Cyclin CLB2 mRNA localization determines efficient protein synthesis to orchestrate bud growth and cell cycle progression

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Abstract

mRNA localization to subcellular compartments has been reported across all kingdoms of life and it is generally believed to promote asymmetric protein synthesis and localization. In striking contrast to previous observations, we show that in *S. cerevisiae* the B-type cyclin *CLB2* mRNA is localized and translated in the yeast bud, while the Clb2 protein, a key regulator of mitosis progression, is concentrated in the mother nucleus. Using single-molecule RNA imaging in fixed (smFISH) and living cells (MS2 system), we show that the *CLB2* mRNA is transported to the yeast bud by the She2-She3 complex, via an mRNA ZIP-code situated in the coding sequence. In *CLB2* mRNA localization mutants, Clb2 protein synthesis in the bud is decreased resulting in changes in cell cycle distribution and genetic instability. Altogether, we propose that *CLB2* mRNA localization acts as a sensor for bud development to couple cell growth and cell cycle progression, revealing a novel function for mRNA localization.

Introduction

Over the past decades, RNA imaging technologies revealed that hundreds of mRNAs localize to various subcellular compartments, from bacteria to multicellular eukaryotic organisms, suggesting that mRNA trafficking is a conserved and integral part of gene expression regulation^{1–7}. However, for many mRNAs, the physiological function of their localization remains uncertain.

Current studies suggest that the primary role of mRNA trafficking is to control asymmetric protein distribution to sustain local functions such as cell migration and polarity^{5,8}. Even in the single-cell organism *S. cerevisiae*, dozens of mRNAs localize to the endoplasmic reticulum, mitochondria, and the growing bud⁹. The best-characterized localized mRNA is *ASH1*, which is transported to the yeast bud on actin filaments by the She2-She3 complex and the type V myosin motor Myo4^{10–17}. The RNA binding proteins (RBP) Khd1 and Puf6 bind the *ASH1* mRNA and inhibit its translation until the bud-localized kinases Yck1 and CK2 phosphorylate Khd1 and Puf6 and thereby release the inhibition and allow local translation to occur^{18–23}. The Ash1 protein is subsequently asymmetrically segregated into the daughter nucleus, where it controls the mating-type switching program^{11,24,25}. An additional kinase-RBP pair, Cbk1-Ssd1, has been shown to localize to the bud²⁶ and tune the translation of specific mRNA targets^{27–29}. The coordination between these translation regulators remains unclear.

Besides *ASH1*, multiple mRNAs have been found to interact with the She2-She3-Myo4 complex³⁰. Among these mRNAs is *CLB2*, which encodes a conserved nuclear-localized B-type cyclin, interacting with and controlling the substrate specificity of the cyclin-dependent kinase Cdk1^{31–41}. Clb2-Cdk1 regulates entry and progression throughout mitosis in a threshold-dependent manner^{32,42–45}, by phosphorylating transcriptional and post-transcriptional regulators^{34,46,47}. This triggers a positive feedback loop leading to the transcription of the *CLB2* cluster^{36,48–50}, a set of 35 genes including *CLB2*, expressed during the G2/M phase transition⁵⁰. Furthermore, Cdk1-Clb2 controls spindle pole bodies elongation and in turn chromosome segregation and genome stability^{51,52}. Aberrant Clb2 expression -depletion or over-expression- results in abnormal mitotic

1 progression and cell size alteration^{31,32,34,52}. To achieve accurate periodic Clb2 expression, cells
2 combine cell-cycle-dependent mRNA synthesis^{53,54}, controlled mRNA decay⁵⁵, and proteasome-
3 dependent protein degradation^{56,57}. While the molecular events controlling *CLB2* transcription and
4 protein degradation are well characterized, as well as Clb2 function during cell cycle progression,
5 it remains unclear whether and how *CLB2* mRNA translation and Clb2 protein levels are
6 modulated in response to changes in cell growth that require adaptation of cell cycle progression.
7 To address this question, we combined single-molecule mRNA fluorescence *in situ* hybridization
8 (smFISH)^{58,59} and immunofluorescence (IF)^{60–64} to simultaneously detect *CLB2* mRNA and its
9 protein product in individual cells. Furthermore, to study dynamic gene expression changes in
10 intact living cells^{65,66}, we utilized the MS2 system (MBSV6) optimized to endogenously tag
11 unstable mRNAs in *S. cerevisiae*^{67–69}. Our work shows that *CLB2* mRNAs are efficiently localized
12 in the bud during the G2/M phase, while the Clb2 protein is localized to the mother nucleus. *CLB2*
13 mRNAs are transported to the bud by the She2-She3 complex recognizing a single ZIP-code in
14 the coding sequence necessary for localization. We find that the *CLB2* mRNA is preferentially
15 translated in the bud, and that this localized translation does not require translation inhibition by
16 Puf6, Khd1 or Ssd1 during transport. Consistent with these observations, lack of *CLB2* mRNA
17 localization results in reduced Clb2 protein synthesis, leading to cell cycle and growth defects.
18 Altogether, we propose that *CLB2* mRNA localization regulates protein synthesis and acts as a
19 cellular timer to couple bud growth and cell cycle progression.

Results

CLB2 mRNAs localize in the bud from S phase to Mitosis

To precisely quantify *CLB2* mRNA expression throughout the *S. cerevisiae* cell cycle, we combined smFISH and IF^{64,70,71}. To monitor cell cycle progression, nuclear localization of the transcription factor Whi5 was used to classify early G1 phase⁷², while G2 and mitotic cells were identified by staining tubulin (Tub1) and monitoring microtubules stretching between the mother and the daughter mitotic spindles⁷³ (**Fig. 1a**). *CLB2* smFISH revealed that mRNAs are detected from late S phase, when the bud emerges from the mother cell, until the end of anaphase. Quantification of *CLB2* mRNA spots showed that *CLB2* mRNAs are found in 60.7% of cells in an unsynchronized population (**Fig. 1Sa**). The expression peak occurred during G2 (average 10.2 ± 5.7 mRNAs/cell) when about 50% of the cells showed an active transcription site (**Fig. 1b-c**) with on average 2.9 ± 1.5 nascent mRNAs per transcription site, similar to previous studies⁵⁵(**Fig. 1Sb**). Furthermore, in expressing cells, the *CLB2* gene showed Poissonian transcription kinetics typical of constitutive genes^{74,75}, suggesting that this cell-cycle regulated gene is likely transcribed in a single activation event with a fixed initiation rate. From late S phase until anaphase, we observed that *CLB2* mRNAs localize to the bud from the first stages of bud formation. Throughout the budded phases, we measured up to 65.6% of mRNA in the bud, as compared to the distribution of the control mRNA *MDN1*, where only 17.2% of mRNAs are found in the bud (**Fig. 1b, d, 1Sc-d**). *CLB2* mRNA bud localization is independent of the *S. cerevisiae* background since we observed it both in BY4741, used throughout this study, as well as in the W303 background (**Fig. 1Se**).

CLB2 mRNAs efficiently localize in the bud of living S. cerevisiae cells

To investigate *CLB2* mRNA localization dynamics in living cells, we used an MS2 system optimized for yeast mRNA tagging (MS2 binding sites V6, MBSV6)^{67,68,76}. We inserted 24xMBSV6 in the 3' UTR of the endogenous *CLB2* locus (**Fig. 2Sa**). To confirm that mRNA tagging with

MBSV6 did not alter *CLB2* mRNA expression and degradation, unlike with previous MS2 variants^{67,77,78}, we performed two-color smFISH with probes targeting either the coding sequence (CDS) or the MBSV6 loops^{67,68}, to compare the expression of the endogenous and the tagged *CLB2* mRNA. This confirmed that MS2-tagged mRNAs are full-length and correctly localized in the bud (**Fig. 2Sb**). Furthermore, comparable mRNA levels were observed whether the mRNA was MS2-tagged, with or without GFP-tagged MS2 coat protein (MCP-GFP), which is used to detect mRNAs in living cells (**Fig. 2Sc-e**).

To monitor cell cycle progression and bud emergence in living cells, we endogenously tagged the bud neck protein Cdc10 with tdTomato in the *CLB2*-MS2-tagged strain (**Fig. 2a**). We performed time-lapse imaging every 2 minutes and measured *CLB2* mRNA expression throughout the cell cycle by acquiring z-stacks encompassing the cell volume (**Video 1**). To reduce perturbations in gene expression due to synchronization protocols^{79,80}, we quantified *CLB2* mRNA expression in unsynchronized cells, using the bud neck marker expression to compare cells. This revealed that up to 62.9% of *CLB2* mRNAs localized in the bud (**Fig. 2b-c**), consistent with the smFISH quantifications (**Fig. 1e**). Furthermore, mRNAs are degraded before the end of mitosis with a half-life of 3.8 ± 1.4 min, similar to previous measure performed for untagged *CLB2* mRNA⁵⁵, demonstrating that the MS2 system does not affect *CLB2* mRNA stability (**Fig. 2b-d; 2Sf**).

Interestingly, imaging of mother-daughter pairs for more than one cell cycle showed that the daughter cell initiated *CLB2* mRNA expression about 20 minutes after the mother (**Fig. 2d**). This observation is consistent with previous evidence showing that *S. cerevisiae* daughter cells are born significantly smaller than mothers and that cell size control occurring during G1 regulates the entry into the next cell cycle^{34,81,82}. High frame-rate imaging every 100 ms revealed that, as the bud grows, the number of *CLB2* mRNAs localized in the bud rapidly increases (**Fig 2e, Video 2**). Altogether, these results show that *CLB2* mRNAs are efficiently transported to the bud, consistent with previous measurements estimating directed mRNA transport velocity in eukaryotic cells to be about $1 \mu\text{m/s}$ ⁸³, suggesting that *CLB2* mRNAs reach the bud within seconds (**Fig 2e**).

The She2-She3 complex independently transport the CLB2 and ASH1 mRNAs to the bud

To elucidate the function of *CLB2* mRNA localization, we first investigated *CLB2* mRNA transport. We performed smFISH-IF throughout the cell cycle for the *CLB2* mRNA in *SHE2* or *SHE3* gene deletion strains to test whether the She2-She3 complex, required for *ASH1* mRNA transport^{10–17}, is also involved in *CLB2* mRNA localization. This revealed that in $\Delta she2$ and $\Delta she3$ strains, localization is strongly affected (**Fig. 3a, 3Sa**). We also observed that during mitosis, when the bud reaches its maximum size, only up to 24.5% and 23.6% of *CLB2* mRNAs are found in the bud of the $\Delta she2$ and $\Delta she3$ strains, respectively (**Fig. 3Sb-c**). Even though the *CLB2* and *ASH1* mRNAs are transported by the same complex, we do not observe co-transport, possibly because *CLB2* expression peak precedes *ASH1* occurrence during late anaphase (**Fig. 3Sd-g** and **Online Methods**). Furthermore, we observed that *CLB2* mRNAs are mostly single-molecules (**Fig. 3Sh**), suggesting that *CLB2* and *ASH1* mRNAs are independently localized to the bud by the She2-She3 complex.

The CLB2 mRNA has a conserved ZIP-code in the coding sequence

As the She2-She3 complex is required for *CLB2* mRNA localization, we hypothesized that the *CLB2* mRNA might possess a ZIP-code akin to the *ASH1* ZIP-code. Previous work defined the sequence and structure of the *ASH1* mRNA ZIP-code bound by She2^{13,84–86}. Based on sequence and structure similarity, a pattern search was performed to predict occurrences within the *CLB2* mRNA (see **Online Methods**). We identified one high-confidence site in the CDS at position 1111-1145 (**Fig. 3b-c**). To test the role of the predicted site, we generated a *CLB2* synonymized mutant whereby the CDS was mutagenized at nine bases to destroy the ZIP-code structure, while keeping the protein sequence and the codon optimization index unaltered (*ZIP mut*, **Fig. 3d**). A pattern search confirmed that the ZIP-code was destroyed upon synonymization. smFISH revealed that the *CLB2* mRNA bud localization was lost in the *CLB2* ZIP-code mutant (**Fig. 3e**). This was further confirmed by quantifying the *CLB2* mRNA bud-mother distribution (**Fig. 3f**),

thereby demonstrating that the ZIP-code in the CDS of the *CLB2* mRNA is sufficient to control bud mRNA localization, possibly by recruiting the She proteins. To further characterize *CLB2* mRNA localization, we quantified the mRNA peripheral distribution index (PDI) in budded cells using the RNA Distribution Index Calculator⁸⁷ (see **Online Methods**). The PDI measures the location of the mRNA in relation to the nucleus and it allows to compare the localization of multiple mRNA species. An index value equals 1 for diffusely distributed mRNAs or >1 if the mRNA has a polarized pattern^{87,88} (**Fig 3g**). This analysis revealed a PDI of 1.9 ± 0.42 for the *CLB2* mRNA, similar to the index value of the control mRNA *ASH1* (PDI = 2.2 ± 0.43) (**Fig 3h**). The PDI value was significantly reduced for *CLB2* in the $\Delta she2$ (PDI = 0.5 ± 0.2), $\Delta she3$ (PDI = 0.4 ± 0.13) and *CLB2* ZIP-code mutant strain (PDI = 0.5 ± 0.16) (ANOVA statistical test: $F(4, 185) = 15.74$, $p < 0.0001$), with PDI values similar to the non-localized mRNA *MDN1* (PDI = 0.6 ± 0.18) (**Fig. 3h, 1Sb**). Thus, the She2-3 complex is required to transport *CLB2* mRNAs to the bud via a conserved ZIP-code sequence.

Lack of CLB2 mRNA localization affects Clb2 protein expression

To elucidate whether *CLB2* mRNA localization influences its expression, we measured *CLB2* mRNA and protein levels in the localization mutants. Using smFISH, we found no significant difference in the number of mature or nascent RNAs in the $\Delta she2$, $\Delta she3$, or *CLB2* ZIP-code mutant strains compared to WT cells (**Fig. 4a-b**). Conversely, a western blot of the endogenously modified myc-tagged Clb2 protein showed that the protein expression in $\Delta she2$ or $\Delta she3$ and, even more, in the *CLB2* ZIP-code mutant was strongly reduced compared to WT cells (**Fig. 4c-d, 4Sa**). To test whether the decrease in protein expression was due to a change in protein degradation, we performed stability assays by treating WT and localization mutants with the translation inhibitor cycloheximide and measured the protein abundance over time (**Fig. 4e-f, 4Sb-c**). No significant difference was observed in the stability of the localization mutants

compared to WT cells, suggesting that *CLB2* mRNA localization controls Clb2 protein synthesis, rather than the stability of *CLB2* mRNA or protein.

Lack of CLB2 mRNA localization does not affect Clb2 protein localization

Next, we investigated whether mRNA localization affected Clb2 protein localization. To this end, *CLB2* was endogenously tagged with yeast optimized GFP (yeGFP⁸⁹) in WT and *CLB2* mRNA localization mutants. We observed that Clb2 is predominantly found in the nucleus in WT and mRNA localization mutants (**Fig. 4g**), as previously reported^{38,39,41}. Consistent with the western blot results (**Fig. 4c-d**), the fluorescence of the *CLB2* ZIP-code mutant was below detection level by live imaging (**Fig. 4g**). Furthermore, comparing Clb2 expression during a complete cell cycle in living WT and $\Delta she2$ cells (**Fig. 4Sd-e**), revealed that in contrast to WT cells, where a rapid Clb2 increase proportional to bud growth is observed, in $\Delta she2$ cells a slower protein accumulation was measured, accounting for the decrease in Clb2 expression. In addition, Clb2 was observed in the mother nucleus already during the G2 phase (**Fig. 4g**, top panels), when the mRNAs are already localized to the bud (**Fig. 1b, 2b, 3a**). Altogether, these results suggest that *CLB2* mRNA localization is not used to segregate the Clb2 protein in the daughter cell, as instead is observed for Ash1^{11,24,25}, but rather to control *CLB2* mRNA translation efficiency in the bud before the protein is imported back to the mother nucleus.

CLB2 mRNAs and protein co-localization suggests preferential translation in the bud.

To detect *CLB2* mRNAs and their site of translation in single cells, we generated a yeast strain where 25 Myc tags were inserted at the N-terminus of the *CLB2* endogenous gene (**Fig. 5Sa-b**). This amplification strategy increases the fluorescent signal of Clb2 proteins without affecting the strain growth (**Fig. 4Sa**). Next, we combined smFISH and IF to simultaneously detect *CLB2* mRNAs and proteins in fixed cells. In addition, IF against tubulin was used to score the cell cycle phases. This approach revealed that the bulk of Clb2 proteins accumulated in the mother (M) nucleus from G2 to mitosis (**Fig. 5a-b**), while the mRNA was preferentially found in the bud (B)

(**Fig. 5a, c**), suggesting that Clb2 proteins were efficiently imported back to the mother nucleus, as observed by live imaging (**Fig. 4g**), and as shown previously^{38–41}. Interestingly, from G2 to mitosis, Clb2 protein foci were also found in the bud in close proximity of *CLB2* mRNAs, suggesting that these foci may represent the site of mRNA translation (**Fig. 5a**, yellow arrowheads). Quantification of co-localized single mRNAs and protein foci within 250 nm distance (i.e. the resolution of our system), revealed that in WT cells, more mRNA-protein foci were found in the bud, while in the localization mutant $\Delta she2$, mRNA-protein foci are preferentially found in the mother cell where the bulk of mRNAs is localized (**Fig. 5d-e, Fig. 3a**). Furthermore, we found a reduction of the percentage of bud-localized mRNAs co-localized with protein foci in $\Delta she2$ cells compared to WT cells (**Fig. 5f**), suggesting that in localization mutants *CLB2* mRNA translation efficiency may be reduced. This analysis could not be performed with the *CLB2* ZIP-code mutant because the protein signal was too weak (**Fig. 4g**). It is interesting to note that even in WT cells, only about 25% of the bud-localized mRNAs are found in close proximity to protein foci (**Fig. 5f**), suggesting that *CLB2* mRNAs are poorly translated, as previously reported⁹⁰.

Finally, we quantified the accumulation of mRNA-protein foci in WT cells exposed for a short period of time to the translation elongation inhibitor cycloheximide (CHX, 20 minutes at 100 μ g/ml). This revealed that translation inhibition leads to an increase in *CLB2* mRNA levels accompanied by an accumulation of mRNAs in the mother cell (**Fig. 5Sc-e**). Furthermore, we observed a reduction of the Clb2 protein foci in the bud (**Fig. 5Sf**), compared to control conditions (**Fig. 5b**), consistent with a decrease in protein synthesis. Furthermore, the increased amount of *CLB2* mRNAs found in the mother upon CHX treatment suggested that mRNA translation may play a role in the asymmetric distribution of *CLB2* mRNAs in the bud, possibly by slowing down the diffusion kinetics of mRNAs bound to ribosomes, as previously shown in mammalian cells^{61,91,92}. To test this hypothesis, we simulated the distribution of *CLB2* mRNAs upon their localization in the bud of a G2 cell, with bud and mother volumes based on our measurements. We included measured *CLB2* mRNA decay rates and assumed an apparent mRNA diffusion

coefficient based on previously reported measurements performed in eukaryotic cells⁹¹ (see **Online Methods**). A fast coefficient of 0.4 $\mu\text{m}^2/\text{s}$ was previously measured for non-translated mRNAs, and a slower coefficient of 0.1 $\mu\text{m}^2/\text{s}$ was measured for translated mRNAs⁹¹. Interestingly, our model suggests that if we assume either a slow or a fast apparent mRNA diffusion coefficient of 0.1 $\mu\text{m}^2/\text{s}$ or 0.4 $\mu\text{m}^2/\text{s}$, respectively, we do not obtain the expected enrichment of the *CLB2* mRNA in the bud (**Fig. 5Sg-h**). To predict the accumulation of about 65% of the mRNA in the bud observed during the G2 phase, we need to include in the simulation the presence of a high-affinity anchoring factor promoting *CLB2* mRNA segregation in the bud (**Fig. 5Si**). Altogether, these data suggest that in WT cells, *CLB2* mRNAs are preferentially translated in the bud where the mRNA is actively transported and localized via an unknown anchoring mechanism, which may include the association with ribosomes and other yet unidentified factors. Furthermore, both in $\Delta she2$ cells and in translationally repressed cells (CHX), we observed an increase of protein foci in the mother cell (**Fig. 5d, 5Sf**), despite protein levels being decrease under these conditions (**Fig. 4c, 4e-f**), suggesting that translation in the mother cell may be less efficient, resulting in reduced Clb2 protein levels in the localization mutants.

Clb2 protein expression is not affected by the translation regulators Puf6, Ssd1 and Khd1

To investigate whether *CLB2* mRNAs are preferentially translated in the bud as a result of translation repression prior to localization, we tested if the RNA binding proteins Puf6^{18,19,23}, Khd1^{20–22} and Ssd1^{27–29}, previously shown to inhibit translation of bud-localized mRNAs, influenced Clb2 protein levels. We performed a western blot of Myc-Clb2 in strains lacking *SSD1*, *KHD1* or *PUF6* genes, to investigate whether an increase in Clb2 protein could be observed (**Fig. 6a**). This revealed no significant difference in protein levels between the WT and the mutant strains (ANOVA statistical test: $F(3, 8) = 0.7677$, $p = 0.3$; **Fig. 6b**). Furthermore, we analyzed by smFISH whether *CLB2* mRNA localization or abundance were affected in $\Delta ssd1$, $\Delta khd1$ or $\Delta puf6$ strains. This showed that the *CLB2* mRNAs are still localized in the bud, suggesting that these RBPs are not required for transport nor bud localization (**Fig. 6c**). Interestingly, we found a

significant increase of *CLB2* mRNA counts in the $\Delta puf6$ strain compared to WT (6.86 ± 1.23 and 4.27 ± 0.52 mRNA/cell, respectively, $p < 0.0001$) (**Fig. 6d, 6Sa**). This rise was not caused by an increased RNA synthesis, measured by the number of nascent RNAs per transcription site (**Fig. 6e**), but by a 25% increase in the number of cells with two active transcription sites (**Fig. 6Sb-c**). Since in WT haploid cells, we only observed one *CLB2* allele transcribed during the G2/M phase, when two copies of the gene are present, this indicated that Puf6 may regulate *CLB2* gene dosage by repressing the transcription of *CLB2* second allele. Altogether, these results suggest that *CLB2* mRNAs are not translationally repressed by factors controlling *ASH1* mRNA expression. While it is possible that other, yet unidentified factors exist, our data suggest the possibility that *CLB2* mRNA may be translated outside of the bud, albeit at a reduced rate.

CLB2 mRNA localization mutants display cell cycle progression defects

Our results so far indicated that the yeast bud promotes *CLB2* mRNA translation and a rapid increase of Clb2 protein levels proportional to bud growth (**Fig. 4c-d, 4Sb**). This suggested that *CLB2* mRNA localization may act as a cellular signal reporting on bud growth to the mother nucleus and coordinating cell growth and division. To test whether the decrease of Clb2 protein levels observed in *CLB2* mRNA localization mutants affected cell cycle progression and growth, we performed growth assays under different nutrient availabilities. In rich media (Synthetic complete medium with 2% glucose), the *CLB2* ZIP-code mutant clones did not show a growth defective phenotype. However, in the presence of a limiting carbon source (Synthetic complete medium with 0.1% glucose), it became apparent that the *CLB2* ZIP code mutant showed varying growth phenotypes with a marked clonal variability (**Fig. 7a**), suggesting incomplete phenotypic penetrance of the mutation. We proceeded with the analysis of three independently isolated clones (*ZIP cl1*, *ZIP cl2*, *ZIP cl3*).

We first tested by spot assay whether the localization mutants showed a growth defect at different temperatures (26°C, 30°C, 37°C) and growth limiting conditions set by the presence of different carbon sources (0.1% glucose or 2% glycerol/ethanol compared to 2% glucose). While the She

mutants grew like the WT strain, the ZIP-code mutants showed a clone-dependent growth defect, with the *ZIP c1* showing reduced growth in carbon-limiting conditions when compared to the other clones and WT cells (**Fig. 7b**). Dynamic growth measurements of cells transitioned from rich liquid media to either liquid media containing 0.1% glucose or 2% glycerol/ethanol (**Fig. 7c**), demonstrated a complete growth arrest for *ZIP c1*, while the other ZIP clones (**Fig. 7c**) and the She mutants (**Fig. 7Sa**) did not show reduced growth.

Finally, to evaluate the impact of the ZIP-code mutation on cell cycle progression, we performed a cell cycle distribution analysis by DNA staining with Sytox Orange coupled with flow cytometry (see **Online Methods**). Analysis of cells grown in rich media revealed a reduction of the G2/M population for *ZIP c2*, *ZIP c3*, but not *ZIP c1* compared to the WT cells (**Fig. 7d**), suggesting that in rich conditions, even when growth was not affected for the ZIP-code mutants, the distribution of cell through the cell cycle was altered. Furthermore, cell cycle analysis of cells grown for 20 hours in presence of 0.1% glucose or 2% glycerol/ethanol, revealed that all the ZIP-code clones behaved differently from WT cells, mostly displaying a G2/M phase delay, demonstrated by a relative increase in the number of cells in this phase (**Fig. 7e-f**). On the other hand, *ZIP c1* grown on 2% glycerol/ethanol displayed an arrest in G1 (**Fig. 7f**) and an increase in cell size (**Fig. 7Sb**), consistent with the growth defect observed both on agar plates and in liquid cultures (**Fig. 7b-c**). Thus, the ZIP-code mutants demonstrated complex clonal phenotypes, possibly triggered by critically low Clb2 protein levels. In most of the cases, the ZIP-code mutants behaved like *ZIP c2* and *c3*, and displayed altered cell cycle distribution but not a growth defect, possibly due to compensatory effects played by G1 growth checkpoints^{34,93}. However, at least three clones were independently isolated behaving like *ZIP c1*, suggesting that critically low levels of Clb2 may trigger the accumulation of secondary mutations and a loss of coordination between cell cycle and cell size control. To test this hypothesis, we investigated whether the *ZIP c1* could be rescued by the presence of a WT copy of the *CLB2* gene. To this end, we generated diploid

strains where the WT or the *ZIP cl1* mutant were crossed with a WT strain (**Fig. 7Sc**). Liquid growth in presence of 0.1% glucose or 2% glycerol/ethanol revealed a growth rescue for *ZIP cl1*. Altogether, our data suggest that in growth-limiting conditions, when bud growth is slowed down by the presence of suboptimal nutrients and in turn ribosomes and other key metabolites may be limiting in this compartment, *CLB2* mRNA localization and protein transport back to the nucleus may act as a biochemical signal adjusting the cell cycle in response to cell growth changes.

Discussion

Numerous instances in prokaryotic and eukaryotic organisms revealed that subcellular localization of mRNAs regulates the synthesis and asymmetric localization of proteins⁸. Here, we show that the B-type cyclin *CLB2* mRNA is efficiently localized to the yeast bud in a cell cycle-dependent manner, while the Clb2 protein is imported back to the mother nucleus, demonstrating the first example of mRNA and protein localization uncoupling. We characterized a new function for mRNA localization, which is to coordinate cell growth and cell cycle progression, possibly by sensing the bud translation capacity via the transport and local translation of the *CLB2* mRNA. We propose that by shuttling back to the mother nucleus, Clb2 signals to the mother cell when the bud is ready for mitosis, establishing a biochemical-based communication between distinct subcellular compartments.

Here, we combined single molecule RNA FISH-IF in fixed cells and a yeast-optimized MS2 tagging system for single RNA visualization in living cells to quantify, for the first time, the complete lifecycle of the *CLB2* mRNA and its protein product in intact cells. This approach revealed that *CLB2* mRNAs are transported to the bud as soon as this compartment is formed during late S phase (**Fig. 1,2,3**). Interestingly, previous reports showed that the B-type cyclin B1 is also asymmetrically localized in higher eukaryotes such as in the *Xenopus*⁹⁴ and *Zebrafish*^{95,96} oocytes as well as in *Drosophila* embryos¹, suggesting that the spatiotemporal regulation of Clb2 expression is a conserved mechanism.

The She2-She3 complex, previously shown to localize other mRNAs such as *ASH1*^{10–17}, *IST2*, *TCB2*, and *TCB3*^{12,30}, is also required to localize *CLB2* mRNAs to the bud (**Fig. 3**). Furthermore, by combining imaging and modelling, we suggested that the efficient asymmetric localization of *CLB2* mRNAs is likely produced by a combination of active transport and bud anchoring via yet unknown RBPs (**Fig. 5S**). To elucidate the function of *CLB2* mRNA localization we identified the cis-acting element required for transport. A single RNA ZIP-code in the mRNA CDS is sufficient to ensure localization in the bud (**Fig. 3**). This ZIP-code has a sequence and predicted structure similar to the *ASH1* ZIP-code, supporting the notion that cis-elements bound by the She2 RBP are conserved^{25,30,84}. Mutation of the ZIP-code RNA sequence caused an mRNA localization defect and correlated with a strong decrease in Clb2 protein expression (**Fig. 4**). Simultaneous visualization of the *CLB2* mRNA and proteins in fixed cells using the myc-tag reporter suggested that *CLB2* mRNAs are preferentially translated in the bud (**Fig. 5**). While this compartment promotes *CLB2* mRNA translation, we could not demonstrate that known mRNA translation inhibitors such as Puf6, Ssd1 and Khd1, prevent *CLB2* mRNA translation prior localization (**Fig. 6**). Unlike the Ash1 protein, the Clb2 protein is not restricted in the bud, possibly explaining why *CLB2* may not require translation inhibition. Yet unidentified translation inhibitors may also be involved in this process.

Interestingly, the *CLB2* ZIP-code mutant showed a further reduction in protein expression compared to the single SHE mutants, even though mRNA localization was equally impaired (**Fig. 4**). This suggested that the destruction of the ZIP-code may prevent not only the binding of the She proteins required for transport, but also the recruitment of translation factors that may travel together with the mRNA to the bud. Further investigations will be focused on identifying factors involved in controlling *CLB2* mRNA local translation.

We found that before the nucleus is divided during yeast closed mitosis, most of the Clb2 protein is efficiently imported back to the mother nucleus (**Fig. 4**), consistent with previous reports^{38–41}. The best characterized function of Clb2 is to trigger mitotic entry via the phosphorylation of key

targets controlling transcription of mitotic genes, including *CLB2* itself, and spindle pole bodies formation. In line with these observations, *CLB2* deletion mutants have been shown to be defective in DNA repair, genome stability and cell size control^{31,32,52}. Consistently, *CLB2* ZIP-code mutants display phenotypes including altered cell cycle distribution, the inability to grow under suboptimal nutrient conditions combined with changes in cell size. The strong clonal variability observed in our study also suggests that critical low levels of Clb2 may trigger genome instability and secondary mutations. Previous work showed that, when overexpressed^{38,39} or when nuclear import is blocked⁴¹, Clb2 can be found also at the bud neck. In our experimental settings, both in living and fixed cells and without overexpression, we did not observe Clb2 at this site. Nevertheless, we cannot exclude that a transient localization of Clb2 at the bud neck may have spatially-defined functions that may contribute to the observed phenotypes.

Altogether, our results suggest that *CLB2* mRNA localization in the bud regulates Clb2 protein synthesis to ensure coupling between cellular growth and cell cycle. An elegant mathematical model previously predicted a similar outcome, and suggested that *CLB2* mRNA localization may act as a bud sizer during the G2/M phase checkpoint⁹⁷. Even though we did not observe specific bud size defects in the *CLB2* localization mutants, future studies may elucidate the role of *CLB2* localization during yeast cell size and cell cycle coordination. Thanks to improved imaging technologies and the advent of spatial transcriptomic, thousands of localized mRNAs have been identified in many model organisms⁴. We predict that additional functions for mRNA localization will emerge, revealing the importance of this process in controlling the spatiotemporal synthesis of proteins, from single-cell to multicellular organisms.

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Online methods

Yeast strains construction

Yeast strains were constructed in the BY4741 background as detailed in⁶⁸. All strains where a gene of interest was tagged with MBSs in the 3'-UTR right after the STOP codon, were prepared as follow: PCR amplification of the MBS insert (see plasmids in Resource Table) followed by the kanamycin resistance gene, flanked by LoxP sequences, was performed with oligos (see Resource Table) containing homology sequences (70 nt) for the specific gene. For all strains, the Kanamycin resistance gene was removed by expressing the CRE recombinase under the control of the *GAL1* promoter (Resource Table, plasmids). Genomic DNA was extracted using standard techniques and PCR amplification of the 3'-UTR was loaded on a gel and sent for sequencing to verify the size and the sequence of the insert.

Plasmids construction

The synonymized *CLB2* region was synthesized as a DNA fragment by Genescript® with restriction endonuclease sites BglII and ClaI restriction sites. This fragment was used to replace the *CLB2* WT sequence in a plasmid encoding the *CLB2* promoter, CDS and UTRs. The promoter was preceded by the *URA3* marker flanked by LoxP sites, which were used to remove the marker upon integration into the yeast genome (pET531). 70-100 nucleotides *CLB2* homology sequences for insertion into the genome were cloned as well in the vector. Insertion of 5-Myc (synonymized multimer) or 25-Myc tags in the *CLB2* coding sequence was performed by restriction digestion into the BamHI site placed after the ATG codon.

Yeast cell cultures

All strains described are derived either by the *S. cerevisiae* background BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) or W303 (MATa; ura3-1; trp1Δ 2; leu2-3,112; his3-11,15; ade2-1; can1-100). Strains are listed in the Resource Table. Yeast cultures were exponentially grown in 6.7 g/L Yeast Nitrogen Base medium (YNB) with 2% glucose and the appropriate amino acids to

complement auxotrophies (either Synthetic Complete (SC), or Drop-Out (DO) media). Cells were grown at the indicated temperature using constant shaking at 210 rpm. For spot-test experiments (Fig. 7), cells were grown overnight in SC or DO media with 2% glucose (see figure legends). In the morning cells were diluted to an OD₆₀₀ of 0.8. Five ten-fold dilutions in water were prepared for each strains. 7 µL were spotted for each dilution on the indicated agar plates (20 g/L agar in the specific medium). For smFISH and live imaging, the details of the cell cultures are described in the Method sections below.

smFISH probes design

CLB2 probes were designed using the Stellaris™ Probe Designer by LGC Biosearch Technologies and purchased from Biosearch Technologies. *ASH1*, *MDN1* and *MBSV6* probes design was previously described in^{67,68}. Probes sequence and fluorophores are provided in the Resource Table.

Single molecule fluorescence in situ hybridization (smFISH)

Single-molecule FISH (smFISH) was performed as follows. Yeast strains were grown overnight at 26°C in synthetic medium with 2% glucose and containing the appropriate amino acids to complement the strain auxotrophies. In the morning, cells were diluted to OD₆₀₀ 0.1 and allowed to grow until OD₆₀₀ 0.3-0.4. Cells were fixed by adding paraformaldehyde (32% solution, EM grade; Electron Microscopy Science #15714) to a final concentration of 4% and gently shaken at room temperature for 45 minutes. Cells were then washed three times with buffer B (1.2 M sorbitol and 100 mM potassium phosphate buffer pH=7.5) and resuspended in 500 µL of spheroplast buffer (buffer B containing 20 mM VRC (Ribonucleoside–vanadyl complex NEB #S1402S), and 25 U of Lyticase enzyme (Sigma #L2524) per OD of cells (~10⁷ cells) for about 7-8 minutes at 30°C. Digested cells were washed once with buffer B and resuspended in 1 mL of buffer B. 150 µL of cells were seeded on 18 mm poly-L-lysine treated coverslips and incubated at 4°C for 30 minutes. Coverslips were washed once with buffer B, gently covered with ice-cold 70% ethanol

and stored at -20°C. For hybridization, coverslips were rehydrated by adding 2xSSC at room temperature twice for 5 minutes. Coverslips were pre-hybridized with a mix containing 10% formamide (ACROS organics #205821000)/2xSSC, at room temperature for 30 minutes. For each coverslip, the probe mix (to obtain a final concentration in the hybridization mix of 125 nM) was added to 5 µL of 10 mg/mL *E. coli* tRNA/ ssDNA (1:1) mix and dried with a speed-vac. The dried mix was resuspended in 25 µL of hybridization mix (10% formamide, 2×SSC, 1 mg/ml BSA, 10 mM VRC, 5 mM NaHPO₄ pH 7.5) and heated at 95°C for 3 minutes. Cells were then hybridized at 37°C for 3 hours in the dark. Upon hybridization, coverslips were washed twice with pre-hybridization mix for 30 minutes at 37°C, once with 0.1% Triton X-100 in 2xSSC for 10 minutes at room temperature, once with 1xSSC for 10 minutes at room temperature. Coverslips were mounted on glass slides using ProLong Gold antifade (4',6-diamidino-2-phenylindole) DAPI to counterstain the nuclei (Thermofisher).

smFISH-IF

smFISH-IF was performed as previously described in^{70,71}. In brief, smFISH-IF was performed in a similar way as smFISH, described above. After the last 1xPBS wash of the smFISH, IF was performed on the same coverslips. The smFISH was fixed in 4% PFA in PBS for 10 minutes at room temperature and then washed for 5 min at room temperature with 1x PBS. The coverslips were blocked with 1xPBS, 0.1% RNase-free Bovine Serum Albumin for 30 minutes at room temperature before being incubated with primary antibodies (Thermofisher, mouse anti-tubulin, 1:1000; Sigma mouse monoclonal anti-Myc clone 9E10, 1:1000; Covance, mouse monoclonal anti-HA, 1:1000) in 1xPBS, 0.1% RNase-free Bovine Serum Albumin for 45 minutes. After being washed with 1xPBS for 5 minutes at room temperature, the coverslips were incubated with the secondary antibody (goat anti-mouse Alexa 647 1:1500, or goat anti-mouse Alexa 488 1:1500) in 1xPBS, 0.1% RNase-free Bovine Serum Albumin for 45 minutes at room temperature. Next, the coverslips were washed with 1x PBS three times for 5 minutes to remove excess antibody.

Coverslips were dehydrated by dipping them into 100% ethanol and letting them dry before being mounted onto glass slides using ProLong Gold antifade mounting solution with DAPI.

smFISH/ smFISH-IF image acquisition and analysis

Images were acquired using an Olympus BX63 wide-field epi-fluorescence microscope with a 100X/1.35NA UPlanApo objective. Samples were visualized using an X-Cite 120 PC lamp (EXFO) and the ORCA-R2 Digital CCD camera (Hamamatsu). Image pixel size: XY, 64.5 nm. Metamorph software (Molecular Devices) was used for acquisition. Z-sections were acquired at 200 nm intervals over an optical range of 8.0 μ m. FISH images were analyzed using FISHQUANT⁹⁸. Briefly, after background subtraction, the FISH spots in the cytoplasm were fit to a three-dimensional (3D) Gaussian to determine the coordinates of the mRNAs. The intensity and width of the 3D Gaussian were thresholded to exclude nonspecific signal. The average intensity of all the mRNAs was used to determine the intensity of each transcription site.

Quantification of peripheral distribution index

The peripheral distribution index (PDI) was quantified as described in⁸⁷. Briefly, the Matlab-based software RDI (RNA dispersion index) calculator was used to calculate the peripheral distribution index for each cell by identifying cellular RNAs and describing their distribution in relation to the nucleus. Prior to analysis with the RDI calculator, the RNA channel was processed using a 3D Laplacian of Gaussian filter of radius=5 and standard deviation=1. The cell and nucleus channels were processed using the brightness/contrast function in ImageJ to enhance the contrast between the object and the background, as advised in⁸⁷.

Co-localization analysis

The RNA-RNA co-localization analysis reported in **Fig. 3S f-g** was performed using FISH-quant as described in⁹⁹. Briefly, FISH-quant performs the co-localization analysis in each cell separately by treating the assignment as a Linear Assignment Problem (LAP). The two spot detection results (x, y, z positions) are considered as 3D point clouds. The Hungarian algorithm solving the LAP

finds the best possible global assignment between these two points-clouds such that for each point in the first channel, the closest point in the second channel is found. LAP has the important property that assignment is exclusive; one point from the first channel can be linked to at most one point from the other channel, and conversely. The linking is also globally optimal because the sum of the squared distance is minimized. This analysis is implemented using the Matlab functions `hungarianlinker2` and `munkres3`.

For the co-localization of *CLB2* mRNA and protein foci, the FISH-quant data for the individual molecules were used as x, y, z coordinates and euclidean distances for all protein - mRNA molecule combinations were calculated in the mother and daughter cells. Protein and mRNA molecules closer than 250 nm were considered to be in a translation complex. Multiple protein molecules can be within 250 nm of a single mRNA molecule, and this would still be considered a single translation complex.

PDE solution for mRNA diffusion

We use a modified diffusion equation at steady state to model the mRNA movement in terms of diffusion of a concentration $c(x,y,z)$ in 3 spatial dimensions, and include binding to ribosomes (uniformly spread) leading to the formation of complexes $b(x,y,z)$:

$$0 = D \cdot \nabla^2 c(x, y, z) - k_d \cdot c(x, y, z) + k_p - k_{on} \cdot c(x, y, z) + k_{off} \cdot b(x, y, z)$$

$$0 = k_{on} \cdot c(x, y, z) - k_{off} \cdot b(x, y, z) - k_d \cdot b(x, y, z)$$

With decay constant $k_d = \text{Ln}(2)/t_{0.5} = \text{Ln}(2)/240 \text{ s}^{-1}$, $k_{on} = 0.25 (k_{off} + k_d) = 0.0035$ and $k_{off} = 1/90 = 0.011$ chosen to reflect a half-life of 240 s, a 90 s mean lifetime of ribosome-bound complexes, and that approximately 20% of mRNAs appear bound at steady state. In the high-binding scenario k_{on} was increase by a factor of 125. For the numerical implementation, the production constant is represented by a small non-zero spread around the bud centre using a smooth step-function of which the volume integral is normalised to 1:

$$k_p = \frac{k_{\max} \cdot e^{s \cdot m}}{e^{m \cdot r} + e^{s \cdot m}}$$

For the simulation results shown in Fig. 5S, the values chosen are $s = 5$ and $m = 10$, and the normalization results in $k_{\max} = 0.0019$. Although the exact value of this constant affects the absolute concentration of mRNA it does not affect the ratio of mother to bud RNA. The PDE is solved in three Cartesian coordinates using the FEM implementation in Wolfram Mathematica (Wolfram Research, Inc., Mathematica, Version 12.3.1, Champaign, IL).

Ellipsoid fitting to mother and bud DIC images

Differential interference contrast (DIC) images were analysed in Mathematica to fit 3D ellipsoids to mother and bud models. X- and y-axes length were measured for the cells and the short axis was used as estimation of the z-axis. The z-axis origin value was aligned with the z-stack images by maximising the Fish-Quant mRNA and protein point inclusions.

Pattern search to predict ZIP-codes and synonymization

To identify potential ZIP-codes in the *CLB2* mRNA, we performed a targeted pattern search (Seiler et al., manuscript in preparation). In the first step, we leniently screened for two nested pairs of inverted repeats with a minimal length of four nucleotides that framed an asymmetric bulge region as found in the E3 ZIP-code in the *ASH1* mRNA⁸⁴. We also checked for the presence of a CGA motif and a singular cytosine on the opposite strand with a defined distance of six nucleotides¹⁰⁰. The search was performed on the complete *CLB2* mRNA with 1476 nt of CDS (YPR119W; genomic coordinates: chromosome XVI, 771653-773128, +, genome version S288C; *Saccharomyces* Genome Database, <https://www.yeastgenome.org/locus/S000006323>). We further added 366 nt 3' UTR and 346 nt 5' UTR as previously determined by⁵⁵. In the second step, the minimum free energy (MFE) folds of all initial instances were analyzed using RNAfold with and without including a constraint on the nested pairs of inverted repeats^{101,102}. Fold prediction was performed at 28°C with 80 nt RNA sequence fragments centered on each instance. Instances

were only kept if (i) at least one of inverted repeat pairs was present in the MFE structure without constraints, and (ii) the free energy (ΔG) of the constraint structure did not differ by more than 20% from the MFE structure without constraint. The latter accounts for energetic benefits from interaction with the She2-She3 proteins. The pattern search predicted a single ZIP-code at nucleotide positions +1111 to +1145 of the CDS (genomic coordinates: chromosome XVI, 772763-772797, +). **Fig. 4c** displays the predicted fold with constraint using RNAfold of ZIP-code instance plus ± 5 nt flanking sequence. Visualization of the predicted structure in dot-bracket notation was generated using VARNA¹⁰³. Repeating the pattern search described above on the synonymized ZIP-code mutant did not retrieve any hits. The complete sequences of the synonymized ZIP-code mutant are provided in the Resource Table.

Sample preparation for live yeast fluorescence imaging

Yeast cells were grown at 26°C in synthetic selective medium. Exponentially growing cells (OD₆₀₀ 0.2-0.4) were plated on coated Delta-T dishes (Bioptech 04200417C). The dishes coating was done by incubating with Concanavalin A 1mg/ml (Cayman chemical company) for 10 minutes at room temperature. Excess liquid was aspirated and dishes were dried at room temperature. To activate Concanavalin A, dishes were incubated for 10 minutes at room temperature with a 50 mM CaCl₂ 50 mM MnCl₂ solution. Excess was removed and dishes dried at room temperature. Finally, dishes were washed once with ultrapure water (Invitrogen) and completely dried at room temperature. Cell attachment was performed by gravity for 20 minutes at room temperature, excess liquid removed and substitution with fresh media. Cells were diluted to OD₆₀₀ 0.1 and grown until OD₆₀₀ 0.3-0.4. before being plated on Concanavalin A coated dish.

Live cell fluorescence imaging and image analysis

The two-color simultaneous imaging of mRNAs and the appropriate cellular marker was performed on a modified version of the home-built microscope described in^{67,68}. Briefly, the microscope was built around an IX71 stand (Olympus). For excitation, a 491 nm laser

(CalypsoTM, Cobolt) and a 561 nm laser (JiveTM, Cobolt) were combined and controlled by an acoustic-optic tunable filter (AOTF, AOTFnC-400.650-TN, AA Opto-electronic) before coupled into a single mode optical fiber (Qioptiq). The output of the fiber was collimated and delivered through the back port of the microscope and reflected into an Olympus 150x 1.45 N.A. Oil immersion objective lens with a dichroic mirror (zt405/488/561rpc, 2mm substrate, Chroma). The tube lens (180 mm focal length) was removed from the microscope and placed outside of the right port. A triple band notch emission filter (zet405/488/561m) was used to filter the scattered laser light. A dichroic mirror (T560LPXR, 3mm substrate, Chroma) was used to split the fluorescence onto two precisely aligned EMCCDs (Andor iXon3, Model DU897) mounted on alignment stages (x, y, z, θ - and ϕ - angle). Emission filters FF03-525/50-25 and FF01-607/70-25 (Semrock) were placed in front of green and red channel cameras, respectively. The two cameras were triggered for exposure with a TTL pulse generated on a DAQ board (Measurement Computing). The microscope was equipped with a piezo stage (ASI) for fast z-stack and a Delta-T incubation system (Biotech) for live-cell imaging. The microscope (AOTF, DAQ, Stage and Cameras) was automated with the software Metamorph (Molecular Devices). For two-color live-cell imaging, yeast cells were streamed at 50 ms, Z plane was streamed, and z-stacks acquired every 0.5 μ m. Single-molecule analysis was done on maximal projected images using Fiji. Maximally projected images were filtered using the Maxican Hat filter (Radius=2) in Fiji. Spots were identified and counted using the spot detection plugin integrated in TrackMate. LoG detector was used for the spot identification, object diameter= 3 and Quality threshold = 2500. Files were exported as csv files and plotted using GraphPad Prism.

Deconvolution algorithm

To reduce imaging artifacts arising from noise and optics of the microscope, we used the Huygens software v3.6, where a Classic Maximum Likelihood Estimation (CMLE) algorithm was applied as a restoration method to deconvolve the images used for protein-mRNA foci co-localization (**Fig.**

5). CMLE assumes the photon noise to be governed by Poisson statistics and optimizes the likelihood of an estimate of an object in the input 3D image while taking the point spread function into consideration. The CMLE deconvolution method was chosen since it is suited for images with low signal-to-noise ratio and to restore point-like objects. The result is a more accurate identification of the location of the object, which in our case is the fluorescently labeled mRNA and protein molecules. The restoration parameters used with the CMLE deconvolution algorithm was 99 iterations, a quality stop criterion of 0.01, and a signal-to-noise ratio of 15.

CLB2 mRNA bud localization quantification in living cells

For the analysis reported in **Fig. 2e**, the ImageJ plugin Labkit (<https://imagej.net/Labkit>) was manually used to segment cells and RNAs. Segmented cells were used as input for training the deep learning program Stardist in 2 dimensions. Stardist was used to automatically detect and segment cells and single mRNAs from live imaging movie frames (Cell Detection with Star-convex Polygons, <https://arxiv.org/pdf/1806.03535.pdf>). Cell and RNA segmentation was imported into R using the RImageJROI package. In R, the cell size, number of mRNAs in the bud and the distance of each bud localized mRNA to the periphery was calculated and plotted over time using the R packages Spatial Data and PBSmapping¹⁰⁴. The Stardist segmentations were used to plot the RNAs and the cell's periphery onto the live imaging movie using the FFmpeg wrapper function for the FFmpeg multimedia framework (<https://ffmpeg.org/>).

Protein extraction and Western blot

Yeast strains were grown overnight at 26°C in yeast extract peptone dextrose (YEPD) medium with 2% glucose. In the morning, cells were diluted to OD₆₀₀ 0.1 and allowed to grow until OD₆₀₀ 0.5-1. Cell lysis was performed by adding 1 ml H₂O with 150 µl of Yex-lysis buffer (1.85 M NaOH, 7.5% 2-mercaptoethanol) to the pellet of 3-5 ODs of cells (~3x10⁷) and kept 10 minutes on ice. Proteins were precipitated by the addition of 150 µl of TCA 50% for 10 minutes on ice. Cells were pelleted and resuspended in 100 µl of 1X sample buffer (1 M Tris-HCl pH 6.8, 8 M Urea, 20%

SDS, 0.5 M EDTA, 1% 2-mercaptoethanol, 0.05% bromophenol blue). Total protein extracts were fractioned on SDS-PAGE and examined by Western blot with mouse anti-Myc (Sigma), mouse anti-Pgk1 (Thermofisher). For quantitative Western blot analyses, fluorescent secondary α -Mouse (IRDye 800CW) and α -Rabbit (IRDye 680RD) antibodies were used. The signals were revealed using the LYCOR[®] scanner and quantified using LITE[®] Software.

Growth curves setup and analysis

Cells were grown overnight at 30°C in SC-complete or Drop-out medium with 2% glucose. Cells in mid-log phase were spun down, the supernatant was removed and cells were resuspended at a final OD₆₀₀ of about 0.1 in test medium containing different carbon sources, as indicated in the figure legend. In 48-well plates with flat bottom, 400 μ l were plated per well. At least 3 well replicates were done per experiment. Cells were grown for the indicated time, at 30°C. OD₆₀₀ measurements were taken every 5 minutes, with 700 rpm shaking between time-points using a CLARIOstar[®] plate reader (BMG Labtech). Growth curves analysis was performed using an adaptation of the R package Growthcurver¹⁰⁵ and plotted using the R package ggplot2¹⁰⁶, tidyverse¹⁰⁷, RColorBrewer¹⁰⁸, dplyr¹⁰⁹. Growthcurver fits a basic form of the logistic equation to experimental growth curve data. The logistic equation gives the number of cells N_t at time t .

$$N_t = \frac{K}{1 + \left(\frac{K - N_0}{N_0}\right) e^{-rt}}$$

The population size at the beginning of the growth curve is given by N_0 . The maximum possible population size in a particular environment, or the carrying capacity, is given by K . The intrinsic growth rate of the population, r , is the growth rate that would occur if there were no restrictions imposed on total population size. Growthcurver finds the best values of K , r , and N_0 for the growth curve data using the implementation of the non-linear least-squares Levenberg-Marquardt algorithm. The carrying capacity and growth rate values (K and r) are used to compare the growth dynamics of strains.

Flow cytometry sample preparation and analysis

Cells were grown overnight at 30°C in SC-complete medium with 2% glucose. Cells were grown to mid-log phase (OD₆₀₀ 0.3-0.4) with constant shaking (220 pm) at 30°C. Next, they were spun down, the supernatant was removed and cells were resuspended at a final OD₆₀₀ of about 0.1 in test medium containing different carbon sources, as indicated in the figure legend. At the indicated time-points, 1 mL of culture was transferred to a 1.5 mL Eppendorf tube and centrifuged for 3 minutes at 3000 rpm. The supernatant was removed and cells were resuspended in 70% ethanol and incubated overnight at 4°C. Cells were washed once with 1xPBS pH 7.4 and resuspended in 500 µl of 1xPBS with 1 µl of RNase A 1 mg/mL and incubated at 37°C for at least 3 hours. Cells were then washed with 1 mL of 1xPBS and resuspended in 500 µl of 1xPBS. 100 µl of cells were then incubated with 3 µl of a 5 µM solution of Sytox Orange and incubated in a water bath at 37°C for 3 hours covered from the light. Cells were then washed 3 times with 1xPBS and resuspended in 500 µl of 1xPBS. The cells were then analyzed with the Beckman Coulter Flow cytometer CytoFLEX S System (B2-R0-V2-Y2). A 561 nm laser was used to excite the fluorescent dye and a band pass filter was used to filter the emitted fluorescence. 50'000 cells were collected per sample. Analysis and plotting was performed using R Studio and the following R packages: ggplot2¹⁰⁶; tidyverse¹⁰⁷, RColorBrewer¹⁰⁸, dplyr¹⁰⁹, mixtools¹¹⁰.

Quantifications and statistical analysis

FISH-quant was used to quantify single mRNA molecules and protein foci in fixed samples. Fiji was used to quantify single mRNA molecules in living cells. GraphPad Prism was used to calculate the mean and the standard deviation (SD) of all the data and perform statistical analysis. Flow cytometry data, growth curves analysis was performed in R Studio, as detailed in previous paragraphs. For each experiment, the number of biological replicates, the number of cells analyzed (n), statistical analysis applied and significance (P<0.05 for significant differences) is indicated in the figure legend or in the main text.

Figure Legends

Figure 1. CLB2 mRNAs localize to the bud in a cell-cycle dependent manner.

(a) Schematic of *CLB2* mRNA expression during the cell cycle. Green dots represent *CLB2* mRNAs. The Whi5 protein (cyan) accumulates in the nucleus during early G1 phase^{72,111,112}. Tubulin (magenta) is a major component of microtubules and the mitotic spindle. Bud emergence starts during S phase and ends with the formation of the daughter cell. During anaphase, the microtubules stretch between the mother and the daughter cell. **(b)** Top panels: MERGE Maximal projections of IF anti-HA (Whi5) (cyan) and DAPI (blue) merged to a single differential interference contrast (DIC) section (grey). Bottom panels: MERGE Maximal projections of *CLB2* mRNA smFISH (green), anti-tubulin IF (magenta) and DAPI (blue) merged to a single DIC section (grey). The corresponding cell cycle phase is indicated on the panels. Scale bars 3 μ m. **(c)** smFISH quantifications of *CLB2* mRNA expression during the different cell cycle phases determined using the markers shown in (b). Dots correspond to individual cells (2083 cells, from two replicates). The black bar indicates the average (G1 early= 1.2 ± 1.9 ; G1 late= 1.2 ± 1.9 ; S= 3.8 ± 3.0 ; G2= 10.3 ± 5.7 ; M= 8.0 ± 6.5 mRNAs/cell mean $\pm$ standard deviation [SD]). **(d)** Relative bud vs mother distribution of the *CLB2* mRNA in WT budded cells based on the smFISH-IF data shown in (b) (mean $\pm$ SD).

Supplementary Figure 1. CLB2 mRNAs are synthesized and localized in the bud in a cell-cycle dependent manner.

(a) Quantification of *CLB2* mRNA smFISH shown in Figure 1b reported as relative frequency distribution of mature mRNAs per cell. Data from two replicates (n=2083). **(b)** Quantification of *CLB2* nascent RNAs at transcriptions sites (TS) from smFISH shown in Figure 1b reported as relative frequency distribution of nascent RNAs per TS. Data from two replicates (n=2083). **(c)** MDN1 smFISH maximal projection (green), and DAPI merged to a single DIC section (grey). Scale bar 5 μ m **(d)** Relative bud vs mother distribution of *MDN1* mRNA in budded cells based on

the smFISH data shown in (b). Data from two replicates (n=2011; mean $\pm$ SD). **(e)** smFISH in the *S. cerevisiae* background W303. MERGE Maximal projections of *CLB2* mRNA smFISH (green) and DAPI (blue) merged to a single DIC section (grey). Scale bar 3 μ m.

Figure 2. CLB2 mRNA imaging in living cells reveals a rapid mRNA synthesis and degradation and localization to the bud.

(a) Schematic of *CLB2* localization during the cell cycle. The *CLB2* mRNA (green) is expressed from late S-phase and localizes to the emerging bud. Bud emergence as well as the separation of the bud from the mother can be identified by endogenously tagging the bud neck protein Cdc10 with tdTomato (magenta). The *CLB2* mRNA is rapidly degraded during mitosis. **(b)** *CLB2* endogenously tagged with 24xMBSV6 to enable visualization of the mRNA (black) in live cells. The bud neck protein Cdc10 is tagged with tdTomato (magenta). Cell A is the mother of cell B. Time point from start of acquisition is indicated in the upper left corner of each time frame. Scale bar 3 μ m. **(c)** Percentage of *CLB2* mRNAs localized in the bud over time from bud appearance. **(d)** Number of *CLB2* mRNAs per cell over time in cell A and cell B. **(e)** Snap shots from live cell imaging of *CLB2* mRNA endogenously tagged with 24xMBSV6 (green). Approximate cell outline identified from fluorescent background (orange). A single Z plane was acquired every 100 ms. Scale bars 3 μ m **(f)** Average cell area monitored over time in live cells (n=3). A single Z plane was acquired every 100 ms. Lower right insert shows cell size of a single cell acquired every 2 min. Red line in insert indicates when the 100 ms images were acquired. Pink, purple and green lines represent time points at which the snapshots in (e) were taken. **(g)** Average number of bud localized mRNAs per cell over time as monitored from 3 single cells. Pink, purple and green lines represent the time points at which the snapshots in (e) were taken.

Supplementary Figure 2. CLB2 mRNAs endogenously tagged with the MBSV6 reporter recapitulate the expression of the endogenous CLB2 mRNA.

(a) Schematic of *CLB2* locus endogenously tagged with 24xMBSV6 inserted in the 3' UTR after the STOP codon. Dotted lines represent smFISH probe positions targeting the CDS (green) or MBS sequences (magenta). **(b)** Two-color smFISH for cells expressing tagged *CLB2* mRNAs. Top panels (MBSV6), cells expressing the control vector (YcpLac111). Bottom panels (MBSV6+MCP) cells expressing MCP (YcpLac111 CYC1p-MCP-NLS-2xyeGFP). DIC/MERGE shows the overlap of the DAPI signal in the nucleus (blue), smFISH for the *CLB2* CDS (green) and the MBS (magenta) with the differential interference contrast (DIC) image. Scale bars 3 μ m. **(c)** Quantification of smFISH represented in 4b as well as untagged WT cells, with CDS probes (green plots) or MBS probes (magenta) reported as distribution of mature mRNAs per cell. Mean of three biological replicates, (*CLB2* MBSV6: *CLB2* probes n= 862, mean $\pm$ SD 4.9 $\pm$ 5.8 mRNA/cell; MBSV6 probes n=738, mean $\pm$ SD 5.1 $\pm$ 5.6 mRNA/cell; *CLB2* MBSV6+MCP: *CLB2* probes n = 752, mean $\pm$ SD 4.7 $\pm$ 5.3 mRNA/cell, MBSV6 probes n=974, mean $\pm$ SD 4.7 $\pm$ 4.9 mRNA/cell; WT cells: *CLB2* probes n = 791). **(d-e)** Correlation between the number of single CDS and MBSV6 molecules per cell in presence or absence of MCP. Pearson *r* values calculated by combining two independent experiments (n=1908 and 2284, respectively). **(f)** Average number of *CLB2* mRNA per cell tagged with 24xMBSV6 monitored over time. Grey represents SD from average mRNA per cell (purple).

Figure 3. The She2-3 complex and an RNA ZIP code in CLB2 mRNA CDS are required for bud localization.

(a) smFISH-IF in WT (top row), Δ *she2* (middle row), and Δ *she3* (bottom row) cells. MERGE Maximal projections of *CLB2* mRNA smFISH (green), anti-tubulin IF (magenta) and DAPI (blue) merged to a single DIC section (grey). The corresponding cell cycle phase is indicated on top of the panel. Scale bars 3 μ m. **(b)** Schematic of *CLB2* mRNA coding sequence. The blue box

represents the ZIP-code at nucleotides 1111-1145 (relative to START codon). **(c)** Predicted secondary structure of ZIP-code (blue box) with flanking sequence (nt 1089- 1168). The free energy (ΔG) of the mRNA folding is indicated. **(d)** Synonymized ZIP code (*ZIP-mut*). Top sequence is *CLB2* WT. Bottom sequence is the synonymized sequence. Mutated nucleotides are indicated in red. Below is the corresponding Clb2 protein amino acid sequence, which is identical for both the WT and synonymized strains. Rare amino acid codons were avoided to maintain the same codon optimization index = 0.74. **(e)** smFISH in WT and ZIP-code mutant strain. Maximal projections of smFISH with *CLB2* probes (green) and DAPI (blue) and fluorescence images overlapped to a single DIC section (MERGE). Scale bars 3 μ m. **(f)** Relative bud vs mother distribution of the *CLB2* mRNA in WT and ZIP-code mutant strain based on smFISH-IF data shown in (e). **(g)** Schematic representation of mRNA peripheral distribution index (PDI). Black dots represent mRNA. Blue is the nucleus. A PDI close to 0 indicates that the RNA of interest is localized near the nucleus. A PDI of 1 indicates that the RNA is diffusely dispersed throughout the cell. As the PDI value grows > 1, the polarization of the mRNA increases. **(h)** PDI in WT cells for *ASH1*, *CLB2* and *MDN1* mRNAs and in $\Delta she2$, $\Delta she3$ and ZIP-code mutant strains for the *CLB2* mRNA. Index values are calculated from smFISH-IF experiments shown in (e) and **Fig. 3Sa**.

Supplementary Figure 3. *CLB2* is not co-localized to the bud together with the *ASH1* mRNA.

(a) WT, $\Delta she2$ and $\Delta she3$ cells: Left panels, MERGE Maximal projections of *CLB2* mRNA smFISH (green), anti-tubulin IF (magenta) and DAPI (blue) merged to a single DIC section (grey). Right panels, MERGE Maximal projections of IF anti-HA (Whi5) (cyan) and DAPI (blue) merged to a single DIC section (grey). Scale bars 5 μ m. **(b)** Relative bud vs mother distribution of the *CLB2* mRNA in $\Delta she2$ budded cells based on the smFISH-IF data shown in **Fig.3a** and **Fig.3Sa**. **(c)** Relative bud vs mother distribution of the *CLB2* mRNA in $\Delta she3$ budded cells based on the smFISH-IF data shown in **Fig. 3a** and in **Fig. 3Sa**. **(d)** Schematic of *CLB2* and *ASH1* mRNAs

1 expression during the cell cycle. Green dots represent *CLB2* mRNAs. Magenta dots represent
2 *ASH1* mRNAs. Tubulin (grey) co-localizes with the spindle pole body which is duplicated during
3 S phase. The bud emergence starts during S phase and ends with the formation of the daughter
4 cell. During anaphase, the microtubules stretch between the mother and the daughter cell **(e)**
5 MERGE Maximal projections of *CLB2* mRNA smFISH (green), *ASH1* mRNA smFISH (magenta),
6 anti-tubulin IF (white) and DAPI (blue) merged to a single DIC section (grey). The corresponding
7 cell cycle phase is indicated on top of the panel. Scale bars 3 μ m. **(f)** Percentage of co-localized
8 *ASH1* and *CLB2* mRNAs (<250 nm) over total amount of *ASH1* and *CLB2* mRNA, from S phase
9 to mitosis (in budded cells co-expressing both mRNAs) (mean $\pm$ SD). We found 10.3% of *ASH1*
10 mRNA and 7.7% of *CLB2* mRNAs to co-localize, suggesting that *CLB2* and *ASH1* mRNAs are
11 not co-transported. **(g)** Example of 2-color smFISH colocalization analysis performed using FISH-
12 quant V3, FQ_DualColor_colocalization function⁹⁹. Colocalization between the two channels
13 was calculated as a linear assignment problem (LAP) solved with the Hungarian algorithm using
14 the Matlab function "hungarianlinker" and "munkres". We defined the maximum 3D allowed
15 distance between two spots to be considered co-localized to 250 nm (~3 pixels). Left, MERGE
16 maximal projection of filtered smFISH channels, *CLB2* (green) and *ASH1* (magenta). Right, color-
17 coded localization of mRNAs by smFISH: Blue, co-localized (co-loc); Red, not-co-localized
18 mRNAs; Yellow, not-co-localized in the other channel. **(h)** Previous structural work demonstrated
19 that the She2-She3 complex comprises two She2 and She3 dimers, which can bind two mRNA
20 targets simultaneously⁸⁴. Comparison of RNA spots intensities distributions by smFISH in WT or
21 Δ she2 and Δ she3 mutants was used to examine whether *CLB2* mRNAs are transported to the
22 bud two-by-two by. Two mRNAs in close proximity (<250 nm) would be detected as a single bright
23 spot due to the microscope diffraction limit. A drop in *CLB2* mRNA spot intensities in the She
24 mutants would indicate co-transported mRNAs. Relative frequency distribution of *CLB2* mRNA
25 spot intensities in WT (n= 3004), Δ she2 (n= 2619) and Δ she3 (n= 2731) mutant strains (median
26 WT = 45.7, lower 95% CI=45.0, upper 95% CI=46.8; Δ she2=40.4, lower 95% CI=39.1, upper 95%

CI=41.2; $\Delta she3$ =42.65, lower 95% CI=41.8, upper 95% CI=43.3). Results are shown for a representative experiment where the three strains were collected, processed and imaged side by side. A 3D Laplacian of Gaussian filter was applied to all the images using FISH-quant. The mean and SD of spot intensities are expressed as arbitrary units (a.u.).

Figure 4. CLB2 mRNA mislocalization affects Clb2 protein expression but not mRNA levels, protein stability or localization.

(a) smFISH quantifications of *CLB2* mRNA expression in WT and localization mutants. Dots correspond to individual cells (~1000 cells per replicate, from at least 2 replicates). The black bar indicates the average (mRNAs/cell mean $\pm$ SD). **(b)** Quantification of nascent *CLB2* RNAs at transcription sites (TS) from smFISH. Dots correspond to individual cells (~1000 cells per replicate, from at least 2 replicates). Visualization as in (a). **(c)** Western blot analysis using anti-myc antibody against Clb2 protein endogenously tagged with 5 myc tags in WT, $\Delta she2$, $\Delta she3$ and ZIP-code mutant cells. First lane is the control untagged strain. Endogenous Pgk1 was used as loading control **(d)** Quantification of western blot in (c). Myc signal normalized to Pgk1 loading control. Protein levels relative to WT indicated in lower bar. Mean $\pm$ SD from 3-7 replicates. **(e)** Clb2 protein stability assay in WT, $\Delta she2$ and $\Delta she3$ cells. Western blot was performed using an anti-myc antibody to target Clb2 protein tagged with 5 myc tags in cells treated with 100 μ g/ml cycloheximide for 0, 5, 10, 20, 30 45, or 60 minutes. Pgk1 was used as loading control. **(f)** Clb2 protein stability assay in WT and ZIP-code mutant. Western blot was performed in the same way as in (c). **(g)** Clb2 fused to yeGFP in WT, $\Delta she2$, $\Delta she3$ and ZIP code mutant cells. Maximal projections of Clb2-GFP (green) overlapped to a single DIC section. Scale bars 2 μ m.

Supplementary Figure 4. CLB2 mRNA mislocalization is not affecting protein stability but it correlates with delayed Clb2 protein accumulation.

(a) Growth curves of strains with Myc-tagged Clb2 protein performed in SC-complete 2% glucose at 26°C. Growth rates (r), calculated by fitting the data to a logistic curve, are provided in the

associate table. **(b)** Example of western blot of Clb2 protein stability assay in WT, Δ she2 and Δ she3 cells. Western blot was performed using an anti-myc antibody to target Myc-tagged Clb2 protein tagged in cells treated with 100 μ g/ml cycloheximide for 0, 5, 10, 20, 30 45, or 60 minutes. Pgk1 was used as loading control. Quantifications are reported in **Fig. 4e**. **(c)** Example of western blot of Clb2 protein stability assay in WT and ZIP-code mutant cells. Western blot was performed using an anti-myc antibody to target Clb2 protein tagged with 5 myc tags in cells treated with 100 μ g/ml cycloheximide for 0, 5, 10, 20, 30 45, or 60 minutes. Pgk1 was used as loading control. Quantifications are reported in **Fig. 4f**. **(d)** Quantification of Clb2-GFP expression measured in living WT and Δ she2 cells. Cells were grown in SC-complete 2% glucose at 26°C. Images were collected every 5 minutes, 13 Z planes were acquired every 0.5 μ m, with an exposure time of 50 ms. Maximally projected images were used for cell segmentation and quantification of GFP integrated intensity (expressed as arbitrary units a.u.). **(e)** Correlation of Clb2-GFP expression and bud volume measured in living WT and Δ she2 cells.

Figure 5. CLB2 mRNA and protein colocalization indicates preferential translation in the bud.

(a) smFISH-IF in WT cells. Top panels: MERGE Maximal projections of IF anti-tubulin (cyan) and DAPI (blue) merged to a single DIC section (grey). Second panel row from the top: MERGE Maximal projections of IF anti-myc-Clb2 protein (magenta), *CLB2* mRNA smFISH (green) and DAPI (blue) merged to a single DIC section (grey). The third and fourth panels from the top are the *CLB2* mRNA smFISH and Clb2 protein IF, respectively. Scale bars 3 μ m. **(b)** Quantification of protein clusters in WT bud (B) and mother (M) cells from IF experiments shown in (a). Cell cycle classification was performed using tubulin and DAPI as markers. **(c)** Quantification of mRNA in WT bud (B) and mother (M) cells from smFISH experiments shown in (a). Cell cycle classification was performed as in (b). **(d)** smFISH-IF in Δ she2 cells. Maximal projections of IF anti-myc-Clb2 protein (magenta), *CLB2* mRNA smFISH (green) and DAPI (blue) merged to a single DIC section (grey). Panels description as in (a). Scale bars 2 μ m. **(e)** Quantification of *CLB2*

1 mRNA-protein clusters found in close proximity (<250 nm) by smFISH-IF experiments performed
2 in bud (B) and mother (M) cells of WT and Δ she2 cells shown in panels (a) and (d). **(f)**
3 Quantification of bud-localized mRNAs co-localizing with a protein cluster (<250 nm) in WT and
4 Δ she2 cells shown in panels (a) and (d).

5 *Supplementary Figure 5. Translation inhibition with cycloheximide causes CLB2 mRNA*
6 *stabilization and correlates with reduced mRNA localization in the bud*

7 **(a)** Schematic of the *CLB2* endogenous gene tagging to simultaneously visualize single mRNA
8 by smFISH and Clb2 proteins by IF in fixed cells. 25 myc tags are inserted into the 5' end of the
9 *CLB2* CDS. smFISH is performed against the *CLB2* CDS (top panel). As the mRNA is being
10 translated the myc tags will be first to emerge from the ribosome and will be detectable with IF
11 (bottom panel). By performing smFISH against the *CLB2* mRNA and IF against the 25xmyc-
12 tagged Clb2 protein, translation can be detected by the co-localization of the two signals. **(b)**
13 Western blot analysis of CLB2 Myc tagged strains. Top row is the myc signal. Bottom row is the
14 Pkg1 loading control. First lane is untagged cells. Second and third lanes are the Clb2 protein
15 tagged with 5 and 25 myc tags, respectively. **(c)** smFISH-IF in WT cells treated with 100 μ g/mL
16 of translation elongation inhibitor cycloheximide (CHX) for 20 min. Top panels: MERGE Maximal
17 projections of IF anti-tubulin (cyan) and DAPI (blue) merged to a single DIC section (grey). Second
18 panel row from the top: MERGE Maximal projections of IF anti-myc-Clb2 protein (magenta), *CLB2*
19 mRNA smFISH (green) and DAPI (blue) merged to a single DIC section (grey). The third and
20 fourth panels from the top are the *CLB2* mRNA smFISH and Clb2 protein IF, respectively. Scale
21 bars 3 μ m. **(d)** smFISH quantifications of *CLB2* mRNA expression of cells exposed to CHX shown
22 in (b). Dots correspond to individual cells (46 cells, from two replicates). **(e)** smFISH
23 quantifications of *CLB2* mRNA expression in bud (B) and mother (M) cells exposed to CHX shown
24 in (b). Dots correspond to individual cells (46 cells, from two replicates). **(f)** Quantification of *CLB2*
25 mRNA-protein clusters found in close proximity (<250 nm) by smFISH-IF experiments performed

in buds (B) and mothers (M) of WT cell exposed to CHX shown in (b). **(f)** PDE simulation results of ribosome binding scenarios using concentration distribution at steady state for normal binding at diffusion $D = 400'000 \text{ nm}^2/\text{s}$; **(g)** at $D = 100'000 \text{ nm}^2/\text{s}$. **(h)** Concentration distribution at steady state for high binding and $D = 100'000 \text{ nm}^2/\text{s}$. Units: length nm, time s, concentration $1/\text{nm}^3$. Note that the colors have been log-scaled.

Figure 6. Known translation inhibitors are not involved in regulating Clb2 protein expression

(a) Western blot analysis using anti-myc antibody against Clb2 protein tagged with 25 myc tags in WT, Δssd1 , Δkhd1 and Δpuf6 cells. Pgk1 was used as loading control. First lane is untagged cells. **(b)** Quantification of (a). Mutant strain signal is normalized to WT signal. Each color corresponds to one replicate experiment. Black bar indicates mean $\pm$ SD (WT= 100; Δssd1 = 88.3 ± 15.6 ; Δkhd1 = 98.8 ± 13.5 ; Δpuf6 = 83.9 ± 10.4). **(c)** Merge maximal projections of *CLB2* smFISH (green), DIC (gray), and DAPI (blue) in WT, Δssd1 , Δkhd1 and Δpuf6 cells. Scale bars 5 μm . **(d)** Quantification of *CLB2* smFISH in WT, Δssd1 , Δkhd1 and Δpuf6 cells. Each dot corresponds to a single cell (500-1500 cells from two to four replicates). Each color corresponds to one replicate. Black bar indicates the average (WT= 4.3 ± 0.5 ; Δssd1 = 4.8 ± 0.8 ; Δkhd1 = 4.2 ± 0.7 ; Δpuf6 = 6.9 ± 1.2 mRNAs/cell mean $\pm$ SD). **(e)** smFISH quantification of nascent *CLB2* RNA expression per transcription site in WT, Δssd1 , Δkhd1 and Δpuf6 cells. Dots correspond to individual cells (500-1500 cells from two to four replicates). Each color corresponds to one replicate. Black bar indicates the average (WT= 2.4 ± 1.1 ; Δssd1 = 2.6 ± 1.0 ; Δkhd1 = 2.1 ± 0.7 ; Δpuf6 = 2.5 ± 1.1 mRNAs/transcription site $\pm$ SD).

Supplementary Figure 6. Known translation inhibitors are not involved in regulating CLB2 local translation, but Puf6 regulates CLB2 transcription.

(a) Relative frequency distribution of mature *CLB2* mRNAs per cell. **(b)** Merge maximal projection of *CLB2* smFISH (green), DIC (gray), and DAPI (blue) in a Δpuf6 cell. Yellow arrows indicate

transcription sites. Scale bar 2 μ m. **(c)** Quantification of transcription sites in transcriptionally active WT and $\Delta puf6$ cells (n = 1340 and 1583 cells, respectively) based on *CLB2* mRNA smFISH.

Figure 7. The CLB2 mRNA ZIP code mutants show clone-dependent growth defect and cell cycle defect.

(a) Top, single *CLB2* ZIP-code mutant clones grown on SC-complete 2% glucose at 30°C for 3 days. Bottom, *CLB2* ZIP-code mutant clones from top plate were grown on SC-complete 0.1% glucose at 30°C for 3 days. **(b)** Tenfold serial dilutions of the indicated strains were spotted onto SC-complete plates at the indicated carbon sources and temperatures. **(c)** Growth curves of *CLB2* ZIP-code mutant clones (*ZIP cl1*, *cl2* and *cl3*) performed at 30°C in SC-complete at the indicated carbon sources. Optical density measurements (OD₆₀₀) were collected every 5 min. Triplicate experiments are shown for each strain. **(d)** Cell cycle analysis by DNA content estimation with flow cytometry in the ZIP-code mutants grown at the indicated conditions. DNA was stained with Sytox Orange. Mixed Gaussian fitting (black lines) was used to estimate the indicated G1 and G2/M components. Percentages are reported for one representative experiment.

Supplementary Figure 7. CLB2 ZIP code mutant cells shows a growth defect and clonal variability under nutrient limited conditions

(a) Growth curves of *SHE* deleted strains ($\Delta she2$, $\Delta she3$) performed at 30°C in SC-complete at the indicated carbon sources. Optical density measurements (OD₆₀₀) were collected every 5 min. Triplicate experiments are shown for each strain. **(b)** Left, Bright-field images of WT or *CLB2* ZIP-*cl1* mutant grown in SC-complete 2% glucose at 30°C. Right, quantification of WT and *CLB2* ZIP-code *cl1* mutant cell areas. **(c)** Growth curves of Diploid strains generated by crossing WT cells (mating type *alpha*) with either (mating type *a*) WT or the *CLB2* ZIP-code *cl1* mutant, performed at 30°C in SC-complete at the indicated carbon sources. Triplicate experiments are reported for each strain.

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- 5

Figure 1

a

b

c

d

Synonymized ZIP-code mutant

CLB2 WT	1111	GCAGATGACTACGATATACAGTCTCGAACTCTTGC	1145
CLB2 Zip Mut		GCCGATGATTATGATATACAGAGCGAAGCCTAGC	
Clb2 protein		A D D Y D I Q S R T L A	

e

f

g

h

a

b

c

d

CLB2 and *ASH1* mRNA expression and localization during the cell cycle

e

f

g

h

a

b

c

Figure 7S