Novel Protein and Coding Determinants of Protein Biosynthesis Speed and Accuracy:

The Role of Scp160p in Translational Efficiency and Fidelity of Suboptimally tRNA Re-Use Encoded Yeast mRNAs

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Abstract

In balancing *accuracy* versus *speed* in protein synthesis, numerous parameters serve to increase translational efficiency by optimizing the binding kinetics of translation factors to the ribosome. On the one hand, use of *frequent codons*, recognized by higher-abundance tRNAs, correlates with *lower mistranslation* rates. On the other hand, *autocorrelation*, the re-use of tRNAs by different synonymous codons, is a proven predictor of translational *velocity*. Factors that limit diffusion of ribosome interacting molecules, e.g. aminoacyl tRNA synthetases, can also contribute to higher efficiency. Additionally, accumulation of positively charged amino acids in the peptide exit tunnel is a reliable predictor of translational stalling.

The budding yeast multi-KH domain RNA-binding protein Scp160p binds to over 1000 mRNAs as well as polyribosomes, and its mammalian homolog vigilin binds tRNAs and translation elongation factor EF1alpha. Despite its implication in translation, studies on Scp160p's molecular function are lacking to date. Our lab previously applied translational profiling approaches and demonstrated that the association of a specific subset of mRNAs with ribosomes or heavy polysomes depends on Scp160p. Interaction of Scp160p with these mRNAs requires the conserved K homology (KH) domains 13 and 14. Transfer RNA pairing index (TPI) analysis of Scp160p target mRNAs had indicated a higher degree of consecutive use of iso-decoding codons in more depletion-affected messages. As shown previously for one target mRNA encoding the glycoprotein Pry3p, Scp160p depletion results in translational downregulation but increased association with polysomes, suggesting that it is required for efficient translation elongation.

In my study, depletion of Scp160p decreased the relative abundance of ribosome-associated tRNAs whose codons show low potential for autocorrelation on depletion-affected mRNAs. Conversely, tRNAs with highly autocorrelated codons in mRNAs are less impaired. The data indicates that Scp160p might increase the efficiency of tRNA recharge, or prevent diffusion of discharged tRNAs, both of which were also proposed to be the likely basis for the translational fitness effect of codon autocorrelation.

Yeast Scp160p also interacts with Asc1p, the homolog of mammalian RACK1, on the ribosomal

small subunit - which in turn has been shown to interact physically with kinases, and genetically with aminoacyl-tRNA synthetases. After analyzing the role of Scp160p and Asc1p in translational efficiency and fidelity in yeast in my study, more insights were gained: *scp160Δ* and *asc1Δ* mutants decode stop codons with increased fidelity, consistent with the proteins' roles in promoting elongation velocity. Loss of Scp160p or Asc1p increases rates of both programmed and non-programmed -1 ribosomal frameshifting but not +1 frameshifting, consistent with roles for both these proteins in enhancing elongation speeds. Differential mistranslation of codons for tRNAs with low or high abundances by the respective mutants on the other hand matches their proposed roles in limiting tRNA diffusion and boosting tRNA recycling, respectively.

In conjunction with their known physical interactions with other components of the ribosome and trans-acting factors, a biophysical model is presented describing how these two proteins might stabilize polysomes and help to channel tRNAs between successive ribosomes during translation elongation.

Keywords

Translational fidelity, translational efficiency, codon autocorrelation, tRNA recycling, Vigilin proteins

Introduction

The elongation step of translation - a recently developing direction for research

Translation is one of the most costly processes inside the eukaryotic cell (reviewed in Watson: Molecular Biology of the Cell, CSHP 2008). A large part of the energy consumption stems from the need for tight regulation, as witnessed in the complicated interplay of loading factors, where GTP hydrolysis is used to give a „rhythm" to tripartite assemblies (reviewed in GEBAUER and HENTZE 2004). The pivots of regulation are eIF4E and eIF2, and both continue to be points of interest: eIF2 due to connections with amino acid starvation signaling via GCN4 in yeasts (HINNEBUSCH 2005), and with diabetes in metazoa (YONG/GRANQVIST/HAN/KAUFMAN 2014). eIF4E on the other hand was found to be important for circularization of mRNAs (WELLS&SACHS 1998), connects to decapping mechanisms via Dhh1 (SWEET/KOVALAK/COLLER 2012) and is part of the Processing Body/ Stress Granule interplay of storage

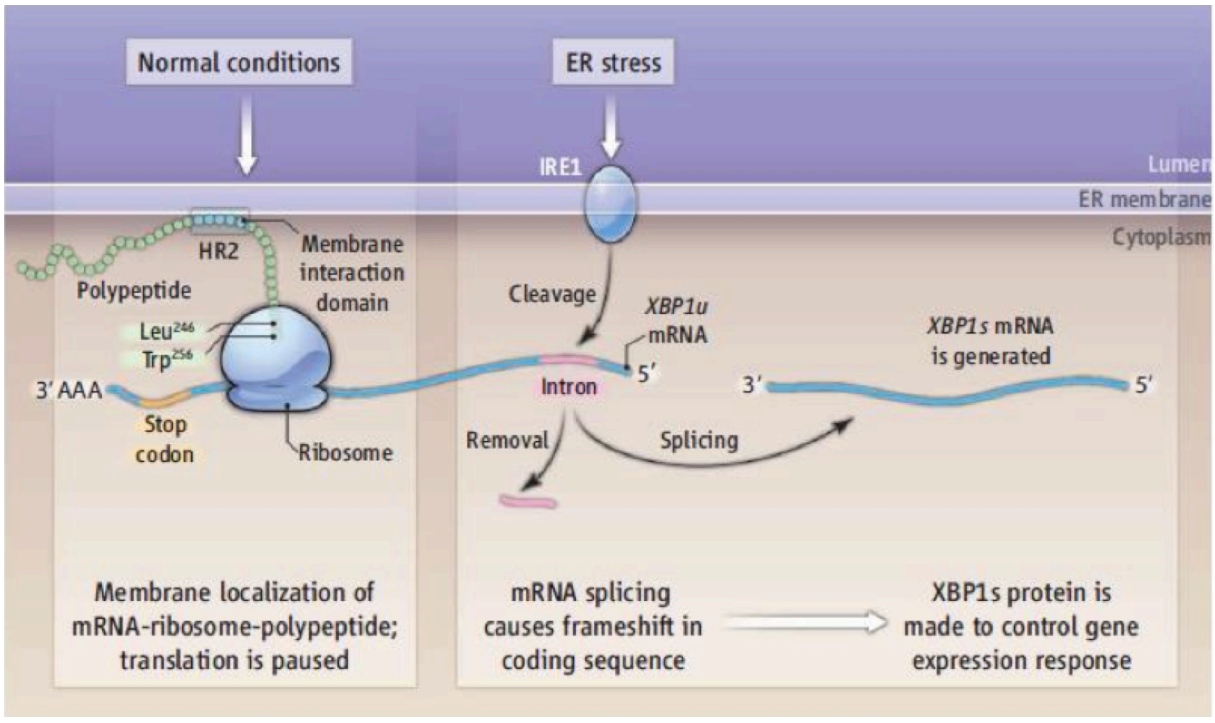


Fig. I: The mechanism of translation slowdown-mediated cleavage in unfolded protein stress. XBP1 mRNA is recruited to ER using a membrane interaction domain. The probability of this event rises with slower translation within a more 3`motif, which is caused by low tRNA availability. Consequently, the ER-vicinal mRNA is more susceptible to cleavage by IRE1, conclusively mediating unfolded protein stress response via translation of an alternative XBP1 variant protein product. *Image from RON&ITO 2011.*

and degradation of mRNA (reviewed in BUCHAN/PARKER 2009). This leads us to another important part in the control of gene expression: The homeostasis of mRNA levels, which has long since been studied, but recently received new twists due to the discovery of miRNA-mediated silencing (reviewed in JONAS & IZAURRALDE 2015), localized translation (reviewed in JANSEN, BAUMANN, NISSING, FELDBRÜGGE 2014), and initiation-preventing RNA-binding proteins like Khd1 (HASEGAWA&GERBER 2008).

However, starting with pivotal work on the elongation control of XBP-1 (a transcription factor, spliced due to slowed elongation which causes IRE1 recruitment of a hydrophobic region, in response to ER stress – cf. **Fig. I**, reviewed in RON&ITO 2011), it has become evident that translation's elongation is also a point of leverage for cellular control. Pioneering work on RACK1/Asc1p's potential as a „platform for signaling molecules on the ribosome" (NILSSON/SENGUPTA/NISSEN 2004) showed that nutrient-sensing protein kinases and ribosomes might interact.

Following work on abiotic concentration clouds as a drive for the origins of cells and nuclei (NEWMAN/FORGACS 2006), researchers began to connect these principles to evolutionarily younger phenomena such as protein synthesis, which emerged after a presumed „RNA world" (reviewed in ATKINS/GESTELAND/CECH, *CSHP 2005*). As the theory of molecular crowding (McGUFFEE/ELCOCK 2010) is catching on, early notions about how local high-concentration „clouds" of substrates around an enzyme or multi-enzyme-complex (i.e. here, ribosomes or polysomes) may boost efficiency of translation being „just another enzymatic reaction" (NEGRUTSKII&DEUTSCHER 1994) are revived. While these early studies indicated that ribosomes somehow favor „self-made" over „ready-made" aminoacyl-tRNA, recent evidence is emerging that ribosomal recruitment of aminoacyl tRNA synthetases may more be the rule than the exception in archaea (MIKULCIC/BAN 2014), mammals (DAVID/YEWDELL 2011) and yeasts (BECKER 2009). This would explain the benefit of using „selfmade", vicinal tRNAs.

Coding determinants of translation elongation and tRNA abundance - emerging fields

Just as the machinery around the ribosome may be host to hithero unexpected coalitions of proteins, which may hail back to primordial times, the genetic code itself, always thought to be monolithic in its conservation yet puzzling in its synonym-richness and the erstwhile enigmatic differences in abundance of codons (SHARP&LI 1987) and tRNAs (PERCUDANI/OTTONELLO/PAVESI 1997), which are higher in highly-expressed genes respectively, may contain programs that connect mRNA composition bias with tRNA

recycling.

However, recent research (AGASHE&MARX 2012) reveals that a presumed benefit of „popular" codons, recognized by „abundant" tRNAs, lies not in translation speed. Rather, „popular" codons tend to be more secondary-structure-rich in mRNAs - stemming from their more refined pairing sterics (reviewed in DINMAN, KINZY 2007). The aforementioned study confirmed that indeed, „popular" codons are read with higher fidelity. Both the „secondary structure-inducing" and „high-fidelity" ideas, as opposed to the „speed" hypothesis, were recently independently verified based on ribosome footprinting data (POP/INGOLIA/PHZIZCKY/WEISSMAN 2015). It turns out that the abundance of „popular" codons in highly expressed genes may indeed protect the cell from mistranslation-caused bad folding homeostasis, with implications for ER stress in immune cell survival (GILCHER 2007) and, eventually, development of cancer

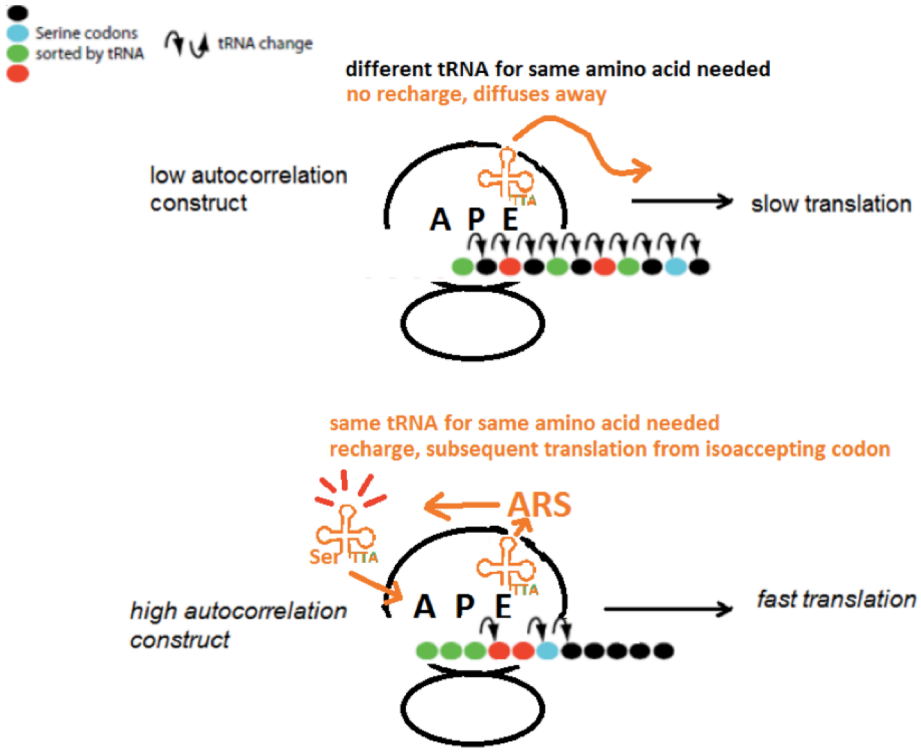


Fig. II: Autocorrelation, i.e. the successive re-use of isodecoding tRNAs for translating same amino acids encoded from same or different codons. If a subsequent codon for the same amino acid within a sequence is encoded by a codons read off the same isodecoding tRNA, the translational processivity of the sequence rises, as witnessed experimentally. The most parsimonious explanation is that faster re-use limits diffusion of empty tRNAs, contributing to faster recycling. This observation build upon the substrate channeling hypothesis (NEGRUTSKII&DEUTSCHER 1991), which was put forth to explain preferential use of "selfmade" tRNAs by ribosomes. *Image from CANNAROZZI&BARRAL 2010, adapted for this thesis.*

(CUBILLOS-RUIZ 2015).

It is important to note the contribution of initiation control on the „on/off" rates of translation, for example via the initiation strength of AUG-vicinal sequences (KOZAK 1991). This aspect is also the focus of recent research into the ratio fine-tuning of products from viral genes bicistronically embedded in overlapping reading frames, but read from eukaryotic host ribosomes (MATSUDA&DREHER 2006): Scanning was previously thought of as a „take-it-or-leave-it" process; however, the discovery of closely spaced AUGs competing for initiation hinted at a „one-step-forward, two-steps-back" behavior of the ribosome. This example also nicely illustrates the apparent power of evolution in accomodating regulation precisely *by, not in spite of,* the ambiguity and synonym-richness of codons, and varying interplays of reading frames (on-going research by T. DREHER and B. BURTON, by personal communication). However, as considered for the „machinery" part, i.e. ribosomes/proteins in the above paragraph, the same is true from the „software" side, i.e. genes/mRNAs: The ambiguity of the code may represent a finer, more gradual way of attuning the synthesis demands of cells to shifting needs.

While cryptic „codon pairs" in eukaryotic genes have been observed before without making apparent sense (reviewed in AGAHSE/MARX 2012 and DEDON/BEGLEY 2014), a recently developed metric, the tRNA pairing index, takes into account non-direct proximal re-dialing of isodecoding tRNAs within genes encoding two instances of one amino acid from different (sic!) isoaccepting codons (FRIBERG/GONNET/BARRAL 2006). Of note, this so-called „autocorrelation" effect (**Fig. II**) was found *in silico* in plants, mammals and

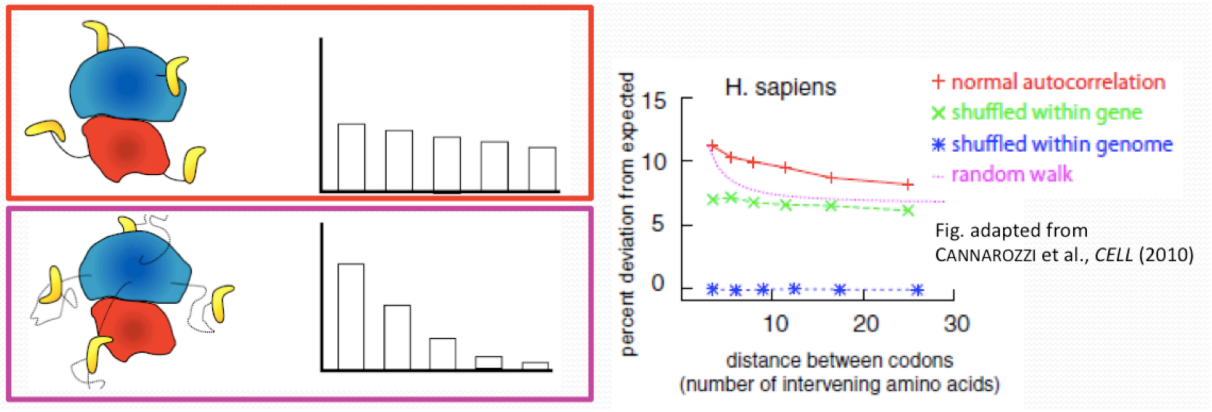


Fig. III: Autocorrelation has been modeled for distance decay of its non-random observability to best fit with tight (red), not loose (purple), coherence of tRNAs and ribosomes. Whether the hypothetical tRNA retention is due to diffusion-limiting proteins around the protein, is still subject to investigation. However, ribosomal recruitment of aminoacyl-tRNA synthetases has been witnessed in mammals (DAVID&YEWDELL 2011) and archaea (MIKULCIC&BAN 2014).

Image from CANNAROZZI&BARRAL 2010, adapted for this thesis.

yeasts, as well as measured by *in vitro* translation experiments to positively affect read-through of reporter gene expression (CANNAROZZI & BARRAL 2010).

The authors of said study also conducted three-dimensional random-walk computations, that show that the observed slow decay of the effect with increasing distance between codons not only matches to predictions of a presumed loose, but tight coherence between tRNAs and ribosomes (**Fig. III**). This fits with considerations made by MCGUFFEE&ELCOCK (2010), which show that the ribosome, being heavy, sits in too much of a cytosolic „draft" as to be able to efficiently pick up substrates, i.e. aminoacyl-tRNAs, from a random diffusion to account for the observed speed of translation.

The role of Scp160p in translational efficiency of suboptimally tRNA re-use-encoded mRNAs

RNA binding proteins (RBPs) form a multi-layered regulatory network that tightly controls post-transcriptional gene regulation. In the yeast *Saccharomyces cerevisiae*, approximately 600 proteins have been predicted to possess RNA-binding activity (HOGAN 2008). To process and regulate the more than 6700 different transcripts within a yeast cell, combinatorial action of these proteins is required. According to

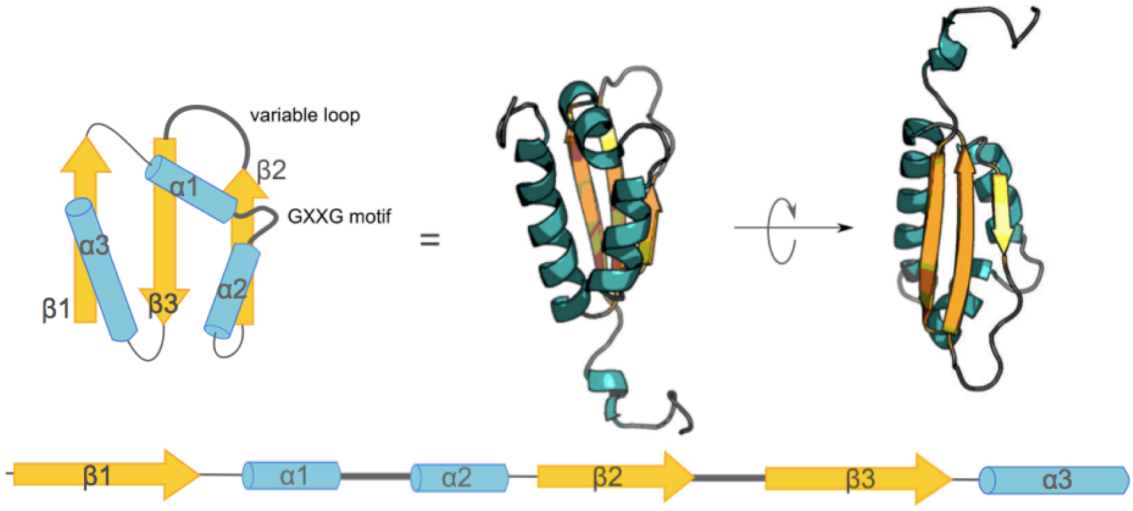


Fig. IV: The array of α-helices and β-sheets characteristic of KH domains, as found in Vigilin proteins. While past research emphasized the presence/absence of a GXXG motif (FRIDOVICH-KEIL 2007) to determine prototypicity, recent analyses have focused more on RNA binding (HOGAN 2008) and secondary structure prediction (this study).

Image courtesy of Jan Overbeck.

the recently proposed 'RNA-operon' model, control occurs in a concerted manner for specific groups of mRNAs (KEENE 2007, and references therein).

Scp160p is the yeast ortholog of the evolutionary conserved vigilin protein family that is characterized by its unique repetitive array of 14 RNA-binding hnRNP KH domains (DODSON/SHAPIRO 1997, WEBER/WINTERSBERGER 1997). Only seven of these represent canonical KH domains (**Fig. IV**), while the remaining seven have insertions or deletions in a characteristic GXXG motif and are therefore termed diverged KH domains (BRYKAILO&FRIDOVICH-KEIL 2007).

Two microarray-based studies have been carried out to identify mRNAs associated with Scp160p. A first approach discovered five mRNA targets, *DHH1*, *BIK1*, *NAM8*, *YOR338W*, and *YOL155C* (LI&FRIDOVICH-KEIL 2003). A more recent study identified >1000 mRNA targets of Scp160p that cluster in groups encoding proteins of cell wall, plasma membrane, endoplasmic reticulum and nucleolus (HOGAN 2008). However, the biological function of these Scp160p – mRNA interactions remains to be elucidated. Since its first description, Scp160p has been implicated in various processes, ranging from the control of cellular ploidy (WINTERSBERGER, KARWAN 1995) to mating response (GUO&DOHLMANN 2003, GELIN-LICHT&GERST 2012), telomeric silencing (BATTLE, MARSELLACH&AZORÍN 2006), and control of spindle pole body biogenesis (SEZEN/SEEDORF/SCHIEBEL 2009). In many of these processes, Scp160p seems to be involved in translational regulation of target proteins. In addition, *scp160* deletion strains are sensitive against translation inhibitors such as cycloheximide and hygromycin B (BAUM/BITTINS/FREY/SEEDORF 2004) and Scp160p is known to associate with cytosolic and membrane-bound polysomes, from which it is released by EDTA treatment as a component of mRNP complexes (FREY/POOL/SEEDORF 2001). Crosslinking experiments indicate that Scp160p binds to the ribosomal 40S subunit close to the mRNA binding site, and that this association is partially dependent on the interaction with Asc1p, the yeast homolog of mammalian RACK1 (BAUM/BITTINS/FREY/SEEDORF 2004). Scp160p acts in concert with the eIF4E-binding protein Eap1p in the so-called SESA network in order to inhibit translation of *POM34* mRNA in response to spindle pole body duplication defects (SEZEN/SEEDORF/SCHIEBEL 2009). In addition, there is evidence that Scp160p is involved in the elongation step of translation, since it can form chemical crosslinks with elongation factor 1A (eEF1A) (BAUM/BITTINS/FREY/SEEDORF 2004). Finally, the mammalian Scp160p homolog vigilin co-purifies in a complex with eEF1A and tRNA (KRUSE/GRÜNWELLER 1998). The latter provides an interesting link between Scp160p and proteins that belong to the “hardware” basis of translation.

When considering translation, the ambiguity of the genetic code “software” enables regulation when

resolving beyond the amino acid level. This means that the same amino acid sequence may be translated with varying speed, depending on the nucleic acid composition of the coding mRNA. Just as the AUG context is an important fine-tuning capacity of eukaryotic initiation (KOZAK 1992), elongation can be boosted by choosing between different synonymous codons (PLOTKIN&KUDLA 2011). Codon ordering also has a beneficial effect on translational fitness (CANNAROZZI&BARRAL 2010).

The two most important predictive parameters of translational fitness that work beyond the initiation stage are codon usage and tRNA autocorrelation. Highly expressed genes with large mRNA copy numbers tend to use a restricted subset of synonymous codons (SHARP&LI 1987) whose decoding tRNAs are more abundant, are recognized faster, and incorporated during translation with higher fidelity (PERCUDANI/OTTONELLO/PAVESI 1997, CHARNESKY&HURST 2013). This can be measured with the codon adaptation index (CAI), the bias of a gene to favor "popular" (or frequent) over "unpopular" (infrequent) codons. On the other hand, it has been found that the re-use of the same tRNA by synonymous codons at successive occurrences of the same amino acid in an mRNA, termed "autocorrelation" and

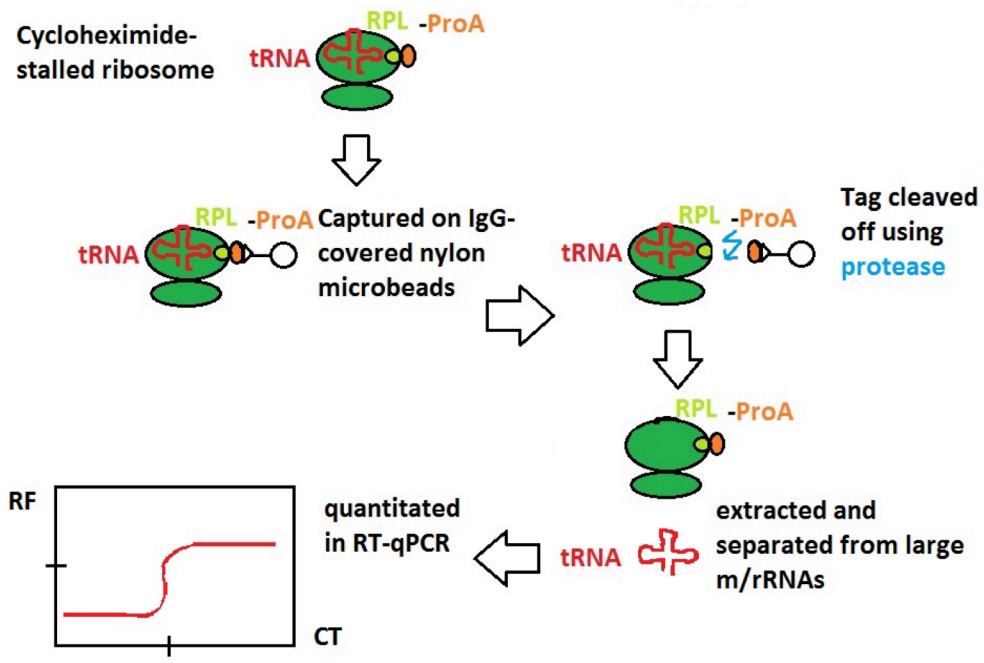


Fig. V: The ribosome-affinity purification (RAP) procedure quantitates tRNAs from ribosomes harvested under different conditions, e.g. wild-type versus knock-out yeast cells. Using cycloheximide for ribosome stalling ensures that on-going translation is arrested, and ribosomes prevented from dissembly. A protease-cleavable tag liberates ribosomes from affinity beads. RNA isolation enables separating into large (m-) and small (t-) RNAs. RT-qPCR (quantitative PCR after Reverse Transcription) quantitates eluted RNAs. *Method pioneered by others in (HALBEISEN & GERBER 2009), adapted for RT-qPCR and tRNA detection by me for this project.*

measured with the tRNA pairing index (TPI), can significantly boost translational efficiency (CANNAROZZI&BARRAL 2010). Based on random walk computations compared with the distance decay of this effect, autocorrelation's benefit on translation (CANNAROZZI/BARRAL 2010) is thought to be caused by local molecular crowding (McGUFFEE&ELCOCK 2010) of tRNAs for recharging near the ribosome, as suggested by the substrate channeling hypothesis of translation (NEGRUTSKII&DEUTSCHER 1994). Of note, this tRNA "re-use" effect is independent of the abundance of recognized codons or their number of synonyms. For example, amino acid starvation genes were found to markedly use "unpopular" (infrequent) synonymous codons but autocorrelate ("codon-cluster") them to make use of a less depleted pool of charged aminoacyl-tRNAs (CANNAROZZI&BARRAL 2010).

To investigate the potential role of Scp160p in translational control, our lab previously followed a translational profiling approach (SCHRECK 2010) to identify mRNAs whose translation is regulated by Scp160p. This identified a set of mRNAs that shift their position within polyribosome gradients upon Scp160p depletion, indicating changes in their translation rates. As the coding optimization of transcripts predicts their translational fitness, in the first part of this study I hoped to elucidate possible connections between Scp160p's action and optimized translation by bioinformatic analysis of its bound mRNAs. I found that mRNAs bound by Scp160p, and translationally affected by its depletion, show a higher frequency of "autocorrelated" successive synonymous codons recognizing the same tRNA. By quantifying tRNAs associated with ribosomes in the presence and absence of Scp160p, using the RAP procedure pioneered in (HALBEISEN and GERBER 2009, cf. **Fig. V**) after adapting it to tRNA detection (**Fig. VI**), I was able to

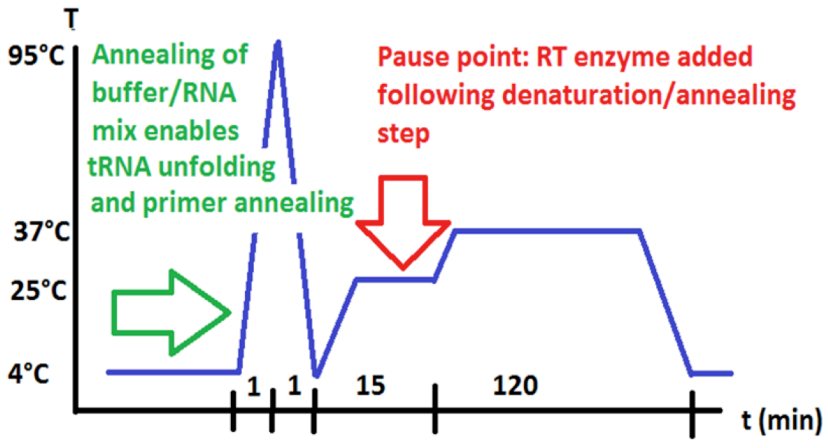


Fig. VI: Modified Reverse Transcription (RT) conditions for tRNA detection. Pre-incubating the RNA with primers before RT enzyme is added allows annealing efficiently at the same time as using standard enzymes without risk of denaturing.

show that loss of Scp160p results in stronger depletion of a specific subgroup of tRNAs. Surprisingly, these tRNAs recognize codons with *low* autocorrelation in Scp160-bound mRNAs. This shows that Scp160p's benefit on translation of its target mRNAs is *synergistic with, but independent of*, codon autocorrelation and might represent a novel mode of translation optimization. My data thus suggest that Scp160p is required for efficient translation of a subset of mRNAs via tRNA recycling or reduction of tRNA diffusion from the ribosome, that counters inherent suboptimal coding for re-dialing.

Relationships between fidelity and efficiency of translation, and Scp160p and Asc1p

The protein biosynthetic machinery is a complex system composed of a multitude of proteins and nucleic acids. Despite its complex nature it is able to rapidly and accurately translate the genetic code. Such high performance is ensured by quality control systems operating at many levels. These include safeguarding that only high quality mRNAs are used (LYKKE-ANDERSEN and BENNETT 2014), that the correct start and stop codons are properly identified (HINNEBUSCH 2011; KISSELEV et al. 2003), that cognate mRNA:tRNA interactions are distinguished from near-and non-cognate interactions (PLANT et al. 2007), that translational reading frame is maintained during elongation (DINMAN 2012), and that nascent peptides are properly folded (RODNINA 2012; LYKKE-ANDERSEN and BENNETT 2014). While quality control has been extensively studied in many systems, our understanding of translational efficiency has been mainly restricted to kinetic analyses of the interactions that occur between ribosomes and *trans*-acting factors, and of the biochemical reactions that occur during translation elongation (RODNINA 2012).

In contrast, elucidation of additional kinetic limitations on translational efficiency has not progressed as far. For example, free diffusion of the substrates for protein synthesis throughout the cell would limit protein synthetic rates to the simple thermodynamics of Brownian motion; this is clearly not the case. With that in mind, "substrate channeling", especially of tRNAs during translation, was suggested to be employed to increase translation rates (NEGRUTSKII and DEUTSCHER 1991). Despite the fact that this concept was proposed nearly a quarter century ago, a molecular elucidation of tRNA channeling has remained elusive.

If tRNA channeling is occurring, it is likely to be facilitated by a complex of factors associated with elongating ribosomes. Several ribosome-associated proteins participate in translation elongation including the 40S subunit binding proteins RACK1/Asc1p (receptor for activated C kinase 1) and vigilin/Scp160p. RACK1 (the human protein) and its yeast ortholog Asc1p are Gβ-like WD40-repeat proteins that bind to several proteins including protein kinase C (PKC) and the 40S ribosomal subunit close to the mRNA exit

site (YATIME 2011, ADAMS et al. 2011). It plays significant roles in intracellular signaling, transcription and translation, and also interacts with several cell surface receptors and with proteins in the nucleus (ADAMS et al. 2011). In translation, RACK1/Asc1p promotes regulated subunit joining by providing a platform for PKC1 to phosphorylate the anti-association factor eIF6 (CECI et al. 2003) and contributes to release of the translation inhibitor ZBP1 from ribosomes on β-actin mRNA (CECI et al. 2012). Pertinent to the current study, RACK1/Asc1p provides a binding platform for association of Scp160p with polysomes (BAUM et al. 2004). Scp160p is the yeast ortholog of the evolutionary conserved vigilin proteins characterized by a unique repetitive array of 14 RNA-binding hnRNP KH domains (heterologous nuclear RNA-binding protein K homology) (DODSON and SHAPIRO 1997; WEBER et al. 1997). Loss of Scp160p impacts diverse processes including cellular ploidy, mating response, and spindle pole body biogenesis (GUO et al. 2003; SEZEN et al. 2009; WINTERSBERGER et al. 1995), suggesting that this protein participates in a fundamental molecular process such as protein synthesis. Consistent with this, loss of Scp160p leads to reduced binding of specific tRNAs to translating ribosomes (HIRSCHMANN et al. 2014). Its association with cytosolic and membrane-bound polysomes depends on Asc1p and Bfr1p, another ribosome-associated protein that physically interacts with Scp160p (LANG et al. 2001). Crosslinking studies demonstrate that Scp160p also interacts with elongation factor 1A (eEF1A), the critical *trans*-acting factor that delivers aminoacylated tRNAs to elongating ribosomes (BAUM et al. 2004). Vigilin has also been shown to co-purify in a complex with eEF1A and tRNA (KRUSE et al. 1998).

If Asc1p and Scp160p function to enhance translational efficiency by channeling tRNAs through polysome complexes, then their absence would alter the kinetics of tRNA utilization. The second part of my study tested this hypothesis by assaying the effects of the absence of these proteins on various aspects of translational fidelity. Deletion of either Asc1p or of Scp160p results in the same effects on some aspects of translational fidelity, while conferring different effects on others. Analysis of these similarities and differences, combined with knowledge of previously identified Asc1p and Scp160p interacting partners suggests a model in which both molecules are part of a complex of proteins that increases translational efficiency by physically linking elongating ribosomes together and recycling tRNAs in the vicinity of their cognate codons. Additional co-immunoprecipitation data implicate Scp160p in buffering diffusion of used tRNAs from ribosomes, whereas ribosome affinity purification in knockouts of Arc1p suggests that Asc1p may be part of a higher order complex of tRNA recycling.

Results

Exploring the Relationships between KH Domains within an Organism, and between Vigilin Homologs from Different Organisms

Scp160p is a protein composed of 14 repeats of the K-homology or „KH" domain motif. These domains generally fold into the same tertiary shape due to the same secondary structure elements, as human Fragile-X Mental Retardation Protein FMRP or the namesake Khd1p protein do, namely β1-α1-α2-β2-β3-α3 (NICASTRO 2015). A GXXG motif is found in the KH1 sequence between α1 and α2, while a variable loop connects β2 with β3. hnRNP K also contains KH domains.

Different functions have been attributed to different domains within Scp160p. The N-terminus is important for ribosome binding (BAUM 2004), while the C-terminal KH domains 13 and 14 are involved in the interaction with mRNA and with polysomes via Asc1p (GILBERT 2010; SCHRECK *Ludwig-Maximilians-Universität München* 2010). As vigilins are conserved from fungi to mammals, it is interesting to examine the structural similarities and divergences on the primary and secondary levels to generate further

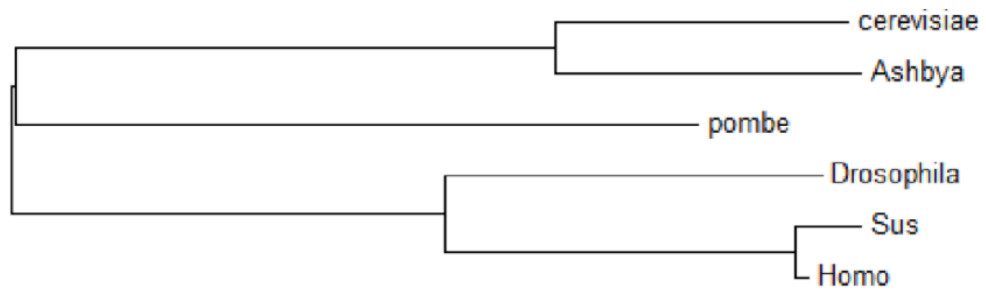


Fig. 1 A: Vigilins from fungi over metazoan to mammalians follow established relationships between species, indicative of conserved sequence of the gene, implying conserved function of the protein.

RefSeq obtained by BLAST of *Saccharomyces* vigilin from ENTREZ and SGD were ClustalW-aligned in MEGA 4.0 and used to construct a neighbor-joining tree.

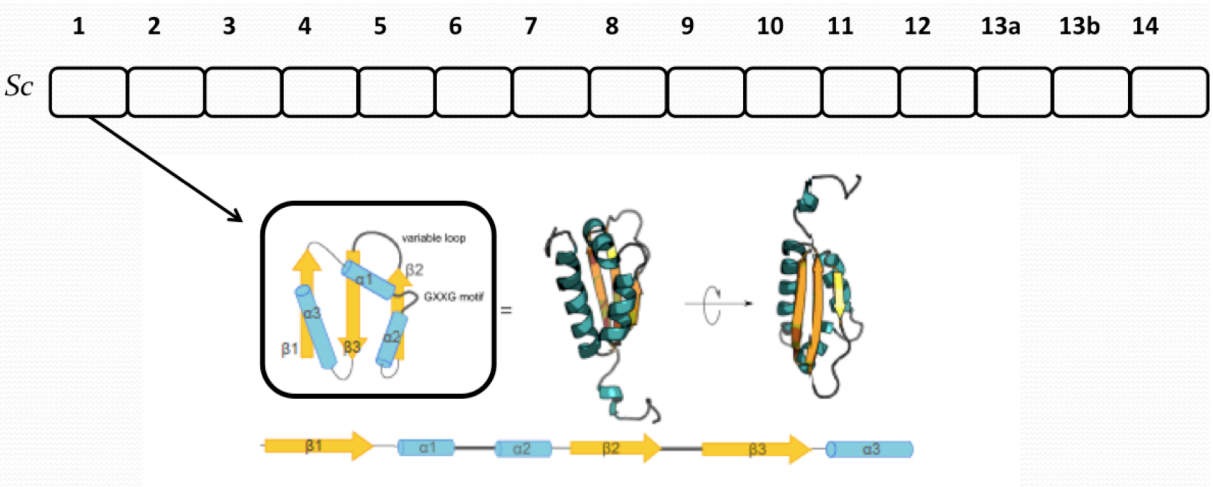


Fig. 1 B: Vigilins consist of a repetitive array of 14 similar but not identical KH domains, which are each made up by a motif of β1-α1-α2-β2-β3-α3 respectively.

Image courtesy of Jan Overbeck, modified for this thesis.

hypotheses. Since functions attributed to various members of the vigilin family mirror each other, e.g. mammalian vigilin also binds to the *ASC1* homolog RACK1, and is part of tRNA-containing RNPs (KRUSE&GRÜNWELLER 1998), commonalities and differences across clades may help distinguish conserved from diverged functions.

To shed light on the conservation and prototypicity of the KH domains within *SCP160* an its higher homologs, an evolutionary bioinformatical approach was selected.

Previous literature (e.g. BRYKAILO/CORBETT/FRIDOVICH-KEIL 2007) had tended to overemphasize the role of conserved GXXG motifs, and disregarded secondary structure in order to delimit and assign KH domains in vigilins, leading to seeming discrepancies in spite of largely conserved amino acid sequence. It thus appeared necessary to complement the hithero existing domain assignments with a novel approach. Then, by threading the non-prototypic behavior of domains onto the linear structure, and comparing these in an alignment according to evoluionary proximity of host organisms, I hoped to generate novel insights.

Interspecies alignments of vigilin protein sequences fit existing assumptions on the lineages of yeasts and mammals

First, vigilin sequences from several yeasts, fruit fly, and different mammalians (S*chizosaccharomyces pombe, Ashbya gossypii, Saccharomyces cerevisiae, Drosophila melanogaster, Sus scrofa*, and *Homo sapiens*) were identified by BLAST searches from databases, and homologous vigilin sequences were aligned using the MEGA suite with ClustalW algorithms. Alignments were subsequently used to build a neighbor-joining phylogenetic tree. As (**Fig. 1A**) shows, the vigilins closely follow the established relationships between the chosen species. This can be interpreted to mean that vigilins are evolutionarily conserved proteins with functions that co-evolve within their organisms.

Intraspecies alignments-based phylogenies show divergent evolution of KH domains within organisms

In order to illustrate the similarity between the 14 domains within the vigilin sequence (**Fig. 1B**), intraspecies alignments between the different KH domains were performed as above, but now using secondary structure predictions by PSIPRED (JONES 1999) from online servers (BUCHAN et al 2013) to delineate the domain limits.

As (**Fig. 2 A-H**) illustrate, KH domains within each organism have a distinct similarity pattern, yet closely related species tend to group corresponding domains as neighbors.

Assessment of „prototypicity" shows clear covariance with domain similarity only in *Saccharomyces cerevisiae*

As another layer of analysis, the presence or absence of „prototypical" GXXG consensus motifs between α1 and α2 was established (marked as yellow and pink in the above phenograms). Two observations can be made. First, the GXXG motif tends to „disappear" and „re-appear" quite loosely across clades. This can be interpreted to mean it is not paramount to function. Second, only for *S. cerevisiae* does GXXG presence correlate with a whole branch of the phylogenetic tree for a within-species alignment (**Fig. 2C**). This could mean that either Scp160p is outstanding in function, or that it is most ancestral. While a general pattern ex-

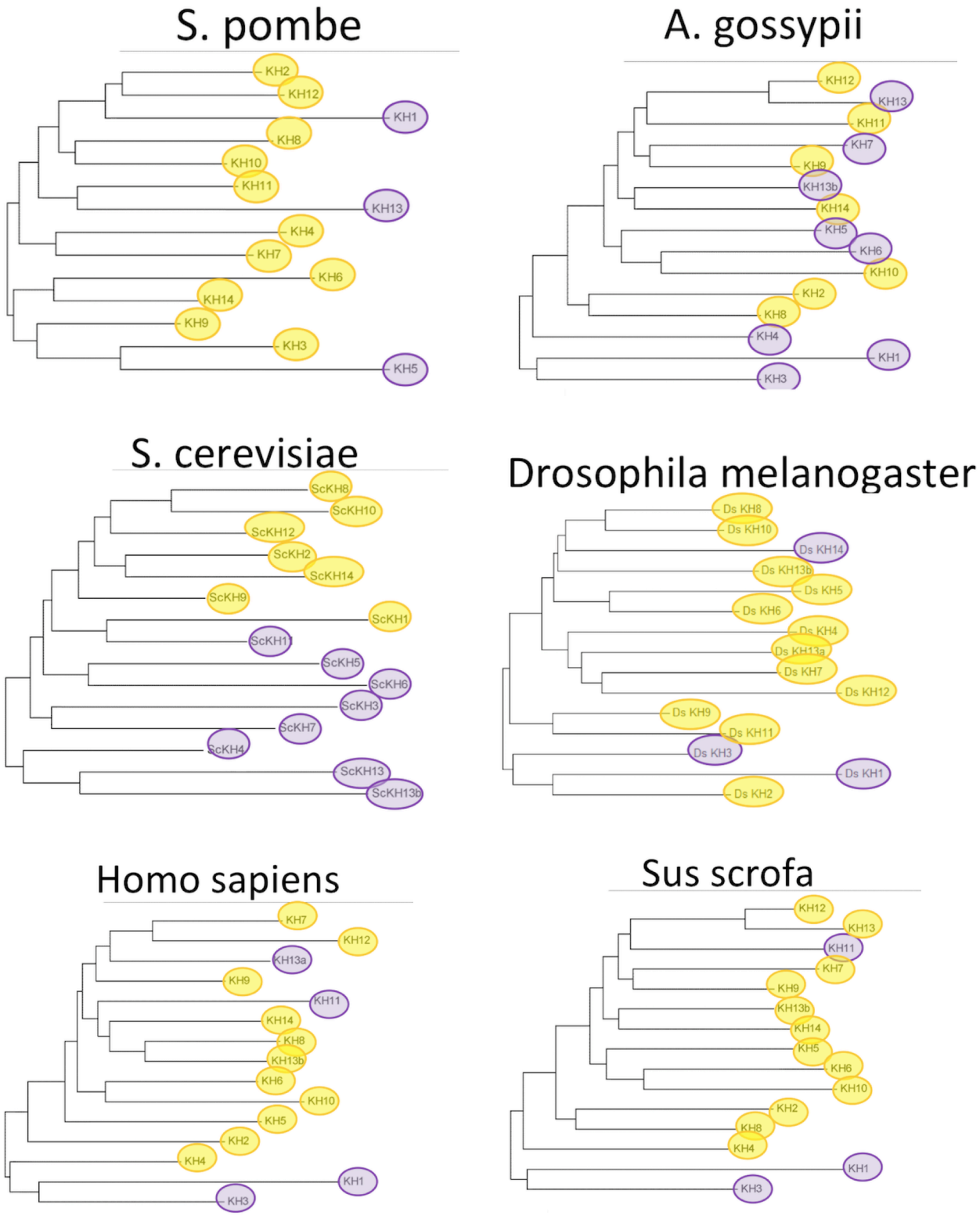


Fig. 2 (A-H): KH domains within each organism have a distinct similarity pattern, yet closely related species tend to group corresponding domains as neighbors.

PSIPRED predictions were used to delineate domain boundaries within each sequence. Then, all 14 KH domains were treated as individual sequences to obtain a Neighbor-Joining tree following a ClustalW alignment in MEGA 4.0 This enabled estimating within-species similarity between the different KH domains.

ists, namely that of GXXG presence being lost around the N- and C- termini, the similarity within clades as opposed to between clades (e.g. *S. cerevisia* and *A. gossypii* versus *D. melanogaster*) governs a more drastically typical behavior (**Fig. 3**).

Secondary structure prediction identifies several hotspots of evolutionary and structural dynamics across species

The PSIPRED secondary-structure predictions that had already enabled partitioning of KH domains for subsequent alignment, were further scrutinized for adherence to or divergence from similarity with the original KH domain. As shown in (**Fig. 4**), the domains within the sequence show a diverse pattern of secondary-structure loss and gain, that could have direct implications for either a loss or an explorative diversification of function. Three distinct patterns could be identified.

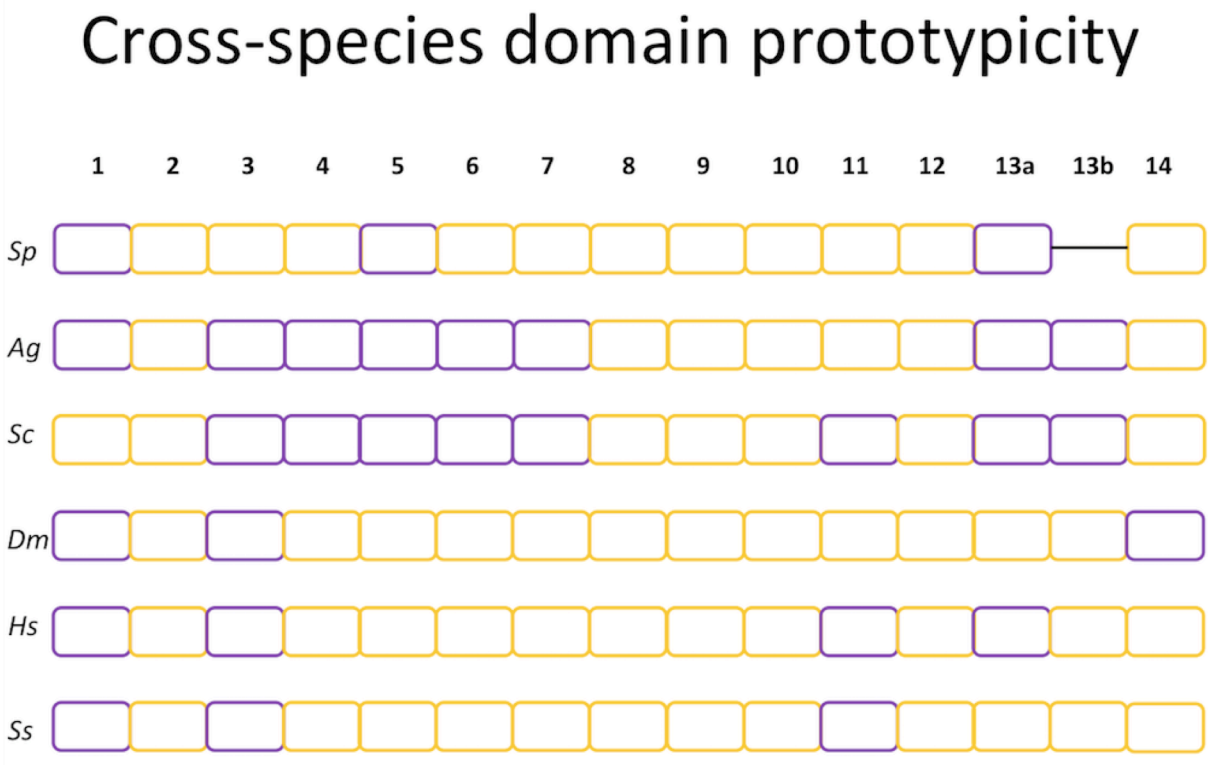


Fig. 3: While a general pattern of GXXG presence being lost around the N- and C- termini exists, the similarity within clades as opposed to between them governs a more drastically typical behavior, which is within clades.

Prototypicity of KH domains, compared across species examined, ordered by phylogenetic closeness. Yellow, "conserved" GXXG motif between α1 and α2. Purple, "diverged" different motif in this position.

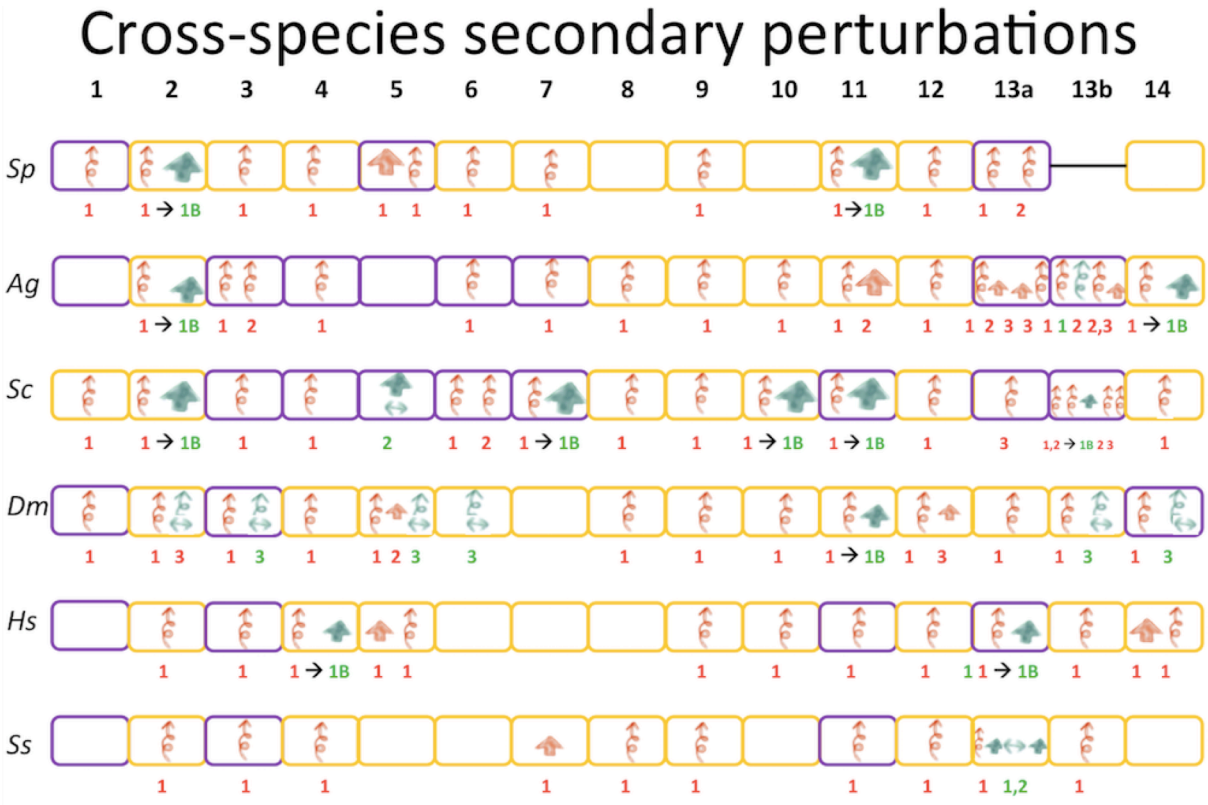


Fig. 4: The domains within the sequence show a diverse pattern of secondary-structure perturbation and rescue, with three common motifs: α1 loss, β „1B" gain, or distance increase.

PSIPRED predictions were used to compare "ideal" prototypicity as witnessed in Khd1p, and charted using a cartoon shorthand. Red, loss. Green, gain. Spring, α helix. Arrow, β sheet. Number, order within domain. Outward horizontal arrow, expansion of element or distance length.

The most common pattern is for a KH domain to lose the α1 helix. Interestingly this occurs without detectable preference to the position within the 14-KH array. Note that this is not the only, but the most prominent structural prediction feature that seems to have no covariance whatsoever with the presence or absence of a GXXG motif.

The second most common behavior is for a lost α1 helix to be replaced with a „1B" β sheet that wouldn't be present in the original model. This motif seems to be more confined to the N- and C-terminal regions of the sequence, across clades. These regions have also been shown to be important for ribosome binding.

The third curious motif is an expansion of either a motif or the distance between two motifs. In the case of *Drosophila*, α helixes in domains 2, 3; 5, 6; and 13b, 14 tend to expand in length. In the case of *Saccharomyces,* a β sheet in domain 5 expands. In the case of *Sus*, the distance between the first two β sheets in domain 13a expands. Whether this represents a teleological „drive" towards emergent „higher" functions, or a „within-clade" effect of group-specific diversification, is beyond the scope of this investigation.

However, the sum behavior of the organisms' vigilins suggests several conclusions. First, the secondary

structure mutability at the N- and C-termini in higher organisms is coincident with high functional importance of the same regions in yeast. This could mean a yeast-prototypical function has been expanded in higher organisms.

Second, for the similarity of KH domains within one organism, the *Saccharomyces cerevisiae* - exclusive phylogenetic bifurcation of GXXG-containing versus -less KH domains could mean two things: Either *Saccharomyces* is most ancestral, or another function was explored in a sidetrack that is absent from other lineages.

Either way, the initial findings merit further scrutiny of KH domains' similarities and differences, probing with *in vitro* experiments, after this lead generated *in silico*.

Using Ribosome-Affinity Purification to Assess Translation Behavior of Target mRNAs whose Levels Decrease in SCP160 Deletions

Scp160p is a protein proposed to interact with close to 1000 mRNA targets which seem to be functionally related according to a recent publication (HOGAN 2008), on top of being an mRNP component (WINTERSBERGER 1997, LANG/FRIDOVICH-KEIL 2000). An appreciable fraction of Scp160p target mRNAs is transcriptome-differential, i.e. either increases or decreases in RNA content, upon deletion of the *SCP160* gene.

As translational stalling can lead to no-go decay (INADA et al. 2013), it is interesting to ask whether all or some of the transcriptome-differential mRNAs are transcriptome-decreased following *SCP160* knockout due to translational stalling. Since autocorrelation, i.e. the successive re-use of different or same codons accepted by one tRNA (cf. Introduction), is a predictor of translational efficiency (CANNAROZZI 2010), it makes sense to include verification of this effect in an investigation.

Following up on a successful double-flanked approach using leads generated in a Diploma thesis at our lab (HIRSCHMANN, *Eberhard-Karls-Universität Tübingen* 2011), I decided to continuously complement experimental measurements with bioinformatical approaches, in a constant feedback loop between *in vitro* and *in silico*.

Tab. 1: The candidate genes shown to be transcriptome-differential upon SCP160 deletion in a previous thesis and paper. Data extraction and matching of candidate lists performed by me for this thesis. Bold, target genes queried during my analysis in mRNA-RAP and detected by qRT-PCR.

MA XCR: transcript level up- or downregulation as witnessed by polysome profiles from sucrose gradients quantitated via microarray in (SCHRECK, *Ludwig-Maximilians-Universität München* 2010).

SAM: significance analysis of microarray – conflated reproducibility and significance of microarray-quantitated myc-IP of mRNAs with Scp160p in (HOGAN 2008).

Global TPI: tRNA pairing index, a measure of isodecoding tRNA re-use propensity by same or different codons, across all amino acids within the transcript, according to my analysis, using programming by Gina Cannarozzi.

p-Val: Monte-Carlo-sampling-based t Test, giving probability of yielding equally non-random counts from 100,000 random pickings in the yeast genome with equal group member number, my analysis, programming by Gina Cannarozzi.

ORF	Gene	MA XCR>	qPCR XCR>	SAM	global TPI	p-Val
YHR087W		0,21		-3,94		
YLR297W		0,33		-2,44		
YLR327C	TMA10	0,28	1,01	-2,04		
YDR222W		0,39		-2,02		
YFR015C	GSY1	0,23		-1,69		
YER067W		0,05		-1,67		
YHL036W	**MUP3**	**0,23**	**0,95**	**-1,23**		
YGR008C	STF2	0,26		-1,2		
YEL011W	GLC3	0,3		-1,18		
YGR142W	BTN2	0,33		-0,9		
YPR157W		0,24		-0,75		
YJL133C		0,39		-0,65		
YBR183W	YPC1	0,36		-0,43		
YOR173W	DCS2	0,39		-0,41		
YMR240W	GAD1	0,3		-0,37		
YER130C		0,39		-0,32		
YJL052W		0,28		-0,25		
YJL052W		0,32		-0,25		
YMR104C	YPK2	0,27		-0,22		
YPL240C	HSP82	0,34		-0,21		
YJL144W		0,18		-0,15		
YBR054W	YRO2	0,24	0,89	0,006		
YER053C		0,1		0,01		
YER053C	PIC2	0,17		0,013		
YOR273C	TPO4	0,29		0,05		
YCR005C	CIT2	0,39		0,07		
YDR171W	**HSP42**	**0,13**	**0,92**	**0,08**		
YML128C	MSC1	0,34		0,23		
YBR169C	SSE2	0,36	0,77	0,24	0.446786	0.008160
YER037W	PHM8	0,35		0,54		
YCL040W	GLK	0,34		0,6		
YDR258C	HSP78	0,31		0,74		
YGR249W	MGA1	0,25	0,83	0,75		
YER150W	SPI1	0,19		0,84		
YGR248W	SOL4	0,29		0,86	0.337300	0.253350
YBR214W	SDS24	0,35		1,29		
YMR081C	ISF1	0,3		1,31		
YCR021C	**HSP30**	**0,04**		**1,32**		
YLL026W	**HSP104**	**0,25**	**0,89**	**1,52**		
YHL021C		0,3		1,8		
YPL014W		0,11		2,04		
YJL080C	SCP160	0,11	0,07	6,5		
YNR034W		0,14				
YOR134W	BAG7	0,33				
YLR142W	PUT1	0,35				
YMR084W		0,36				
YOR178C	GAC1	0,36				
YGR088W	CTT1	0,39				
YMR085C		0,4			0.532361	0.017490
global					0.458202	0.000520

In order to assess whether the mRNAs that change their abundance upon *SCP160* deletion are also translationally affected in this condition, four candidates from the transcriptome-decreased, and four candidates from the transcriptome-increased list, previously compiled from microarray data by Heidrun Schreck for a PhD thesis in our lab (SCHRECK, *Ludwig-Maximilians-Universität München* 2010), were picked for biochemical analysis.

Transcriptome-decreased mRNAs fall into distinct sub-classes of binding reproducibility, that exhibit different autocorrelation (tRNA re-use) strength

To assess whether two well-known aspects of translational fidelity - codon popularity, or codon autocorrelation - were specifically offset in the candidate groups, the tested mRNAs were divided into two groups, those that decreased in abundance after loss of Scp160p and those that increased. Subsequently, mRNAs were ordered by binding strength to Scp160p according to HOGAN 2008. Subgroups were then subjected to analysis of average codon frequency (CAI, codon adaptation index) and isodecoding-codon-autocorrelation (TPI, tRNA pairing index).

As the transcriptome-increased group was neither significantly offset towards genomic average in terms of codon popularity, nor codon autocorrelation, the remaining analysis in this chapter was set to focus on the other group.

The transcriptome-decreased group was found to fall into two sub-classes, one of high and one of low Scp160p binding strength as determined by SAM (Significance Analysis of Microarrays) Analysis (TUSHER et al. 2011) (**Tab. 1**). Further analysis showed that one group with insignificant „binding reproducibility“ to Scp160p in (HOGAN 2008), was also the one with lower autocorrelation. Inversely, a group with significant „binding reproducibility“ to Scp160p, was also the one with higher autocorrelation.

This means that the motif found for the transcriptome-stable but translatome-unstable mRNAs later also shows with the transcriptome-unstable mRNAs: Namely, that binding reproducibility to Scp160p is correlated with a propensity of mRNAs to be coding-optimized towards tRNA re-use.

mRNAs transcriptome-differential in *SCP160* deletion cells show translational induction and repression upon *SCP160* knockout

In order to experimentally tackle the behavior of said mRNA subgroups, two candidates from the „binding" versus two candidates from the „non-binding"group were subjected to translational efficiency analysis.

Ribosome-Affinity-Purification (RAP), a method established at our lab following pioneering work by HALBEISEN (2010), and adapted by me for RT-qPCR to quantify RNAs, was selected to probe for ribosomal occupancy of mRNAs in wild-type compared to *SCP160* deletion cells.

Following leads generated by investigations in our lab on the translation behaviour of transcriptome-stable mRNAs (SCHRECK *Ludwig-Maximilians-Universität München* 2010, HIRSCHMANN *Eberhard-Karls-Universität Tübingen* 2011), it was not surprising to find an overall pattern that all queried mRNAs were offset towards the wild-type in ribosomal occupancy in *SCP160* deletion cells. An interesting pattern nonetheless emerged from the differential reaction of mRNAs to SCP160 deletion from the „binding-reproducibility" subgroups, when paired with autocorrelation bioinformatics (**Fig. 5**).

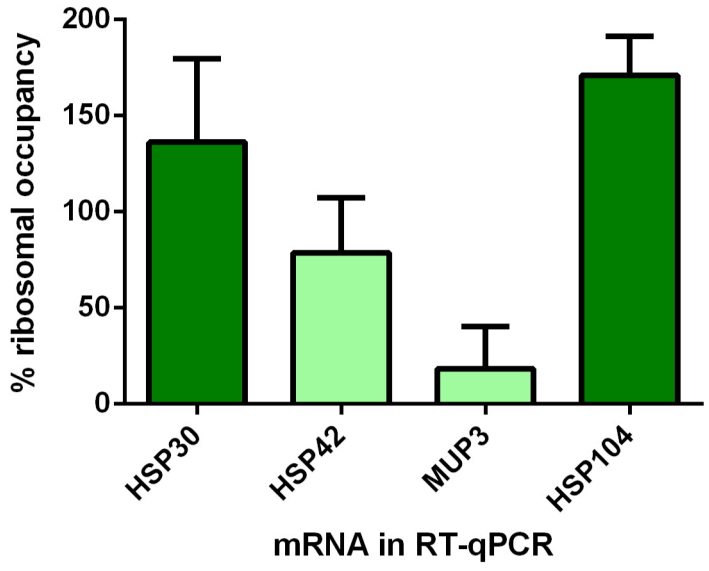


Fig. 5: Ribosomal occupancy of four select target mRNAs in SCP160 deletion cells, quantitated from RAP using RT-qPCR.

Dark green, higher-TPI mRNAs with high SAM score. Light green, lower-TPI mRNAs with low SAM score.

Characteristic features of mRNAs differ between targets translationally repressed or indudced upon SCP160 knockout

The first observation was that two targets - MUP3 and HSP42 - were translationally down regulated in the *SCP160* deletion cells, compared with wild-type levels (**Fig. 5**, light green bars). They were picked from the Scp160p-„binding-insignificant" cohort of transcriptionally depleted mRNA targets, which were on average less optimized for codon autocorrelation (0.42).

The second observation was that two other targets - HSP30 and HSP104 - were translationally upregulated in the *SCP160* deletion cells, compared with wild-type levels (**Fig. 5**, dark green bars). They were picked from the Scp160p-„binding-significant" cohort of transcriptionally depleted mRNA targets, which were on average more optimized for codon autocorrelation (0.53).

This means that the translational behavior following knockout of SCP160 may be tied to the characteristic coding optimization employed by the different subsets of target mRNAs.

Hypotheses towards the fate of transcriptome-differential mRNAs, and the relevance of Scp160 in the efficiency of translation

As elaborated in the Discussion section, no-go decay and down-regulation by decreased gene expression, respectively, are two conceivable explanations for the common result of transcriptional depletion for the two subgroups translationally depleted and increased in *SCP160* deletions, respectively.

This stage of the investigation raised the issue of re-interpreting „binding reproducibility" in this previous large-scale study. While the Discussion section offers a more concise view of this, briefly Scp160p-to-mRNA „binding reproducibility" in HOGAN 2008 may indeed better be viewed as a proxy for Scp160p/mRNA co-localization on the same polysomes. This is justified as prior investigations at our laboratory had suggested that co-immunoprecipitation of mRNAs could also be due to ribosome contaminations being characteristic of preparations of the protein (SCHRECK 2010).

The direction suggested by these results is that while autocorrelation may be a phenomenon synergistically working with the Scp160p protein to ensure optimal translation, badly-autocorrelated mRNAs may rely on a mechanism mediated by Scp160p to keep their translation optimal to a greater extent than well-

autocorrelated mRNAs do.

This motif was further reinforced by the following set of experiments. Here, the focus shifted to the mRNAs transcriptome-*stable* upon *SCP160* deletion, which had previously been analyzed for translational state by polysome profiles from sucrose gradients, quantified using microarrays, in (SCHRECK 2010).

Using Bioinformatics and Ribosome-Affinity Purification of tRNAs to Examine Effects of Scp160p Depletion on Translation

Bioinformatical analysis implies a role for tRNA autocorrelation in the translational down-regulation of mRNAs following Scp160 depletion

The implied translational impairment of mRNAs in Scp160p-deficient cells is consistent with slow growth of these cells, especially when grown on media supplemented with translation elongation inhibitors (SEZEN/SEEDORF/SCHIEBEL 2009, BAUM/BITTINS/FREY/SEEDORF 2001). This suggests that the protein or its interaction partners are at least indirectly linked to efficiency of translational elongation.

To elucidate a possible connection between the above observations, the codon composition of mRNA targets of Scp160p (HOGAN et al. 2008), or those translationally altered upon its knockdown (SCHRECK 2010, Tab. 4), was analyzed for use of popular codons, or re-use of tRNAs, computationally.

The first question I asked was if codon popularity or tRNA re-use would be a characteristic of mRNAs interacting with Scp160p. Therefore the tRNA pairing index (TPI, see FRIBERG/GONNET 2006, CANNAROZZI/BARRAL 2010) was computed for these mRNAs to measure the autocorrelation or "clustering" of codons for all amino acids (Tab. 2).

A marked increase in the average TPI was found for the entire group, with high significance (0.527 compared to 0.124 genomic average, p=0.005 for 100,000 random pickings of equal-sized groups), whereas the codon adaptation index (CAI, SHARP/LI 1987) was not significantly changed (results not shown). This result suggests that Scp160p interacting mRNAs might be biased towards optimized translation not by containing "popular codons" fitting to globally high tRNA abundance, but because "codon clustering" in them could cause locally high tRNA abundance.

Tab. 2: Candidate mRNAs with varying extent of reproducible Scp160p binding as reported by a previous study, as indicated via ordering by falling significance analysis of microarray (SAM) binding scores. *All SAM values presented here were experimented and calculated by Daniel J. Hogan in (HOGAN et al. 2008).* Re-ordering and target picking performed for this thesis.

Gene	ORF	SAM threshold	Gene	ORF	SAM threshold
AGA1	*YNR044W*	*8*	*DOA1*	*YKL213C*	*3*
BMS1	*YPL217C*	*7*	*GOR1*	*YNL274C*	*3*
SPF1	*YEL013W*	*7*	*TOM70*	*YNL121C*	*3*
PMT4	*YJR143C*	*7*	*SLA1*	*YBL007C*	*3*
UTP10	*YJL109C*	*7*	*TRM10*	*YOL093W*	*3*
ERG4	*YGL012W*	*7*	*CRN1*	*YLR429W*	*3*
FUS3	*YBL016W*	*6*	*ILV1*	*YER086W*	*3*
CRH1	*YGR189C*	*6*	*SEC31*	*YDL195W*	*3*
SCP160	*YJL080C*	*6*	*CSI2*	*YOL007C*	*3*
FAS1	*YKL182W*	*6*	*CCW14*	*YLR390W-A*	*3*
PRP19	*YLL036C*	*6*	*MSB2*	*YGR014W*	*3*
LSG1	*YGL099W*	*6*	*POM34*	*YLR018C*	*3*
SVL3	*YPL032C*	*6*	*MRPL3*	*YMR024W*	*2*
IMH1	*YLR309C*	*6*	*PMD1*	*YER132C*	*2*
NGR1	*YBR212W*	*6*	*-*	*YPL260W*	*2*
FEN1	*YCR034W*	*6*	*LTV1*	*YKL143W*	*2*
FKS1	*YLR342W*	*6*	*LPX1*	*YOR084W*	*2*
ROX1	*YPR065W*	*6*	*DNM1*	*YLL001W*	*2*
GNP1	*YDR508C*	*6*	*DHR2*	*YKL078W*	*2*
CDC48	*YDL126C*	*5*	*TYS1*	*YGR185C*	*2*
RPG1	*YBR079C*	*5*	*MRP4*	*YHL004W*	*2*
-	*YJR015W*	*5*	*GRC3*	*YLL035W*	*2*
KAP123	*YER110C*	*5*	*PDX1*	*YGR193C*	*1*
ILS1	*YBL076C*	*5*	*PDA1*	*YER178W*	*1*
HNM1	*YGL077C*	*5*	*-*	*YFL042C*	*1*
STO1	*YMR125W*	*5*	*PXL1*	*YKR090W*	*1*
ALO1	*YML086C*	*5*	*MUD2*	*YKL074C*	*1*
SED1	*YDR077W*	*5*	*DUR1,2*	*YBR208C*	*0*
NCP1	*YHR042W*	*5*	*ULA1*	*YPL003W*	*0*
PMT3	*YOR321W*	*4*	*GRX3*	*YDR098C*	*0*
ELP3	*YPL086C*	*4*	*RPS11A*	*YDR025W*	*0*
ASN2	*YGR124W*	*4*	*RER1*	*YCL001W*	*0*
MPT5	*YGL178W*	*4*			
KEM1	*YGL173C*	*4*			
- (eIF2A)	*YGR054W*	*4*			
SPT6	*YGR116W*	*4*			
CHA1	*YCL064C*	*4*			
RPD3	*YNL330C*	*4*			
SRP68	*YPL243W*	*4*			
PRY3	*YJL078C*	*4*			
APL6	*YGR261C*	*3*			

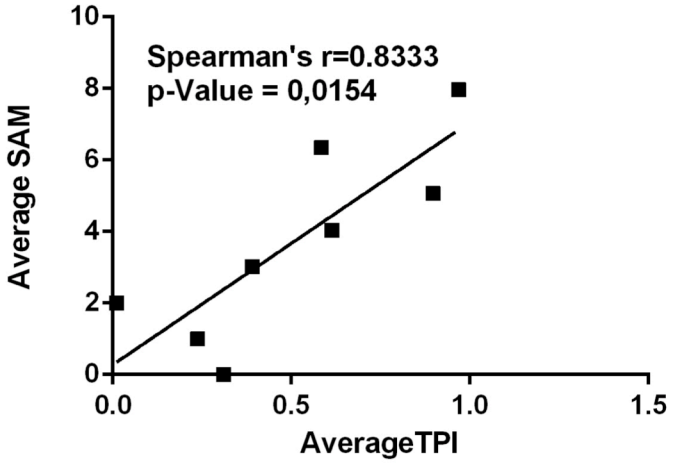


Fig. 6: High global tRNA re-use as implied by autocorrelation is a characteristic feature of mRNAs reproducibly interacting with Scp160p.

Average use of isoaccepting codons within subgroups of Scp160p-bound mRNAs (TPI, my analysis, programming by G.C.) shows pronounced nonlinear correlation with average binding reproducibility in a previous study (SAM, from HOGAN et al. 2008). *Image re-done; original analysis in my Diploma thesis (Univ. Tübingen 2011).*

The next objective was to determine if the observed deviation from genomic averages for "codon clustering" would be directional above the average non-randomness proven above. To this end subgroups of these mRNAs were created, ordered by rising binding reproducibility, subjected to within-group average calculations, and queried by t-test if the subgroups are significantly distinct. This analysis found that candidate subgroups ordered for binding reproducibility have a pronounced nonlinear correlation of high significance with codon autocorrelation (Spearman's r = 0.833, p = 0.015; **Fig. 6**), with the subgroups' averages significantly distinct (**Tab. 3**). This means that the more reproducibly mRNAs are found to associate with Scp160p, the more they are optimized for efficient translation.

Next, I asked whether the bias of Scp160p-interacting mRNAs to be skewed for "codon clustering", could be a key to explaining their shift to presumably "slow polysomes" in sucrose gradients as witnessed by a higher „translational state change" in previous work by another member of the lab (TSC, cf. **Tab. 4**). To this end, the TPI of mRNAs that had shifted to different degrees towards heavy polysome fractions after loss of Scp160p was calculated. The data were grouped into subgroups constructed by taking all members within distinct thresholds of TSC. This way, a rising 'translational depletion' threshold would select subgroups of decreasing size, raising the statistical threshold they would have to overcome to prove the significance of their elevated 'codon clustering': The smaller a set of genes, the less likely they are skewed by chance. For each subgroup, average TSC was plotted against average TPI as a measure of across-amino acid autocorrelation, and a pronounced nonlinear correlation of high significance was found (Spearman's r = 0.893, p=0.012, **Fig. 7**), with the subgroups' averages being significantly distinct (**Tab. 5**). The same tendency was observed for select amino acids when autocorrelation was resolved at within-amino acid level for the subgroups of mRNAs differently affected by Scp160p depletion (**Tab. 6**), albeit with the TPI only

Tab. 3: Average TPI (codon autocorrelation, my analysis, programming by G.C.) and SAM (Scp160p binding reproducibility, calculated by Hogan et al.) within subgroups from the HOGAN et al. 2008 study. P-values indicate significance of independent average values from neighboring subgroups.

Table re-done; original analysis in my Diploma thesis (Univ. Tübingen 2011).

TPI	SAM	p-Value
0,969	7,962	1,20E-02
0,584	6,350	6,11E-14
0,897	5,059	1,01E-16
0,614	4,037	5,62E-24
0,391	3,020	1,95E-19
0,010	2,005	2,68E-31
0,238	0,999	7,98E-21

promoting tRNA recycling and/or preventing tRNA diffusion being significantly distinct from random genomic pickings of equal-sized groups in some cases.

This means that the mRNA targets bound by Scp160p, and translationally affected more drastically by its knockdown, rely more heavily on 'codon clustering' (autocorrelation), which has been proposed to work by (FRIBERG/GONNET 2006, CANNAROZZI/BARRAL 2010).

Ribosome affinity purification followed by RT-qPCR enables quantitative detection of different tRNAs at the ribosome

The above-described results indicate that Scp160p might be involved in recruiting specific mRNAs with unique coding optimization to ribosomes. Alternatively, it would be conceivable that Scp160p is implicated in the correct execution of autocorrelation itself, i.e. at its mechanistic basis, e.g. via preventing diffusion of tRNAs. Since both scenarios are in line with the bioinformatical meta-studies (see above), an experimental setup was conceived to distinguish between both models. It was hypothesized that if the protein worked by recruiting mRNAs of biased codon composition to ribosomes, or ensuring correct tRNA "re-use" for these, loss of Scp160p should decrease the ribosome occupancy of such tRNAs that have a potential for pronounced recycling on target mRNAs (KRUSE/MÜLLER 1996, CANNAROZZI/BARRAL 2010, KRUSE/MÜLLER 1998). Successful measurement of such changes would speak in favour of the substrate channelling hypothesis, which holds that re-use of discharged tRNAs and their circulation near the ribosome boosts translational efficiency (KOZAK 1992, NEGRUTSKII/DEUTSCHER 1991).

Tab. 4: Candidate mRNAs from a previous thesis with varying extent of translational depletion following Scp160p depletion, as indicated via ordering by falling translational state change (TSC) values. *All TSC values shown here were experimented and calculated by Heidrun Schreck in (SCHRECK 2010).*

Gene	ORF	TSC	Gene	ORF	TSC
	YPR159C-A	3,71	*MMS21*	YEL019C	1,82
	YHR213W	2,67	*TIS11*	YLR136C	1,82
PRY3	YJL078C	2,66	*MSB2*	YGR014W	1,81
FIG2	YCR089W	2,6		YOR394C-A	1,81
SLS1	YLR139C	2,6	*SCD5*	YOR329C	1,81
	YAL065C	2,59	*MGA1*	YGR249W	1,8
DAN4	YJR151C	2,42		YDR034C-A	0,55
PTH1	YHR189W	2,4		YGR109W-A	0,55
	YAL065C	2,29		YDL156W	0,55
HKR1	YDR420W	2,23	*EPL1*	YFL024C	0,52
MUC1	YIR019C	2,21		YFL068W	0,52
SEC16	YPL085W	2,18	*BUD25*	YER014C-A	0,52
AGA1	YNR044W	2,17		YIR018C-A	0,51
VRP1	YLR337C	2,17	*HUR1*	YGL168W	0,47
BNI1	YNL271C	2,17		YFR035C	0,47
GAT3	YLR013W	2,15		YGR240C-A	0,46
FLO1	YAR050W	2,09		YGR035W-A	0,45
	YAR009C	2,07		YDR524W-A	0,36
FLO10	YKR102W	2,05	*CFT1*	YDR301W	0
	YJL216C	2,05	*POM34*	YLR018C	-
LAS17	YOR181W	2,01			
AAR2	YBL074C	2,01			
	YOL019W-A	2			
MOT3	YOL019W-A	1,97			
NKP2	YLR315W	1,95			
	YOR316C-A	1,95			
	YBR072C-A	1,94			
	YNL277W-A	1,94			
FKH2	YNL068C	1,91			
	YCR108C	1,91			
ATG15	YCR068W	1,9			
PRM7	YDL039C	1,9			
PMD1	YER132C	1,89			
MTL1	YGR023W	1,88			
	YBL044W	1,87			
YPT53	YNL093W	1,87			
UTR5	YEL035C	1,85			
CCW14	YLR390W-A	1,84			
	YNL034W	1,84			
ATP10	YLR393W	1,83			
SEC31	YDL195W	1,83			

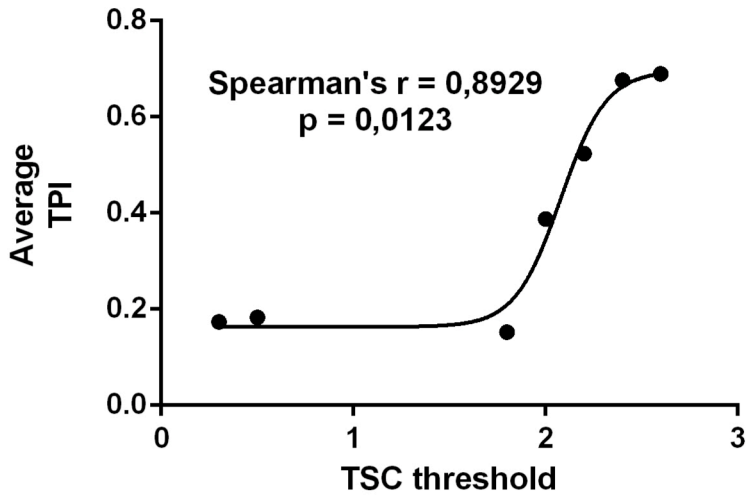


Fig. 7: High codon autocorrelation is a characteristic feature of mRNAs more drastically affected by Scp160p depletion in a previous thesis in our lab.

Average use of isoaccepting codons within subgroups of Scp160p-bound mRNAs (my analysis, programmed by G.C.) shows pronounced non-linear correlation with average translational repression threshold in polysome assay (experimented and calculated in SCHRECK 2010).

Therefore, a protocol for detection of tRNAs co-precipitating with ribosomes was established, to verify *in vivo* that the translational decrease observed in mRNAs upon Scp160p depletion is interconnected with tRNA autocorrelation, as predicted by bioinformatics. Translating ribosomes were arrested by cycloheximide before applying RAP This way, the translocation step was inhibited, stalling the ribosomes without dissociating them. tRNAs co-precipitating with ribosomes were reverse-transcribed and quantitated by qPCR. Extensive homology searching and multiple sequence alignments ascertained maximum uniqueness for annealing region recognition. In order to cover a broad spectrum of different tRNA classes, analyses included one 'synonym-poor' tRNA (that decodes only one codon) and one 'synonym-rich' tRNA (that decodes more than one codon), for four different amino acids (Ser, Leu, Thr, Val) respectively (8 tRNAs in total). For each amino acid, certain combinations of tRNAs using 'rare' (low RSCU, i.e. low genomic frequency) versus 'popular' (high RSCU, i.e. high genomic frequency) codons with or without capacity to recognize different synonymous isodecoding codons were chosen. Rare or popular is defined using the RSCU (relative synonymous codon usage, (PLOTKIN/KUDLA 2011, SHARP/ LI 1987). The RSCU was computed and the highest and the lowest taken as popular or non-popular, respectively. (Tab. 7) sums up the array of tRNA gene classes investigated, together with the number and popularity of synonymous codons. For each homologous tRNA class, one representative member was used as the eponym for the whole block used in multiple sequence alignments to construct qPCR primers (see Methods). tS_E and tL_L represent high-copy gene families for tRNAs (Ser2, Leu1) recognizing a popular codon, whereas tS_F and tL_G denote low-copy gene families encoding tRNAs (Ser4, Leu3) each recognizing several unpopular codons. tT_J and tV_M represent high-copy gene families for tRNAs (Thr1, Val1) recognizing several popular codons, whereas tT_K and tV_D denote low-copy gene families encoding tRNAs (Thr3, Val2)

recognizing an unpopular codon. Thus, all possible combinations were assessed to distinguish between effects due to codon popularity and/or synonym-richness.

As will be elaborated below, in contrast to synonym-richness, codon clustering cannot be inferred from the tRNA, but has to be computed from the usage in mRNA targets of interest. In this aspect it is similar to codon popularity. However, codon clustering, in contrast to codon popularity, is a "local" effect. These respective values stem from a subset of mRNAs affected by *SCP160* depletion and were amended at a later point in the study (**Tab. 8**).

As a preliminary experiment, yeast cells expressing a temperature-sensitive mutant of the gamma subunit of translational elongation factor eEF1B (*yef3-F650S*; (ANAND/KINZY 2003) was grown at restrictive temperature and the ribosomal occupancy of tRNAs from this mutant was compared with that of wild-type cells to assess the impact of impaired translation elongation on ribosomal occupancy. As a general pattern, a more drastic decrease in ribosomal occupancy was found for popular codon-reading tRNAs than for unpopular ones within a given amino acid. At the same time, ribosomal occupancy across all investigated amino acids dropped uniformly compared to wild type as shown for Serine and Leucine (**Fig. 8A, B**). This is consistent with impaired translation elongation and consequently, loss of tRNAs from the ribosome.

To support this finding, ribosome-associated tRNAs coding for two amino acids from the set were also quantitated in an alternative translational stress condition, amino acid depletion. Again, this led to a uniformly higher drop in ribosome association of tRNAs recognizing popular codons as opposed to

Tab. 5: Querying for higher translational impairment in a previous thesis upon Scp160p depletion does not impair significance of elevated autocorrelation, in spite of smaller sub-groups.

Average TPI (corresponds to codon autocorrelation) and CAI (corresponds to codon frequency above genomic average) at mRNA level across all amino acids, for rising TSC (equivalent to degree of mRNA distribution shift in polysome gradients) threshold.

P-values indicate significance by comparison with 100 000 random genomic pickings of equally sized groups. TPI and CAI were calculated for the whole set, respectively, up to the indicated TSC threshold. TSC as reported by SCHRECK 2010. TPI, CAI my analysis, programmed by G.C.

TSC threshold	Number in group	TPI	p-Value	CAI	p-Value
0,3	45	0,174	3,15E-01	0,146	3,69E-03
0,5	43	0,183	2,89E-01	0,149	8,98E-03
1,8	39	0,153	4,01E-01	0,150	1,79E-02
2,0	22	0,388	3,51E-03	0,162	2,02E-01
2,2	11	0,523	2,49E-02	0,160	3,02E-01
2,4	8	0,676	8,42E-03	0,154	2,82E-01
2,6	5	0,690	2,98E-02	0,161	4,40E-01

unpopular codons for Threonine and Valine (**Fig. 8 C, D**). As the most highly expressed genes are also uniformly biased towards popular codons (SHARP/LI 1987), this experiment confirms that global translational deficits are mirrored in our tRNA-RAP measurements.

Ribosomal occupancy of most synonym-rich tRNAs decreases upon Scp160p depletion, regardless of popularity of cognate codons

Next, the method was applied to monitor tRNA ribosomal occupancy to Scp160p depleted cells. Again, in comparison to wild-type, the level of both assayed tRNA species of a given amino acid dropped upon depletion of Scp160p (**Fig. 9 A-F**). Yet the pattern was different than the one observed for *yef3-F650S* cells. For three of the four amino acids investigated (Serine, Threonine, Valine), loss of Scp160p more drastically decreased ribosomal occupancy of "synonym-rich" tRNAs recognizing several synonymous codons. Conversely, "synonym-poor" tRNAs read by only one synonymous codon were still co-precipitating with ribosomes at levels similar to wild-type.

Of note, this effect was independent of the popularity of the codons recognized by said tRNAs, in contrast to the pattern observed in the *yef3-F650S* mutant. For instance for Serine, the unpopular, synonym-rich Ser4 species was significantly ($p<0.1$) more depleted (30.4 +/-

Tab. 6: Average TPI for individual amino acids (= codon autocorrelation at amino acid level), in mRNA target subgroups ordered by TSC (degree of mRNA distribution shift in polysome gradients) threshold, from Fig. 5.

P-values indicate significance by comparison with 100,000 random genomic pickings of equally sized groups. TPI was calculated for the whole set respectively, up to the indicated TSC threshold. TSC as reported by SCHRECK 2010. TPI, CAI my analysis, programmed by G.C.

	Serine		Leucine	
TSC threshold	TPI	p-Val	TPI	p-Val
0.3	0.116	0.277	0.175	0.397
0.5	0.132	0.343	0.166	0.433
1.8	0.0830	0.187	0.122	0.378
2.0	0.128	0.374	0.124	0.413
2.2	0.441	0.067	0.204	0.388
2.4	0.598	0.018	0.276	0.283
2.6	0.535	0.089	0.181	0.464

	Threonine		Valine	
TSC threshold	TPI	p-Val	TPI	p-Val
0.3	0.293	0.00418	0.0546	0.403
0.5	0.319	0.00189	0.0567	0.394
1.8	0.324	0.00248	0.0560	0.405
2.0	0.576	0.00002	0.180	0.133
2.2	0.652	0.00043	0.103	0.353
2.4	0.600	0.00490	0.177	0.258
2.6	0.573	0.02880	0.301	0.174

3.3% residual occupancy) than the popular, synonym-poor Ser2 species (64.3 +/-18.7% residual occupancy, **Fig. 9A**). In contrast, for valine, the popular, synonym-rich Val1 species showed a highly significant ($p<0.01$) greater depletion (46.8 +/-1.2% residual occupancy) than the unpopular, synonym-poor Val2 species (107.2 +/-7.9% residual occupancy, **Fig. 9D**).

In summary, following Scp160p depletion, the decrease in ribosomal occupancy of synonym-rich tRNAs was much more pronounced than the respective decrease for synonym-poor tRNAs. This unique pattern of tRNA depletion from the ribosome cannot be explained by a general translational depletion, and speaks in favour of a specialized role for Scp160p in translational efficiency.

Scp160p can boost tRNA 're-use' independent of, but synergistic with, mRNA optimization at the codon level

Intuitively, one might speculate that synonym-rich tRNAs might have a higher chance to "recycle" and thus would be more prone to be used in "codon clusters". This might lead to the premature assumption that the ribosomal occupancy drop of synonym-rich tRNAs upon knockdown already means that Scp160p has a function in autocorrelation itself. However, no such clear-cut prediction of codon clustering from synonym-richness is possible, as autocorrelation, just like codon popularity and positive charge stalling, is an evolutionarily entrenched effect (PERCUDANI et al. 1997, CANNARozzI et al. 2010, CHARNESKY/HURST 2013). Entrenchment is characterized by constant feedback between divergent mutation and confining selection (MAYR 1953, reviewed in NIKLAS/

Tab. 7: tRNA species in experimental analysis.

tRNA gene family names, common tRNA names, anticodon used, codons recognized and RSCU for the candidate pairs contrasting within the four amino acids experimentally assessed (nomenclature according to PERCUDANI et al. 1987). RSCU values for codon frequency analogous to SHARP, LI 1986, as published online, courtesy of Institut Pasteur webservers (http://www.pasteur.fr/~tekaia/HYG/hemiasrscu.html).

(tRNA) gene family	*tS_E (Ser2)*	*tS_F (Ser4)*	*tL_L (Leu1)*	*tL_G (Leu3)*
anticodon	UGA	GCU	UAA	GAG
codons	uca	agu, agc	uua	cuu, cuc
frequent? (RSCU)	y (1.27)	n, n (0.97 / 0.67)	y (1.65)	n, n (0.79 / 0.35)

(tRNA) gene family	*tT_J (Thr1)*	*tT_K (Thr3)*	*tV_M (Val1)*	*tV_D (Val2)*
anticodon	AGU	CGU	AAC	GAC
codons	acu, acc	acg	guu, guc	gua
frequent? (RSCU)	y, n (1.36 / 0.85)	n (0.56)	y, n (1.55 / 0.80)	n (0.86)

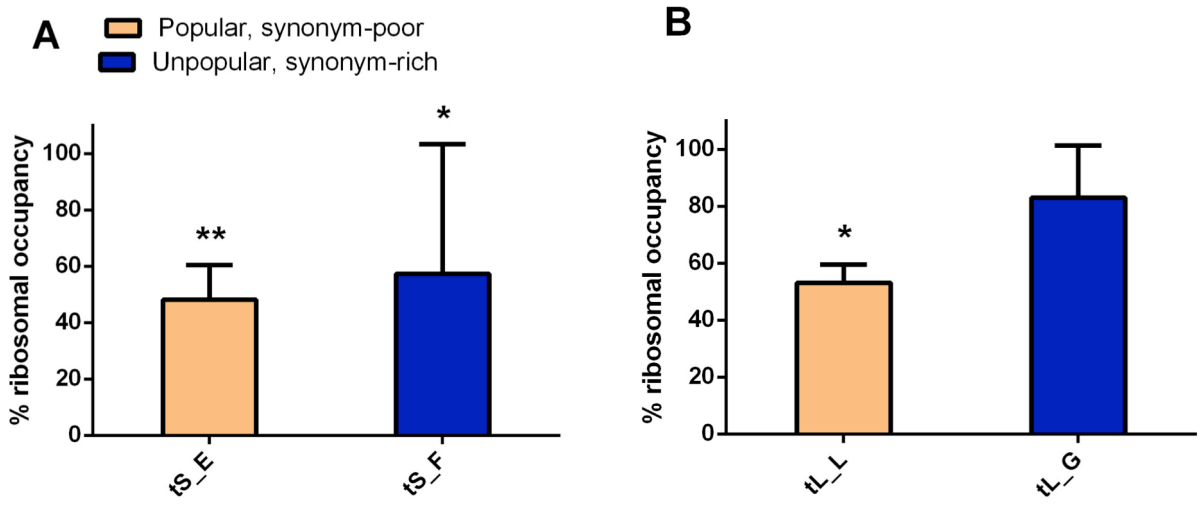


Fig. 8 A, B: Ribosomal occupancy of tRNAs recognizing frequent codons drops more severely upon loss of eEF1a function.

Displayed is the percentage of ribosomal occupancy (ribocc) of indicated tRNAs in yef3(F650S) as percentage of ribocc found in wild-type cells. The standard deviation between at least two biological replicates with two technical replicates each is indicated as bars, while * denotes p-Values <0.1 and ** denotes p- Values <0.01 for significance of independent average values between restrictive and permissive temperature levels (above columns).

Residual ribosomal occupancy (ribocc) of tRNA pairs for amino acids serine and leucine after temperature shift of a temperature-sensitive eEF1a mutant (yef3(F650S)).

(A) Serine, comparing Ser2 and Ser4: tRNAs recognizing only one frequent (orange) versus several infrequent (blue) codons. (B) Leucine, comparing Leu1 and Leu3: tRNAs recognizing only one frequent versus several infrequent codons.

KUTSCHERA 2004), and leads to a selection of „what works best“, in spite of original distinctions between erstwhile equal choices in evolution.

Two considerations follow: First, synonym-poor tRNAs can also benefit from codon clustering if compared with equal amino acid choice but from a different tRNA. Second, autocorrelation varies in extent across mRNAs in the whole genome. However, similar autocorrelation patterning seems to fit with ontological similarities within mRNAs, as was evidenced for unpopular-codon-exclusive autocorrelation in amino acid starvation genes that seem to make use of a rare „shelf-warmer“ pool (CANNARozzI et al. 2010).

To elucidate the basis of how Scp160p is beneficial to optimal translation that isn't caused by global use of "popular" codons, or local general tRNA re-use, the analysis thus had to be improved at the amino acid average level (**Tab. 6**) by performing a similar analysis at the resolution of individual tRNAs (**Tab. 8**), to find out which tRNAs are indeed the most and least dependent on coding-optimization by 'codon clustering'.

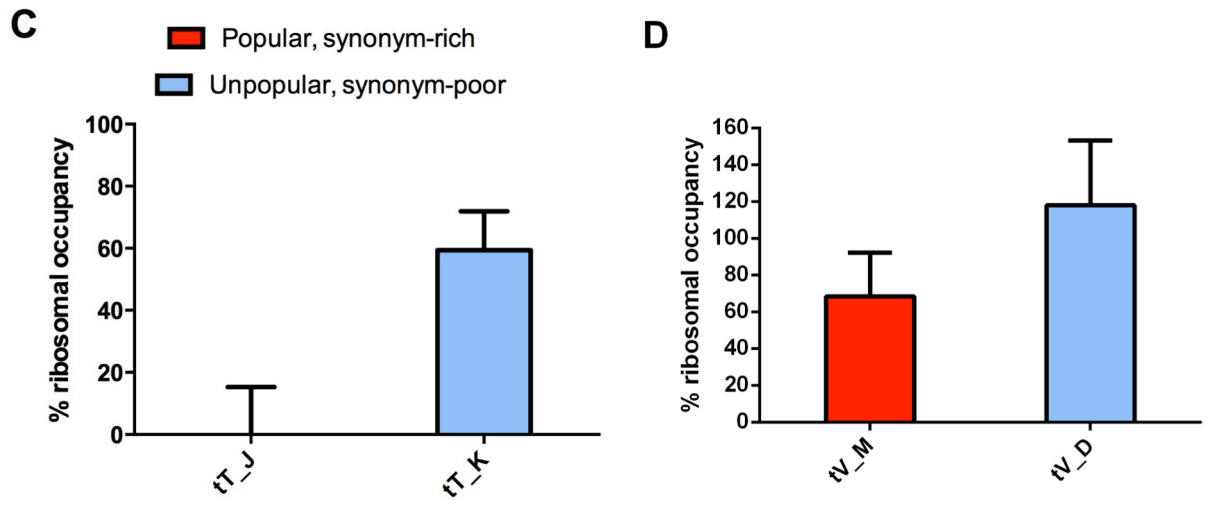


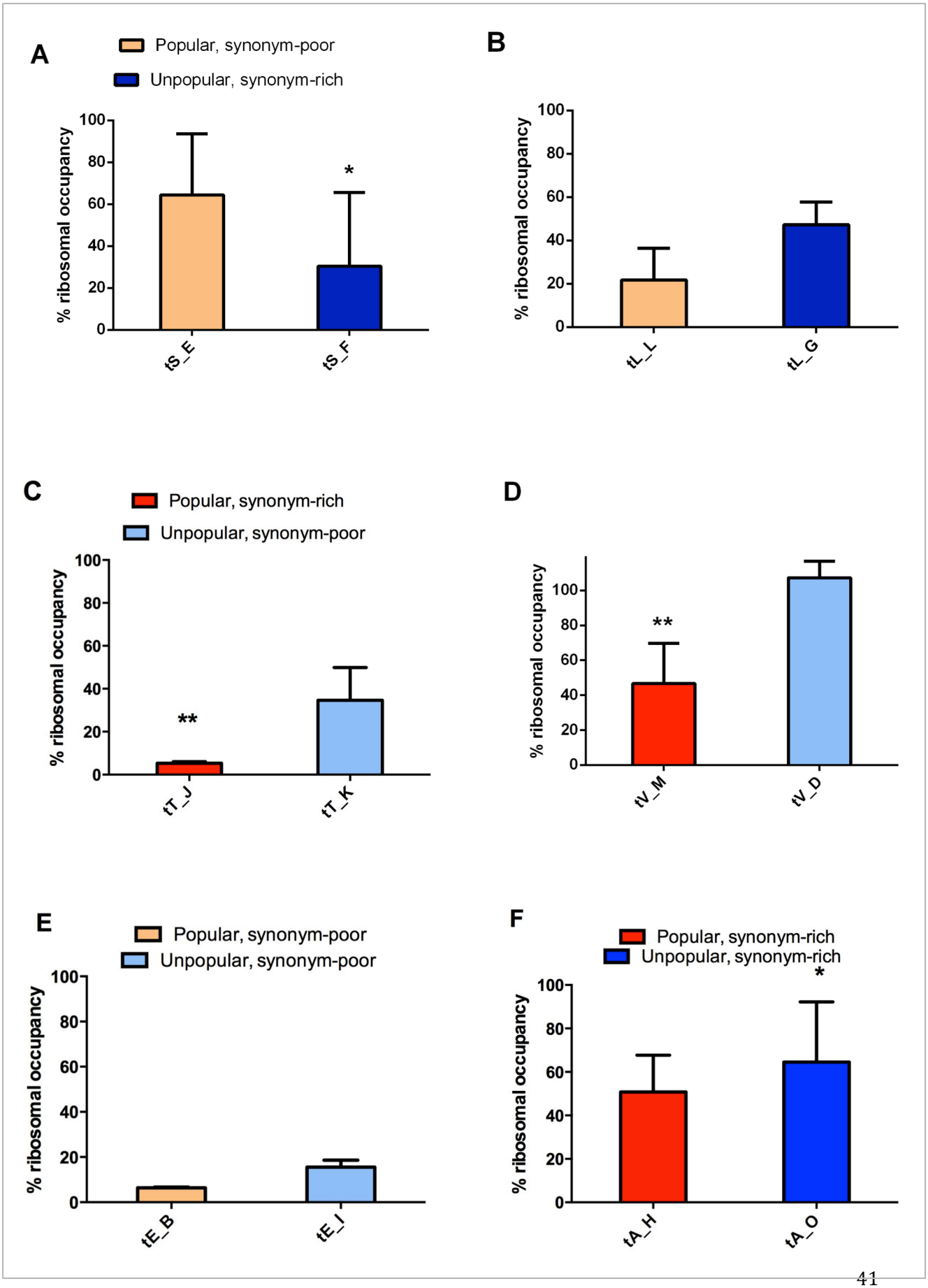
Fig. 8 C, D: Ribosomal occupancy of tRNAs recognizing frequent codons drops more severely after amino acid depletion.

Displayed is the percentage of ribosomal occupancy (ribocc) of indicated tRNAs in yef3(F650S) as percentage of ribocc found in wild-type cells. The standard deviation between at least two biological replicates with two technical replicates each is indicated as bars, while * denotes p-Values <0.1 and ** denotes p- Values <0.01 for significance of independent average values between restrictive and permissive temperature levels (above columns).

Residual ribosomal occupancy (ribocc) of tRNA pairs for amino acids threonine and leucine after shift from amino acid-rich (synthetic complete dextrose) to amino acid-poor (synthetic minimal dextrose) media. (C) Threonine, comparing Thr1 and Thr3: tRNAs recognizing several frequent (red) versus only one infrequent (light blue) codon. (D) Valine, comparing Val1 and Val2: tRNAs recognizing several frequent versus only one infrequent codon.

When calculating the autocorrelation ('codon clustering') of mRNA subgroups that are to varying degrees translationally affected by Scp160p depletion at a tRNA resolution, it turned out that the tRNAs *least impaired* in ribosomal occupancy indeed are *most codon-clustering* within the mRNA groups specifically affected by Scp160p depletion, i.e. those with a high TSC. Conversely, the tRNAs *most affected* by Scp160p depletion are in contrast the ones that are the *least codon-clustering* in the mRNA groups most affected - in spite of a general tendency of these targets to be 're-use'-optimized at within-amino acid resolution, presumably by virtue of the other tRNA (**Tab. 6**). At the same time, the setup of tRNA classes assessed rules out contributions imparted by other effects, i.e. codon popularity or synonym-richness of tRNAs.

Thus, the observed ribosomal occupancy changes of tRNAs cannot be explained by impaired recruitment of clustering-biased mRNAs to the ribosome. This finding also immediately rejects another hypothesis, namely that Scp160p would be involved in the correct execution of robust autocorrelation. In contrasts, it indicates that Scp160p could provide the same benefits in translational fitness as autocorrelation


A
Popular, synonym-poor
Unpopular, synonym-rich
% ribosomal occupancy
100
80
60
40
20
0
*
tS_E
tS_F
B
% ribosomal occupancy
100
80
60
40
20
0
tL_L
tL_G
C
Popular, synonym-rich
Unpopular, synonym-poor
% ribosomal occupancy
100
80
60
40
20
0
**
tT_J
tT_K
D
% ribosomal occupancy
100
80
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20
0
**
tV_M
tV_D
E
Popular, synonym-poor
Unpopular, synonym-poor
% ribosomal occupancy
100
80
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40
20
0
tE_B
tE_I
F
Popular, synonym-rich
Unpopular, synonym-rich
% ribosomal occupancy
100
80
60
40
20
0
*
tA_H
tA_O

Fig. 9 (A-F): A specific pattern of ribosomal tRNA occupancy is observed upon Scp160p depletion, that is not connected to the popularity or number of codons recognized by corresponding codons.

Residual ribocc of tRNA pairs for amino acids on Scp160p depletion, displayed as percentage of wild-type levels. The standard deviation between at least two biological replicates with two technical replicates each is indicated, while *, P <0.1 and **, P <0.01 for significance of inde- pendent average values between knockdown and wild-type (above columns). **A**, Serine, comparing Ser2 and Ser4: tRNAs recognizing one frequent (orange) versus several infrequent (dark blue) codons. **B**, Leucine, comparing Leu1 and Leu3: tRNAs recognizing one frequent versus several infrequent codons. **C**, Threonine, comparing Thr1 and Thr3: tRNAs recognizing several frequent (red) versus one infrequent (light blue) codon. **D**, Valine, comparing Val1 and Val2: tRNAs recognizing several frequent versus only one infrequent codon. **E,** Glutamate, comparing Glu1 and Glu2: tRNAs recognizing one popular versus one unpopular codon. **F,** Alanine, comparing Ala1 and Ala2: tRNAs recognizing several popular versus several unpopular codons.

optimization does, i.e. by preventing used tRNAs' diffusion or aiding used tRNAs' recycling (**Fig. 10**). Furthermore, autocorrelation seems to be a robust, independent mechanism, which however relies upon an intact molecular setup of the ribosome with accessory beneficial factors, of which Scp160p might be a part.

Tab. 8: The tRNAs recognizing codons *less* autocorrelated in Scp160p target mRNAs are *more* depleted from ribosomes following Scp160 depletion. This effect in independent both of the *number* of synonymous codons, or their *frequency* in the genome.

The tRNAs more depleted from ribosomes upon Scp160p deletion are recognizing the same codons that are the least optimized for autocorrelation in the mRNA groups most translation-depleted in the same condition in a previous study.

Z-score percentage (equals to codon autocorrelation at the tRNA level; my analysis, programming by G.C.) in mRNA target subgroups ordered by TSC (equivalent to degree of mRNA distribution shift in polysome gradients, from a previous thesis by SCHRECK 2010) threshold.

Z-scores were calculated individually for each indicated set up to the indicated TSC threshold. All tRNAs analyzed are shown. For enhanced clarity, underlinings of average Z-score values corres- pond to color-coding of the respective tRNA species analyzed experimentally in the above figures, with red versus blue shades for popular or unpopular codons, and bold versus faint shades for synonym-rich or synonym-poor tRNAs.

(tRNA) gene	tS_E (Ser2)	tS_F (Ser4)	tL_L (Leu1)	tL_G (Leu3)
TSC threshold	Z% (#)	Z% (#)	Z% (#)	Z% (#)
0.3	18.45 (299)	2.78 (334)	0.10 (150)	10.65 (118)
0.5	18.46 (297)	2.73 (331)	-0.06 (150)	10.17 (115)
1.8	17.33 (205)	1.68 (309)	-2.08 (141)	5.44 (100)
2.0	23.6 (194)	-4.6 (201)	-4.97 (86)	12.54 (70)
2.2	34.04 (112)	-7.51 (105)	-5.81 (25)	22.49 (34)
2.4	39.05 (69)	10.12 (72)	3.89 (18)	28.52 (25)
2.6	26.60 (63)	3.65 (43)	-4.58 (14)	26.57 (23)
average	17.75 (302)	2.57 (345)	-0.06 (163)	9.95 (121)

(tRNA) gene	tT_J (Thr1)	tT_K (Thr3)	tV_M (Val1)	tV_D (Val2)
TSC threshold	Z% (#)	Z% (#)	Z% (#)	Z% (#)
0.3	8.3 (1405)	-23.72 (89)	7.00 (609)	29.17 (128)
0.5	8.25 (1405)	116.36 (87)	7.09 (607)	28.71 (125)
1.8	8.04 (1380)	117.08 (83)	7.57 (583)	30.43 (122)
2.0	8.64 (1168)	168.8 (69)	9.66 (436)	47.68 (99)
2.2	8.35 (663)	213.81 (58)	14.88 (196)	59.42 (68)
2.4	4.63 (398)	27.98 (10)	10.64 (132)	48.23 (16)
2.6	6.63 (202)	22.61 (9)	8.98 (71)	36.31 (13)
average	7.91 (1433)	115.05 (89)	6.15 (642)	28.01 (131)

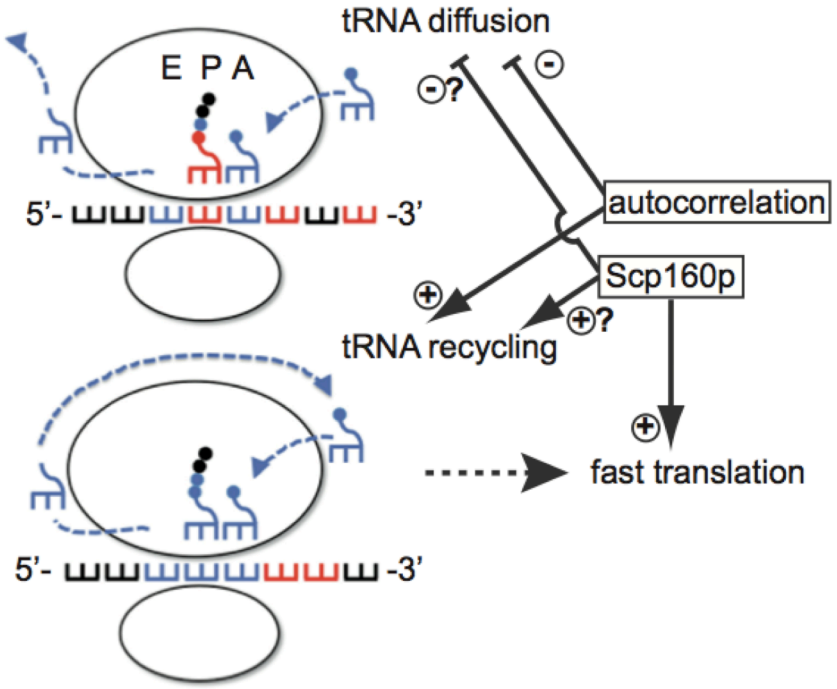

Fig. 10: A model for the partial synergism between Scp160p action and mRNA autocorrelation on jointly preventing diffusion of used tRNAs from ribosomes. *Image courtesy of Ralf-Peter Jansen, as published in (Hirschmann, Westendorf, Mayer, Cramer, Cannarozzi, Jansen 2014).*

Dual Luciferase Assays Reveal Roles for Both Scp160p and Asc1p in Efficiency and Accuracy of Translation

Read-through in reporter constructs is impaired in knockouts of both SCP160 and ASC1

Asc1p is one of the ribosomal interactors of Scp160p on the yeast ribosome (GILBERT/ DOUDNA 2009). It is present in cells equimolar to ribosomes, and falls off of polysomes in stationary yeast (BAUM/FREY/SEEDORF 2004). Its mammalian homolog has been shown to promote 80S formation (CECI et al. 2003), and both the yeast and mammalian protein are interactors of protein kinase C (GERBASI et al. 2004) while the yeast homolog ASC1 is a negative genetic interactor of the ALA1 aminoacyl-tRNA synthetase (TKACH/ COSTANZO 2010). Thus, it would be conceivable that Scp160p and Asc1p work together to promote tRNA recycling, e.g. via growth-dependent ribosomal recruitment of Scp160p and/or Ala1p to Asc1p.

Anyhow, if Scp160p and Asc1p really function to increase elongation rates, e.g. by enhancing tRNA recycling, then changes in the kinetics of elongation by their absence should differentially affect translational fidelity depending upon which choice of ligands the ribosome is presented with. To test this, a series of dual-luciferase reporters designed to monitor different aspects of translational fidelity and ligand choice were employed. Wild-type cells recognize the three termination codons with very high efficiency, misreading them at rates on the order of 0.3 – 0.8% (**Fig. 11A**). In comparison, termination codon recognition is up to two-fold more efficient in both *scp160Δ* and *asc1Δ* cells (**Fig. 11B**). This is consistent with a kinetic partitioning model (DINMAN and LIAO 2008): Decreasing tRNA utilization rates while maintaining the rate of release factor selection shifts the equilibrium in favor of release factor selection resulting in increased fidelity of termination codon decoding.

Missense reading of tRNAs is increased in knockouts of both SCP160 and ASC1; however, the effects vary for abundant versus rare tRNAs

These observations raise the following question: how do Scp160p and Asc1p influence tRNA utilization in the context of codon frequency and tRNA abundance? To examine this, a series of dual-luciferase "missense" reporter plasmids were employed (PLANT et al. 2007). All three of the reporters require misreading of Serine codons by Arginyl-tRNAs, which normally only pair with Arginine codons, at codon

218 of the firefly luciferase mRNA in order to restore enzymatic activity of the protein. However, they differ in codon frequency and tRNA abundance. The UCU missense codon is highly used (relative synonymous

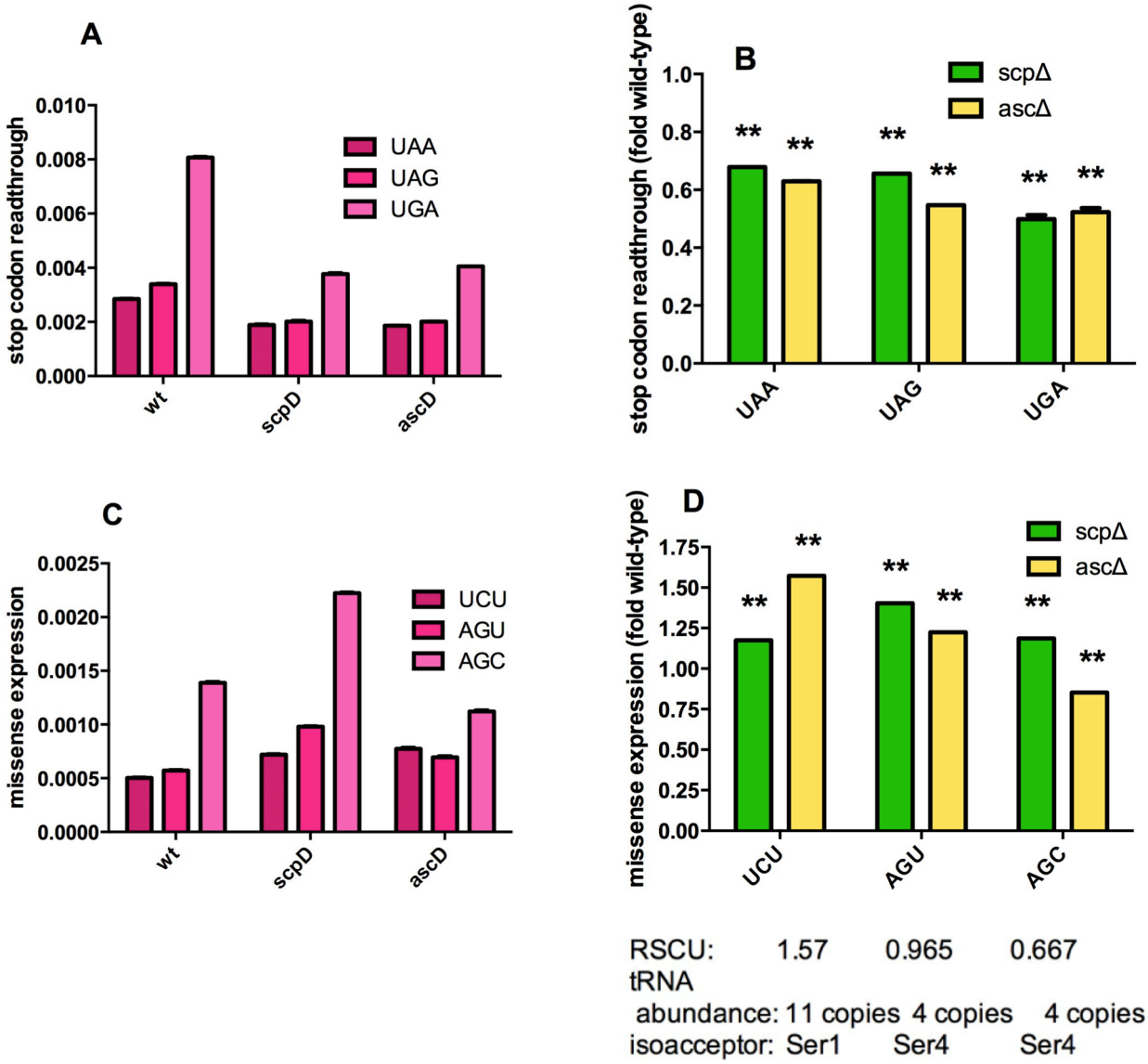

Fig. 11 (A-D): Loss of SCP160 or ASC1 promote decreased readthrough of stop-codons and suppression of missense codons. (A) Individual levels of stop codon read-through in isogenic WT, scp160Δ, and asc1Δ cells, normalized to a control lacking a stop codon. (B) Relative levels of stop codon readthrough in deletion strains, compared to wildtype, each individually normalized to a readthrough control plasmid. Readthrough in (A) and (B) is defined as ratio of firefly to Renilla luciferase expression from a dual reporter mRNA. (C) Individual levels of mistranslation in WT, scp160Δ, and asc1Δ cells. (D) Relative levels of mistranslation in deletion strains, compared to wildtype, each individually normalized to a readthrough control plasmid. Mistranslation in (C) and (D) is defined as ratio of firefly to Renilla luciferase expression from a dual reporter plasmid. This requires deliberate Arg for Ser mistranslation at firefly luciferase active site. Three different codons recognized by two different tRNAs (Ser1, Ser4) were analyzed by individual reporter plasmids. Ser1 tRNA recognizes one non-cognate UCU codon (RSCU=1.57). Ser4 tRNA recognizes two near-cognate AGC, AGU codons (RSCU = 0.67, 0.97).

codon usage; RSCU = 1.57), and its isoacceptor Ser1 tRNA is encoded by 11 copies in the yeast genome (PERCUDANI et al. 1997). While the AGU and AGC missense codons are both decoded by Ser4 tRNA (4 genomic copies), the former represents the mid-range of codon frequency, while the latter is rarely used (RSCU = 0.965 and 0.667 respectively). Consistent with prior observations (PLANT et al. 2007) there is a correlation between codon frequency and translational accuracy, i.e. more frequently used codons are

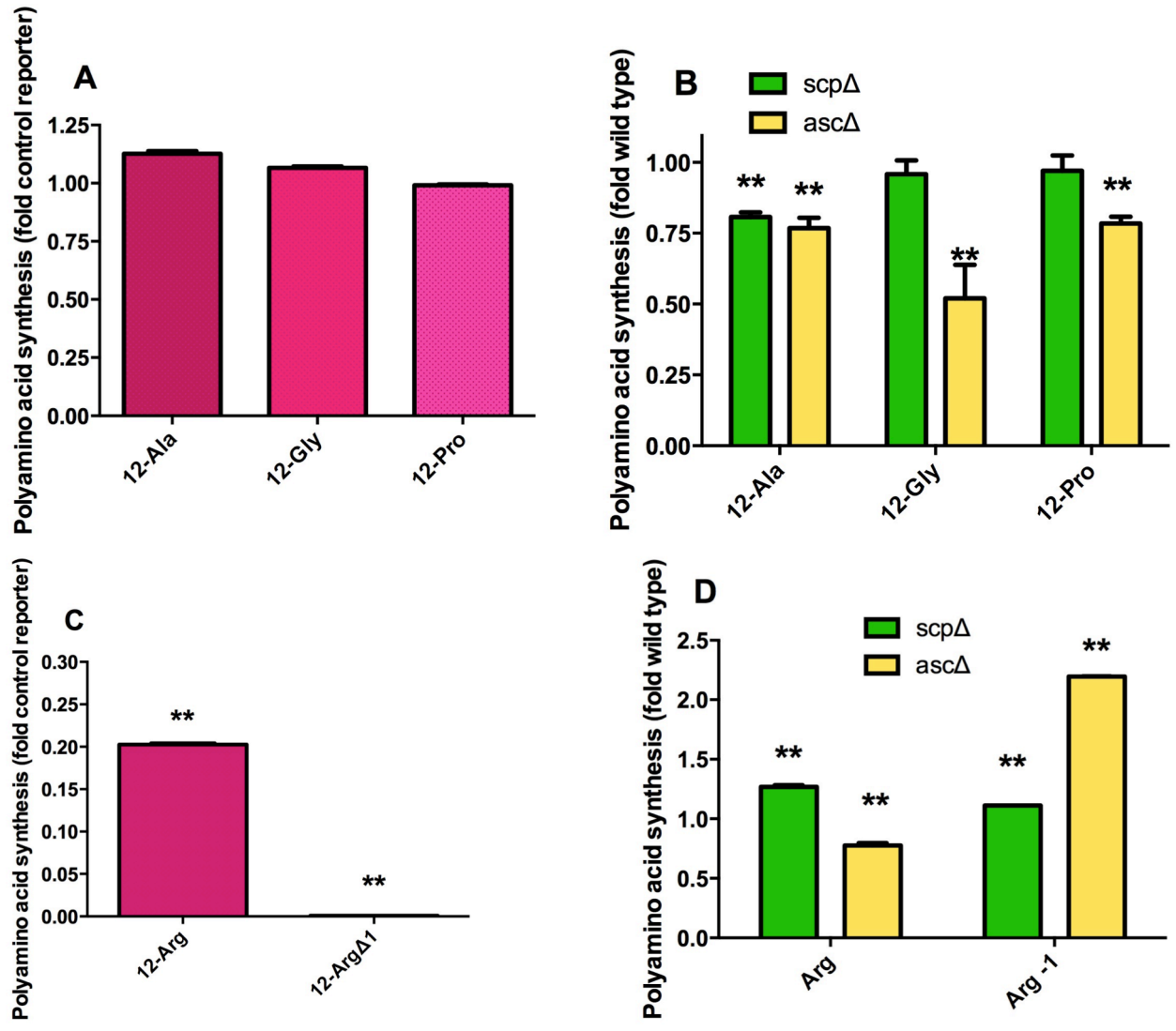

Fig. 12 (A-D): Roles of Scp160p and Asc1p on decoding stretches of auto- and anti-correlative stretches of codons.

(A) Individual levels of poly-amino acid synthesis in isogenic WT, scp160D, and asc1D cells, normalized to a readthrough control. (B) Relative levels of poly-amino acid synthesis in deletion strains, compared to isogenic WT, each individually normalized to a readthrough control plasmid. (C) and (D) Elucidating roles of Scp160p and Asc1p on ribosome processivity using Poly-Arg. (C) and (D) Accumulation of positively charged amino acids decreases translational efficiency. Poly-amino acid synthesis in (C) and (D) is defined as ratio of firefly per Renilla luciferase expression from a dual reporter plasmid. (D) Relative levels of poly-amino acids synthesis in deletion strains, compared to wildtype, each individually normalized to a readthrough control plasmid.

translated more accurately than less abundant codons, irrespective of tRNA abundance (**Fig. 11C**). Loss of Scp160p generally amplified this trend while loss of Asc1p generally ablated it (**Fig. 11D**).

These data suggest that Scp160p aids in recycling tRNAs to low abundance codons, e.g. by minimizing tRNA diffusion away from the ribosome. In contrast, Asc1p conceivably helps ribosomes recycle tRNAs to abundant codons, i.e. minimizing competition with nearby polysomes translating highly expressed genes by enhancing local retention of tRNAs.

Roles of Scp160p and Asc1p in translating repeated stretches of amino acids, i.e. autocorrelation boost and polybasic stalling proxies

Autocorrelation is the re-use of an isoacceptor tRNA by subsequent synonymous codons (FRIBERG/GONNET 2004). To query autocorrelation, a series of dual luciferase reporters were employed containing contiguous stretches of 12 Alanine (GCU), 12 Glycine (GGU), or 12 Proline (CCA) codons. To examine anticorrelation, i.e. the re-use of same tRNAs by synonymous codons that may be different but isoaccepting (FRIBERG/GONNET 2006), a reporter containing 12 repeated Arginine codons alternating between CGG and CGA was constructed. Additionally, since stretches of positively charged amino acids have been shown to promote ribosome stalling on mRNAs (CHARNESKI/HURST 2013; DIMITROVA/KUROHA/INADA 2009), a 12 Arginine reporter harboring a one base deletion was constructed to monitor rates at which ribosomes escape stalling by non-programmed ribosomal frameshifting.

Wild type cells translate autocorrelative mRNAs as well as if not better than regular mRNAs (**Fig. 12A**). However, in the absence of Asc1p, translation of autocorrelative mRNAs is significantly decreased (**Fig. 12B**). The strongest effect was observed with the 12-Gly reporter, which encodes the most frequently used codon of the three (SHARP/LI 1987). These findings are consistent with the proposed role of Asc1p in recycling tRNAs to abundant codons. In contrast, autocorrelation is generally not affected by the absence of Scp160p. The exception is highlighted by the 12-Ala construct, the least frequently used of the three codons (SHARP/LI 1987). This observation is consistent with a proposed role of Scp160p in recycling tRNAs to low abundance codons by limiting diffusion of uncharged tRNAs from polysomes. **Figure 12C, left** shows that the 12-Arg construct is translated approximately 5-fold less efficiently than a control reporter, in line with the ability of polybasic peptides to promote ribosome stalling. As previously reported (DINMAN/ICHO/WICKNER 1991), moving the downstream firefly luciferase reporter into the -1 frame by deletion of one base (12-ArgΔ1) reduced its synthesis by 4 orders of magnitude (0.08%) (**Fig. 12C, right**).

In *scp160Δ* cells, both 12-Arg and 12-ArgΔ1 utilization were slightly, but significantly enhanced as compared to wild-type cells (**Fig. 12D**). Based on the results shown in **11D**, we suggest that this may be caused by enhanced mistranslation of Arginine codons by near-cognate Ser-tRNAs. The absence of Asc1p had opposing effects in this context. Specifically, while the ability to translate the 12-Arg reporter was significantly decreased in *asc1Δ* cells as compared to wild-type, utilization of the 12-ArgΔ1 reporter was enhanced more than two-fold. These findings suggest that tRNA recycling defects exacerbate positive charge mediated ribosome stalling, and that these increased stall times provide ribosomes with more time to spontaneously shift reading frame.

Frame maintenance assays and the kinetic partitioning model point towards a role for Scp160p in the clearance of de-acylated tRNAs from the E-site

As demonstrated by the 12-ArgΔ1 reporter, the rate at which ribosomes spontaneously slip out of reading frame is exceedingly low. However, in response to specific *cis*-acting mRNA elements, ribosomes can be programmed to shift reading frame 2 - 3 orders of magnitude more frequently. This is called programmed ribosomal frameshift, or PRF (DINMAN 2012). While all known varieties of PRF can be explained by

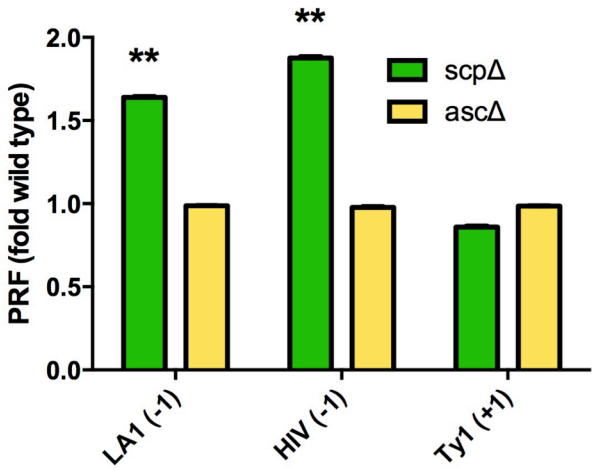

Fig. 13: Deletion of SCP160 promotes increased rates of programmed -1 ribosomal frameshifting in RNAs of viral origin.

Bars indicate relative levels of programmed ribosomal frameshifting (PRF) in deletion strains, compared to wildtype, each individually normalized to a readthrough control plasmid. Sequences derived from the yeast L-A virus and from HIV-1 were used to monitor rates of -1 PRF, and sequence from the yeast Ty1 retrotransposable element was used to monitor +1 PRF.

kinetic partitioning, different types of PRF signals can be used as specific probes of ribosome function (HARGER/MESKAUSKAS/DINMAN 2002). Programmed -1 ribosomal frameshifting (-1 PRF) promoted by sequences in the yeast L-A and HIV-1 viruses require simultaneous slippage of tRNAs in both the ribosomal A- and P-sites. In general, there is a correlation between the amount of time ribosomes with tRNAs in these sites spend paused at -1 PRF signals and rates of -1 PRF (LIAO 2011). This can theoretically happen after accommodation of aa-tRNA into the A-site (as evidenced by the ability of anisomycin, an inhibitor of this process, to inhibit -1 PRF (DINMAN/RUIZ/PELTZ 1997) and up to completion of translocation (CHEN/PUGLISI 2014; CALISKAN/RODNINA 2014; HEKMAN/GOMEZ 2012; ORTIZ/ULLOQUE/KINZY 2006). In contrast, Ty*1* mediated +1 PRF is driven by the availability of a low abundance tRNA in the ribosomal A-site, i.e. it must occur after translocation and before aa-tRNA accommodation (BELCOURT/FARABOUGH 1990; LIAO/GUPTA/LEE 2008). Neither -1 or +1 PRF was affected in *asc1Δ* cells, indicating that these processes are independent of tRNA recycling. However, -1 PRF rates were specifically increased in *scp160Δ* cells (**Figure 13**). If Scp160p plays a role in clearance and channeling of deacylated tRNAs away from the E-site, then in its absence, ribosomes having deacylated tRNA in the E-site, peptidyl-tRNA in the P-site and aa-tRNA in the A-site would accumulate, thus increasing the concentration of substrates for -1 PRF. The lack of effect on +1 PRF indicates that Scp160p is not involved in delivery of aminoacylated tRNAs to the A-site.

Concludingly, the data add weight to a view of both Scp160p and Asc1p working to ensure speed and fidelity of translation.

tRNAs Co-precipitate With myc-Scp160p During Active Translation

The prior experiments have revealed a synergism between autocorrelation of synonymous codons in mRNAs, and Scp160p, in yeast cells via indirect evidence (ribosome-affinity purification and bioinformatics, **Fig. 9**). The interpretation that Scp160p is pivotal to correct autocorrelation was confirmed in different

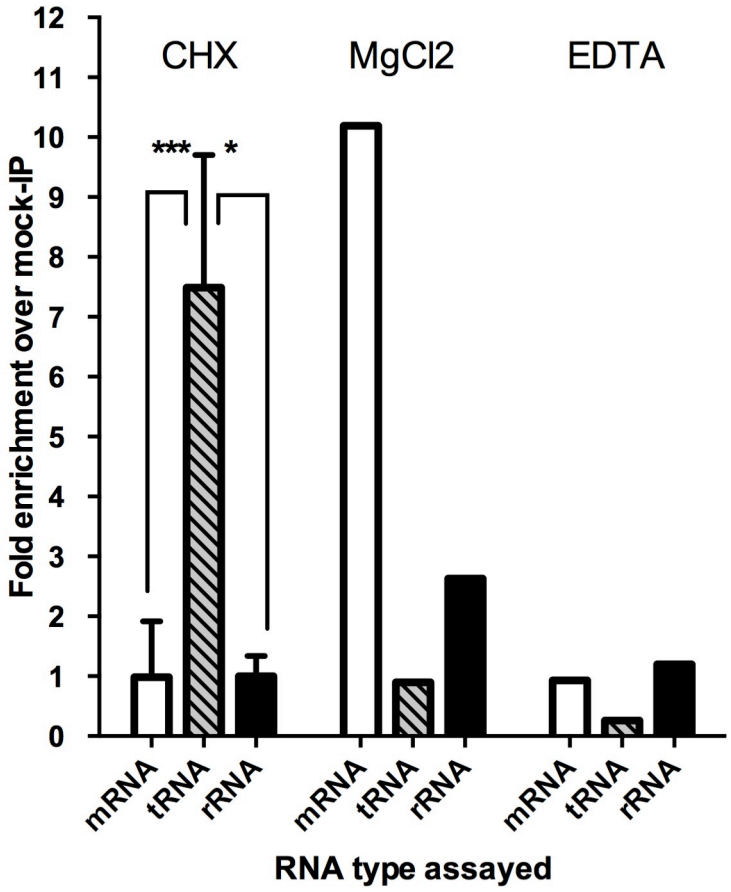

Fig. 14: Immunoprecipitation of Scp16op-myc and subsequent RNA isolation and detection yield divergent patterns, depending on the conditions. White bars, mRNA; grey bars, tRNA; black bars, rRNA.

Left group: Only tRNA reproducibly co-immunoprecipitates when active translation is frozen via cycloheximide administration in harvesting and lysis buffers.

Middle group: Mainly mRNA and rRNA co-immunoprecipitates when Magnesium Chloride is administered in harvesting and lysis buffers to enable run-off of intact ribosomes.

Right group: Neither RNA species appreciably co-immunoprecipitates if EDTA is administered in harvesting and lysis buffers to disrupt ribosomes.

experiments demonstrating impaired read-through of autocorrelation constructs in *scp160Δ* cells (**Fig. 13A, B**).

I therefore further investigated the hypothesis that Scp160p may be a limiting factor of diffusion of used tRNAs at or around the ribosome. To this end the tRNA quantitation method (using reverse transcription and quantitative PCR) employed previously downstream of ribosome purification, was now applied downstream of myc-immunoprecipitations of Scp160p itself.

Buffers containing Cycloheximide were used to stall translating ribosomes during elongation. In these conditions, high, significant and reproducible binding of myc-Scp160p to tRNA, but only residual binding of mRNA and rRNA, was found. (**Fig. 14**, first column group).

In case magnesium chloride was added to buffers to enable run-off of ribosomes, binding of mRNA and rRNA but not tRNA to Scp160p was observed. (**Fig. 14**, second column group). However, the level of mRNA precipitation cannot be explained by ribosome co-immunoprecipitation, as the pulldown efficiency for mRNA is many times that for rRNA.

Adding EDTA in order to completely disrupt ribosomes, led to no immunoprecipitation of either mRNA, tRNA or rRNA (**Fig. 14**, third group column).

These results suggest that Scp160p may buffer tRNAs for subsequent re-use during ongoing translation, and that an elongation block results in an accumulation of a tRNA-binding form of Scp160p.

Probing Translation Roles of Additional Potentially Interacting Proteins

tRNA-RAP shows higher ribosomal depletion of both rare Ala and Glu tRNAs in ARC1 knockout cells

Glu1p or (GluRS, the Glutamate-aminoacyl-tRNA synthetase) is a known physical interactor of Arc1p (DEINERT et al. 2001), a protein found on the large subunit of yeast ribosomes (BECKER 2009). Ala1p/AlaRS is a known physical interactor of Asc1p (COSTANZO 2012), a protein found on the small subunit of yeast ribosomes.

Both Arc1p and Asc1p have been shown to relocalize from polysomes and ribosomes in times of stress or altered metabolism: Arc1p has been shown to relocalize from ribosomes to mitochondria in stationary yeast (LAPORTE & BECKER 2014), liberating part of a mitochondrial Glutamyl-tRNA synthetase (FRECHIN & BECKER 2014), while under the same conditions Asc1p falls off polysomes (BAUM/ FREY 2004). Additionally, Asc1p has been shown to relocalize to the cytosol, off of ribosomes, and be indirectly involved in transcriptional up-regulation of gene targets of respiration, in stationary yeast recently (RACHFALL & BRAUS 2013). The fact that they may be part of a physical interaction network with Scp160p, which has connectivity to both small and large ribosomal subunits, raises the possibility of a ribosomal complex of accessory factors of elongation. The fact that Asc1p and Arc1p also each interact with specific aminoacyl-tRNA synthetases raises the possibility that their positioning on the ribosome may connect re-charging of used tRNAs with locally optimal substrate intake of the ribosome. If ribosomal recruitment of specific aminoacyl-tRNA synthetases was key to a proposed mode action of Arc1p and Asc1p in translation, then local molecular crowding of tRNAs should be disrupted upon knockout of the interactors, and would be measurable via ribosome-affinity purification.

In order to test whether knockout of ARC1 has an effect on ribosomal occupancy of all, some, or distinct tRNAs, tRNA quantitation after ribosome-affinity purification (RAP) from wild-type or *arc1Δ* deletion cells was performed.

Interestingly, only Alanyl and Glutamyl tRNAs were reliably quantifiable from the deletion samples in a way offset from wild-type levels, indicating that the deletion may only affect ribosome association of these two groups (**Fig. 15A, B**).

Comparing this result with the tRNA quantitations in *YEF3* deletion cells harboring a temperature-sensitive add-back mutant under repressive and permissive temperatures (**Fig. 8A, B**), it is remarkable that the

relationship of the depletions in ribosomal levels (popular codon tRNA being more depleted) is similar. This means that the extent to which cells react to knockouts of ARC1 is comparable to the effects of an elongation factor knockout. If the two conditions have similar effects, it is conceivable that they also have a common functional basis, i.e. helping with elongation.

Taking into account that both autocorrelation of isoaccepting codons, and ribosomal association of aminoacyl-tRNA synthetases, were found in archaeal (MIKULCIC/BAN 2014) and mammalian cells (CANNAROZZI et al. 2010/ DAVID, YEWDELL 2011), a tantalizing possibility is that ribosomally recruited super-complexes boosting tRNA re-use may in fact be the molecular basis of all substrate channeling at ribosomes (NEGRUTSKII&DEUTSCHER 1991). A possible complex of Alanyl and Glutamyl tRNA synthetases, or their respective ribosomal recruitment factors Asc1p and Arc1p, would be in line with these previous findings. To verify the above hypotheses, future experiments should be conducted in this direction.

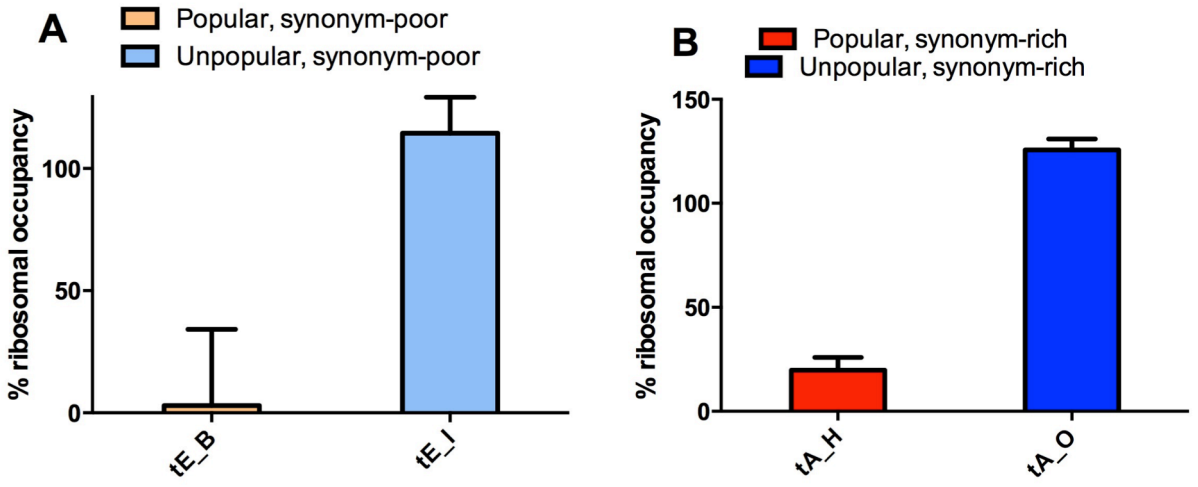

Fig. 15 (A-B): Ribosome affinity purification shows divergent ribosomal occupancy from wild-type of Glutamyl- and Alanyl-tRNAs from ribosomes in ARC1 deletion cells. The popular codon tRNA is depleted and the unpopular codon tRNA increased, independent of synonym-richness.

Discussion

Codon Autocorrelation, an Emergent Property of the Genetic Code?

Since autocorrelation was discovered as a characteristic of mRNAs from fungi to mammals, and proposed as a factor of translational fitness (CANNAROZZI et al. 2010), there have been several publications emphasizing its role in yeasts and archaea (HIRSCHMANN et al. 2014, MIKULCIC/BAN 2014). However, in a recent article Hussmann and Press (HUSSMANN&PRESS 2014) have criticized the concept, based on theoretical considerations. Their experimental setup examined the extent to which idiosyncracies in repetitive isoaccepting codon choice may be caused by inherent properties in the genetic code, in mRNAs re-dialing different but isoaccepting tRNAs for subsequent occurances of an amino acid. In other words, the similarity of codons accepted by the same tRNA may explain mutations favoring different but isoaccepting codons. This finding is trivial: Third-base wobble, due to anticodon pairing using rare bases like inositol, has long been known to cause the ability of tRNAs accepting two almost-similar codons (reviewed in KINZY, DINMAN 2003 and BEGLEY, DEDON 2013).

The authors go further, suggesting that due to the inherent favoring of isoacceptors by numerics, evolution as a driving force was irrelevant for this phenomenon:

‚Having presented this control, it should be noted that Cannarozzi et al.'s argument that „if the correlation effect was simply due to the accumulation of frequent codons in genes with biased codon composition, this effect should also be highest for frequent codons and not observed for rare codon'" misstates the effect that local bias in codon composition has on correlation effects. The effect will be highest for codons whose location-specific frequency exhibits the most variation around its average frequency in the genome, not those whose average frequency is highest.'

This accusation is flawed in many ways. First, it ignores the effort the autocorrelation statistic makes to eliminate the very inherent unfairness within genes the paragraph refers to. In other words, they overlook the prior existence of a control which they themselves suggest as additional.

Their line of thought also completely ignores the widely accepted notion of evolutionary entrenchment, i.e. the propensity of biological systems to stick with a working solution, however arbitrary the initial choice may have been. This is because universal rules, such as the general genetic code which is thought to date back to

the RNA world (reviewed in GESTELAND/ATKINS 2012), or the exclusive use of L-amino acids for building proteins in all organisms (reviewed in STRYER 2012), make spreading of genes and recycling of building blocks between organisms more efficient, respectively.

The article by Hussmann and Press is also a bona fide example of unjustified recursion. For example, although inherent natural constants dictate the aggregation behavior of atoms, the existence of the phenomenon is real nonetheless. Also, even if the mind is an emergent property of the brain, its existence cannot be rejected. Especially the example of the mind underscores that in evolution, for all we know there is no chicken or egg first, but ever-increasing feedback between them, i.e. selection and genes. The correctness of these notions is reflected in the interplay between divergent mutation and confining selection, which is part of staple literature on evolution (proposed by MAYR 1953, reviewed in NIKLAS, KUTSCHERA 2004).

On top of this, the tendency of rare codons to make marked use of autocorrelation inductively inferred from combinatorics by HUSSMANN and PRESS 2014, was also found deductively and experimentally in CANNAROZZI et al 2010. In other words, in contrast to the authors, there is no conflict but actually a synergism of explanations, which in my view has been hampered by jargon peculiarities and attitude differences between bioinformaticians and biomathematicians. What the authors perceive as a disagreement is actually two complementing views working together to describe a full picture.

If anything, as long as the bulk of pre-existing literature is correct, the findings put forth by HUSSMANN and PRESS actually allow for an excellent explanation as to how genes can easily adapt towards increased autocorrelation to make use of molecular crowding for boosting their translation efficiency. They also directly predict that autocorrelation should in principle work across all kingdoms of life. Careful scrutiny of the literature in this field indeed shows that evidence in this direction has accumulated over the years:

Genetic evidence for autocorrelation, as well as experimental evidence for protein-based mechanisms that explain it with molecular crowing around ribosomes, was concomitantly found in mammals (YEWDELL& DAVID 2011, NOVOA, PAVON-ETERNOD & YEWDELL 2013), yeast (CANNAROZZI et al. 2010, HIRSCHMANN et al. 2014, FRECHIN/SENGER/BECKER 2009), archaea (MIKULCIC/BAN 2014) and bacteria (SHAO/ZHANG/FENG 2012; AGASHE et al. 2013). Autocorrelation is thus enforced as an interesting lever of translation elongation control, where genetic and experimental analysis come together to reach an explanation. The strongest value of the investigation by HUSSMANN and PRESS, in my eyes, is to point out the underestimated role inherent combinatorics may play in evolution generally.

Appreciating the Variability of KH Domains in the Course of Evolution

In order to enable future studies on Scp160p to rely on correct domain borders and numbering, a bioinformatic effort was done to overhaul these. Hence, secondary-structure prediction was used to delineate the borders between successive, similar KH domains in the protein sequence. The secondary structure prototypicity, as compared with the namesake Khd1p protein (**Fig. 1B**), was concomitantly estimated and charted.

The existing structures were used to construct a neighbor-joining evolutionary tree of vigilins (**Fig. 1A**), based on a multiple-sequence alignment. Additionally, within-species alignments between KH domains were used to construct neighbor joining trees, to easily visualize their similarity to each other (**Fig. 2, A-H**). Also, presence/absence of a GXXG motif (**Fig. 3**), as well as prototypicity and changes in secondary structure (**Fig. 4**), were threaded onto a diagram to visualize characteristics in successive domains horizontally, compared with evoluionary history vertically.

Intraspecies alignments of Vigilin sequences from yeasts over insects to mammals reveal the importance of secondary structure for estimating prototoypicity of KH domains

Following interspecies alignment of vigilin sequences from multiple fungi, insects, and mammals, one observation is that the vigilins closely follow the established relationships between the species. This can be interpreted to mean that vigil ins are evolutionarily conserved proteins with functions that co-evolve within their organisms.

In contrast, in intraspecies alignments, KH domains within each organism have a distinct pattern of similarity with each other, where closely related species tend to group corresponding domains as neighbors, whereas more distant species don't. This speaks for an organism-specific plasticity, possibly to accommodate functional needs in connection with the different patterns of gene expression.

Finally, following secondary-structure prediction of individual KH domains and comparison with prototypic architecture, high secondary structure domain plasticity was observed. Predictions show that almost all domains tend to reduce or expand alpha helices or beta sheets away from the optimal architecture; this points towards a highly plastic gene which may underscore a character of adaptability against the backdrop of general conservation due to physiological relevance.

Understanding the evolution of KH domains in vigilins to discover mutation hot-spots and generate leads for future functional analyses

In any case, as the protein sequences demonstrate an ability to retain secondary structure elements in spite of divergent primary structure, it seems advisable to shift the focus of future studies towards bioinformatical and biochemical analysis of secondary structure and tertiary fold, as opposed to simply delving into primary sequences. One day this may lead to a better understanding of the structure determinants of the functions of this protein.

While it is clear that the specific and concerted behavior of vigilin KH domains within a species, as well as the similarities and differences across clades is far from being understood concisely, this field should merit future interest. Future studies may be needed to verify if and how the evolution of individual domains generates effects such as ribosome binding and tRNA diffusion limitation. Identification of structural determinants may one day advance the complete understanding of this protein and its properties.

Insights from the Translation Behavior of mRNAs whose Transcript Level Decreases in Deletions of SCP160

A study by HOGAN et al. (2006) reported over 1000 mRNAs to interact with Scp160p., only probed for mRNAs co-immunoprecipitating with Scp160p-TAP. The question whether this interaction is direct is still subject to debate. Previous work in our lab (SCHRECK, *Ludwig-Maximilians-Universität München* 2010) showed that ribosomal proteins are part of the co-IP profile of Scp160p IPs. Further work by rotation students however implied that purified Scp160p may indeed constitute an RNP even in absence of ribosomal proteins, as an appreciable amount of RNA is found in nanodrop and size exclusion chromatography spectra from these samples (results not shown). Taking into account that Scp160p has been shown to interact with polysomes and ribosomes by virtue of KH domains on its C- and N-termini and the Asc1p and Bfr1p proteins, respecitively (GILBERT 2010/ FRIDOVICH-KEIL 2001), the following view is conceivable: mRNAs co-immunoprecipitating with Scp160p in the HOGAN et al. (2006) study may be most parsimoniously viewed as mRNAs from co-immunoprecipitating ribosomes engaged in active translation. Yet, direct mRNA or tRNA binding may also contribute to the protein's wholesome function spectrum.

It follows that actually, sub-groups of Scp160p mRNA targets showing strong or weak „binding reproducibility" might simply be enriched or depleted in subpopulations of polysomes which are strongly or weakly doted with Asc1p, and resultantly Scp160p. While Asc1p is roughly equimolar with ribosomes in yeast cells weighing in at 100,000 copies (BAUM et al. 2004 and WARNER 1999), Scp160p is much less abundant at around 2,600 copies (CHONG 2015). Both have been shown to be released from polysomes in yeast cells approaching stationary phase (BAUM et al. 2004). Thus, the different „Scp160p binding reproducibility" behaviors might alternatively constitute examples of preferential doting of ribosomes with Asc1p and Scp160p, matching growth states of the cell, in order to benefit optimal translation of repsonse mRNA targets, whose coding is class-specific and dependent on accessory factors.

mRNAs transcriptome-differential in SCP160 deletion cells fall into distinct sub-classes of binding reproducibility and significance

The fact that a transcriptome-differential group of mRNA targets of Scp160p exists (**Tab. 1**), which was excluded from past analyses (SCHRECK, *LMU München* 2010), led to the design of experiments on the translational state of representative targets from these subsets.

Briefly, bioinformatical analyses of the whole group of transcriptome-dfferential mRNA targets revealed that while the subset transcriptome-*increased* upon knockout is not significantly deviating from random genomic mRNAs in any characteristic, the subset transcriptome-*decreased* upon knockout is significantly above average in re-use of codons recognizing same tRNAs, or autocorrelation. Further scrutiny showed that the part of the transcriptome-decreased subset with *insignificant* binding reproducibility to Scp160p in a previous study (HOGAN/BROWN 2008), was less optimized for autocorrelation, while the part of the transcriptome-decreased subset with *significant* binding reproducibility was more optimized.

Translational activity in the form of ribosomal occupancy was measured for several mRNAs from either subset, using ribosome-affinity purification (RAP) coupled with downstream quantitation of mRNAs by two-step RT-qPCR, yielding percentage counts of ribosomal occupancy in *SCP160* deletion versus wild-type cells. Here, mRNAs were either boosted or dampened in ribosomal occupancy, interestingly matching the sub-sets of binding strength.

Different sub-classes show translational induction or repression upon SCP160 knockout, respectively

mRNAs from the transcriptome-*decreased* subgroup, within the group found to be transcriptome-differential following Scp160p depletion, were analyzed by ribosome-affinity purification and subsequent determination of ribosomal occupancy via RT-qPCR. They divided into two classes: One translationally repressed, and one translationally activated group.

The mRNAs translationally repressed during *SCP160* deletion (**Fig. 5**), are also from a subgroup less optimized for autocorrelation (**Tab. 1**).

As translational arrest has been shown to promote No-Go Decay of mRNAs (INADA et al. 2013), it is conceivable that this mechanism might be the reason for the transcriptional depletion, concomitant with a translational depletion, of specific mRNAs upon *SCP160* deletion. This way, mRNAs recognized as „unproductive“ or „stalling“, presumably due to a lack of Scp160p making their translation less efficient, would be marked for degradation.

The mRNAs translationally increased during SCP160 deletion (**Fig. 5**), are also from a subgroup better optimized for autocorrelation (**Tab. 1**).

Aberrant protein levels have been hypothesized to be kept in homeostatic check by regulation of gene expression (SALLIE 2004). It is thus conceivable that this mechanisms might be the reason for the transcriptional depletion observed here, in spite of a translational increase, of specific mRNAs upon SCP160 deletion.

If this were true, protein levels recognized as „aberrantly high", presumably due to decreased competition with different mRNAs, and resultingly higher use of raw materials in translation, would be targeted by degradation, or decrease in transcription, to keep protein levels normal.

The following part of this work will now take the focus of considerations from the transcriptome to the translatome level.

Biochemical and Bioinformatical Data Position the Translation Role of Scp160p in Close Synergy with Codon Autocorrelation

So far several efforts in the group of Ralf-Peter Jansen (SCHRECK *LMU München* 2010, HIRSCHMANN and WESTENDORF 2014) had shown that Scp160p is a yeast protein determining the translational state of several target mRNAs (**Tab. 4**), and that at least for one such mRNA, *PRY3*, upon depletion of Scp160p a shift towards polysome fractions corresponds to less protein being produced. Ribosome affinity purification and detection of other target mRNAs to measure ribosomal occupancy and thus translational activity, including CCW14 and PTH1, indicates that this may be a general pattern (HIRSCHMANN and WESTENDORF 2014).

These results shed new light on prior observations, such as the seeming implication of Scp160p in the control of ploidy, chromatin remodeling, or cell wall integrity. The most parsimonious interpretation is that translation of Scp160p's mRNA targets (**Tab. 2**) with ontologies fitting these processes is the basis of its indirect influence in these fields, without direct physical interactions between protein and mRNA necessary.

A correlation was found between the translational shift of target mRNAs as seen by their shift in polysome gradients, and their potential for improved translation by tRNA autocorrelation (**Fig. 7, Tab. 5**). This led to a further investigation of the link between Scp160p function, translational fitness and tRNA recycling.

The hypothesis that ordering of synonymous codons in a transcript prevents tRNA species change during translation elongation is explained by presumably increasing ribosomal economy and thus the mRNA's translational fitness (CANNARozzI et al. 2010). mRNA targets of Scp160p have been found to be sginificantly skewed towards mRNAs with coding optimization by autocorrelation (**Fig. 6 and Tab. 3**). Thus, it was initially hypothesized that the preferential recruitment of ontologically important, codon-optimized mRNA targets to the ribosome depends on the presence of Scp160p. This would especially be the case in times of ample resources and exponential growth, since Scp160p is recruited to the ribosome by the adapter protein Asc1p/RACK1, which itself is found to associate with the ribosome only in exponentially growing cells (BAUM/BITTINS/FREY/SEEDORF 2004). An alternative model could be a direct involvement of Scp160p in tRNA recycling (NEGRUTSKII/DEUTSCHER 1991, STAPULIONIS/DEUTSCHER 1995).

To address the question which of these models is valid, a protocol was developed to detect tRNAs (**Tab. 7**) that co-precipitate with translating ribosomes. With this protocol, the expected global depletion of tRNAs from ribosomes in elongation factor mutants and after amino acid starvation or elongation factor depletion was successfully detected (**Fig. 8**). To the best of my knowledge, this is the first PCR-based quantitative

measurement of tRNAs on the translating ribosome using non-spectrometric methods. When tRNA ribosomal occupancy was monitored in Scp160p depleted cells, it turned out that the tRNA species the most depleted from the ribosome (**Fig. 9**) are the ones that are less optimized for tRNA recycling by codon order autocorrelation, within Scp160p target mRNAs that suffer in translation from its absence (**Tab. 8**). This pattern was independent of the number of synonymous codons per corresponding tRNA („synonym richness"), or their frequency of use („popularity"). A likely interpretation of these findings is that Scp160p has a beneficial effect on translational fitness that is similar to, but independent of, tRNA autocorrelation at the mRNA coding level.

From the observation that tRNAs that are more optimized for re-use were less depleted from ribosomes (**Fig. 9**), it can be interpreted that autocorrelation is robust and deterministic. However, autocorrelation relies on synergism with independent accessory factors that maximize its effect. The above results indicate that Scp160p might constitute one of these factors. Scp160p associated mRNAs are already partially optimized for autocorrelation of codons (**Tab. *5* and 6**), which boosts efficiency synergistic with but largely independent of Scp160p. Loss of Scp160p might therefore decrease translation of target mRNAs due to impaired autocorrelation-independent tRNA recycling. Scp160p's mode of action, just as codon autocorrelation itself, may make use of molecular crowding phenomena. Together with the substrate channeling hypothesis for the ribosome (STAPULONIS/DEUTSCHER 1995, NEGRUTSKII/DEUTSCHER 1991), a role for a tight association between ribosomes, tRNAs and their aminoacyl synthetases as tRNA recycling factors has been proposed by physical modeling to be the basis of autocorrelation in eukarya (CANNAROZZI et al. 2010). This is supported by experimental evidence in human cell culture and archaea (DAVID/YEWDELL 2011, MIKULCIC/DJURASEVIC/BAN 2014). It is possible to hypothesize that Scp160p may exert its benefits on translation by either consolidating these associations, or preventing the dissociation of tRNAs from the ribosome (**Fig. 10**). In any case, the above quantitation of tRNAs at the ribosome in the presence and absence of Scp160p in combination with the bioinformatics analysis of the codon composition of mRNAs rules out a third hypothesis: That Scp160p recruits only those mRNAs to ribosomes, which are optimized for translational efficiency by tRNA re-use due to codon autocorrelation. This is because, due to the general optimization of Scp160p target mRNAs for tRNA re-use, abrogation of hypothetical ribosomal recruitment caused by Scp160p depletion would be expected to more drastically affect tRNA species thus optimized, not the ones less optimized.

Zooming in on Roles for Scp160p and Asc1p in Translation

If the roles of Scp160p and Asc1p are related to the translation apparatus, a knockout of the said genes would be expected to alter the performance of the ribosome. In contrast to ribosome affinity purification, an altogether different view would be enabled by measuring the read-through rates of transcript constructs, in wild-type versus knockout cells. Such a system enables challenging ribosomes with obstacles like stop codons, deliberate miscoding, or uniform stretches of same amino acids encoded by same codons. Dual luciferase expressing plasmids (HARGER&DINMAN 2003) are such a system. These encode two different luciferase enzmes, one N- and one C-terminal, that react differently to reaction-stopping reagents, but are part of one peptide, and separated by a stretch of „translation obstacles" (e.g. stop codons, codon stretches, frameshifting signals...) The system allows for correcting for per-cell copy numbers and background levels, and estimate read-through rates by relating the expression of an N-terminal versus a C-terminal luciferase protein. The big advantage of the system is the high throughput that can be generated in a limited time, enhancing statistical robustness; and the possibility of in-luminometer automated pipetting of exciting and stopping solutions.

Hence, another set of experiments, using dual luciferase assays, was started to further elucidate the connections of Scp160p and Asc1p with translational efficiency and fidelity, in hopes of pinpointing possible modes of action. This was performed in collaboration with Prof. Jon Dinman's group of the University of Maryland (College Park).

Generally, upon knockout of each SCP160 or ASC1 small but significant effects were observed, on characteristics as diverse as translational frameshifting, misincorporation of amino acids, stop-codon readthrough, and readthrough efficiency of amino-acid stretches, were observed.

The overall observations of the effects seen upon *SCP160* deletion on cells, concerning translational nonsense, missense, and autocorrelation read-through assays, allowed me to reach the following conclusions.

The general propensity of *SCP160* deletion cells to recognize nonsense codons with increased fidelity (**Fig. 11A, B**) can be interpreted to mean that in these cells the net speed of elongation is impaired, thus alleviating the normal "thrust" on stop-codon read-through that would otherwise „push through" a translation event. The finding that mistranslation was pronouncedly increased in *SCP160* deletion cells (**Fig.**

11,C D) is interesting as mistranslation rises e.g. when building blocks (i.e. cognate tRNAs) become scarce. Taking into account the relative usage of the codons examined, and the tRNA gene copy number of cognate tRNAs involved, the observation can be extended. The fact that codons recognized by the less-abundant Ser4 tRNA were more pronouncedly mistranslated correlates with the fact that the said codons are less popular, in accordance with previous studies (DINMAN&KINZY 2008; AGASHE&MARX 2013) that demonstrated that rare codons are in general more prone to mistranslation, and that more popular codons in the same amino acid sequence increase amount of functional protein but not yield in an enzyme. In contrast, the extent of above-wildtype mistranslation for the more-abundant Ser1 tRNA was relatively smaller, correlating with a higher tRNA gene copy number and a more popular codon (**Fig. 11D**). This can be interpreted to mean that Scp160p matters more to rare codon tRNAs, presumably because these tend to get lost more easily in the cytosol if not immediately recycled and re-used, from the perspective of single ribosomes. This goes along with prior findings (HIRSCHMANN et al. 2014), which demonstrated that a failure of mRNAs to re-use codons depletes corresponding tRNAs from ribosomes even more drastically in SCP160 deletion versus wild-type cells than imperfect autocorrelation alone would do. One can interpret these findings to amount to increasing evidence for a role of the Scp160p protein in the interplay of fidelity and efficiency of translation elongation.

Pertaining to the constructs where auto- or anti-correlated repeats of same amino acids were used, **Fig. 12A** demonstrates that there is no relationship between codon popularity or tRNA copy number and the benefit towards control constructs in wild-type cells. However, the benefit itself is small but significant, and underscores autocorrelation as a predictor of translational fitness, in accordance with results from CANNAROZZI et al 2010. In addition, the decrease in reporter translational level in SCP160 deletion relative to wild-type cells is also independent of influences of codon choice (**Fig. 12B**). The appreciable decrease of efficiency in scp160Δ cells however speaks for an involvement of Scp160p in correct execution of autocorrelation, in accordance with CANNAROZZI et al 2010.

The finding that -1, but not +1 frameshifting, is elevated in deletions of SCP160 (**Fig. 13**) is in line with the kinetic partitioning model (LIAO&DINMAN 2011), emphasizing the role presumably played by Scp160p in correct elongation. Specifically, this tendency means that deletion cells have problems in E-site clearance, not A-site delivery. This would speak for a role for Scp160p to help clear deacetylated tRNAs away from the E-site. Presumably this might work in concert with a proposed molecular crowding effect (MCGUFFEE & et al. 2013), whereby vicinal aminoacyl-tRNA synthetases could boost recycling of re-usable tRNAs, provided their diffusion is limited. This would be most pivotal for rare tRNAs. Note that in this model view, a synergism between the independently proposed and evidenced models of tRNA channeling to the ribosome (NEGRUTSKII & DEUTSCHER 1991), molecular crowding (MCGUFFEE/ELCOCK 2010), codon autocorrelation (CANNAROZZI et al. 2010) and ribosomal aminoacyl-tRNA recruitment (DAVID &

YEWDELL 2011) would be mutually explanatory, not mutually exclusive.

Finally, the finding of dramatically decreased translational read-through in codon-anticorrelated polybasic arginine stretches (**Fig. 12C, left**) is in line with prior findings by others, as both characteristics (i.e., anticorrelated codons and polybasic amino-acid products) have been proposed and evidenced to decrease translational speed (CANNARozzi et al 2010; DIMITROVA/INADA et al 2008, CHARNESKI&HURST 2013, KOUTMOU&GREEN 2015, ARTHUR&GREEN 2015). Introducing a base deletion on top of this, which requires -1 frameshift to be overcome, all but completely abrogated read-through in a second construct (**Fig. 12C, right**). The ability of SCP160 deletion cells to overcome both these obstacles and not only restore but surpass wild-type readthrough levels in both constructs (**Fig. 12D**) can be seen as evidence for both the RNA-signal-independent mistranslation, highest for rare codons, and the -1 frameshifting of *SCP160* deletion cells, that were observed earlier using frameshift-inducing RNA signal constructs. Each deficiency in this special setup would actually enable escape of readthrough from the chief obstacle of polybasic stalling, albeit at the price of reduced fidelity. We are currently planning future investigations to determine if such escape effects might constitute a general pattern in situations where cells are challenged with obstacles, e.g. long stretches of rare codons. This would be another addition to the arsenal of evolutionary population bottlenecks (AMBROSE 1998, reviewed in DAWKINS 2004), whereby detrimental situations may paradoxically trigger and eventually select for otherwise slightly disadvantageous mechanisms in a survival bottleneck, a popular example being idiosyncracies in human immune cell „defects“ that explain present-day HIV resistance and are thought to be caused by adaptations enabling medieval plague survival (LAAYOUNI 2014). Similar escape routes have been proposed for missense suppression (HEKMAN 2012), ribosomopathies (SULIMA&DINMAN 2013), and ploidy versus aggregation (KAISER/RICHTER 2013) in yeast.

Encouragingly, my experiences with tRNA quantitation that led to my favoring the „slow polysome“ hypothesis were reinforced by ribosome footprinting results (POP/INGOLIA/PHYZYCKY/WEISSMAN 2015) that were motivated by my 2014 publication in Nucleic Acids Research.

The RNA Co-Immunoprecipitation Profile of Purified Scp160p

In order to verify the conceivable tRNA-buffering role of Scp160p, a set of immunoprecipitation experiments was conducted, to examine binding of different RNA species.

Examining the results from co-immunoprecipitations of RNA with myc-Scp160p (**Fig. 14**), I propose the following interpretation. The fact that Scp160p co-precipitates tRNAs significantly only when translation is stalled with cycloheximide could mean that the binding of tRNAs is transient and not stable. This would mean that Scp160p's role is not to sequester tRNAs away from the ribosome (as tight binding would suggest), or not influence it at all (as no binding would suggest). Cycloheximide stalling may instead illustrate the situation where residual tRNAs are stochastically "caught in the act" of on-going translation. Medium-strong binding would not bias individual tRNAs, and benefit all amino acids equally – this effect may be the most parsimonious explanation for the already known synergism with autocorrelation, which was previously established (HIRSCHMANN et al 2014). The absence of detectable mRNA and rRNA speaks against a counterhypothesis where the detected tRNAs are associated with ribosomes that co-IP with an mRNA to which Scp160p should allegedly bind.

The control conditions illustrate this view. Disrupting ribosomes during Scp160p immunoprecitipation apparently abrogates tRNA binding and increases mRNA and rRNA binding, which may be interpreted to be less specific, "idleness-born". As magnesium chloride should favor run-off and subsequent natural disassembly, and puromycin should elicit synthetic disassembly, both conditions have in common that they act on ongoing translation. This might explain the residual mRNA and rRNA binding. In contrast, the complete abrogation of nucleic acid binding using EDTA can be explained by the compound's capacity to totally disassemble ribosomes even before (in addition to during) translation, resulting in less probability for a vicinity of Scp160p, mRNA, rRNA, and ribosomal proteins in total.

Conclusively, the simplest conceivable explanation is that Scp160p may indeed be a protein serving to limit diffusion of tRNAs away from the ribosome. The already established synergism of Scp160p's presence on ribosomes with more optimally-composed mRNAs opens the interesting perspective of multi-layered, adaptable translation. Hypothetical coalitions between mRNA-binding proteins delivering specific messages to ribosomes, and accessory factors such as Stm1p and Asc1p, and possibly also Scp160p, sitting on

ribosomes could enable temporal or spatial control of fine-tuned translation. As much as this view is thrilling, it is clear that additional experiments beyond the scope of this investigation are needed to expand and refine this model.

The Ribosomal Vicinity of Asc1p and Possible Roles in Translation

ARC1 and *ASC1* are negative *genetic* interactors of each other (WILKES 2008), i.e. knockout of both genes affects cells more than knockout of either would do. Thus it is conceivable that both proteins, although found in different positions on the large and small subunit of the ribosome (BECKER 2006/ GILBERT 2010), are genetic interactors due to a presumed *physical* interaction.

Glu1p Glutamyl- amnoacyl tRNA synthetase is a physical interactor of Arc1p in yeast (DEINERT 2001), and Alanyl- aminoacyl tRNA synthetase *ALA1* is a negative genetic interactor of *ASC1* in yeast (COSTANZO 2010). It has been suggested in the case of Arc1p (BECKER 2006), and may mean in the case of Asc1p, that the respective aminoacyl-tRNA synthetases interact physically with these ribosome-recruited proteins in order to facilitate charging of ribosomes with the corresponding aminoacyl-tRNAs in yeast, as was shown for different synthetases in mammalians (DAVID/YEWDELL 2011) and archaea (MIKULCIC/BAN 2014).

If said interaction were conducive to function due to increased recycling via molecular crowding, it should result in ribosomal depletion of only the „cognate" amino acid's tRNAs. However, if also the two synthetases, or their ribosomal adapters, interacted, not only the „cognate" tRNA of the proven interactor's synthetase, but also the other's should be depleted, upon a knockout of either protein. Following a tentative interpretation of preliminary, indirect experiments, both may be the case:

Hypothetical physical interactions between Arc1p and Asc1p, or AlaRS and GluRS, may further contribute to substrate channeling at ribosomes

In order to test whether knockouts of *ARC1* have an effect on ribosomal occupancy of all, some, or distinct tRNAs, tRNA quantitation following ribosome-affinity purification (RAP) of wild-type versus *ARC1* deletion cells was performed. All tRNA species coding for Alanine, Glutamate, Serine, Leucine, Threonin and Valin were quantitated from eluted ribosomes, to calculate the shift of ribosomes in knockout cells relative to wild-type in ribosomal occupancy.

Glutamate and Alanine were the only species reliably quantitatable in knockout cells that were offset to wild-type level.

The first experimental finding was that popular codons tended to be depleted, and rare codons increased, in ribosomal occupancy upon *ARC1* knockout (**Fig. 15**). This can be interpreted to mean that first, the higher

drop in popular codons within one amino acid makes sense given that a recruiting enzyme would be expected to matter more to those species (cf. dedicated parahraph).

Secondly, not only Glumatyl-tRNAs (**Fig. 15A**), but also Alanyl-tRNAs (**Fig. 15B**), were depleted from ribosomes, following *ARC1* deletion. Thus, there seems indeed to be evidence of a functional cross-talk between Arc1p an Asc1p, as also levels of the amino acid's tRNAs whose aminoacyl synthetase (*ALA1*) interacts with another gene genetically (*ASC1*) were decreased following *ARC1* knockout, on top of the „cognate" one (*GLU1*).

In any case, the similarity of this observation with the knockout of the elongation factor *YEF3* in a previous chapter is striking. It could be argued that, as Yef3p has been shown to play a pivotal role in unimpeded, efficient translation (KINZY 2008), the similar depletion pattern for *ARC1* points in the direction of this protein being similarly meaningful for it.

Furthermore, the finding that not only Glutamyl, but also Alanyl tRNAs are depleted in ribosomal occupancy in deletions of *ARC1*, can be seen as indirect evidence towards possible physical interactions between Arc1p and Asc1p and/or AlaRS, or GluRS and Asc1p and/or AlaRS. While further studies are necessary to illuminate this question, the possible existence of efficiency-enhancing multi-protein complexes around the ribosome is tantalizing. It would not only fit well with previous findings across all kingdoms of ribosomal localization of ARSes (DAVID/YEWDELL 2010, MIKULCIC/BAN 2014), but also provide an easy avenue towards a molecular framework between the molecular crowding/ substrate channeling notion of the ribosome put forth by NEGRUTSKII/DEUTSCHER (1991).

A Network of Ribosome-Associated Factors that Increase Molecular Crowding and Contribute to Substate Channeling

There is a 1000-fold difference between the volumes of a yeast cell (~42 μm^3) (Tyson et al. 1979) and a tRNA (~45 nm^3, based on the average diagonal distance between the 3' tail and the anticodon loop ~76Å). Given that tRNAs must interact with three different partners in the proper temporal order (an appropriate aminoacyl-tRNA synthetase, eEF1A•GTP and a translating ribosome) to deliver an amino acid to a growing polypeptide, a mechanism relying on simple Brownian diffusion unlikely accounts for measured rates of translation elongation, which are ~4.5 sec^{-1} (SIWIAK and ZIELENKIEWICZ 2010). Indeed, the tendency of ribosome crystals to pack as asymmetric dimers (BEN-SHEM et al. 2011) and the presence of multimerized ribosomes in cryo-EM studies (BRANDT&HARTL 2010 2010, VIERO et al. 2015) suggests that ribosomes may naturally interact with one another.

My data provides corroborating evidence for two hypothetical models of cooperation between RNA-binding proteins and the ribosome. The RNA operon model (HALBEISEN/SCHERRER/GERBER 2009, KEENE 2007) holds that shifting coalitions of interactions between RNA-binding proteins and their mRNA targets might be the key to understanding the complex networks of post-transcriptional regulation phenomena. Scp160p has already been implicated in one such network, the SESA (*SMY2/EAP1/SCP160/ASC1*) network, in the function of a translational activator (SEZEN/SEEDORF/SCHIEBEL 2009). It binds ribosomes via its interaction partner Asc1p (BAUM/FREY 2004), whose mammalian homolog RACK1 binds activated kinases, promotes 80S joining and might integrate growth or proliferation signaling cues (CECI et al. 2003, RACHFALL/BRAUS 2013). In an unrelated second model, efficiency of translation *in vivo* is explained by the observation of active aminoacyl tRNA synthetase recruitment to translating ribosomes by interaction with the ribosome itself or ribosome-associated proteins (GONNET/BERNARDIN 2000, DAVID/YEWDELL 2011). Along these lines, Scp160p with its multitude of RNA-binding domains could serve as a facilitator of tRNA recycling, possibly by preventing diffusion from an associated ribosome, or 'catching' tRNAs given off by a ribosome that has advanced further on the same transcript. Its adapter Asc1p on the other hand has been shown to interact with aminoacyl tRNA synthetases, although these re-charge tRNAs different from those assayed in this study (FRIBERG/GONNET 2006, DAVID/YEWDELL 2011, CANNAROZZI et al. 2010, SHARP/LI 1987). Both proteins could function hand-in-hand to facilitate fast tRNA recycling, Asc1 by recruiting aminoacyl-tRNA synthetases and Scp160p by ensuring that empty tRNAs are not lost from one translating ribosome to

the next. In this scenario, Scp160p might also aid mRNAs not-yet perfectly-composed to make the most use of autocorrelation because evolutionary entrenchment hasn't yet succeeded in consolidating their optimal coding - or because their dependency on accessory factors would give the cell a means of leverage on functionally related sets of targets with a common mechanism, a motif elaborated in later chapters for considerations of different growth phases and diverse accessory proteins.

Here, I would like to present a model which combines the genetic data described in this study with known biophysical interactions (http://www.yeastgenome.org/; http://funcoup.sbc.su.se/search/; GAVIN et al. 2006) to explain how local molecular crowding may channel tRNAs from ribosome to ribosome during translation elongation (**Fig. 16**). I propose that Scp160p tethers elongating ribosomes together through its interactions with ribosomal proteins S5, L30 and L27 located on the E-site face of the leading ribosome, and with L10, L20 and L4 on the A-site face of the lagging ribosome. Scp160p also interacts with the ribosomal stalk proteins P0 and P2, eEF2, and the gamma subunit of translational elongation factor eEF1B (which helps eEF1A exchange GTP for GDP and aminoacyl-tRNA synthetases). Asc1p interacts with Scp160p and S5,

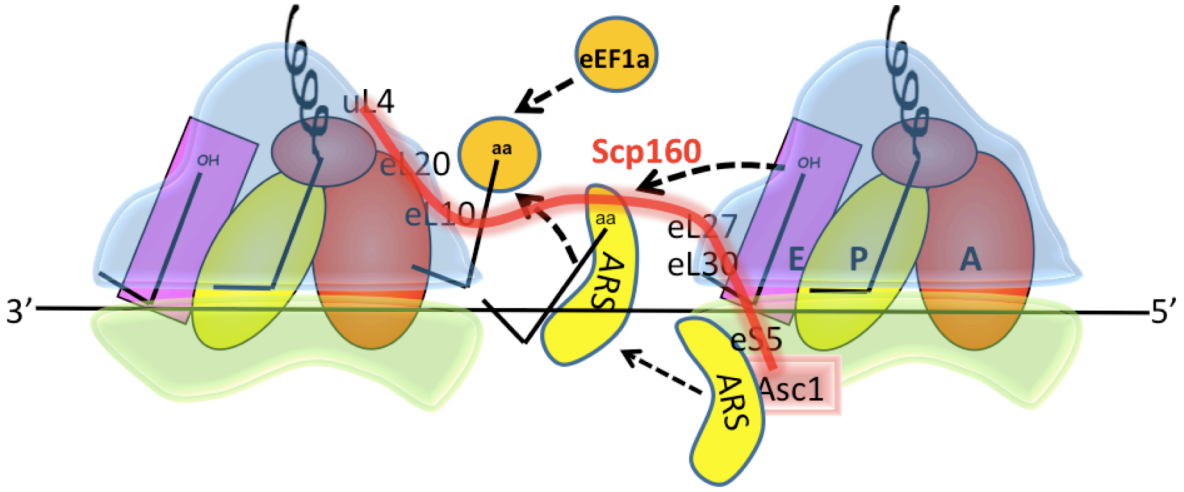

Fig. 16: A network of ribosome-associated factors stabilizes polysomes and enables efficient recycling of tRNAs.

Based on known interactions between Scp160p, Asc1p, ribosomal proteins and soluble trans-acting factors, I propose that Scp160p functions to tether elongating ribosomes together and/or bind tRNAs, while Asc1p serves to recruit and reycle aminoacyl tRNA synthetases and consolidates ribosomal tethering. Local crowding effects prevent escape of deacylated tRNAs, especially rare species. In general, this enhances polysome formation and elongation rates.

Image courtesy of Jon Dinman, as submitted in HIRSCHMANN, KENDRA, WATANABE, DINMAN, JANSEN 2015 (in review).

as well as with aa-tRNA synthetases. In addition, Bfr1p (a coiled-coil protein with structural homology to tropomyosin) interacts with L11 and Scp160p. I propose that these interactions constitute the scaffold upon which the tRNA channeling complex is built. Starting with the leading ribosome, I suggest that the Bfr1p/L11 interaction channels the deacylated tRNAs away from the E-site. Simultaneously, Asc1p presumably delivers aa-tRNA synthetases to Scp160p where the deacylated tRNAs are channeled to the aa-tRNA synthetases. Once charged, the aa-tRNAs are handed off to eEF1A, where they are delivered to the A-site of the following ribosome. This model explains how a specific tRNA, its aa-tRNA synthetase and its cognate codon can all be retained in the same vicinity during translation elongation. Similarly, the interaction between Scp160p and eEF2 enhances translation elongation by delivering the translocase to the A-site.

This model also easily explains the translational fidelity defects, or effects, respectively observed in *scp160Δ* and *asc1Δ* cells. Decreased elongation rates in the absence of Scp160p or Asc1p would allow the release factor eRF1 more time to interact with ribosomes paused at stop codons, promoting increased termination fidelity. Increased rates of misreading of rare codons are also consistent with increased pause times. Likewise, lack of Scp160p would generally decrease rates of tRNA clearance from ribosomes, giving more time for tRNAs to slip at -1 PRF sites (LIAO et al. 2010). In contrast, this is not predicted to affect +1 PRF (LIAO et al. 2008), which fits my observations. The differential mistranslation of codons for tRNAs with different abundances by both mutants is also consistent with their proposed roles in limiting tRNA diffusion and boosting tRNA recycling, respectively. Decreased readthrough of highly-autocorrelated Ala and Gly repeats in both *scp160Δ* and *asc1Δ* cells is consistent with their proposed roles in boosting coding-optimized elongation speed and supports of the roles of autocorrelation and positive charged amino acids in translational efficiency (CANNAROZZI et al. 2010, HIRSCHMANN et al. 2014, INADA et al. 2013, KOUTMOU & GREEN 2015). Interestingly, stalling promoted by the anticorrelated poly-Arg reporter was partly rescued in *scp160Δ* cells. We suggest that this is due to increased rates of mistranslation of the Arg codon, which would relieve ribosome stalling at this stretch of amino acids. In addition to independently verifying these important predictors of efficient translation, this model begins to illuminate a complex network of factors function together to optimize the speed and accuracy of translation elongation.

An important consideration in the case of autocorrelated constructs, and the differing effects of deleting *SCP160* and *ASC1*, is competition between discharged and charged tRNAs of various global abundance, but locally around ribosomes. While the need for a diffusion limitation on rare tRNAs easily explains their higher rate of mistranslation in knockouts of *SCP160*, the problems encountered by cells upon *ASC1*

deletions are less straightforward. I assume that, as popular tRNAs are under competition with neighboring polysomes reading off different transcripts, they would suffer more in read-through when *ASC1* is deleted if a physical coherence between Alanyl-tRNA synthetase (AlaRS) and Asc1p and thus ribosomes is assumed. The fact that mistranslation increases most for popular codons in *ASC1* deletions can be explained by assuming that a loss of proximal recycling increases local pile-up of discharged tRNAs, thus extending the time-frame for correct accommodation of cognate aminoacyl-tRNAs by competition (due to recognizing the same codon), and enabling near-cognate aminoacyl-tRNAs „slipping a foot in the door". (Popular tRNAs have been shown to translate with higher fidelity, but not faster, by KINZY/DINMAN 2007 and POP/INGOLIA/WEISSMANN/PHYZICKY 2015).

Conversely, a „tRNA diffusion limiting" function of Scp160p would be expected to chiefly affect rare tRNAs, as they are in more danger of being lost due to diffusion. A partial synergism with autocorrelated genes may be expected here.

Conclusively, these observations add weight to an emerging view of the ribosome governed by the laws of supply and demand (WINTERSBERGER/KARWAN 1995, PECHMANN/FRYDMAN 2012), possibly due to kinetic effects imposed by the substrate channeling (BELLI/HERERO 1998, NEGRUTSKII/DEUTSCHER 1991) and molecular crowding phenomena (RUEPP/MÜNSTERKÖTTER 2004, MCGUFFEE/ELCOCK 2010). This work also describes the first *in-vivo* method to prove the predictions of the bioinformatical parameter of "tRNA Pairing Index" (FRIBERG/GONNET 2006, CANNAROZZI et al. 2010) both on the mRNA and tRNA level, reinforcing it as a novel predictor of translational fitness.

Encouragingly, my experiences with ASC1 deletions in relation to mistranslation along partly repetitive mRNA sequences were partly complemented by the findings of a recent study, albeit using different sequences and methods (WOLF & GRAYHACK et al. 2015).

SUMMARY: Scp160p - a Protein Connecting Autocorrelation, tRNA Buffering, and mRNA Operons?

mRNA classes with different codon composition and functions of Scp160p: Egoistic genes, optimized genes, and regulated genes

Coming back to the observations from the first two sets of experiments, some conclusions can be reached when comparing the behavior of distinct cohorts of mRNAs upon *SCP160* deletion.

For the first major group, the mRNAs that are transriptome-decreased upon deletion according to SCHRECK *LMU München* 2010 and HIRSCHMANN et al. 2014, fall into two subclasses, that I want to call „profiteers" and „stringents".

The „profiteers" show little reproducible binding to Scp160p or polysomes carrying it in (HOGAN et al. 2008), and also lower autocorrelation within their genes. As a result, if they come across an Scp160p-doted polysome by chance, in my model view they capitalize on the protein's implied tRNA-buffering function to reach local crowding effects akin to autocorrelation-rich targets. Upon knockout of the gene however, they tend to fall short in translation as witnessed by a relative ribosomal occupancy drop in RAP assays, presumably because the Scp160p-mediated buffering function is their only means of achieving molecular crowding.

The mirror image are the „stringents". Their binding to Scp160p-doted polysomes is high in (HOGAN et al. 2008), and also their sequences show higher autocorrelation. As a result, they do not rely as heavily on the protein in translation, as their globally-high autocorrelation can make them achieve molecular crowding of tRNAs on its own. As a result, their translation increases upon knockout, presumably because the „helper" function of Scp160p on imperfect mRNAs sucks less building blocks (empty tRNAs) away to other polysomes.

The second major group contains the mRNAs that are transcriptome-stable upon deletion according to SCHRECK *LMU München* 2010 and HIRSCHMANN et al. 2014, but shift to polysome fractions in sucrose gradients to varying degrees. I call them „regulatables". As shown in (HIRSCHMANN et al. 2014), Western

Blots for two target mRNAs' products show that the increased polysomes are probably slow ones, as their high-mass shift is concomitant with a decrease in the corresponding protein's level. Several of these targets also show decreased ribosomal occupancy in RAP assays.

Does regulation of translation elongation connect to growth behavior and tumor models?

When examining their mRNA sequences, it was striking that they are globally optimized for autocorrelation, with the exception of specific codons using several tRNAs for specific amino acids. As demonstrated in the Results section and (HIRSCHMANN et al. 2014), it is the very tRNAs within one amino acid that are more depleted from ribosomes in RAP followed by RT-qPCR quantitation in *SCP160* knockdown cells, that are also less-perfectly autocorrelated at a genetic level, with the highest tendency in the mRNA subsets most polysome-shifted in (HALBEISEN et al. 2010). These targets are a representative subset of the targets identified in (HOGAN et al. 2008), and show ontological enrichment to similar functions and localizations. Hence it is conceivable that in their case, a deliberately suboptimal coding makes them regulatable in translation elongation as they need buffering prevention to compete with highly-optimized mRNAs to achieve the same molecular crowding of tRNAs. In this view, it would be no coincidence that Asc1p, and resultingly Scp160p, fall off polysomes in stationary yeast (BAUM/FREY 2004 and FRIDOVICH-KEIL 2001). Together with the observed de-recruitment of Arc1p, an interactor of MetRS and AlaRS in the same conditions (FRECHIN/SENGER/BECKER 2009), a model is conceivable. What if yeast cells use deliberate coding discrepancies of mRNAs, in conjunction with variable accessory factors of ribosomes as has been proposed for mammals and yeast (MCINTOSH/WARNER 2007, GILBERT 2011) and evidenced (KOMILI/ SILVER 2007) in other cases, to fine-tune translation of functionally related sets of genes? This notion would go well with the ribosome filter hypothesis (MAURO&EDELMAN 2007), the RNA operon model (KEENE 2007), and the molecular crowding model (MCGUFFEE 2010). Of note, recent work by DEDON and BEGLEY (2014) has found interesting covariances between tRNA modifications and „stretches of codons" in functionally related, co-behaving mRNAs.

OUTLOOK 1: Codon Repeat Diseases Deplete Aminoacy-tRNA Pools, a Situation where Pharmaceutically Elevated Levels of Vigilins May Aid Partly-Autocorrelated mRNAs to Cope

Possbile tantalizing medical connections comprise poly-Glutamine tract mutations caused by repeat expansion via slippery DNA polymerases, which are a major cause of neurodegenerative human diseases, e.g. Chorea Huntington. As recent work shows (GIRSTMAIR et al 2013), depletion of charged tRNA pools can impair high-autocorrelation poly-Glutamine stretch expression, leading to -1 frameshifting. A striking finding was that the very tissues that suffer most from the polyglutamine disease, are also most depleted in Glutamine tRNAs following mutation, and are endogenously poor in Glutamine tRNAs to start with. The implication according to the authors was that the extent of charged aminoacyl tRNA depletion by repeat mutations, its detrimental impact on brain cells, and an endogenous paucity of glutamine tRNAs in these tissues, all covary. According to the authors, this in turn may mean that the tRNA depletion is the cause, not the effect, of the toxicity. Foremost, this result is reminiscent of the independence of highly-autocorrelated mRNAs, and dependence of partly anticorrelated mRNAs, on Scp160p. Cells reacting to its absence by increased -1 frameshifting, in our previous (HIRSCHMANN et al 2014) and present works.

Additionally, manipulation of endogenous Scp160p levels may open an avenue towards leverage on repeat-based diseases, posing Vigilins as possible druggable targets for future therapies: Overexpressing Scp160p or SCP160 or its homologs may be developed as a tractable therapeutic route for the future, if current experiments should verify that higher levels of Vigilins might boost translational efficiency of endogenous, partly-autocorrelated mRNAs in favor of totally-autocorrelated Huntingtin or other repeat-borne, highly autocorrelative constructs. Due to their composition, these could be expected to be independent of the protein's proposed function. Notably, even if the notion held by GIRSTMAIR et al should be correct, no matter if the Huntingtin protein itself is detrimental, or only a marker of toxicity due to tRNA paucity - stopping translation of mutant mRNA should be productive curatively, either way; as should be additional viglilin protein.

Experiments are initiated at our laboratories presently to investigate into these connections, but will have to be elaborated in future publications.

OUTLOOK 2: Outlining a Hypothetical Early Warning System for Malignancy, Based on tRNA Chargeomes

In order to take the insights gained by the study of Scp160p further, it may be worthwhile to extend experiments into cell culture, to explore if there is conservation of effects observed in yeast, so they are is also found in mammals like human. To this end, a bioinformatics and literature search was conducted, to approximate the relative ease of transferring knowledge between experimental setups. While the tRNA sequence conservation situation is paramount to decide whether existing primer sets may be re-used, and still pending, the first step is to examine if codons are conserved in isodecoder magnitude, and genomic usage, between yeast and human.

Back in the days before connections between fidelity of translation and frequent codons were elucidated (DINMAN, KINZY 2003) and autocorrelation was discovered (CANNAROZZI et al. 2010), the finding of popular codons being recognized by tRNAs with higher gene copy number and hence abundance (SHARP, LI 1987) was still attributed to mean faster/more productive protein synthesis, uniformly. It was with this in mind that researchers of a publication (D'ANDREA, CRISTINA 2011) embarked to measure the abundance of codons in human cell lines, as compared with a virus that spreads across the whole body. Their initial finding was that cancer cell lines tend to have markedly distinct codon choice that is different from human-coevolved viruses as well as other eukaryotes like yeast. This finding is important and valid, and will stand the test of time. The same cannot be said for the interpretations reached at the time, as they are outdated meanwhile by younger data, as stated above. Indeed, in my view, the fact that the codon popularity in a whole-body-affecting virus is indistinguishable from the situation in yeasts and other eukaryotes, is most parsimoniously explained by its good *average* adaptation to its host. Similar examples exist for the coevolution of virus and host in herpesviruses (reviewed in STRAUSS, STRAUSS 2006).

As for the divergent measurements in cell lines' transcriptomes, two explanations are possible. First, cell lines' idiosyncracies may represent the codon choice of the host tissues from which the respective tumors were lifted. Second, cell lines' idiosyncracies may represent the codon choice stereotypical of different cancer types, e.g. invasive vs. non-invasive, angiogenetic vs. WARBURG-like, solidifying vs. soft, etc. While it is clear that much more investigation in this field is needed, existing literature on viruses (YEWDELL, PAVON-ETERNOD 2013) and tissues (GESLAIN, PAN 2011) point in the direction that in terms of tRNA usage or even charging state by aminoacylation, virus-infested cells and tissues may divert from genomic average.

If future studies should indeed find codon choice within cancer cell lines specific not only for their surrounding tissues, but also for their type of mutation course or infestation behaviour, e.g. angiogenetic vs. WARBURG-like (reviewed in CAIRNS 2015), then this discovery would single-handedly open a route towards early warning for developing malignancy. For example, if tRNA charging state changes due to usage differences were observable in the informative way speculated on above, it may be one day be possible to use biopsies from across the whole body, in conjunction with tRNA counts, to help people predisposed by certain familial risk genes, to have a much earlier warning flag, possibly even before proteomes react, or cells begin to alter their behavior. If so, hitting early with a highly tailored drug regimen may one day constitute an advance towards preventive care instead of aftercare (e.g. irradiation or surgery). If combined successfully, the new strategy may prove as hard a hit on cancer as H.A.A.R.T. (highly active anti-retroviral therapy) was on HIV in the 1990s.

Of note, the most important observation in the paper by D'ANDREA and CRISTINA also goes along nicely with recently discovered tissue-specific needs for certain ribosomal genes in development, e.g. Hox expression (KONDRASHOV, BARNA 2011). This may interconnect with notions of composition-dynamic ribosomes (KOMILI, SILVER 2007) and paralog choice in autoregulation of ribosome biogenesis (O'LEARY, KENNEDY 2013).

Interestingly, vigilins in higher eukaryotes have also been discussed as possible druggable targets for pancreatic cancer (VOLLBRANDT, WILKOMM, KRUSE 2004) and breast cancer (WOO, XI, LAMB 2011), but dropped due to too complex readouts (personal communication).

It is interesting to note in this context that TOR, which has been proposed to be at the helm of cell-cycle dependent regulation of elongation via EF2 (LEPRIVIER, SORENSEN 2013) and ribosome biogenesis (LEMPIÄINEN and SHORE 2009), is also among the genes whose mRNAs are reacting badly in translation to absence of *SCP160* (SCHRECK, *LMU München* 2010). Its mammalian homolog has been proposed to influence translation quality and quantity control by affecting chaperone level sensing (QIAO, BENNINK, YEWDELL 2010).

The observation of different tRNA repertoires in proliferating versus differentiating cells as observed by GINGOLD, TEHLER and PILPEL in 2014, together with the aforementioned connections between growth states and polysome presence for Asc1, Arc1 and Scp160, may herald a new era of discovery, if more threads prove to be connectable by further experiments.

OUTLOOK 3: Yeast, a Model Organism Frozen in Time between Uni- and Multicellularity

Yeast, in many respects, is already an ideal model organism. Its relatively ease of use is highlighted by growth with standard microbiological techniques in unicellular form that resemble prokaryotic systems, i.e. liquid culture. This stands in stark contrast to the parallels in terms of genome organisation, genomic content, and post-translational modifications, that closely resemble more complex eukaryotes. While yeast can mate and reproduce sexually, it lives just as happily in the laboratory in a unicellular, haploid form. This has led to some skepticism from other disciplines as to how informative data from the yeast system would be as a prototype for e.g. disease models for human.

I would like to put forth some thoughts on this, and argue quite to the contrary. I would like to make a case for yeast's potential to continuously predict behavior in mammals correctly, starting with the unicellularity issue. This is important as a pivotal next step in my eyes is to take the vigilin community to the next level, and pick up where groups like Charlie KRUSE's left off; also I think the modeling done here in yeast merits at least exploration of the generality of tRNA clouding and codon autocorrelation ideas in higher organisms.

If we conceive of yeast as a lab model system, it is important to remember that it usually cycles between several growth states, exponential growth and stationarity. Exponential growth consumes glucose mainly in glycolysis; while approaching stationarity, respiration kicks in. A first tantalizing parallel is that proliferative versus differentiated cells, as well as WARBURG in contrast with angiogenic tumors, resemble these states. Translation regulation has been implicated in the former phenomenon (GINGOLD, TEHLER, PILPEL 2014), while yeast has been proposed as a proxy for the latter one (CAP, PALKOVA 2012).

Two publications from the last years came as somewhat of a surprise to the community: The discovery of apoptosis and multicellularity in yeast. The finding in 2002 by MADEO and others that stationary, hypoxic yeast tends to do apoptosis has spawned a small but successful community that to this day continues to delve into important ramifications, and underline yeast's potential as a model system. Still, apart from witnessing „evolution in action", the finding remained functionally cryptic. In order to keep our thinking sorted, one may ask: „Why should a unicellular organism like yeast develop a mechanism that is only of use much later, in human, to prevent malignancy?" Such thinking, obviously, is flawed by introducing a false teleology and a premature sense of direction. In truth, nearly all biological innovations tend to start as a current solution to a given problem A, that later are adapted to cope with problem B once established. This does not necessitate a „will" or „tendency" toward solution B in the A state at all!

The missing puzzle piece in my view arrived a decade later, when RATCLIFF and TRAVISANO found in 2012 that yeast is not unilaterally unicellular. Indeed, it seems that the attempt to always keep growth conditions optimal for yeast cells in the laboratory ignores a part of the wild-type repertoire that consequently escapes observation. Experienced experimenters are familiar with the tendency of stationary yeast to „clog" or „aggregate", a behavior that also cryptically often follows certain mutations, and is usually remedied by gentle stirring or sonication (SHERMAN 2002). However, the authors of the 2012 study found that yeast could reversibly transition between more aggregated and more dispersed appearances, depending on what amount of vigorous shaking was applied as a selective pressure. It appeared to them that the cells tend to answer hypoxia by establishing diffusion-preventing „biofilms" that make better use of nutrients by exhibiting a more globular shape. Of note, they noticed that the capacity to disperse once shaking is made more vigorous, hinges on increased apoptosis at the breakage points of „blobs". This shows that apoptosis may have developed in yeast as a solution to the respiration problem, only to become adapted towards malignancy control later in humans. In the same vein, this could mean that yeast possesses many more programs that pertain to respiration, and this point so far is completely under-appreciated, *could be coincident with or causal for the emergence of multicellularity in the first place*!

In this respect, our findings of a presumed tRNA buffering role for the yeast vigilin Scp160p may interconnect with several interesting fields. The function may enable concerted regulation in the translation of related sets of mRNAs, possibly integrating growth cues on polysomes as a unit of regulation via Asc1p and its associated kinases. This may relay information on ribosome biogenesis, as the presence of the *yTOR* target in the mRNA list for Scp160p dependence in translation shows.

Accordingly, it follows that the recently discovered phenomena of Asc1p relaying signals towards expressing targets important for respiration (BRAUS et al. 2014), and Arc1p relocalizing to mitochondria in stationarity (BECKER 2009), may not be sophistic isolated observaions, but in fact connect to higher principles - and may offer leverage for their understanding!

Hence, I would like to advocate more research on vigilins and autocorrelation, and Asc1p/Arc1p and crowding, in yeast and human. On top, I would like to suggest that the field may have to prepare for an era where only the concerted efforts of wet lab biochemistry, classical genetics, bioinformatics of translation, and yet-to-be devised cybernetics/ interactomics of gene and protein clusters will have to intersect. If we succeed at integrating the inputs from all these fields, linking the control of growth with the control of translation and the physical basis for its efficiency may usher a new era of answers and drug targets for problems from Chroea Huntington to cancer. The insights to be gained surely merit the effort and investment.

Materials

Consumables

1.5 and 2 mL microcentrifuge cups (Sarstedt)

13 and 50 mL plastic tubes (Sarstedt)

1 and 10 µL inoculation loops (Sarstedt)

6x DNA loading dye (Fermentas)

Carboxylated polystyrene microbeads 0.75 µm #07309-15 (Polysciences)

CryoPure tubes (Sarstedt)

Dynal magnetic beads (Invitrogen)

GeneRuler 1kb DNA ladder mix (Fermentas)

GeneRuler 100bp DNA ladder mix (Fermentas)

Glass beads 2 mm (Roth)

Glass beads 0.4µm (Roth)

MicroAmp Fast Optical 96-well Reaction plate # 4346906 (ABI)

MicroAmp 96-well support base # 4379590 (ABI)

MicroAmp adhesive film applicator # 4333183 (ABI)

MicroAmp optical adhesive film (ABI)

PageRuler Unstained Protein Ladder (Fermentas)

PageRuler Pre-Stained Protein Ladder (Fermentas)

PCR tubes (Sarstedt)

PCR 8-stripes (Sarstedt)

Petri dish (Sarstedt)

Plastic cuvettes (Roth)

Protein assay kit, BRADFORD principle, # 500-0205 (Bio-Rad)

PVDF membranes (GE healthcare)

rTEV protease, 1000 U @ 8U/µL, # E4310-01 (Roboklon)

SybrSafe GelRed (Invitrogen)

RotorGene 36-cup ring and 72- and 101-cup rings with corresponding qPCR tubes (Qiagen)

Whatman papers (GE healthcare)

Chemicals

1,4-Dithiothreitol (Roth)

1-Propanol (Sigma)

2-mercaptoethanol (Roth)

Acetic acid (Merck)

Agarose ULTRA (Roth)

Acryl-Bisacrylamide (Roth)

Bovine Serum Albumin (Fermentas)

Coomassie Brilliant Blue G-250 (Merck)

Cycloheximide (Roth)

dNTPs (Fermentas)

Diethyl pyrocarbonate (Roth)

Dimethyl sulfoxide (Roth)

EDTA-free protease inhibitor (Roche)

Ethanol (Riedel)

Glycerin (Roth)

Heparin (USB)

HPLC-grade H_2O (Fisher)

Leupeptin (AppliChem)

Lithium acetate (Roth)

Magnesium Chloride Hexahydrate (Roth)

Nonfat dried milk powder (AppliChem)

Pepstatin A (AppliChem)

Phenylmethylsulfonylfluoride (Roth)

Polyethylene glycol (Roth)

Potassium Chloride (Roth)

Protein-G-coupled Magnobeads (Invitrogen)

RNasIn (Fermentas)

RQ1 DNaseI (Promega)

Sodium chloride (Sigma)

Sodium dodecyl sulfate, SDS (Roth)

TEMED (Roth)

Trichloracetic acid (Merck)

Tris-HCL (Roth)

Triton X-100 (Roth)

tRNA from E.coli (Roche)

Equipment

Agarose cast (selfmade)

Centrifuge 5415, microcentrifuge size (Eppendorf)

Centrifuge 5702 (Eppendorf)

Decon DeVision DB0X gel documentation (Decon)

Dry Bath FB15103 (Fisher Scientific)

FlexCycler PCR cycler (Jena Analytik)

GeneQuant 1300 spectrophotometer (GE healthcare)

GeneSys 10 Bio spectrophotometer (Thermo Scientific)

Millipore filter system (Sartorius)

Mixing Block MB-102 (BioER)

MyCycler PCR cycler (Bio-Rad)

NanoDrop (ThermoScientific)

Portable spectrophotometer, yellow model (Amersham)

Power supply (Bio-Rad)

Protean Mini Tetra Electrophoresis (Bio-Rad)

Rotor SW40, Rotor F10S (Eppendorf)

RotorGene qPCR cycler (Qiagen)

SNAP-i.d. protein detection system (Millipore)

Sonorec RK100 sonifier (Bandelin)

StepOnePlus qPCR cycler (ABI)

TransBlot TD Semi-Dry Blotter (Bio-Rad)

Ultracentrifuge Discovery (Sorvall)

Ultracentrifuge plastic tubes # 343778 (Beckman)

Ultracentrifuge rotor S140AT-0221 (Sorvall)

Vortex genie 2 (Scientific Ind.)

Commercially available kits

Amersham ECL-plus reagent kit # RPN2132 (GE healthcare)

High Capacity cDNA Reverse Transcription kit (ABI)

NucleoSpin RNA II isolation kit #740955.50 (Macherey&Nagel)

NucleoSpin miRNA isolation kit #740971.50 to separate large and small RNAs (Macherey&Nagel)

PolyLink Protein Coupling Kit using EDAC # 24350-1 (Polysciences)

Power SYBR Green qPCR Master Mix (ABI)

QuiagenQuick PCR extraction kit # 28104 (Quiagen)

rDNase for on-column digestion in RNA II kit #740963 (Macherey & Nagel)

RotorGene qPCR Master Mix (Qiagen)

SilverQuest protein staining kit (Invitrogen)

Enzymes

Herculase II Fusion DNA polymerase (Stratagene)

Hi-Temp RTase (Qiagen)

RNase-free DNaseI (Quiagen)

rTEV-Protease (Roboklon)

Taq polymerase (Genaxxon)

Methods

For all methods described, deionised water was used. Restriction digests, dephosphorylation of fragments, ligations and separation of DNA in agarose gels as well as preparation and handling of SDS gels and Western blots are based on standard techniques (AUSUBEL et al. 2003). Commercially available kits were used according to the manufacturers' instructions.

Molecular biology and genetics

Plasmids used in this study are described in a separate **Supplementary table S1**, and were sequenced by Eurofins MWG Operon (Ebersberg, Germany) before use. Plasmids to monitor programmed -1 and +1 ribosomal frameshifting and termination codon readthrough were described in (Harger and Dinman 2003), and plasmids to monitor misreading of near- and non-cognate codons were described in (Plant et al. 2007). Dual luciferase based plasmids used to monitor auto- and anti-correlation and decoding of 12 consecutive Gly, Ala or Pro codons were based on repeat sequences from (DIMITROVA/INADA et al. 2009) and constructed by site directed mutagenesis and synthetic **DNA sequences** listed in **Supplementary Table S3** by Tatjana F. Watanabe based on sequences by Toshifumi Inada. **Yeast strains (Supplementary Table S2)** were derived from haploid (MATa) or diploid (MATa/MATα) W303 background cells (*ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52*). Chromosomal deletion of yeast *SCP160* and *ASC1* was performed by a PCR-based strategy (Janke et al. 2004) and gene deletion mutants were verified by colony PCR.

Ribosome affinity purification (RAP)

RAP was performed essentially as established (HALBEISEN et al 2009), as previously described in my Diploma thesis (HIRSCHMANN, *University of Tübingen* 2011):

Coupling of microbeads to immunoglobulin using carbodiimide

Coupling of 0.75 µm microbeads (Polysciences) to PP-64K unspecific IgG (Millipore) is performed essentially as prescribed by the manufacturer. Briefly, 12.5 mg of microparticles from 500 µL of a 2.5% w/v suspension are pipetted into a microcentrifuge tube. Particles are pellet at 1,000 rcf for 10 mins, and

supernatant is evacuated. Particles are resuspended in 400 µL of Coupling buffer (50 mM MES pH 5.2, 0.05% Proclin-300), and pelleted again as above. Then the particles are resuspended in 170 µL of Coupling buffer, and a 200mg/mL EDAC (carbodiimide) solution is prepared just before use by dissolving 5 mg EDAC in 25 µL Coupling buffer. 20 µL of the EDAC solution are added to the particle suspension, preceded and followed by brief maximal vortexing. A protein equivalent of 300 µg, e.g. from 30 µL of a 10 mg/mL solution, IgG is added immediately, followed by brief vortexing. Incubation is performed on a thermomixer for 1-1.5h at RT and 850 rpm shaking. End-over-end incubation, e.g. on a spin-wheel, is not recommended. After incubation, the tube is centrifuged as described above and supernatant (~ 220 µL) is kept to determine coupling efficiency. The pellet is resuspended in 400 µL of Washing buffer (10 mM Tris pH 8, 0.05% BSA, 0.05% Proclin-300). Centrifugation and resuspension is repeated as above, so that particles are stored in 400 µL of Washing buffer and ~400 µL of supernatant are retained for coupling efficiency determination. Quantification of protein concentration in supernatants is achieved using a commercial Bradford kit (Bio-Rad) on a GeneSys 10 spectrophotometer (ThermoScientific).

Ribosome-affinity purification (RAP)

RAP was performed essentially as described by HALBEISEN, SCHERRER and GERBER in Methods (2009). The most important alterations concern collection of yeast cells, buffer volume for TEV cleavage. Detection is not performed using microarrays, but with quantitative PCR following reverse transcription. All following recipes concern treatment of 100 mL of one yeast strain, grown to mid-log phase, i.e. to an OD_{600} of 0.5, in complete medium at 30°C while shaking at 150 rpm.

One minute prior to harvest, cells are provided with e.g. 1mL of 10 mg/mL cycloheximide stock added to 100 mL of culture, to achieve translational arrest by an concentration of 0.1 mg/mL CHX. 100 mL of yeast of each strain subject to assay are harvested at OD_{600} of 0.5 in 50 mL Falcon- equivalent tubes (Sarstedt) in aliquots of 50 mL using an Eppendorf 5702 centrifuge. Harvest is performed by centrifuging for 5 mins at 3,000 rpm. Pellets are resuspended with a total of 10 mL of buffer A (20mM Tris–HCl, 140mM KCl, 2mM MgCl2,1% Triton X-100, 0.2 mg/ml heparin, 0.1 mg/ml cycloheximide) in two aliquots of 5 mL. The aliquots are pooled in a 14 mL Corex-equivalent plastic tube (Sarstedt) and again centrifuged as described above. After discarding the supernatant, cells are flash-frozen using liqid nitrogen and stored at -80°C.

For RAP analysis, cells are thawed on ice and resuspended in 1 mL of buffer B (buffer A plus 0.5mM dithiothreitol [DTT], 1mM phenylmethylsulfonylfluoride [PMSF], 0.5 lg/ml Leupeptin, 0.2 lg/ml Pepstatin, 20 U/ml DNase I, 100 U/ml RiboLock (Fermentas)) in their 14 mL plastic tube, and broken using 1/3 vol. of 0.4 µm glass beads (Roth) by four cycles of (20s vortex at max speed – 90s cooling on ice) each. Then, crude extracts are transferred to microcentrifuge tudes and cleared by three subsequent centrifugation steps

at 2,600 – 8,600 – 13,500 rcf at 4°C. Supernatants following the last centrifugation are brought to 1 mL with buffer B. From now on, subsequent steps are performed in the cold room at 4°C to minimize degradation of protein and RNA.

Four aliquots of IgG-coupled 0.75µm microbeads, each amounting to 12.5 mg from 500 µL of a 2.5% w/v suspension, are needed per yeast strain. First, they are blocked to prevent unspecific aggregations: They are collected for 10 mins at 1,000 rcf, resuspended in 1 mL blocking buffer (buffer A supplemented with 0.4 mg/ml heparin, 0.1 mg/ml Escherichia coli tRNA [omitted in yeast tRNA quantitation], and 1% BSA) for each aliquot, and shaken at 850 rpm and RT for 10 mins. Then collection is done by centrifuging for 10 mins at 1,000 rcf.

This whole blocking step is repeated once.

Then, beads are washed once using 500µL of buffer A per aliquot (10 mins, 850 rpm, RT). Prior to centrifugation (10 mins at 1,000 rcf, RT) the four aliquots are pooled in a 2mL microcentrifuge tube. During centrifugation, a thermomixer is pre-incubated to 4°C in the cold room.

250 µL of lysate are secured and stored at -80°C (flash-freezing by placement of microcentrifuge tubes in cold metal blocks) for further analysis as lysate controls. The remaining 750 µL of lysate are batch-incubated with the pelleted beads for 2h at 4°C and 850 rpm in the cold room.

Following batch incubation, beads are collected for 2 mins at 3,000 rcf, 4°C.

Then they are washed four times with 1.5 mL buffer C (20mM Tris–HCl [pH 8.0], 140mM KCl, 2mM MgCl2, 5% glycerol, 0.5mM DTT, 40 U/ml RiboLock (Fermentas)) by shaking at 750 rpm and 4°C for 15 mins. Before the last washing step, the microcentrifuge tube is replaced with a fresh one to prevent unspecific aggregation to tube walls.

Beads are resuspended in 207 µL of buffer C and 23 µL of 8U/µL commercial rTEV protease (Roboklon) are added, amounting to an end concentration of 0.8 U/µL. Then, cleavage is performed for 2h at 15°C and 750 rpm in the cold room.

Following cleavage, beads are pelleted for 5 mins at 13,400 rcf and 4°C. The eluate is secured and stored at -80°C.

Adjustment of procedure:

I introduced two major changes two the protocol. Instead of 50 OD600 units I lysed 100 OD600 units of

cells and omitted tRNA as blocking agents. Quantitation of mRNAs and tRNAs coprecipitating with ribosomes was done by RT-qPCR. Up to five biological replicates were quantitated in duplicate technical replicates, and only observations for high (>2x Tag vs Mock) IP efficiency were pursued. qPCR measurements were discarded if technical replicate Ct value SD exceeded 0.5 or multiple Tm peaks were observed.

mRNA was extracted using a commercially available kit (Macherey&Nagel RNA II kit, Düren, Germany). tRNA and mRNA were separated by size using a similar system (Macherey&Nagel miRNA kit).

For tetracycline repression, appropriate strains were grown for six hours in medium supplemented with 2µg/mL Doxycycline (BELLI/GALLI/HERRERO 1998). For *yef3(F605S)* mutant analysis (ANAND/CHAKRABURTTY/KINZY 2003), yeast cells were grown at permissive temperature (26°C) to optical density at 600 nm of 0.7, then shifted for one hour to restrictive temperature of 37°C before harvesting.

Quantitative RT-PCR of mRNAs and tRNAs following RAP-IP

mRNA and tRNA were extracted from RAP eluates and cell lysates as described above. **qRT-PCR Primers for tRNA (Supplementary Table S3)** were constructed based on zones of low homology using multiple ClustalW sequence alignments of tRNA genes in MEGA software. Reverse Transcription of tRNA was facilitated by introducing a 95° pre-incubation step of RNA and master mix before addition of reverse transcriptase at 4°C, to enable melting of tRNA secondary structures and annealing of primers.

Percent ribosomal occupancy (%ribocc) was calculated according to the ΔCT method as follows: The comparison between different strains (e.g. wt vs *scp160Δ*) was done by comparing quantitative values obtained from eluted RNA. For mRNAs each strain's eluate (E) was normalized to its cognate lysate (L), thus eliminating transcriptome discrepancies from consideration. In accordance with prior publications, transcriptome stability of tRNAs was assumed for further analyses. In all cases however, each translational quantification was also normalized to its own cognate mock-IP strain to eliminate quantification resulting from unspecific adherence of ribosomes to beads.

Calculations of coding determinants of translational fitness

CAI (popularity of codons), TPI (codon autocorrelation across and within amino acid) and Z-scores (expressing the difference between observed counts of codon pairs and those expected as standard

deviations at both codon and tRNA resolution) were computed with the Darwin package (GONNET/BERNARDIN 2000) as described elsewhere (FRIBERG/GONNET 2006; CANNAROZZI et al. 2010; SHARP/LI 1987). P-values were computed for each group of genes by Monte Carlo sampling in which the TPI value of the group was compared to that of 100,000 random groups of the same size chosen from the yeast genome.

ΔCt calculations, averages, standard deviations and p-Values were calculated with appropriate tests, e.g. Student's t, using Microsoft Excel and Prism (Graphpad Software, La Jolla, USA). Prism was also used for calculating Spearman's nonlinear correlation analyses.

Translational fidelity assays and subsequent statistical analyses

Dual luciferase assays to monitor translational fidelity were performed as previously described (Grentzmann et al. 1998; Harger and Dinman 2003; Plant et al. 2007). Briefly, yeast cells were grown in appropriate selective-dropout media until mid-log phase, harvested, and processed as previously described. Four biological replicates were assayed in technical quadruplicates each, using the Promega Stop&Glo© kit (Promega, Madison, WI) on a GloMax Multi-Microplate Luminometer (Promega). Data analysis of luciferase assays was performed essentially as outlined in (Jacobs and Dinman 2004), with the following modifications. After quartile assessment and outlier removal, normality of residues was verified by by D'Agostino-Pearson K2, Shapiro-Wilk, or Kolmogorov-Smirnov test. Depending on the presence or absence of normal distribution, significance analysis was performed with Student's t-test or Wilcoxon's significance test. All analyses were conducted using GraphPad PRISM (LaJolla, CA) software. Wherever indicated, ** equals $p<0.01$.

Immunoprecititation of myc-tagged Scp160p

Immunoprecipitation of Scp160p was carried out using 100mL of yeast grown to optical density at 600nm of 0.5, as previously described (BÖHL et al 2000) with the following minor changes. To protect RNAs, Ribolock RNase inhibitor (Fermentas) was added to breaking buffer (50 mM HEPES/KOH pH 7.3, 20 mM potassium acetate, 0.1% Triton X-100, 5% glycerol, 0.5 mM PMSF,) at 0.8 U/μL. DNA was removed by adding 20U/mL RQ1 DNase (Promega) to breaking buffer. EDTA-free protease inhibitor (Roche) was added at 1x concentration to breaking buffer. Protein G magnetic beads (Invitrogen) were used that had been coupled to anti-myc antibody. For individual experiments, buffers were used with added 100μg/mL Cycloheximide, 10mM MgCl2, or 2mM EDTA pH 8.0.

Subsequent analysis for mRNAs, tRNAs and rRNAs:

RNA was isolated from eluates and separated into large and small (<200nt) species using the Macherey&Nagel miRNA kit at manufacturer's recommendation. For reverse transcription, the High Capacity cDNA kit (Applied Biosystems Inc.) was used. To quantitate mRNA and rRNA, random hexamers were employed in the standard protocol. Fot tRNA quantitation, annealing of primers was facilitated by introducing a 95°C pre-incubation step before adding the RT enzyme, as described previously (HIRSCHMANN et al 2014). Then quantitation was carried out in quantiative real-time polymerase chain reaction (qRT-PCR) using the StepOnePlusTM system and Power SYBR(R) Green PCR master mix (both from Applied Biosystems Inc.) as prescribed by the manufacturer. Subsequent analysis using the deltaCT method, averages, standard errors and P-Values were calculated with appropriate tests, e.g. t-test, using Excel (Microsoft) and Prism (Graphpad, La Jolla, USA) software.

Alignments, secondary structure prediction, and phylogenetic trees

Gene sequences for vigilin genes from S*chizosaccharomyces pombe, Ashbya gossypii, Saccharomyces cerevisiae, Drosophila melanogaster, Sus scrofa*, and *Homo sapiens* were retrieved from ENTREZ. Domain assignment was performed building on insights from the Khd1p PDB stucture (1KHD), according to secondary structure predictions from PSIPRED. ClustalW was used to align vigilin sequences between species, and also between domains within a species, obtaining Neighbor-Joining dendrograms building on the alignments in MEGA 4.0 software.

Table S1: Plasmids used in this study

Number	Name	Short description	Source
pRJ148	pRS316		SIKORSKI/HIETER 1989
pRJ957	pCM182		BELLI et al 1998
pRJ1220	pYM18	9xmyc	JANKE et al 2004
pRJ1464	pMS342	*SCP160* ORF	FREY 2001
pRJ1463	pCM182-*SCP160*	Tet_{off}-*SCP160*	SCHRECK 2010
pRJ1845	pTKB595	p*yef3(F650S)*	ANAND et al 2003
pHS4	pRS316-*SCP160*		SCHRECK 2010
pHS9	pRS316-*SCP160myc9*	Expression of myc9-tagged Scp160	SCHRECK 2010
pHS10	pRS316-*scp160ΔKH13/14-myc9*	Expression of myc9-tagged Scp160 lacking KH domains 13 and 14	SCHRECK 2010
pJD375		Readthrough control	HARGER (2003)
pJD376	LA1	-1 PRF	HARGER (2003)
pJD377	Ty1	+1 PRF	HARGER (2003)
pJD378	HIV	-1 PRF	HARGER (2003)
pJD1457	OAZ1	+1 PRF	This study
pJD431	UAA	Nonsense codon	HARGER (2003)
pJD432	UAG	Nonsense codon	HARGER (2003)
pJD433	UGA	Nonsense codon	HARGER (2003)
pJD642	UCG	Non-cognate Arg missense	PLANT et al (2007)
pJD643	AGC	Near-cognate Arg missense	PLANT et al (2007)
pJD644	AGU	Near-cognate Arg missense	PLANT et al (2007)
pJD1451	12 Ala	Autocorrelated Ala repeat	This study*
pJD1452	12 Gly	Autocorrelated Gly repeat	This study*
pJD1453	12 Pro	Autocorrelated Pro repeat	This study*
pJD1454	12 Arg	Anticorrelated Arg repeat	This study*
pJD1455	12 Arg -1	Anticorrelated Arg repeat; base deletion requires -1 shift to maintain frame	This study*

*pJD1451, pJD1452, pJD1453, pJD1454, pJD1455: For the generation of the 12x repeat plasmids, parent plasmid pJD375 was digested at the SacI and BamHI restriction sites in between the two luciferase sequences. T4 ligase was used to clone the respective insert sequences (see Table S2) into the linearized plasmid. Correct insertion of oligonucleotide sequences was confirmed via sequencing.

Table S2: Yeast strains used in this study

Strain	Relevant genotype	Plasmid	Source
W303a	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*	-	
RJY497	*scp160Δ::klTRP1*	-	SCHRECK 2010
RJY3178	*scp160Δ::HIS3MX6*	-	SCHRECK 2010
RJY3180	*scp160Δ::HIS3MX6* Tet_{off}-*SCP160*	pRJ1463	SCHRECK 2010
RJY3509	*SCP160-myc9::klTRP1*	-	SCHRECK 2010
RJY3652	*CCW14-myc9::KanMX6*	-	SCHRECK 2010
RJY3676	*CCW14-myc9::KanMX6 scp160Δ::HIS3MX6*	pRJ1463	SCHRECK 2010
RJY3677	*CCW14-myc9::KanMX6 scp160Δ::HIS3MX6*	-	SCHRECK 2010
RJY3687	*RPL16a-TEV-ProtA::HIS3MX6*	-	HIRSCHMANN 2011
RJY3688	*RPL16a-TEV-ProtA::KanMX6*	-	HIRSCHMANN 2011
RJY3689	*scp160Δ::klTRP1 RPL16a-TEV-ProtA::HIS3MX6*	-	HIRSCHMANN 2011
RJY4275	*yef3Δ::KanMX6* p*yef3(F650S)*	pRJ1845	this study
RJY4282	*scp160Δ::HIS3MX6* Tet_{off}-*SCP160 RPL16a-TEV-ProtA::KanMX6*	pRJ 1463	this study
RJY4288	*yef3Δ::KanMX6* p*yef3(F650S) RPL16a-TEV-ProtA::HIS3MX6*	pRJ1845	this study
RJY4478	*PTH1-myc9::KanMX6*	-	this study
RJY4481	*PTH1-myc9::KanMX6 scp160Δ::klTRP1*	-	this study
HSY11	*scp160Δ::HIS3MX6* pRS316-*SCP160myc9*	pHS9	SCHRECK 2010
HSY12	*scp160Δ::HIS3MX6* pRS316-*SCP160ΔKH13/14-myc9*	pHS10	SCHRECK 2010

All strains are derived from W303a (*MAT**a** ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*).

Table S3: DNA sequences used in this study

In the following list, only oligos used for plasmid generation, site-directed mutagenesis and RT-qPCR are listed. Oligos used for gene knock-outs, taggings and checking of transformants were generated according to standard protocols (4, 7).

Number	Name	Sequence	Purpose
RJO2920	ACT1 FW	TCAGAGCCCCAGAAGCTTTG	RT-qPCR
RJO2921	ACT1 RV	TTGGTCAATACCGGCAGATTC	RT-qPCR
RJO3188	CFT1 FW	TTGGCCAACGACTTTTATCAGA	RT-qPCR
RJO3189	CFT1 RV	CGCAATGCTTTTCCGTCTATC	RT-qPCR
RJO3198	PRY3 FW	CAAACGAAGGCACCTCTTCC	RT-qPCR
RJO3199	PRY3 RV	TTGCACCTAGGCTTGTGCTG	RT-qPCR
RJO3332	CCW14 FW	CAAGGCTTCTTCCACCGAAT	RT-qPCR
RJO3333	CCW14 RV	GGAAGCTTGCTTGCTCGAAG	RT-qPCR
RJO3373	PTH1 FW	AACCACACCCACCTCAAACC	RT-qPCR
RJO3374	PTH1 RV	TAAAACCGTGGCCGTTGG	RT-qPCR
RJO4139	18S FW	TCAACACGGGGAAACTCACC	RT-qPCR
RJO4140	18S RV	CTAAGAACGGCCATGCACCA	RT-qPCR
RJO4888	HSP30 FW	CGGATTGGTTATGGGCAGTG	RT-qPCR
RJO4889	HSP30 RV	TCAGCTTGAACACCAGTCCA	RT-qPCR
RJO4890	HSP42 FW	TCGAAGACAGAGTGGGCATT	RT-qPCR
RJO4891	HSP42 RV	ACTCCAATTCTTCGTCGGGT	RT-qPCR
RJO4892	MUP3 FW	TGGTCCAAGTACGTCAGCAT	RT-qPCR
RJO4893	MUP3 RV	GAAAAGCGTGTATAGCCCGG	RT-qPCR
RJO4894	HSP104 FW	ATGCCGACTCCACCACTAAA	RT-qPCR
RJO4895	HSP104 RV	CTACGTTCAGCATCAAGGGC	RT-qPCR
RJO4896	VID24 FW	AGCCACAGACCAAACAGACT	RT-qPCR
RJO4897	VID24 RV	TGCGCTTGGTGTAGTAGGAA	RT-qPCR
RJO4898	ZPS1 FW	ATCACCGTCAATCTGCTCCA	RT-qPCR
RJO4899	ZPS1 RV	TACTCATGGTAGGGACGTGC	RT-qPCR
RJO4900	BSC1 FW	TCTGACGGTTGCACAGTTTG	RT-qPCR
RJO4901	BSC1 RV	AAATCCGTTTGTGAGTGGCC	RT-qPCR
RJO4902	MIG2 FW	GCCTGCAACTTCTCCTATGC	RT-qPCR
RJO4903	MIG2 RV	GCTGTGGGATATGTTGCTGG	RT-qPCR
pJD375	12 Arg	GTCGACtcggcgacgacggcgccgccggcgacgacggcgccgccttaagttcGGATCC	Subcloning
pJD375	12 Arg -1	GTCGACggcggcgacgacggcgccgccggcgacgacggcgccgccttaagttcGGATCC	Subcloning
pJD375	12 Gly	GTCGACtggtggtggtggtggtggtggtggtggtggtggtcttaagttcGGATCC	Subcloning
pJD375	12 Ala	GTCGACtgctgctgctgctgctgctgctgctgctgctgctcttaagttcGGATCC	Subcloning
pJD375	12 Pro	GTCGACtccaccaccaccaccaccaccaccaccaccaccaccacttaagttcGGATCC	Subcloning
RJO4624	Ala1 FW	GGGCGTGTGGCGTAGTCG	RT-qPCR
RJO4625	Ala1 RV	AGTCCGGAATCGAACCGGA	RT-qPCR
RJO4629	Ala2 FW	GGGCACATGGCGCAGTTG	RT-qPCR
RJO4630	Ala2 RV	CAACCGGAATCGAACCGAT	RT-qPCR
RJO4632	Glu1 FW	ATAGTGTAACGGCTA	RT-qPCR
RJO4633	Glu1 RV	TACGGGGAGTCGAAC	RT-qPCR
RJO4636	Glu2 FW	GTAGTGTAACGGCTA	RT-qPCR
RJO4637	Glu2 RV	AGCGGGGAGTCGAAC	RT-qPCR
RJO4324	Ser4 FW	ATTTCCTCAGAAAAGCAATTA	RT-qPCR
RJO4325	Ser4 RV	CGTCACAGACAGGATTC	RT-qPCR
RJO4340	Ser2 FW	GGCACTATGGCCGAGT	RT-qPCR
RJO4341	Ser2 RV	CGACACCAGCAGGATTT	RT-qPCR
RJO4336	Leu1 FW	GGAGGGTTGGCCGAGT	RT-qPCR
RJO4337	Leu1 RV	CGCGGACAACCGTCCA	RT-qPCR
RJO4338	Leu3 FW	GGTACTCTGGCCGAGT	RT-qPCR
RJO4339	Leu3 RV	CGCGCCTCCGAAGAGA	RT-qPCR
RJO4393	Thr1 FW	GCCAAGTTGGTAAGGCGCCAC	RT-qPCR
RJO4394	Thr1 RV	CGGATTTGAACCGATGATCT	RT-qPCR
RJO4397	Thr3 FW	GCCAAGTGGTAAGGCATCGCA	RT-qPCR
RJO4398	Thr3 RV	GGGAATTGAACCCACGATCC	RT-qPCR
RJO4399	Val1 FW	GTCTAGTCGGTTATGGCATCT	RT-qPCR
RJO4400	Val1 RV	ATCGAACTGGGGACGTTCTG	RT-qPCR
RJO4401	Val2 FW	GTGTAGCGGCTATCACGTTGC	RT-qPCR
RJO4402	Val2 RV	GATCGAACTCGGGACCTTTG	RT-qPCR
RJO4402	Val2 RV	GATCGAACTCGGGACCTTTG	RT-qPCR

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Parts of this work have been presented at the 2013 Barcelona IRB Research Conference on tRNAs (ESP), and at the 2014 Cold Spring Harbor meeting on Translation (USA), after being selected by the organizers for a poster and an auditorium talk, respectively. Poster PDF/ talk PPT re-prints are available upon request.

Parts of this work have been published in Nucleic Acids Research Magazine (HIRSCHMANN & WESTENDORF 2014), and submitted to Translation Magazine (HIRSCHMANN & KENDRA 2015). This means that parts of the text, figures and tables made by me during the writing of this thesis *before,* were also used in the writing of these publications. Generally, I abstained from specifically indicating self-quoting on each occasion, as this thesis contains only new and original results I obtained for the purpose of and in the course of my PhD work unless specifically indicated otherwise in each case.

As the thesis will appear in public printed form *after* the papers however, re-print permissions were secured from Oxford University Press online on 8/21/2015: Free license for online and print use in the finished thesis was granted using license numbers 3693751172778, 3693751334088 and 3693751392341.

Some parts of the Materials & Methods section, due to identical experimental conditions required, contain direct quotes from my Diploma thesis (HIRSCHMANN, *Eberhard-Karls-Universität Tübingen* 2011) for the sake of consistency and reproducibility.

The Introduction contains images from publications by others, as indicated.

A picture used in the Results section was generated by a rotation student, as indicated. A Figure and Table re-printed for stringency of narration originally appeared in my Diploma thesis before, as indicated.

Model figures used in the Discussion section were generated by collaborators, as indicated.

As large parts of the bioinformatical analysis required meta-studying TSC data from a previous thesis and SAM data from a publication by another group (Heidrun SCHRECK's PhD thesis, *Ludwig-Maximilians-Unviersität München* 2010; a paper by HOGAN et al., PLoS 2008), I have tried my best to credit the sources of this extraneous data. Also, wherever possible the figure and table legends indicate where my analysis required help in the form of tools programmed and provided by Gina Cannarozzi, my bioinformatics collaborator, to calculate bioinformatical values as required by the course of my analysis.

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