# Supplementary Methods

**Strains**

Cln3 mutation was done by CRISPR as described in main materials and methods section. a synthetic double stranded repair product was co-transformed with Cas9 harbouring the mutation. GCT**G**G ( -1016bp upstream) was replaced by GCT**C**G, to match the *S. paradoxus* allele. Introducing the Cln3 mutation was done on BY4741 expressing the cell cycle markers CDC10-YFP and ACS2-CFP(Jonas et al. 2018).

High-copy expression of Clb2 was done as previously described(Hollenhorst et al. 2000). plasmid pRS426 was digested by BamHI restriction enzyme, and was ligated with a genomic amplicon of the CLB2 gene, including 1000bp upstream and 300bp downstream. Ligated plasmid was used for transformation, and colonies were selected by growing on uracil depleted plates.

**Quantifying Cln3 mutation effect on cell cycle**

Quantifying cell cycle effect was done as previously reported(Jonas et al. 2018). Briefly, cells were grown over-night to saturation in SD media, and diluted in morning to fresh SD media until reaching OD~0.2. Cells were mounted for imaging on SD-agar pads in 96-well plate with SD medium. Growth of micro colonies at 30°C was observed with a fully automated Zeiss Axio Observer Z1 inverted microscope equipped with a motorized XY and Z stage, external excitation and emission filter wheels (Prior), IR-based Definite Autofocus from Zeiss and a 63 × oil objective. Fluorescent proteins were detected with the 46 and 47 filter set from Zeiss for YFP and CFP respectively. Exposure times and fluorescent intensity for the YFP or CFP detection were 150 and 100 ms. Images were acquired with the Orca FLASH 4.0 v2 CMOS camera (Hamamatsu). The microscopic setup allowed sequential imaging of bright field (- 1.5 um offset for image analysis), and both fluorescence channels for 20 positions in 3 min and over 6 hr. Image analysis was performed by a custom made matlab software as previously described(Jonas et al. 2018).

**RT-qPCR**

RNA from exponentially growing cells was extracted as describe in main methods section, only using nucleospin® 96 single columns (Macherey-Nagel™ cat 740955.50). cDNA was synthesized using **LunaScript® RT SuperMix Kit (NEB E3010) according to LunaScript® protocol. RT-qPCR reaction was measured in a 96 well plate using StepOnePlus™ Real-Time PCR system, in a 10ul reaction using Fast SYBR™ Green Master Mix.**

**For measuring Ace2 levels, the following primers were used:** ATCCGCAACCCATGGAACTC, CGCCACCTCTTCTTGCTCTTC. For measuring Fkh2 in S. cerevisiae the following primers were used: TACCATCAATCCACAAGCGGCTTC, TCGGCTAGTGAAATAACTCCCTCC. For measuring Fkh2 in S. paradoxus and Fkh2-SWAP strain the following primers were used: ATCAGCGAATGCCTATCCTCAAGC, GCCGGACTTAGCAAACCTGTAG. All results were compared to the house-keeping genes ACT1 (GTGTGATGTCGATGTCCGTA, TTCTTTCTGGAGGAGCAATG), and TAF10 (TGTCGCAGAAAACATGAAGA, ATCGGGAATGATAGGAGGAG).

**ChEC-Seq experiments multi-copy Clb2**

ChEC-seq experiments on strain expressing the multicopy Clb2 plasmid was done as described in main methods section, only using SD -uracil media instead of YPD to prevent plasmid loss.

# References

Hollenhorst PC, Bose ME, Mielke MR, Müller U, Fox CA. 2000. Forkhead genes in transcriptional silencing, cell morphology and the cell cycle. Overlapping and distinct functions for FKH1 and FKH2 in Saccharomyces cerevisiae. Genetics. 154(4):1533–48. https://www.genetics.org/content/154/4/1533.

Jonas F, Soifer I, Barkai N. 2018. A Visual Framework for Classifying Determinants of Cell Size. Cell Rep. 25(12):3519-3529.e2. http://doi.org/10.1016/j.celrep.2018.11.087.