deactivation might be required to activate specialized translational programs and the production of Elongator-independent proteomes. If this potential regulatory circuits existed, the question arises whether it represents a unique feature of Eurotio- mycetes or similar regulatory “bypasses” are present in case of other organisms and other regulatory ATPases.

We analyzed the most crucial charged and hydrophobic residues within the CtKti12 nucleotide-binding pocket and simulated the structural consequences upon mutation of K14. As the side chain of lysine is relatively long, K14A creates a significant breach which may allow water to reach the hydrophobic core of the protein. K14V and K14L substitutions would gradually revert this breach (Fig. 4a). In addition, we compared available structural information from members of the ‘DxTN’ kinase family, named after its Walker B motif (Leipe et al. 2003). Superposition of ATPase domains of eukaryotic Kti12 (PDB ID: 6QP0), archaeal PSTK from Methanocaldococcus janaschii (PDB ID: 3ADB), and T4 bacteriophage PNK (PDB ID: 1LY1) revealed highly similar ATPase folds with a particularly strong conservation of the Walker A motif. While the relative position of K14 and the hydrophobic core is almost identical in the analyzed structures, we failed to identify any archaeal or viral proteins carrying L14. Furthermore, we searched the literature for described deviations from Walker A motifs that would include substitution of signature lysine residues by leucine. Gp28, a terminase from phage mu that allows the phage to pack its genome into the capsid, was described to carry a similar substitution (Mitchell and Rao 2004). The authors mentioned that the function of L70 may be successfully complemented by K64 located in close vicinity. We performed homology-based modeling to obtain a model of the gp28 structure (Fig. 4b). We compared Walker A motif of gp28 with those of CtKti12 and AnKti12 in terms of sequence and structure. Kti12 harbors no lysine in a position that would correspond to a previously described K64 residue in gp28 (Fig. 4c). Furthermore, no other lysine from the ATPase domain can perform instead of K14. According to our homology-based model, all lysine residues are located far away from the Walker A motif (Fig. 4d). We would like to highlight that a critical signature substitution within
the Walker A motif of *Eurotiomycetes* Kti12 protein may have regulatory importance without diminishing its basal ATPase function. To systematically defy the conservation of the Walker A motif, further bioinformatical research is needed to pinpoint similar substitutions occurring in other kinases and proteomes.

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**Author contributions** RK designed and conducted machine-learning approaches, performed all protein purifications and biochemical analyses. ACH produced and purified in vitro transcribed tRNA. ART constructed the yeast strains, performed the in vivo functional assays and did sequence alignments; RK prepared figures with input from SG. RK, KDB and SG wrote the final text of the manuscript with the input from all authors.

**Compliance with ethical standards**

**Conflict of interest** Authors declare no conflict of interests.

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