Distinct cis-acting elements mediate co-localization of mRNAs encoding for co-translational interactors in cytoplasmic clusters

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Abstract

Many newly synthesized proteins assemble co-translationally, providing a vital mechanism to prevent subunit misfolding in the crowded cytoplasm. Initial evidence indicates that the spatial organization of mRNAs aids this assembly, but it is unclear how these mRNAs are organized and how common this mechanism is. We used single-molecule Fluorescence in situ Hybridization to examine the spatial organization of mRNAs encoding subunits of various cytosolic complexes involved in critical cellular functions, such as fatty acid synthesis, glycolysis, translation and amino acid biosynthesis. We found that mRNAs of the same protein complex often co-localize in specific cytoplasmic clusters. Additionally, we observed that the mRNAs encoding enzymes of biosynthetic pathways are organized in cytosolic clusters. Focusing on mRNAs encoding fatty acid synthase complex subunits, we discovered that non-coding cis-elements significantly influence mRNA localization in an additive manner. Inhibiting mRNA co-localization impaired growth when complex activity was essential, highlighting the importance of mRNA spatial organization for cellular survival. Transiently disrupting mRNA translation also affected clustering, indicating that both the nascent chains and mRNA sequence targeting cues are important for spatial organization. In summary, we provide evidence that mRNA co-localization in cytoplasmic foci is coordinated by complementary mechanisms crucial for co-translational assembly, allowing efficient regulation of protein complex formation and entire pathways.

Introduction

Protein synthesis is an intricate process that ensures the efficient assembly of protein complexes. A crucial element is co-translational interactions, where subunits start assembling while still being synthesized. This mechanism ensures timely formation of functional complexes and protects nascent polypeptide chains from misfolding and aggregation as they emerge from the ribosome's exit tunnel. This raises the question: are mRNAs spatially organized within the cytosol to facilitate co-translational assembly interactions? If so, how does this arrangement contribute to the efficiency and accuracy of protein complex assembly?

Co-translational assembly is a well-established phenomenon in eukaryotes, conserved from yeast to humans (Bertolini et al., 2021; Duncan & Mata, 2011; Hampoelz et al., 2019; Kamenova et al., 2019; Lautier et al., 2021; Seidel et al., 2022, 2023; Shiber et al., 2018; Venezian et al., 2024). These intricate processes initiate as nascent polypeptides exit the ribosome, allowing efficient formation of protein complexes. Specific residues, or "hotspots," initiate these interactions through high-energy binding interfaces (Schlee et al., 2019). Molecular chaperones like the Nascent Polypeptide-Associated Complex (NAC) and the Ribosome-Associated Complex (RAC) protect nascent chains from premature or off-pathway interactions, ensuring correct folding and assembly, which maintains proteome organization and cellular homeostasis.

The spatial organization of mRNAs is vital for eukaryotic gene regulation (Cassella & Ephrussi, 2022; Das et al., 2021; Horste et al., 2023; Mikl et al., 2022; Seidel et al., 2022; Weatheritt et al., 2014). While prokaryotes utilize operons for expression of polycistronic mRNAs and co-localized synthesis of complex subunits (Nair et al., 2021; Shieh et al., 2015), eukaryotes employ different strategies for protein synthesis coordination.

Localization of specific mRNAs at subcellular sites is common in eukaryotes. For example, mRNAs encoding for endoplasmic reticulum (ER) and mitochondrial proteins are targeted to the respective organellar membranes (Cohen et al., 2022; Eliyahu et al., 2012; Gadir et al., 2011; Garcia Arceo, 2023; Gerst, 2008; Zabezhinsky et al., 2017). Membrane targeting is facilitated by mechanisms like the Signal Recognition Particle (SRP) pathway (Hermesh & Jansen, 2013; Pyhtila et al., 2008) and RNA-binding proteins that regulate mRNA localization (Lesnik et al., 2015).

In various tissues, subcellular mRNA localization involves active transport by motor proteins and adaptors along cytoskeletal tracks, and localized mRNA degradation (Cassella & Ephrussi, 2022). In neurons, cytosolic mRNAs encoding the Cox7c mitochondrial protein are co-transported with mitochondria along axons, enabling localized translation (Cohen et al., 2022; Eliyahu et al., 2012). During early Drosophila embryonic development, specific mRNAs localize to the oocyte´s poles, establishing morphogen gradients (Gagnon & Mowry, 2011; Johnstone & Lasko, 2003; Lehmann’ & Nüsslein-Volhard’, 1986). Even single cell organisms like yeast

exhibit active mRNA transport, such as the transport of *ASH1* mRNA to the bud tip of a dividing cell for mating-type switching (Paquin & Chartrand, 2008). Together, these examples highlight the diverse roles of mRNA co-localization in cellular functions and spatially regulated gene expression (Seidel et al., 2022).

Another type of co-localization of mRNAs is induced by stress conditions, such as starvation and heat shock, leading to co-localization of mRNAs and RNA-binding proteins in stress granules (SGs). SGs provide a protective environment for transcripts and ensure coordinated translation upon stress recovery. Under glucose deprivation, for example, mRNAs encoding human regulatory subunits Rpt1 and Rpt2 of the proteasome co-localize at distinct subcellular sites. This process depends on the CCR4-NOT1 complex, which regulates multiple aspects of the mRNA lifecycle, including its synthesis, translation, and degradation (Panasenko et al., 2019). In growing yeast cells, specific glycolytic mRNAs localize to granules, which recruit mRNA decay factors to become P-bodies during glucose starvation, suggesting an interplay between mRNA translation and degradation (Balagopal & Parker, 2009; Buchan & Parker, 2009; Haimovich et al., 2016; Hoyle & Ashe, 2008; Lui et al., 2014). Moreover, granules containing mRNAs encoding for translation factors are inherited by daughter cells and contribute to polarized growth, proposing a model where specific mRNAs are directed to granules for localized protein synthesis (Pizzinga et al., 2019).

The importance of mRNA co-localization for protein complex assembly is evident in various findings. For instance, sarcomere pattern establishment in muscle cells requires coordinated expression and assembly of myosin, titin, and other proteins (Fulton & L'ecuyer, 1993). In migrating fibroblasts, mRNA localization to the leading protrusions is crucial for the Arp2/3 complex assembly, influenced by extracellular stimuli and cytoskeletal components (Mingle et al., 2005). mRNA localization together with nucleoporin condensation is also critical for nuclear pore complex (NPC) assembly (Hampoelz et al., 2019). with co-translational events at the nuclear pores ensuring proper assembly and delivery of NPC subunits to target sites (Lautier et al., 2021). For the assembly of the mammalian Transcription factor II D (TFIID) complex, *TAF10* engages *TAF8* co-translationally, and their dimerization is mediated by association of *TAF8* N`-terminal interaction domain exposure (Kamenova et al., 2019).

This study investigates the spatial distribution of mRNAs coding for protein complex subunits and metabolic pathways, focusing on the role of localized translation in co-translational assembly in *S. cerevisiae*. We identify mechanisms supporting mRNA co-localization and examine the impacts of failed mRNA localization on proteome organization, protein assembly and functionality. Our research shows that mRNAs encoding for different subunits of the same protein complex often cluster in cytoplasmic foci. By swapping sequence elements, we identify specific upstream and downstream elements affecting mRNA co-localization and complex formation, suggesting spatial mRNA organization is a key regulatory mechanism in protein

complex assembly. The co-localized synthesis of protein subunits may be essential for maintaining a functional proteome, especially given the instability of orphan subunits.

Results

Co-localization of mRNAs encoding protein complex subunits in diverse pathways

To investigate whether co-translational assembly interactions are facilitated by localized translation, we analyzed the *in vivo* localization of mRNAs encoding subunits of various co-translationally assembling complexes using single-molecule fluorescence in situ hybridization (smFISH) in yeast (Zenklusen et al., 2008). The studied complexes are involved in diverse cytoplasmic processes, including fatty acid synthesis (fatty acid synthase, FAS), glycolysis (Phosphofructokinase, PFK), translation (Translation Initiation Factor eIF2 subcomplex, SUI2 and GCD11), and amino acid biosynthesis (tryptophan and methionine biogenesis) (Supplementary Table 1). As negative controls, we assessed co-localization of *ASH1* mRNA at the bud neck and cytoplasmic *FAS1* mRNA, as well as co-localization of cytoplasmic mRNAs encoding subunits of different complexes, such as *TRP2* and *MES1*. For a positive control for maximum co-localization detectable under our experimental conditions, we labeled the same transcript, *FAS1,* with two different fluorescent probes.

The smFISH analysis of endogenous mRNA distribution (Figure 1) revealed consistent mRNA co-localization patterns for transcripts encoding different subunits of the same protein complex. *FAS1* and *FAS2* mRNAs, encoding the α and β subunits of fatty acid synthase, showed 53% co-localization per cell. Similarly, *PFK1* and *PFK2*, encoding subunits of phosphofructokinase, exhibited 50% co-localization, while *GUS1* and *ARC1*, encoding components of the aminoacyl-tRNA synthetases complex (ARSs), displayed 44.5%. In contrast, negative control pairs *ASH1* and *FAS1*, along with *TRP2* and *MES1*, showed no significant co-localization.

These results indicate that co-localization of mRNAs encoding different subunits of the same protein complex is widespread. This may facilitate co-translational protein-protein interactions to promote proper folding in the crowded cytoplasm and protecting nascent chains from misfolding.

Co-localization of mRNAs encoding interacting proteins within biosynthetic pathways

We expanded our analysis to mRNAs encoding enzymes of three major metabolic pathways: fatty acid synthesis, arginine biosynthesis, and glycolysis (Supplementary Table 2). We previously observed that components of these pathways exhibited co-translational interactions of their nascent polypeptides (Shiber et al., 2018), including components of distinct enzymes within a pathway, indicating that co-localized translation may contribute to pathway regulation.

Supporting our model, mRNAs encoding co-translational protein interactors within the same pathway often show co-localization in cytosolic foci (Figure 2). For example, *FAS1* and *ACC1* mRNAs, which encode Acetyl-CoA Carboxylase (Acc1p) and the β subunit of the FAS complex, catalyze consecutive reactions in lipid synthesis and have been found to transiently interact co-translationally. Our smFISH analysis revealed that *ACC1* and *FAS1* mRNAs are significantly close to each other (Figure 2. C), with a median of 58% co-localized mRNAs per cell. Similarly, PFK2 and PYC2 mRNAs exhibit notable co-localization (Figure 2). PFK2 encodes phosphofructokinase 2, a key glycolytic enzyme, while PYC2 encodes pyruvate carboxylase, which converts pyruvate to oxaloacetate in gluconeogenesis. Despite their involvement in distinct, yet interconnected metabolic pathways, these proteins are functionally coordinated in cellular energy metabolism. smFISH analysis showed that PFK2 and PYC2 mRNAs are in close proximity, with a median of 50% co-localized mRNAs per cell. In contrast, the control pairs *ASH1* and *FAS1*, as well as *TRP2* and *MES1*, showed no significant co-localization.

Our findings connect mRNA co-localization with co-translational interactions of proteins in functional protein networks, potentially coordinating gene expression at the translational level. The regulation of translation, including the formation of mRNA-containing SG condensates, is as a critical mechanism responsive to nutrient availability, stress, and pathogen infection (Leprivier et al., 2013; Levy et al., 2024; Musa et al., 2016). Our results suggest that even under optimal growth conditions with abundant nutrients, cells fine-tune various pathways' functions by regulating the spatial organization of mRNAs encoding key metabolic enzymes.

Co-localization of mRNAs occurs in the cytosol

We examined whether co-localization of functionally related mRNAs begins in the nucleus, close to their transcription sites (Supplementary Table 3), or later in the cytoplasm where translation occurs. Analysis of localization patterns in different compartments revealed that mRNAs encoding co-translational interactors primarily co-localize in the cytosol (Figure 3), with co-localization levels ranging from 11% to 55%, while nuclear mRNA showed a median of 0% co-localization. Notably, we detected high nuclear co-localization for dually labeled *FAS1* mRNAs, indicating that all probes (*FAS1*-Red and *FAS1*-Yellow) target the same transcription site. These results suggest that mRNAs come together in the cytosol rather than in the nucleus, indicating cytoplasmic mechanisms driving co-localization.

mRNA clusters in cytoplasmic foci dedicated to complex assembly

We investigated how many mRNA molecules are present in each cytosolic assembly focus. smFISH´s remarkable sensitivity allows detection of single mRNA molecules and estimation of mRNA levels based on foci fluorescence intensity (Zenklusen et al., 2008). To assess mRNA co-localization within distinct foci, we classified foci into two groups: those with less than 2 mRNAs (<2) and those with 2 or more mRNAs (≥2). Analysis showed that multi-mRNA foci had significantly higher co-localization rates across most mRNA pairs (Figure 4). For example, *FAS1* and *FAS2* co-localized in 75% of multi-mRNA foci, *PFK*1-*PFK2* in 60%, and *GUS1*-*ARC1* in 53.7%. Similarly, analysis of mRNAs encoding different subunits of the same biosynthetic pathways revealed trends such as 66.6% co-localization for *ACC1/FAS1*. These findings suggest that mRNA spatial organization in the cytoplasm is a coordinated, with multiple mRNAs brought to specific subcellular foci to facilitate assembly and biosynthetic pathways formation.

5´ and 3´ UTRs direct co-localization of mRNAs encoding FAS subunits

We investigated which mRNA sequence elements contribute to cytoplasmic foci clustering. Previous studies indicated that mRNA localization often relies on cis-acting elements in their 5' and/or 3` UTRs, though coding regions may also play a role (Berkovits & Mayr, 2015; Cassella & Ephrussi, 2022; Chekulaeva, 2024). Additionally, starvation-induced mRNA foci formation is influenced by promoter regions (Chen et al., 2022), suggesting that localization cues may reside in regulatory elements of the genomic locus that are not transcribed. To investigate the impact of these diverse elements on mRNA co-localization in the context of protein complex assembly, we focused on *FAS1* and *FAS2* mRNAs, which encode the hetero-oligomeric FAS complex. We generated yeast strains with the following sequence elements mutated in the *FAS2* genomic locus: (1) promoter and 5'UTR swap with *NOP1* corresponding sequences (based on Yofe et al., 2016), (2) 3'UTR and terminator swap with *CYC1* corresponding sequences (based on Yofe et al., 2016), (3) *FAS2* ORF swap with *TRP2* ORF (4) deletion of the *FAS2* segment encoding the malonyl/palmitoyl-transferase (MPT) domain (2-282nt), essential for co-translational FAS subunit interactions (*FAS2 ΔMPT*) (Shiber et al., 2018), and (5) swap of the *MPT* domain sequence alone into the *TRP2* locus (*FAS2 MPT* in *TRP2*), in *FAS2* deletion strain (see details in methods).

To assess the impact of *FAS2* mutations on FAS complex formation, we compared growth rates under fatty acid depletion conditions, requiring FAS assembly and function (Figure 5, supplementary Figure 2). Strains lacking essential FAS catalytic domains required fatty acid supplementation for growth, as expected. However, promoter+5'UTR and 3'UTR+terminator swap strains showed growth levels similar to wt (Figure 5B), indicating that these genetic exchanges do not significantly impair FAS complex function, and thus assembly. In contrast, swapping the entire *FAS2* ORF with *TRP2* resulted in an extended lag phase and growth inhibition in fatty acid-deficient media (Figure 5B), suggesting upstream and downstream regulatory elements to the *FAS2* ORF impact FAS assembly and function.

The mutational alterations did not impact the cellular levels of *FAS1* and *FAS2* transcripts, as in strains with swapped *FAS2* cis-elements, mRNA levels were comparable to wt (Figure 5E). We then analyzed the levels of cytosolic co-localization of the *FAS2* and *FAS1* mRNAs (Figure 5D). The distribution pattern of *FAS1* and *FAS2* mRNAs in the *FAS2* promoter+5'UTR and 3'UTR+terminator swap strains was similar to wt, with *FAS2* mRNAs often in close proximity to *FAS1* mRNA. In contrast, swapping the entire *FAS2* ORF resulted in a ~6-fold decrease in clustering with *FAS1*. This indicates that both upstream and downstream elements contribute to *FAS1* mRNA co-localization with *FAS2* mRNA. In *FAS2* ORF mutants (MPT deletion and MPT-alone strains), *FAS2* mRNAs levels were ~3-4-fold lower compared to wt (Supplementary Figure 2). In these strains, the *FAS1* and *FAS2* co-localization levels were much lower than in wt, however the strong impact on transcripts levels prevents direct comparison to wt mRNA distribution.

Taken together, these findings suggest that sequence elements in the 5`and 3`UTRs, along with genomic elements further upstream and downstream to the ORF, such as promoter regions, each contribute to *FAS1* and *FAS2* mRNA co-localization. The role of coding sequences is complex, as ORF mutations significantly affect transcript levels and may impact mRNA stability. Together, these results suggest a combined role of multiple zip codes with compensatory functions, similar to other mRNA targeting pathways, such as OSKAR and neuronal (Gunkel et al., 1998; Mikl et al., 2022). Impairment of these cues compromised FAS complex functionality, highlighting the significance of mRNA localization for complex assembly pathways.

Translation-dependent and independent co-localization of mRNAs encoding FAS subunits

We investigated how translation inhibition affects *FAS* mRNA co-localization. To assess immediate effects, cells were exposed to short-term (20 min) exposure to puromycin, which incorporates into the C-termini of elongating nascent chains, blocking further extension and resulting in premature termination of translation, or subjected cells to severe heat shock at 42°C, which rapidly inhibits translation (Aviner, 2020; Wu, 2003). After treatment, cell s was fixed and analyzed. We compared these treatments to transiently inhibiting translation of fatty acid synthesis genes including *FAS1* and *FAS2*, by adding fatty acids to growth media (Hinnebusch, 1988).

None of the treatments significantly affected transcript levels, allowing us to analyze their impact on *FAS1* and *FAS2* mRNA co-localization. Fatty acid supplementation and heat shock mildly reduced co-localization by 8% and 10%, respectively (Figure 6A, 6B), while puromycin treatment resulted in a 16% decrease. We further explored puromycin´s effects by analyzing the distribution of *FAS1* mRNA in proximity to *FAS2* mRNA as a function of distance (Figure 6B). This analysis demonstrated puromycin significantly impacts co-localization, resulting in decreasing proximity detection between *FAS1* and *FAS2* mRNAs. The greater impact of Puromycin, which causes premature translation termination and nascent chain release, compared to fatty acids and heat shock, suggests that nascent chain interactions may play a role in mRNA clustering in complex-specific translation hubs.

Discussion

In this study, we demonstrate widespread co-localization of mRNAs encoding functionally related proteins that assemble co-translationally, clustering in cytoplasmic foci in *S. cerevisiae*. This pattern appeared across diverse pathways, including fatty acid synthesis and tryptophan synthesis. These findings extend the list of crucial functions of mRNA localization regulation, which emerged in embryonic development, proliferation, stress responses, nutrient sensing, and subcellular compartmentalization (Buchan & Parker, 2009; Cohen et al., 2022; Eliyahu et al., 2012; Gadir et al., 2011; Gagnon & Mowry, 2011; Haimovich et al., 2016; Horste et al., 2023; Hoyle & Ashe, 2008; Johnstone & Lasko, 2003; Lehmann' & Niisslein-Volhard', 1986; Panasenko et al., 2019; Pizzinga et al., 2019; Balagopal & Parker, 2009).

Function-related mRNA co-localization can facilitate early subunit interactions, as soon as interface domains are exposed at the ribosome exit tunnel, as shown by ribosome profiling experiments (Shiber et al., 2018). By enabling timely subunit interaction, co-localized mRNA synthesis can protect emerging polypeptide chains from harmful hydrophobic interactions, which can lead to misfolding and aggregation (Cassella & Ephrussi, 2022; Mingle et al., 2005).

A key challenge in assembling heteromeric protein complexes is that nascent subunits must interact with partner subunits promptly. In fact, ribosome profiling experiments revealed that partner subunit association occurs right upon the exposure of interaction domains at the ribosomal tunnel exit (Shiber et al., 2018). Translational pausing was suggested to aid these timely interactions. In *S. cerevisiae*, 78% of transcripts display transient translation slowdown at specific sites, mediated for example by local enrichment in nonoptimal codons (Sharma et al., 2021). Such specific slowdowns are correlated with the co-translational association of the Signal Recognition Particle (SRP), which promotes the docking of ribosome-nascent chain-SRP complexes to the ER membrane translocon (Mason et al., 2000; Pechmann et al., 2014; Voorhees & Hegde, 2015). However, co-translational assembly pathways of heteromeric complexes often do not involve a slowdown in translation at the onset of interactions (Shiber et al., 2018). Our results propose an alternative mechanism in which co-localized translation reduces the need for pausing by increasing the local enrichment of ribosomes synthesizing interaction partners, enhancing association rates.

Different mRNA features can direct cytoplasmic localization. Zip codes in 5´and 3´UTRs, and also in coding regions, were shown to recruit dedicated localization factors as well as molecular

motors (Jambhekar & DeRisi, 2007). Furthermore, stress-induced mRNA distribution in the cytoplasm was shown to depend on promoter regions (Zid & O'Shea, 2014), suggesting transcription factors could additionally function in cytoplasmic targeting. We analyzed the features of *FAS2* mRNA influencing its cellular distribution, finding that swapping its ORF into the *TRP2* locus strongly reduced co-localization with *FAS1* mRNA and impaired growth, suggesting that co-localization is critical for assembling metabolic complexes. Therefore, our results support the hypothesis that mRNA clusters are vital for the assembly of key metabolic complexes. The upstream and downstream untranslated sequences of the ORF are essential for proper cytoplasmic localization, indicating that multiple zip codes may have evolved to ensue, in a redundant manner, co-localization with functionally related mRNAs encoding for partner subunits. As *FAS2* encoded protein is highly dependent on its partner to achieve correct folding and avoid rapid degradation (Scazzari et al., 2015), co-localized synthesis and assembly may be crucial for the stability of the *FAS2*-encoded protein. Finally, the emerging polypeptide chains may also play a key role in mediating mRNA co-localization in complex specific translation hubs, as evidenced by our finding that puromycin decreases mRNA co-localization levels. Further research will have to clarify if this is mediated via direct nascent chain interactions, or whether these interactions require additional factors, binding both the nascent chain and molecular motors.

We further found widespread mRNA co-localization in key metabolic pathways, including arginine synthesis, glycolysis and fatty acid synthesis. Translational repression of fatty acid synthesis was recently shown to regulate cell survival under glucose starvation in glioblastoma (Levy et al., 2024). Furthermore, metabolic homeostasis regulation by mRNA translation were shown to alleviate effects of excess glucose and fatty acids in tissues such as pancreatic islets and the liver (Schaffer, 2020). Glycolytic enzymes cluster in membrane-less cytoplasmic granules in response to hypoxia (Jin et al. 2017) that rely on the participation of their encoding mRNAs (Fuller et al. 2020). This clustering was suggested to facilitate rapid feedback regulation of the entire pathway translation. Our results suggest that even under physiological conditions, mRNAs clustering occurs, allowing for rapid regulation of fluctuating metabolic needs.

In conclusion, this study indicates that mRNA co-localization is a crucial regulatory mechanism for efficient protein complex assembly, ensuring proper folding and function. This process may significantly impact cellular organization and offer new insights into treating diseases linked to protein misfolding and metabolic dysfunction.

Acknowledgements

We would like to thank all the Shiber lab members for fruitful discussions, and especially Muhammad Makhzumy for his contribution to co-localization analysis. We thank the Technion's Lorry I. Lokey Interdisciplinary Center for Life Sciences and Engineering for the core facility. We are grateful for the professional services provided by Nitsan Dahan and Yael Lupu-Haber from the Imaging and BioAnalysis unit of the LS&E Infrastructure Center of the Technion, Israel. This work was funded by the European Research Council Starting Grant 2031817 (A.S.) and the Israeli Science Foundation grants 2106/20 (Q.S.). B.B. acknowledges a research grant of the European Union (ERC - SyG - 101072047 - CoTransComplex). Views and opinions expressed are however those of the authors only and do not necessarily reflect those of the European Union or the European Research Council. Neither the European Union nor the granting authority can be held responsible for them.

Methods:

Yeast strain constructs

Deletion and various sequence swap strains in the endogenous genomic locus of the *FAS2* gene were generated using homologous recombination, following the method described by Janke (Janke et al., 2004) utilizing SWAT (SWAp-Tag) library strains (Meurer et al., 2018; Weill et al., 2018; Yofe et al., 2016). We compared the *mRNAs* co-localization of wt strain to these swap strains: Promoter and 5'UTR swap, where the *FAS2* native gene promoter was exchanged by the *NOP1* promoter and 5'UTR region, followed by GFP tag from the SWAT library. *FAS2* 3'UTR swap where 3'UTR and terminator elements were exchanged by 3'UTR and terminator sequence, followed by a selection cassette (Meurer et al., 2018; Weill et al., 2018; Yofe et al., 2016). We further generated a strain where *FAS2* ORF was inserted instead of the *TRP2* ORF locus. We next generated a strain deleted for the *FAS2* sequence encoding for malonyl/palmitoyl-transferase (MPT) domain, which was found to direct co-translational assembly interactions (Shiber et al., 2018) (*FAS2* ΔMPT). Lastly, we generated a strain in which the sequence region encoding *FAS2* MPT domain alone was cloned into the *TRP2* genomic endogenous locus (*FAS2* MPT in *TRP2*) was analyzed to evaluate the importance of the genomic context compared to the co-translational interface expressed alone. All experiments were performed in the *S. cerevisiae* BY4741 strain background. *S. cerevisiae.* The primers used for constructing these strains are

provided in Supplementary Table 4. The strains used in this study are listed in Supplementary Table 5.

Growth assays

Yeast cultures were incubated in YPD growth medium, or in YPD supplemented with fatty acids (0.03% myristic acid and 1% Tween 40) to test fatty acid auxotrophy levels (as in https://pubmed.ncbi.nlm.nih.gov/25564609/).

mRNA smFISH

Yeast cells were grown in SD medium (Low-Fluorescence yeast nitrogen base, Formedium) containing 2% glucose to early log phase growth (to ~ OD= 0.4); amino acids composition is indicated per experiment. Growth temperature was 30 °C, unless otherwise indicated. The cells were then fixed with 37% formaldehyde for 15 min, centrifuged at 1,200g for 12 min and re-suspended in 4% paraformaldehyde and 100 mM KPO4 at room temperature for 1 h. After fixation, the cells were sedimented and the pellet was washed then resuspended in 250µl Zymolyase 20T (2 ml Wash Buffer, 1.8µl β-mercaptoethanol, 10mM Vanadyl-ribonucleoside complex, 5 mg Zymolyase 20T). Cells were incubated 40 min at 30° on shaker,then the cells were sedimented, washed and resuspended with 1ml Triton 100X (0.2% triton in 2%BSA in PBS) and incubated 5m at room temperature.and the cells were then washed and incubated overnight in 70% ETOH 1ml at 4°. The cells were then washed and incubated in hybridization buffer (4xSSC, 50% formamide, 10% dextrane sulfate, 125 µg/ µl E. coli tRNA, 500 µg/ml salmon sperm DNA, 1x Denhardts solution, 1mM DDT and 0.4 U/µl rRnasin) 1hr at 37°. thereafter added smFISH probes for labeling. The sequence-specific fluorescent probes (PixelBiotech GmbH) were generated targeting the Open Reading frame (ORF) of the transcript of interest (see Table 3) unless otherwise indicated. To analyze the impact of these gene modifications on mRNA localization, we used smFISH. Probes targeting the last ~600nt of the FAS2 mRNA coding region were used for most strains, except for the FAS2 MPTΔ strain (3'UTR probes) and the MPT alone strain (MPT-specific probes). For each transcript, 32 complementary DNA probes were produced (~17-21nt in length), each conjugated with 3 fluorophores in tandem (Atto647 or Atto565), using a T4 DNA ligase (T4DL) so each mRNA molecule is tagged with 96 fluorophores (Cheng et al., 2017). The nuclear staining was performed using Hoechst (20 ng/µl). The stained cells were resuspended in Mounting medium (55% Glycerol in 1 X SSC and 0.125M EDTA).

Confocal Microscopy

High-sensitivity confocal imaging was performed on a Spinning Disc Confocal microscope at the LS&E Microscopy Core Facility. Cells were imaged with a confocal microscopy with high magnification 100x Oil Objective and high numerical aperture (NA-1.45) and Dual sCMOS Camera, fluorescent filters for DAPI, FITC, Atto-647N and Atto565.

Image processing and analysis

The representative images were processed using FIJI software. The quantification is based on the segmentation of the cell (bright field) and nucleus (405 nm) to identify the cell compartment of the mRNA signal, each mRNA foci detection in two channels: detection at 641 nm, detection at 565 nm. A 3D Gaussian mask is used to identify the centroid for each detected dot and the distance between neighboring dots is estimated centroid to centroid. The mRNA quantification per foci is also estimated in this analysis using accumulative intensity 47. The analysis was validated comparing FAS1 mRNA detection levels utilizing 32 vs. 16 DNA probes, each conjugated to 3 fluorophores.

Figures:

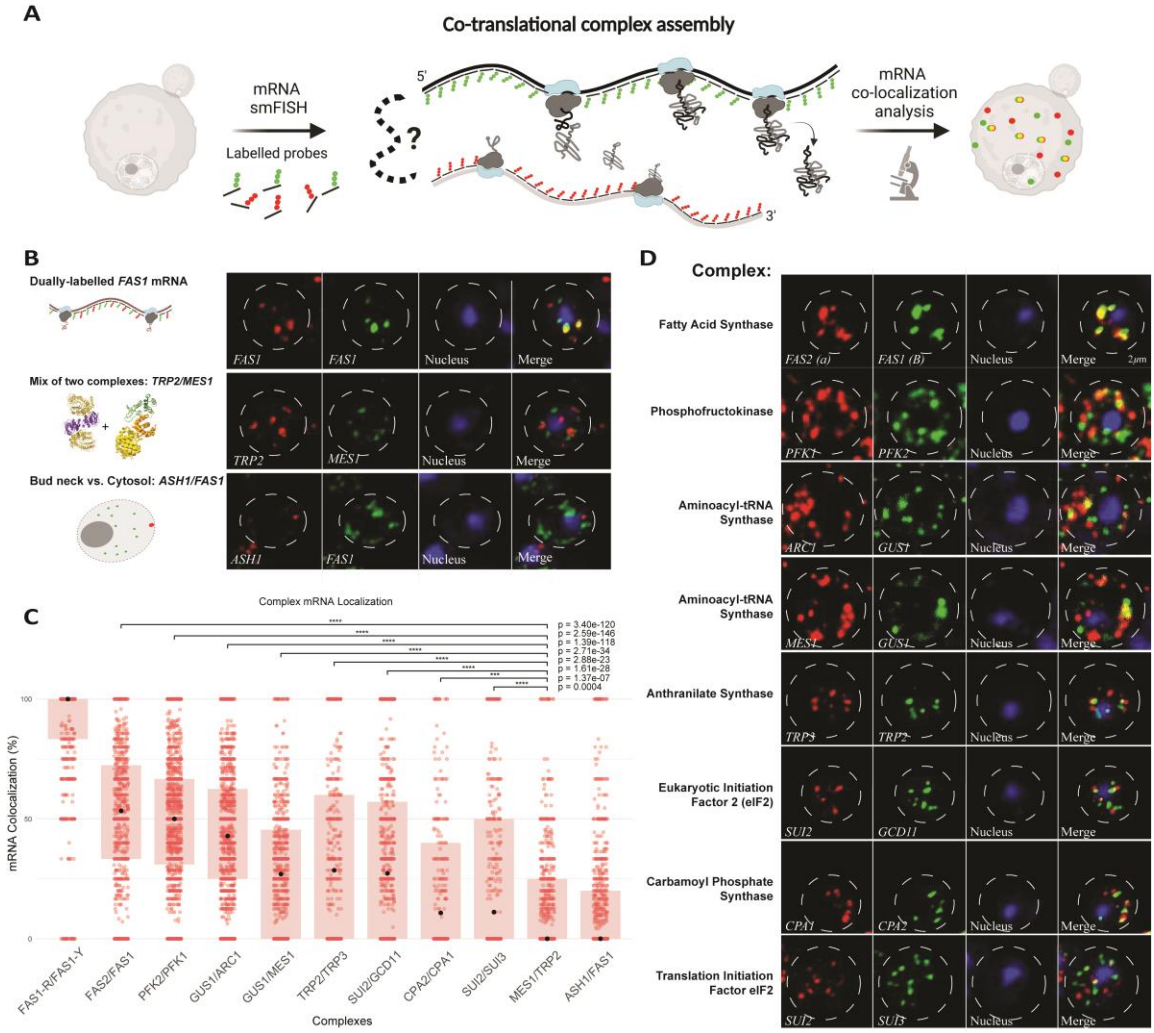


Figure 1. Colocalization of mRNAs encoding complex subunits in diverse pathways. A. Schematic workflow illustrating the smFISH methodology used to analyze the co-localization of mRNAs encoding subunits of a single protein complex: A schematic representation of the smFISH technique used to detect and analyze the co-localization of mRNAs encoding subunits of a single protein complex. **B.** Control Experiments for smFISH Detection. Z-stacked images showing smFISH detection of control conditions in wild-type yeast strains. Positive control: probes of different colors binding the same mRNA. Specificity controls: a mix of unrelated mRNAs (TRP2, FAS) and co-detection of ASH1 with FAS. Yeast cells were grown to log phase, fixed with formaldehyde, and hybridized with smFISH probes in two channels: channel one (red) and channel two (green). Nuclei were stained with Hoechst (blue) for visualization. Scale bar = 2 µm. **C.** Quantification of mRNA co-localization: Boxplot showing the proportion of co-localized smFISH-detected mRNAs per cell was analyzed. Co-localization was assessed pairwise for dually labeled mRNAs encoding subunits of a single protein complex, functionally unrelated mRNAs, and non-co-localized mRNAs. The box represents the interquartile range, with the median indicated by a point. Each data points. **D.** Z-stacked images showing smFISH detection of the indicated mRNAs in wild-type yeast strains. Yeast cells were grown to log phase, fixed with formaldehyde, and hybridized with smFISH probes for mRNA detection in two channels: channel one (red) and channel two (green). Nuclei were stained with Hoechst (blue) for visualization. Scale bar = 2 µm. **C.** represents an individual cell (n > 1036 cells per detection). At least two independent experiments were conducted. Statistical analysis revealed highly significant p-values compared to negative controls.

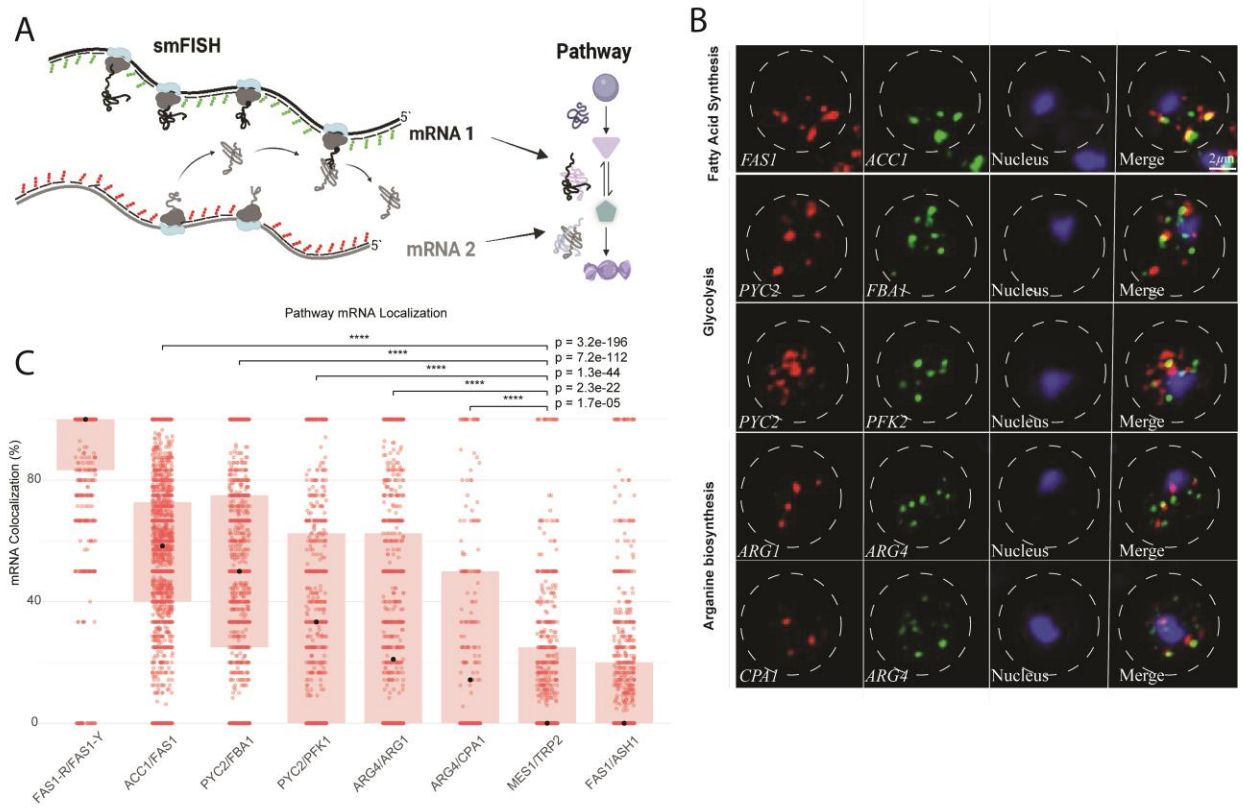


Figure 2. Colocalization of mRNAs encoding interacting proteins of biosynthetic pathways A. Schematic workflow illustrating the smFISH methodology used to analyze the co-localization of mRNAs encoding subunits of a single protein complex: A schematic representation of the smFISH technique used to detect and analyze the co-localization of mRNAs encoding proteins within a single biosynthetic pathway. **B.** Representative Z-stacked smFISH images of yeast cells: Wild-type yeast cells were grown to log phase, fixed with formaldehyde, and hybridized with smFISH probes targeting specific mRNAs. Co-staining was performed in two channels: channel one (red) and channel two (green). Nuclei were visualized using Hoechst staining (blue). Scale bar = 2 µm. **C.** Quantification of mRNA co-localization: Boxplot showing the proportion of co-localized smFISH-detected mRNAs per cell was analyzed. Co-localization was assessed pairwise for dually labeled mRNAs encoding interactors within a single biosynthetic pathway, functionally unrelated mRNAs, and non-co-localized mRNAs. Data are presented as a boxplot, where the box represents the interquartile range, and the black dot indicates the median. Each data point represents an individual cell (n > 778 cells per detection). At least two independent experiments were conducted. Statistical analysis revealed highly significant p-values compared to negative controls.

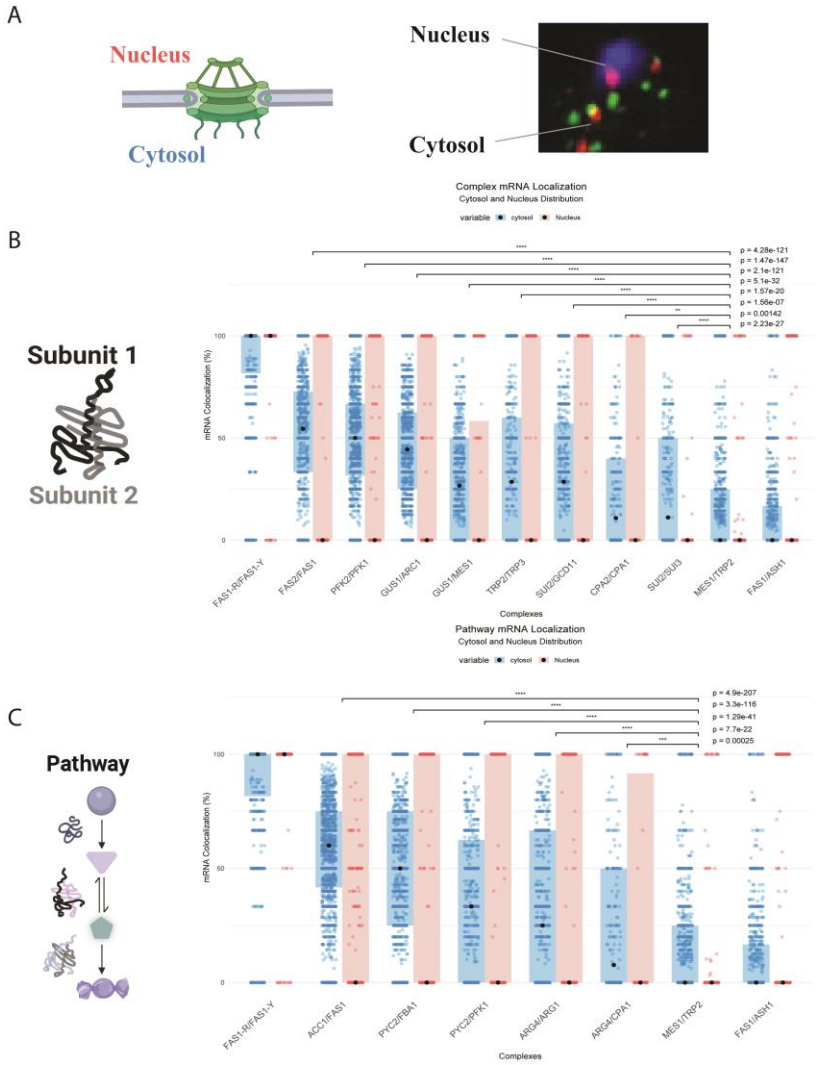


Figure 3: Colocalization of mRNAs encoding for co-translational interactors occurs in the cytoplasm. A. Schematic representation of mRNA localization in the cytoplasm (blue) versus nuclei (light pink): A diagram illustrating the spatial distribution of mRNAs within yeast cells, highlighting mRNA localization in the cytoplasm versus nuclei. **B.** Quantification of mRNA co-localization in the cytoplasm and nuclei for subunits of a single protein complex: Boxplot showing the proportion of co-localized mRNAs per cell detected using smFISH probes. Co-localization was analyzed for mRNAs encoding subunits of a single protein complex in the cytoplasm and nuclei. **C.** Quantification of mRNA co-localization in the cytoplasm and nuclei for biosynthetic pathway proteins: Boxplot depicting the proportion of co-localized mRNAs per cell for mRNAs encoding proteins within a single biosynthetic pathway. Detection was carried out separately in the cytoplasm and nuclei. Data are presented as boxplots, with the box representing the interquartile range and the black dot indicating the median. Each data point corresponds to an individual cell (n > 778 cells per detection). The results are based on at least two independent experiments, showing highly significant p-values compared to negative controls.

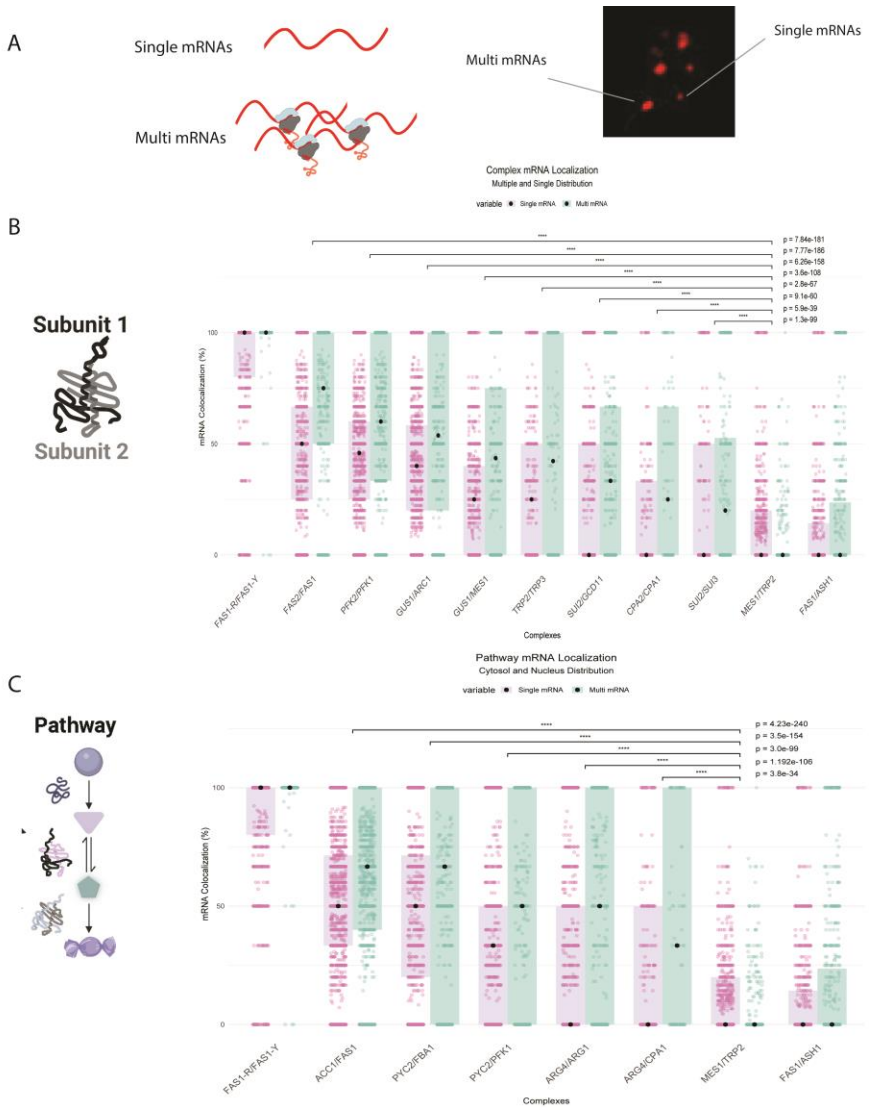


Figure 4. Co-translational interactors` mRNA clusters in cytoplasmic foci: A. Schematic representation of single (purple) and multi-mRNA (green) foci: Diagram illustrating the spatial organization of mRNAs within cytoplasmic foci. Single mRNA foci represent individual mRNAs localized as discrete spots, while multi-mRNA clusters consist of two or more mRNAs grouped together in a single focus. smFISH was performed on wild-type yeast cells, targeting the coding regions of specific mRNAs of interest. **B.** Quantification of mRNA clustering for subunits of a single protein complex: Boxplot showing the proportion of co-localized mRNAs per cell, categorized by the number of mRNAs per focus. Foci with fewer than two mRNAs (<2) were compared to multi-mRNA clusters containing two or more mRNAs (≥2). **C**. Quantification of mRNA clustering for proteins of a biosynthetic pathway: Analyzed similarly to panel B, showing the proportion of co-localized mRNAs per cell for mRNAs encoding proteins within a single biosynthetic pathway. Data are presented as boxplots, with the box representing the interquartile range and the black dot indicating the median. Each data point represents an individual cell. Results from at least two independent experiments indicate highly significant p-values compared to negative controls.

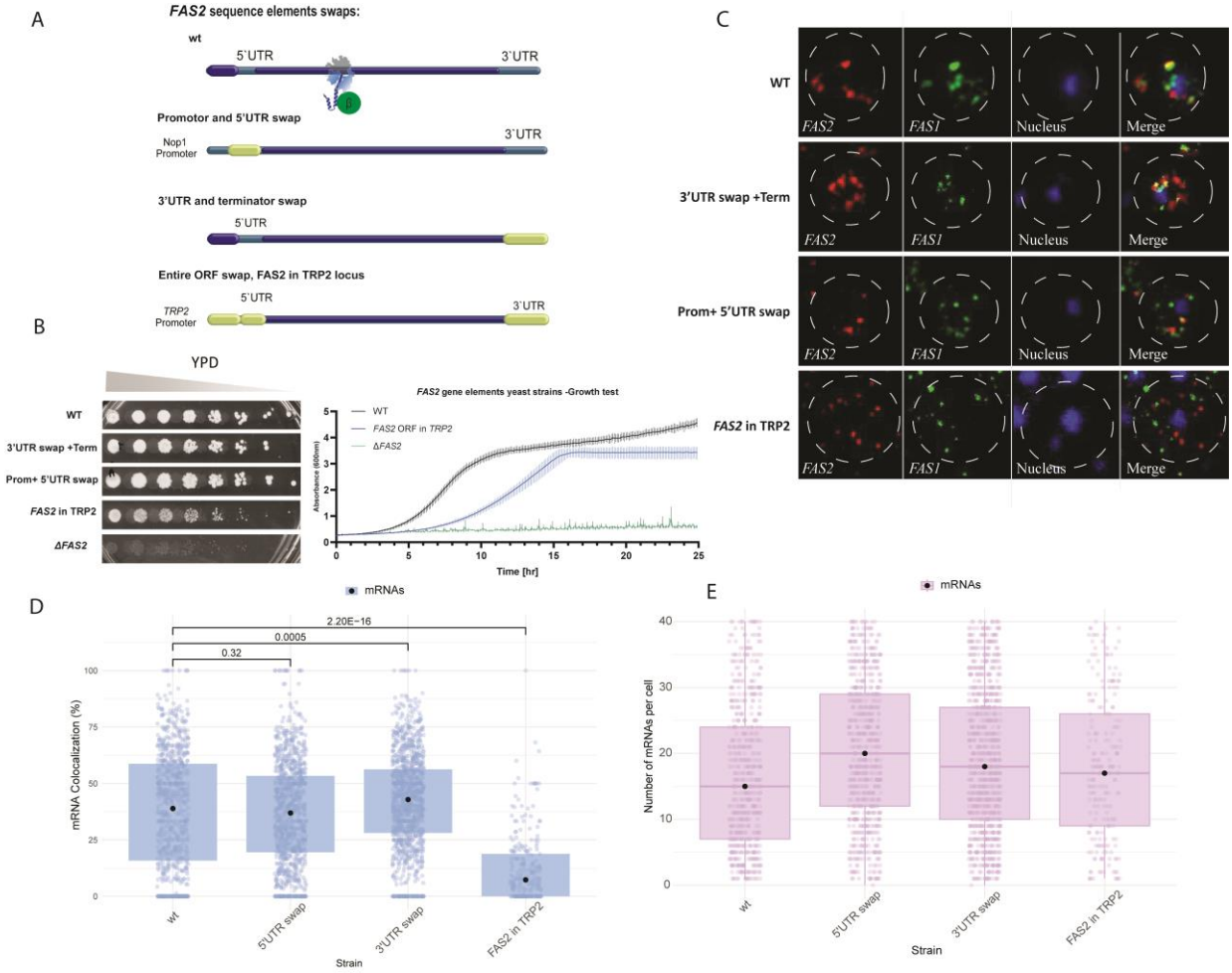


Figure 5. **ORF upstream and downstream sequence elements direct the localization of mRNAs encoding for subunits of the FAS complex. A.** Scheme of *FAS2* gene elements. **B.** Growth analysis of *FAS2* mutant strains. Left: A serial dilution of 2 x 10^{-1} order magnitude was performed from yeast cultures spotted on YPD without Fatty acids supplementation, followed by incubation at 30°C. Right: Growth kinetics analysis by OD_{600} measurements at 10min intervals, over 25hr. **C.** Representative Z-stacked images of smFISH performed the indicated *FAS2* mutant strains compared to wt. Yeast cells were grown to log phase, formaldehyde-fixed, and hybridized with smFISH probes for co-staining of mRNAs in channel one (in red) and channel two (in green). Nuclei (in blue) were visualized using Hoechst staining (Bar size: 2µm). **D.** The proportion of co-localized mRNAs detected per cell by smFISH. Co-localization was assessed in a *FAS2* and *FAS1* pairwise manner, as in figure 1. **E.** The number of *FAS2* mRNAs detected per cell in the indicated strains by smFISH. Box represents the interquartile range and point represents the median. Each data value represents a single cell, n > 700 cells per strain, at least 2 independent experiments.

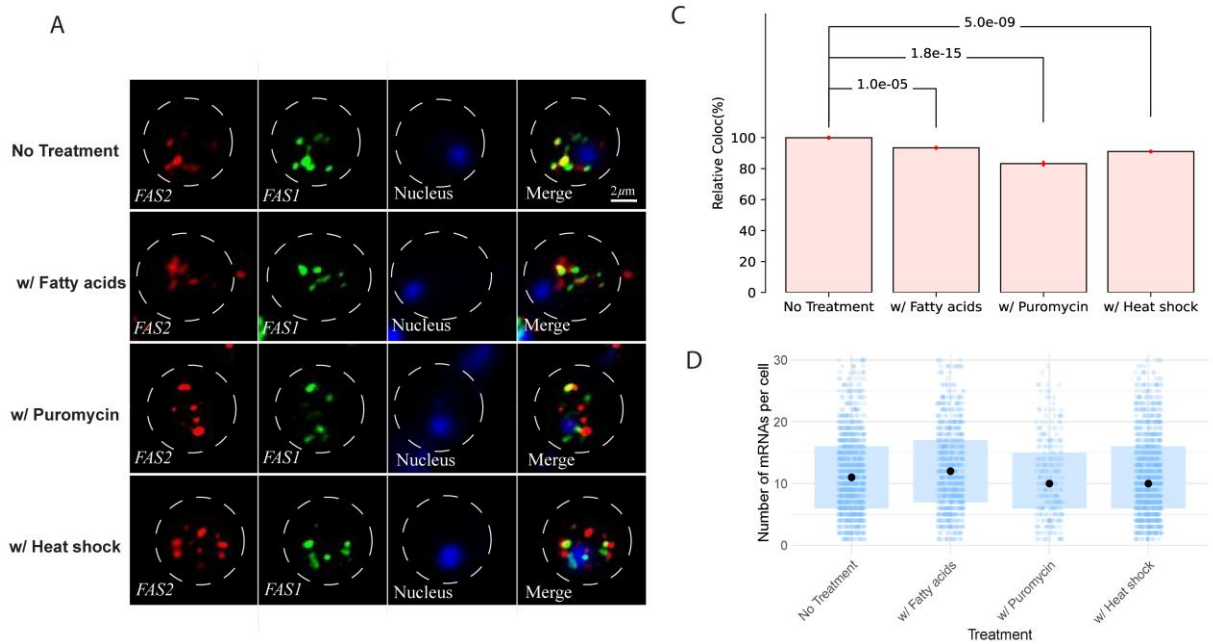


Figure 6. **Spatial Analysis of mRNA Co-localization Within Complexes Under Diverse Translation Inhibiting Treatments**. **A.** Bar plot showing the relative co-localization of the *FAS1*/*FAS2* complex under fatty acids, heat shock, and puromycin treatments. Red circles indicate standard deviation. **B.** Histogram displaying the cumulative frequency of detecting *FAS1* mRNA`s in proximity to *FAS2* mRNA, as a function of distance, under the indicated treatments. Needs elaboration. **C.** Representative Z-stacked images of smFISH performed on wt yeast strains following the indicated treatments. Yeast cells were grown to log phase, treated for 20min as indicated, then immediately fixed, and hybridized with smFISH probes for co-staining of mRNAs in channel one (in red) and channel two (in green). Nuclei (in blue) were visualized using Hoechst staining (Bar size: 2µm). **D**. Boxplot showing the number of mRNAs per cell for *FAS2* mRNAs. Box represents the interquartile range and point represents the median. Each data value represents a single cell, n > 5612 cells per strain, at least 2 independent experiments.

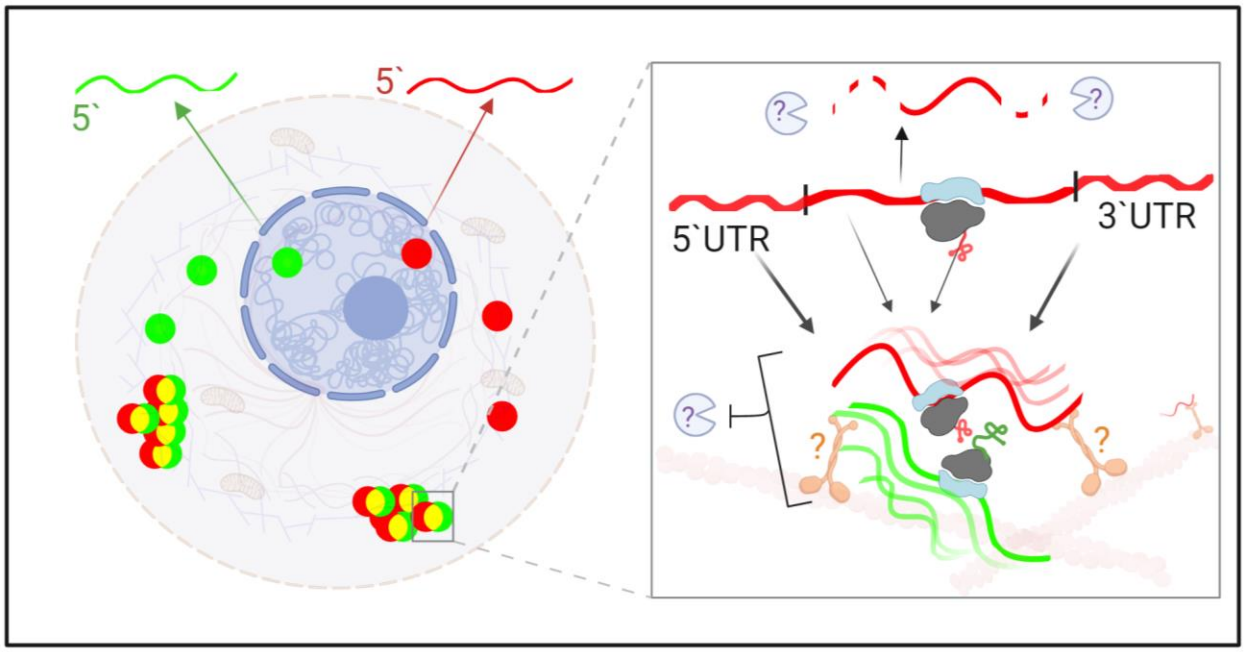


Figure 7: Suggested model of mRNA co-localization mechanisms in Saccharomyces cerevisiae. The left panel illustrates the distribution of mRNAs within the cytoplasm, highlighting the clustering of functionally related mRNAs (green and red) at specific cytoplasmic foci. The right panel provides a detailed view of the structural elements within the mRNAs, including the 5' and 3' untranslated regions (UTRs) that contribute to localization. Potential binding factors and molecular interactions are indicated, suggesting their roles in regulating mRNA translation and co-localization, which facilitate efficient protein complex assembly. The question marks indicate areas where further research is needed to clarify the mechanisms involved.

Supplementary information:

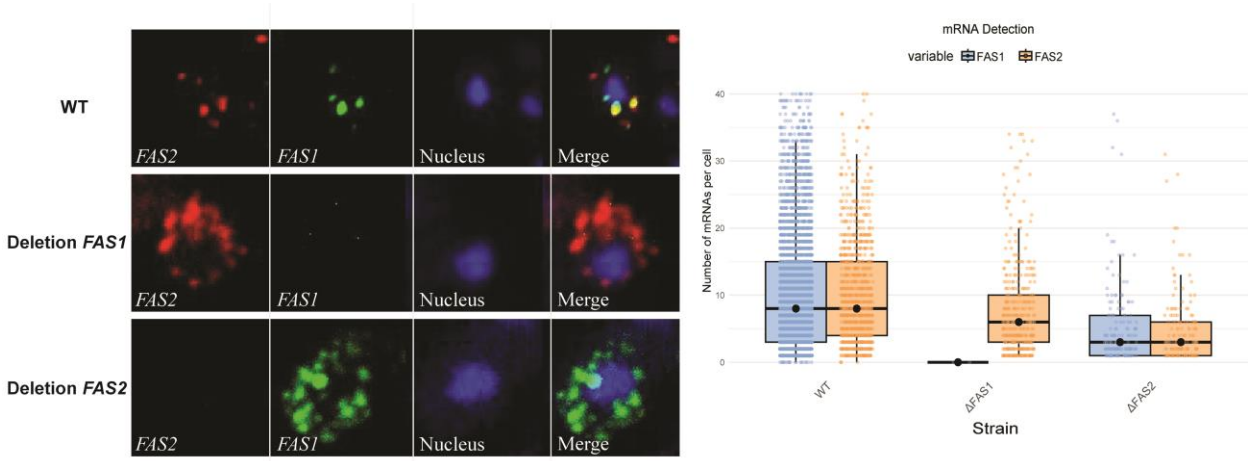
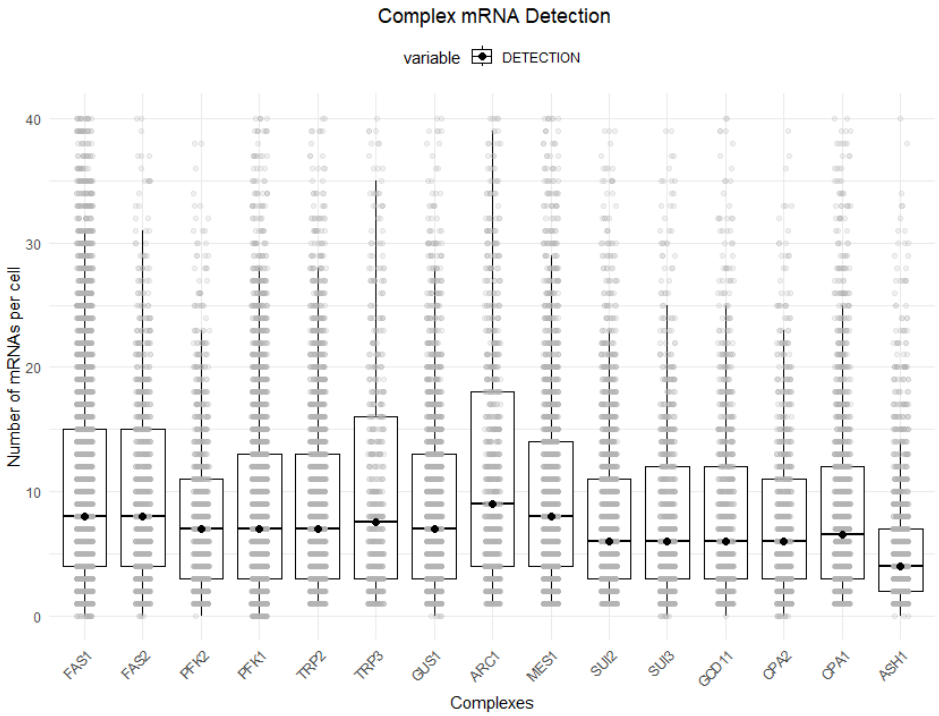


Figure1 Verification of smFISH probes specificity. **A**. Representative Z-stacked images of smFISH performed on wt as well as ΔFAS1, and ΔFAS2 yeast deletion strains for the detection of FAS1 and FAS2 mRNAs under physiological conditions. cells were grown to log phase, formaldehyde-fixed, and hybridized with smFISH probes for co-staining of mRNAs in channel one (red) and channel two (green). Nuclei (blue) were visualized using Hoechst staining. Bar size: 5 µm. FAS2 (red) and FAS1(green); mRNAs encoding for subunits of a single protein complex. **B**. Boxplot showing the number of mRNA molecules detected per cell. Each data value of the plot corresponds to a single cell. n > 390 cells per Single independent experiment.



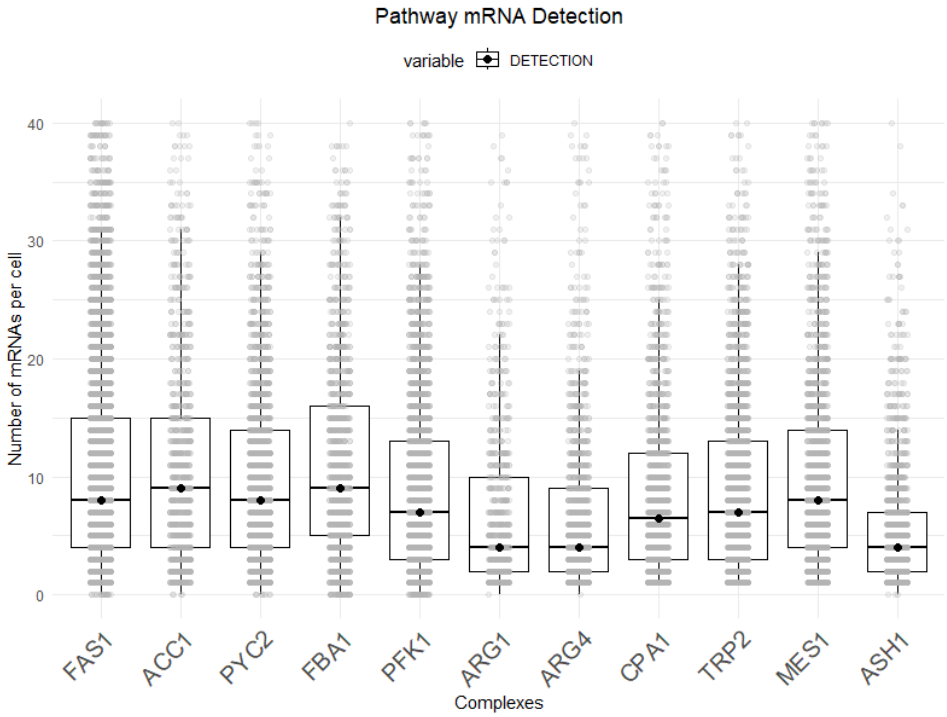


Figure 2. Number of mRNAs per cell detected using smFISH. **A**. Boxplot showing the number of mRNAs per cell for mRNAs encoding for subunits of a single protein complex. **B**. Boxplot showing the number of mRNAs per cell for mRNAs encoding for proteins of a single biosynthetic pathway. Box represents the interquartile range and point represents the median. Each data value represents a single cell, n > 1,319 cells per detection. Two independent experiments.

Table 1. Characteristics of complexes studied, which assemble in a co-translational manner:

Complex	Function	Protein Subunit	mRNA
Fatty Acid Synthase	Fatty acid synthesis	Fas β	*FAS1*
		Fas α	*FAS2*
Aminoacyl-tRNA synthetases complex (ARSs)	Translation	Glutamyl-tRNA synthetase (GluRS)	*GUS1*
		Arclp	*ARC1*
		Methionyl-tRNA synthetase (MetRS)	*MES1*
Phosphofructokinase	Glycolysis	Pfk β	*PFK2*
		Pfk α	*PFK1*
Translation Initiation Factor eIF2 subcomplex	Translation	eIF2 α	*SUI2*
		eIF2 γ	*GCD11*
Carbamoyl Phosphate Synthase A	Arginine biosynthesis	Cpa2p	*CPA2*
		Cpalp	*CPAl*
Anthranilate Synthase	Tryptophan biosynthesis	Trp2p	*TRP2*
		Trp3p	*TRP3*

Table 2. Characteristics of Studied metabolic pathways which transiently interact in a co-translational manner:

Metabolic Pathway	Protein	mRNA
Fatty Acid Synthesis	Fas β	*FAS 1*
	Acc1	*ACC1*
Arginine biosynthesis	Cpa2p	*CPA2*
	Arg4p	*ARG4*
	Arg1p	*ARG1*
Glycolysis	Pfkβ	*PFK2*
	Pyc2	*PYC2*
	Fba1	*FBA1*

Table 3. Chromosomal locations of genes of interest.

Gene	Chromosome
FAS2	16
FAS1	11
PFK2	13
PFK1	7
GUS1	7
ARC1	7
MES1	7
TRP2	5
TRP3	11
SUI2	10
SUI3	16
GCD11	5
CPA1	15
CPA2	10
ACC1	14
PYC2	2
FBA1	6
ARG1	15
ARG4	7
ASH1	11

Table 4. PCR Primers used in this study

Primer	**Sequence**
FAS2_F_in_trp2_L	AGGCCACAATCGATAATTAGCACTGATATTCTGATTGGAAAAAAGGCAAAAAATGAAGCCGGAAGTTGAGCAA
FAS2_R_in_trp2_L	ACTATAAAAATGTCGTCTAAGGGGAAAAAAACAGAGAATGCCCTTTCTATTTCTTAGTAGAAACGGCG
FAS2_94_F_only	ATGCTATTCGAAGGATGCCAAAGAGATTTATTATACCCCAGATCCATCCTAGGCAGGTCGACAACCCTTAAT
FAS2_94_R_only	GAAGCGACACGTTACATATTAAAAGAGGGACTACGTAGTGCTCTCTGCATAGGCCACTAGTGGATCTG
FAS2_94_F_in_trp2_L	AGGCCACAATCGATAATTAGCACTGATATTCTGATTGGAAAAAAGGCAAAAAGCCGCCAGCTGAAGCTTATG
FAS2_94_R_in_trp2_L	ACTATAAAAATGTCGTCTAAGGGGAAAAAAACAGAGAATGCCCTTTGCATAGGCCACTAGTGGATCTG

Table 5. Yeast strains used in this study

Strain	Genotypes	Mating type	Source
wt	his3Δ1, leu2Δ0, met15Δ0, ura3Δ0	a	EUROSCARF
FAS1Δ	his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, *FAS1Δ*:: His5	a	This study
FAS2Δ	his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, *FAS2Δ*:: His5	a	This study
SpNOP1pr-sfGFP-FAS2	his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hphΔn::URA3::SpNOP1pr-sfGFP-FAS2	a	SWAT LIBRARY
FAS2-GFP	his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FAS2-GFP:: hphNT1	a	Shiber et al., 2018
FAS2 in TRP2	his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, *FAS2Δ*:: His5, FAS2:: TRP2	a	This study
FAS2 MPT domain	his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FAS2 MPT domain (1-282nt)-GFP:: hphNT1	a	This study
FAS2 MPT domain in TRP2	his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, *FAS2Δ*:: His5, FAS2 MPT domain (1-282nt)-GFP:: TRP2	a	This study

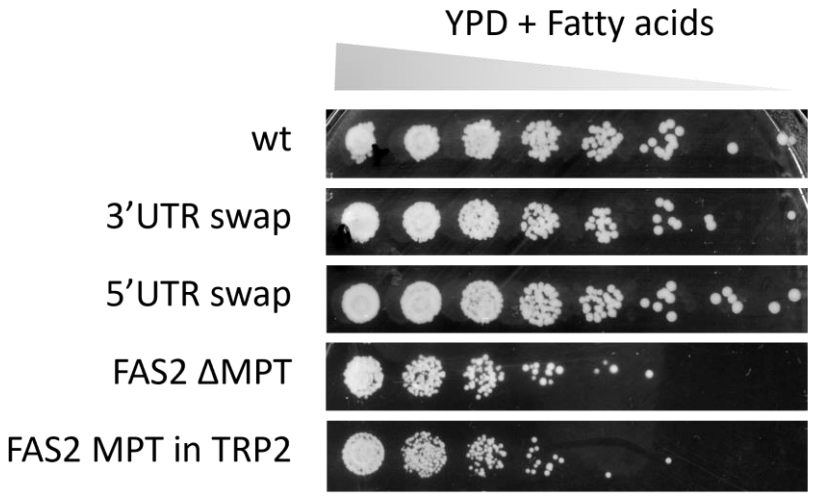


Figure 3. Growth analysis of FAS2 gene-modified yeast strains Fatty acids.

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