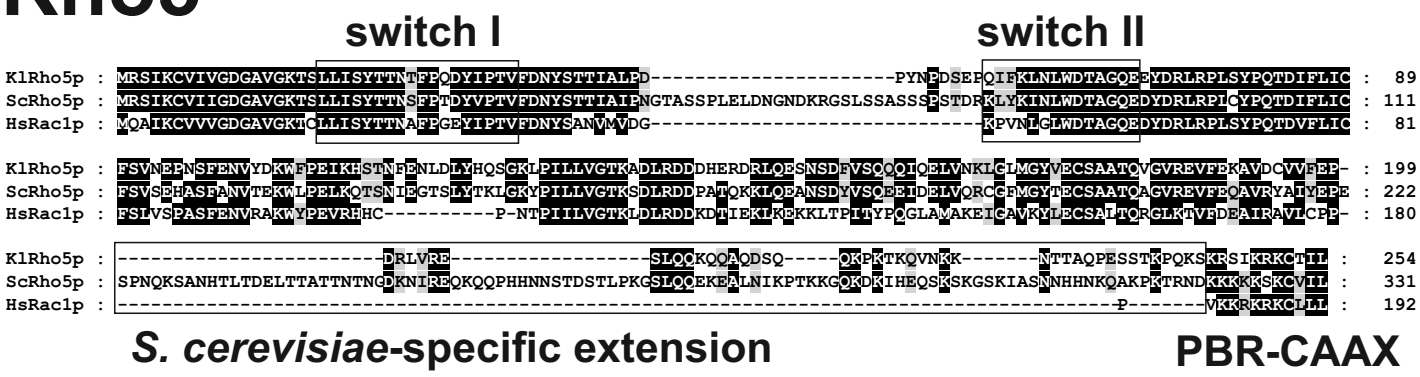
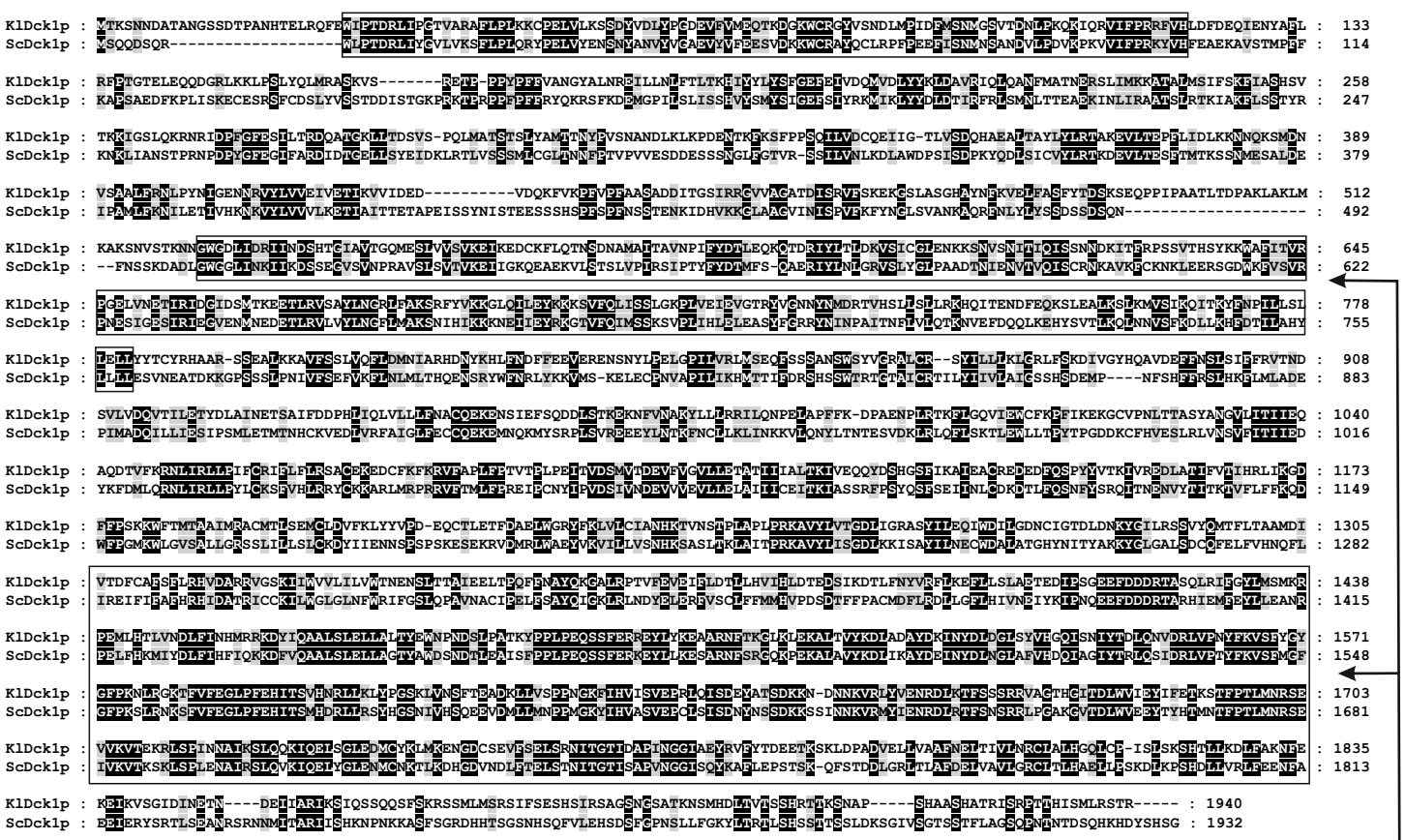
Rho5



Dck1



Lmo1

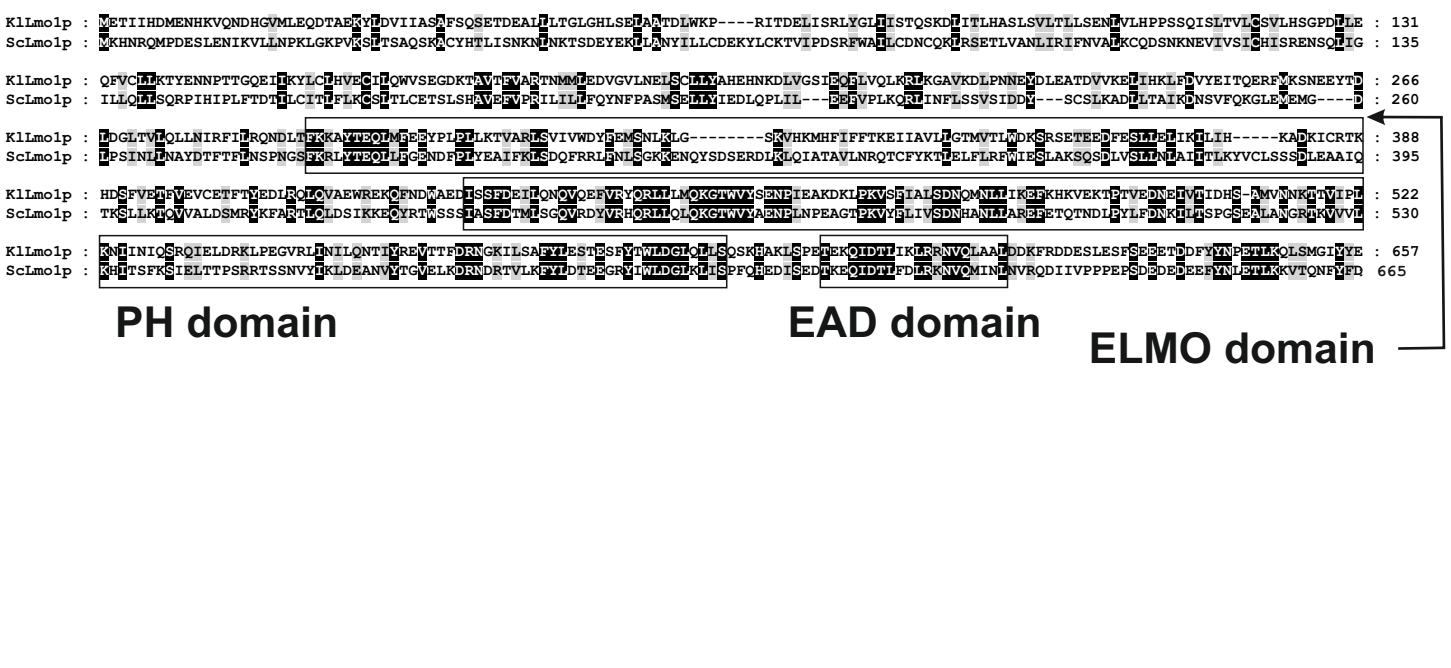
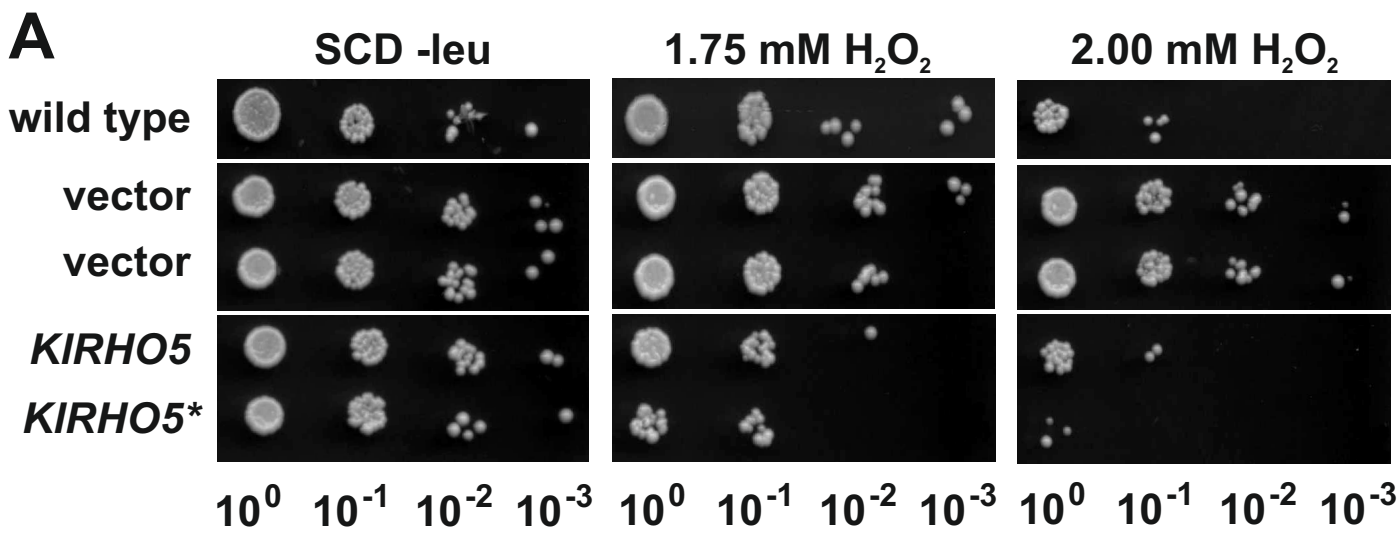


Fig. S1. DLR components of *K. lactis* are conserved in their functional domains with their homologs in *S. cerevisiae*. Identical amino acid residues are highlighted by inverse print, conserved amino acid residues by light gray background. Upper rows: The deduced amino acid sequence of KlRho5 was aligned both to ScRho5 and human Rac1 (HsRac1p). Highly conserved functional domains (switch I, switch II, PBR, and CAAX-box) are indicated by boxes. Of special interest, ScRho5 carries a specific extension not present in HsRac1 and drastically shorter in KlRho5, also marked by a box. Middle rows: Alignment of the homologs of the GEF subunit Dck1 from *K. lactis* and *S. cerevisiae*. Functional domains described for ScDck1 are indicated. Lower rows: Alignment of the homologs of the GEF subunit Lmo1 from *K. lactis* and *S. cerevisiae*. Described functional domains are boxed and designated.



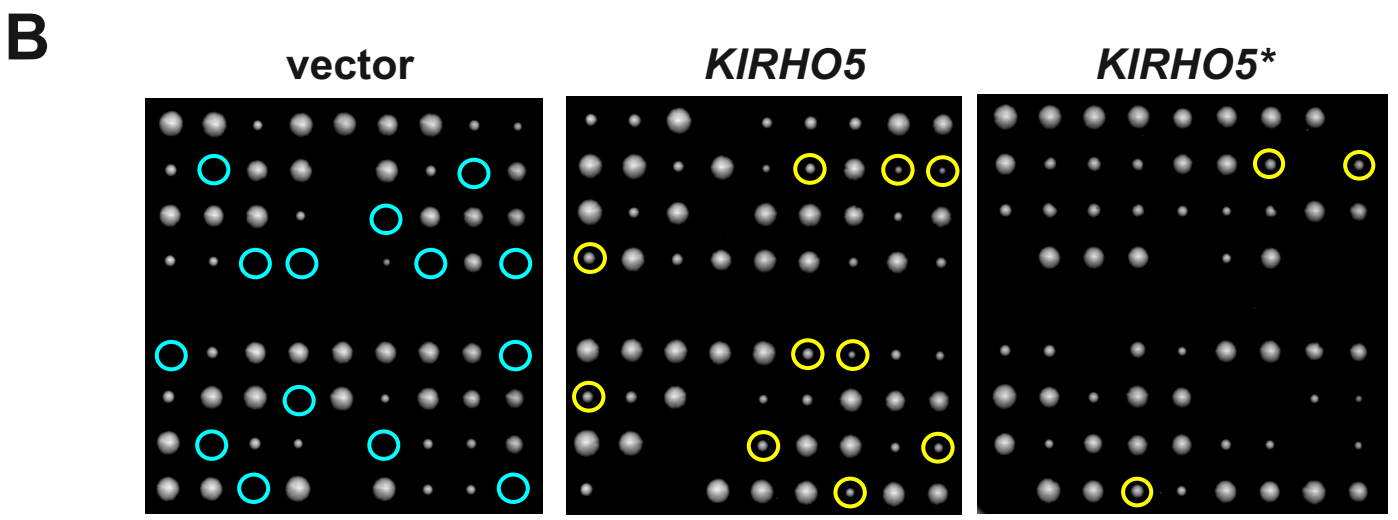


Fig. S2. ***Scrho5* deletion phenotypes are complemented by its homolog *KlRHO5***

A) Drop-dilution assays of HOD342-6D carrying the vector without insertion (vector = YEp181JJH), the same vector with the wild-type allele of the *K. lactis* homolog (*KlRHO5* = pJJH2759), and its activated allele (*KlRHO5** = $KlRHO5^{Q69H}$ = pJJH2760). As a control, the *ScRHO5* wild-type strain HD56-5A was transformed with the vector YEp181JJH and treated in the same way (upper lane). Ten-fold dilutions of logarithmically growing cultures were spotted onto plates lacking leucine with the concentrations of hydrogen peroxide as indicated as described in materials and methods. Growth was monitored by scanning after incubation at 30°C for 3 days.

B) The diploid strain DAJ138 with the heterozygous deletions indicated was used to introduce either the vector (YCplac111), or plasmids carrying the wild-type *KlRHO5* (pJJH2759) and the activated *KlRHO5** (pJJH2760) allele of *K. lactis*. Transformants selected on synthetic medium lacking leucine were sporulated, subjected to tetrad analyses and replica-plated onto drop-out media for detection of the deletion markers and the presence of the plasmid. Blue circles indicate the position of segregants that should carry the *rho5 sch9* double deletion, but are synthetically lethal, yellow circles designate segregants carrying the double deletion and the plasmid with the respective *KlRHO5* allele. Note that *sch9* deletions form smaller colonies, independent of the *RHO5* allele they carry.

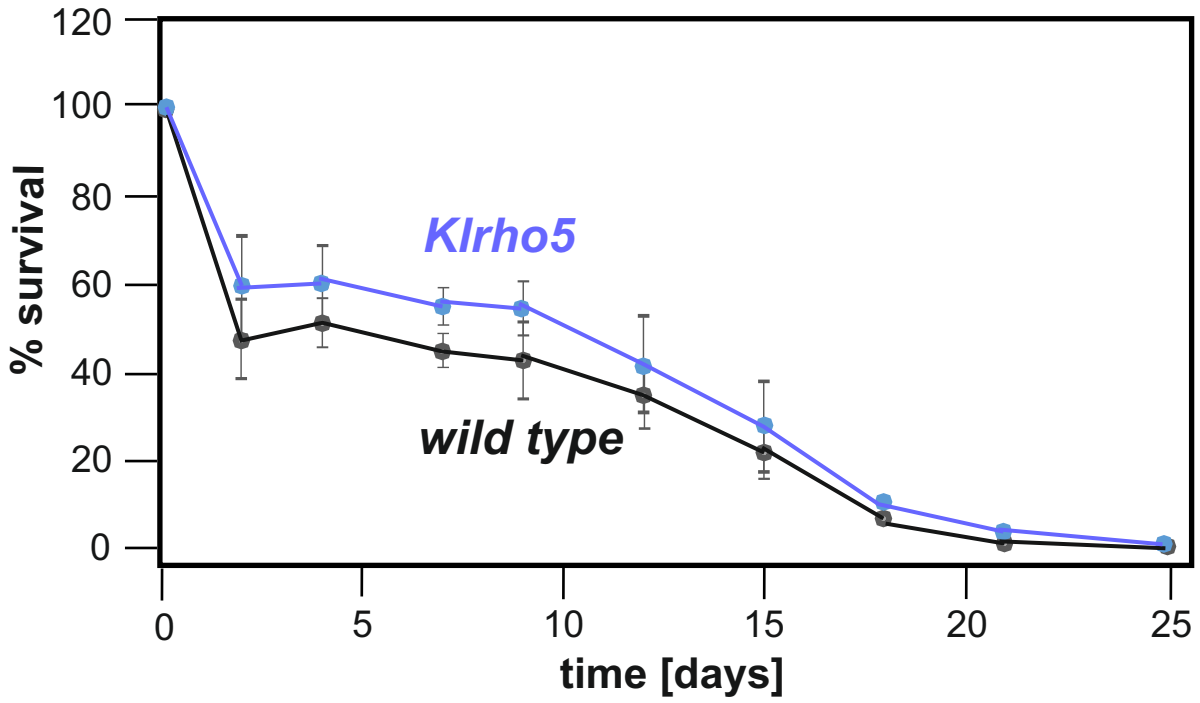


Fig. S3. Deletion of *KlRHO5* does not affect chronological life span. Two cultures of three independent strains, each being either wild-type for *KlRHO5* or carrying a deleted allele, were grown to stationary phase in synthetic selective medium (strains employed: wild-types KHO46-2A, KHO46-6C, KHO46-12B; *Klrho5* deletions KHO208-8B, KHO276-2A, KHO368-3B). Samples were taken at the time points indicated, diluted to produce single colonies, plated on rich medium (YEPD) and incubated for three days to determine the number of colony-forming units (CFU). CFUs were set at 100% at day 0, with starting at approximately $2x10^7$ viable cells/mL. Error bars give the standard deviations of % survivors in the three biological replicates.

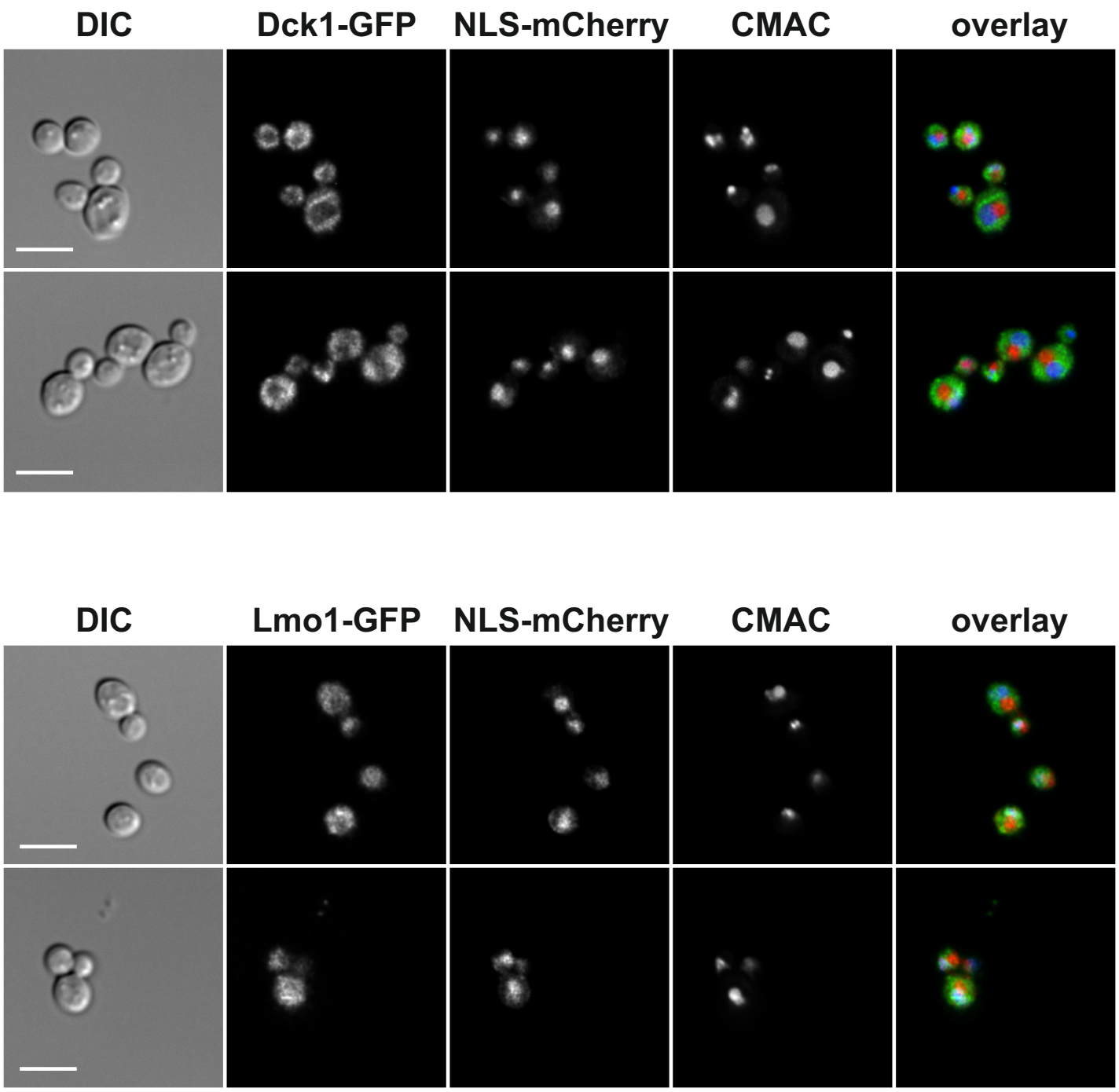


Fig. S4. KlDck1-GFP and KlLmo1-GFP do not strongly associate with the nucleus or vacuoles under standard growth conditions. Strains carrying either the *KlDCK1-GFP* allele or the KlLMO1-GFP allele at their native loci were crossed to a strain encoding a nuclear localization signal fused to mCherry integrated at the *Klleu2* locus, sporulated and subjected to tetrad analyses. Segregants expressing both fluorophore fusions were used for life-cell imaging. Vacuoles were stained with CMAC as described in materials and methods. Images were aquired with 0.5 sec exposures in the respective channels. The size bar in the lower left corner of the DIC images represents 5 µm, applicable to all images in the panels of the same row.

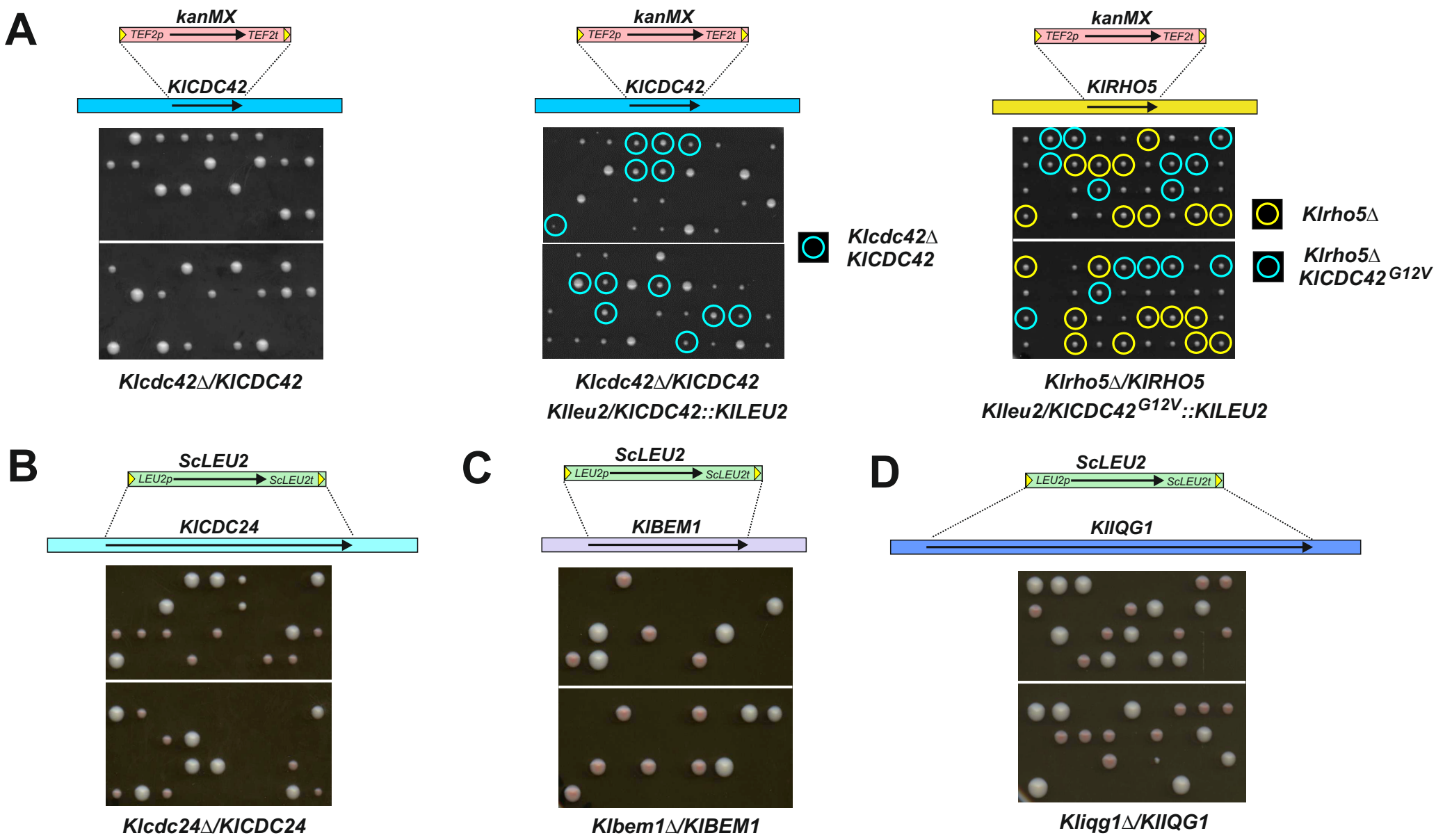


Fig. S5. ***KlCDC42*, *KlCDC24*, *KlBEM1*, and *KlIQG1* are essential genes.** Heterozygous diploids were constructed in the background of strain KHO70 by homologous recombination as indicated by the schematic representations, and subjected to tetrad analyses. Asci were dissected and allowed to germinate on YEPD plates, with 3-4 days incubation at 30°C. Relevant genotypes are indicated below the images (strains with complete genotypes are listed in Table S1). A) Tetrad analyses of strains carrying different *KlCDC42* alleles. The original heterozygous diploid strain KHO70/cdc42 did not yield viable progeny carrying the deletion marker (left). Integration of a wild-type copy at the *Klleu2* locus in this strain (pJJH2918) restored viability to segregants also carrying the deletion (middle, blue circles). No synthetic growth phenotypes were observed if combining the integrated activated $KlCDC42^{G12V}$ allele (pJJH2917) with a *Klrho5* deletion (right, blue circles) as compared to the single *Klrho5* deletion (right, yellow circles). However, morphological defects of the deletion were compensated by the presence of the activated $KlCDC42^{G12V}$ allele (see main text for details). B) to D) Segregants of heterozygous diploid strains carrying deletions of either *KlCDC24*, *KlBEM1*, or *KlIQG1* were not viable, while auxotrophic markers in the viable wild-type colonies segregated as expected (examplified by the red colony colour caused by a *Klade2* deletion in approximately half of the segregants).

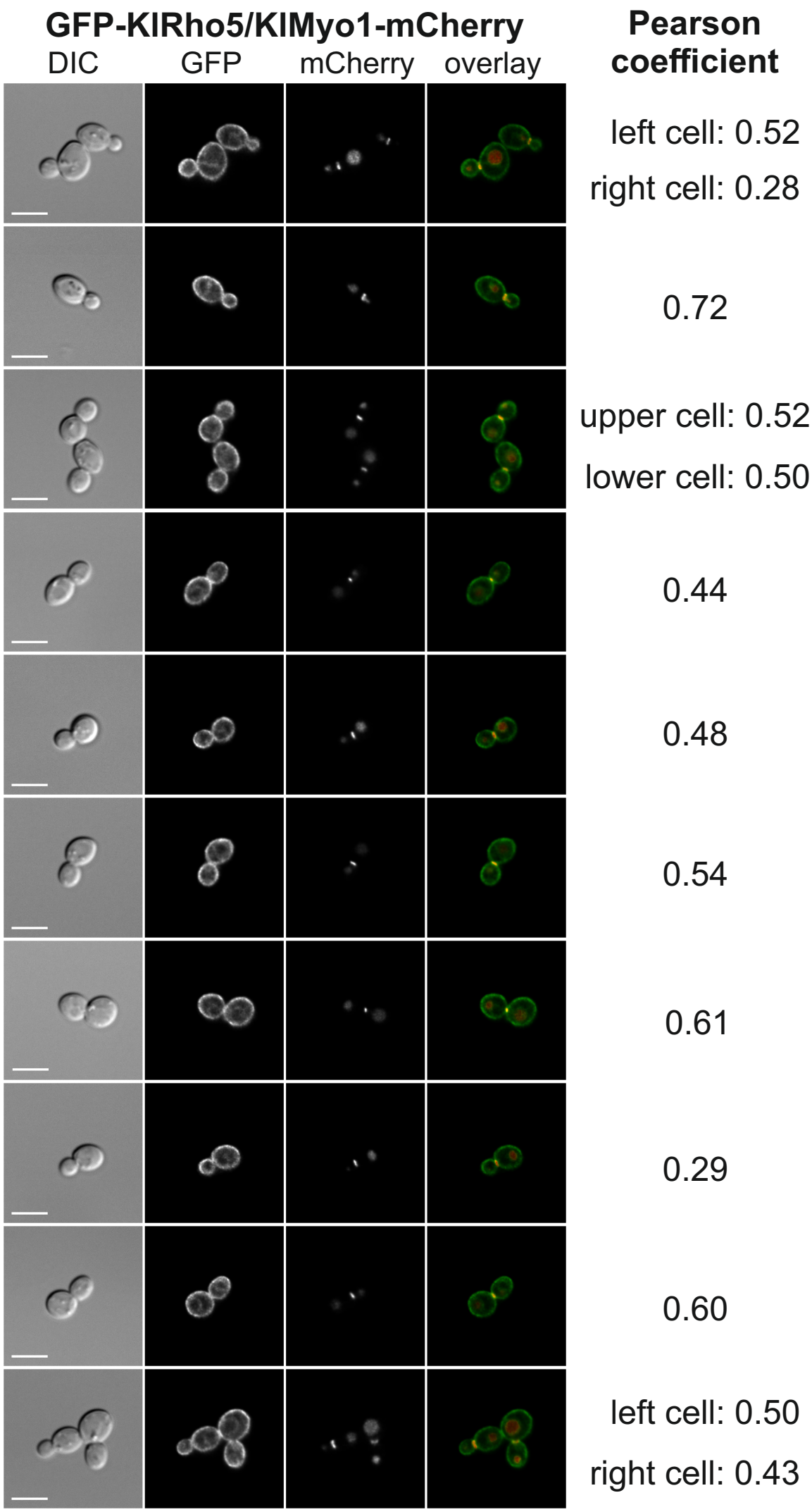


Fig. S6. Additional images showing the colocalization of GFP-KlRho5 and Myo1-mCherry at the bud neck of dividing cells. Strains, growth conditions, image aquisition and calculations of the Pearson coefficient were as described in the legend of Fig. 7.

Table S1. Strains used in this study

Strain	Collection number	Genotype	Reference
***Kluyveromyces lactis* strains** [1]			
CBS2359	Os83	*MATa*	(Kooistra et al., 2004)
KHO70	- [2]	*MATa/MATalpha ura3/ura3 leu2/leu2 his3::loxP/ HIS3 ade2::loxP/ADE2 ku80::loxP/ku80::loxP*	(Heinisch et al., 2010)
KHO70/bem1	Kl1250	same as KHO70 but with *bem1::ScLEU2/BEM1*	this study
KHO70/cdc24	Kl1245	same as KHO70 but with *cdc24::ScLEU2/CDC24*	this study
KHO70/cdc42	Kl1262	same as KHO70 but with *cdc42::ScLEU2/CDC42*	this study
KHO70/iqg1	Kl482	same as KHO70 but with *iqg1::ScLEU2/IQG1*	this study
KHO.01-14C	Kl642	*MATalpha dck1::kanMX*	this study
KHO.03-1D	Kl644	*MATalpha lmo1::kanMX*	this study
KHO208-8A	Kl390	*MATalpha ura3 leu2 his3::loxP ku80::loxP*	this study
KHO208-8B	Kl391	*MATalpha ura3 leu2 his3::loxP rho5::kanMX ku80::loxP*	this study
KHO218-9A	Kl428	*MATa ura3 leu2 his3::loxP dck1::kanMX*	this study
KHO254-1A	Kl1312	*MATalpha ura3 leu2 Kldck1::kanMX*	this study
KHO255-1B	Kl648	*MATalpha ura3 leu2 Kllmo1::kanMX ku80::loxP*	this study
KHO255-3A	Kl649	*MATalpha ura3 leu2 his3::loxP Kllmo1::kanMX ku80 ::loxP*	this study
KHO276-2A	Kl652	*MATa ura3 his3::loxP rho5::kanMX*	this study
KHO277-3D	Kl667	*MATalpha ura3 leu2 Klrho5::kanMX*	this study
KHO46-6C/2751	Kl1073	*MATa ura3 leu2::pJJH2751-LEU2*	this study

KHO347-2B	Kl958	*MATa ura3 leu2 his3::loxP KU80wt DCK1-GFP-SkHIS3 IDP1-mRuby-kanMX*	this study
KHO348-2B	Kl953	*MATa ura3 his3::loxP LMO1-GFP-SkHIS3 IDP1-mRuby-kanMX*	this study
KHO362	- [2]	*MATa/MATalpha ura3/ura3 leu2/leu2 his3::loxP/ HIS3 ade2::loxP/ADE2 ku80::loxP/ku80::loxP Kllmo1::kanMX/KlLMO1*	this study
KHO364	- [2]	*MATa/MATalpha ura3/ura3 leu2/LEU2 his3::loxP/ HIS3 Klrho5::kanMX/KlRHO5*	this study
KHO371	- [2]	*MATa/MATalpha ura3/URA3 leu2/LEU2 his3::loxP/HIS3 Kldck1::kanMX/KlDCK1*	this study
KHO384	- [2]	*MATa/MATalpha ura3/URA3 leu2/leu2 his3::loxP/HIS3 Klrho5::kanMX/KlRHO5 Klgpr1::ScLEU2/KlGPR1*	this study
KHO393	- [2]	*MATa/MATalpha ura3/URA3 leu2/LEU2 his3::loxP/HIS3 Klsch9::kanMX/KlSCH9*	this study
KHO401	- [2]	*MATa/MATalpha ura3/URA3 leu2/LEU2 his3::loxP/ his3::loxP Klsch9::kanMX/KlSCH9 Klrho5::SpHIS3/ KlRHO5*	this study
KHO402	- [2]	*MATa/MATalpha ura3/URA3 leu2/leu2 Klgpr1::ScLEU2/KlGPR1*	this study
Kl017/IDP1mRub	Kl927	*MATalpha ura3 leu2 his3::loxP ku80::loxP IDP1-mRuby-kanMX*	this study
KMO30-4A	MKL237	*MATalpha ura3 leu2 MYO1-mCherry-ScURA3 GFP-RHO5-SkHIS3*	this study
KMO30-6D	MKL238	*MATa ura3 leu2 his3::loxP MYO1-mCherry-ScURA3 GFP-RHO5-SkHIS3*	this study
KMO31-4B	MKL251	*MATa ura3 leu2 his3::loxP GFP-RHO5-SkHIS3 IDP1-mRuby-kanMX*	this study
KMO32-5D	MKL270	*MATalpha ura3 his3::loxP leu2::pRRO297-LEU2 DCK1-GFP-SkHIS3*	this study
KMO33-8A	MKL271	*MATa ura3 his3::loxP leu2::pRRO297-LEU2 LMO1-GFP-SkHIS3*	this study

***Saccharomyces cerevisiae* strains**			
DAJ138	Os1649	*MATa/MATalpha ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 sch9::SkHIS3/SCH9 rho5::kanMX/RHO5*	(Schmitz et al., 2015)
HD56-5A	Os5	*MATalpha ura3-52 his3-11,15 leu2-3,112*	(Kirchrath et al., 2000)
HOD342-6D	Os1483	*MATalpha ura3-52 his3-11,15 leu2-3,112 rho5::SpHIS5*	this study

[1] All strains listed in this part of the table were *K. lactis* so that the prefix "Kl" was omitted for all gene names given and only genes from other species are indicated as such.

[2] Diploid *K. lactis* strains are not kept in glycerol stocks (and therefore have no collection number), due to their tendency to sporulate even in rich medium. As an exception, heterozygous diploids with deletions in essential genes are held in stock.

Table S2. Vectors and plasmids used in this study *

Plasmid	Features	References
YCplac111	centromeric vector with *LEU2* as selection marker in *S. cerevisiae*	(Gietz and Sugino, 1988)
YEp181JJH	multicopy vector with *LEU2* as selection marker in *S. cerevisiae*, and a modified polylinker; derived from YEplac181	(Gietz and Sugino, 1988)
pCXs22	triple shuttle vector for *E. coli/S. cerevisiae/K. lactis*; *CEN/ARS* for *S. cerevisiae*, multicopy for *K. lactis*, *ScURA3* as selection marker for both yeasts	(Heinisch *et al.*, 2010)
pCse24	triple shuttle vector for *E. coli/S. cerevisiae/K. lactis*; *CEN/ARS* for *S. cerevisiae*, multicopy for *K. lactis*, *ScLEU2* as selection marker for both yeasts	(Heinisch *et al.*, 2010)
pUK1921	*E. coli* cloning vector conferring kanamycin resistance	(Heinisch, 1993)
pUG6	template vector for PCR-based deletions carrying the *kanMX* marker cassette	(Gueldener et al., 2002)
pUG27	template vector for PCR-based deletions carrying the *SpHIS5* marker cassette	(Gueldener et al., 1996)

Table S2. continued

pFA6a GFP-SkHIS3	template vector for PCR-based GFP fusions carrying the *SkHIS3* marker cassette	(Longtine et al., 1998)
pKT178-mRuby	template vector for PCR-based mRuby fusions carrying the *kanMX* marker cassette	(Lee et al., 2013)
pJJH955L	template vector for PCR-based deletions carrying the *ScLEU2* marker cassette	(Heinisch et al., 2010)
pJJH1409	plasmid obtained by gap repair of an erroneous *KlDCK1* PCR clone; based on pCse24	this study
pJJH1524	template vector for PCR-based mCherry fusions carrying the *kanMX* marker cassette	this study
pJJH1525	template vector for PCR-based mCherry fusions carrying the *SkHIS3* marker cassette	this study
pJJH1619	template vector for PCR-based GFP fusions carrying the *kanMX* marker cassette	this study
pJJH1620	template vector for PCR-based GFP fusions carrying the *SkHIS3* marker cassette	this study
pJJH2600L	integrative *KlLEU2* vector with polylinker and blue/white screen in *E. coli*	this study
pJJH2751	integrative *KlLEU2* vector encoding a KlLifeAct-mRuby fusion expressed under the control of the *ScPFK2* promoter in conjunction with a GFP-KlRho5 fusion expressed from its native promoter	this study
pJJH2759	*KlRHO5* in YEp181JJH	this study
pJJH2760	$KlRHO5^{Q69H}$ in YEp181JJH	this study
pJJH2917	integrative *KlLEU2* vector with $KlCDC42^{G12V}$ under its native promoter	this study
pJJH2918	integrative *KlLEU2* vector with *KlCDC42* under its native promoter	this study
pMMO5	integrative *KlLEU2* vector with *KlRHO5* coding sequences flanked by its native regions	this study
pMMO6	integrative *KlLEU2* vector with *KlLMO1* coding sequences flanked by its native regions	this study
pMMO7	integrative *KlLEU2* vector with *KlDCK1* coding sequences flanked by its native regions	this study
pMMO22	integrative *KlLEU2* vector with *GFP-CDC42* expressed from the *ScPFK2* promoter	this study
pRRO297	integrative *KlLEU2* vector with a nuclear localization sequence fused to mCherry expressed from the *ScPFK2* promoter	gift from Rosaura Rocidio

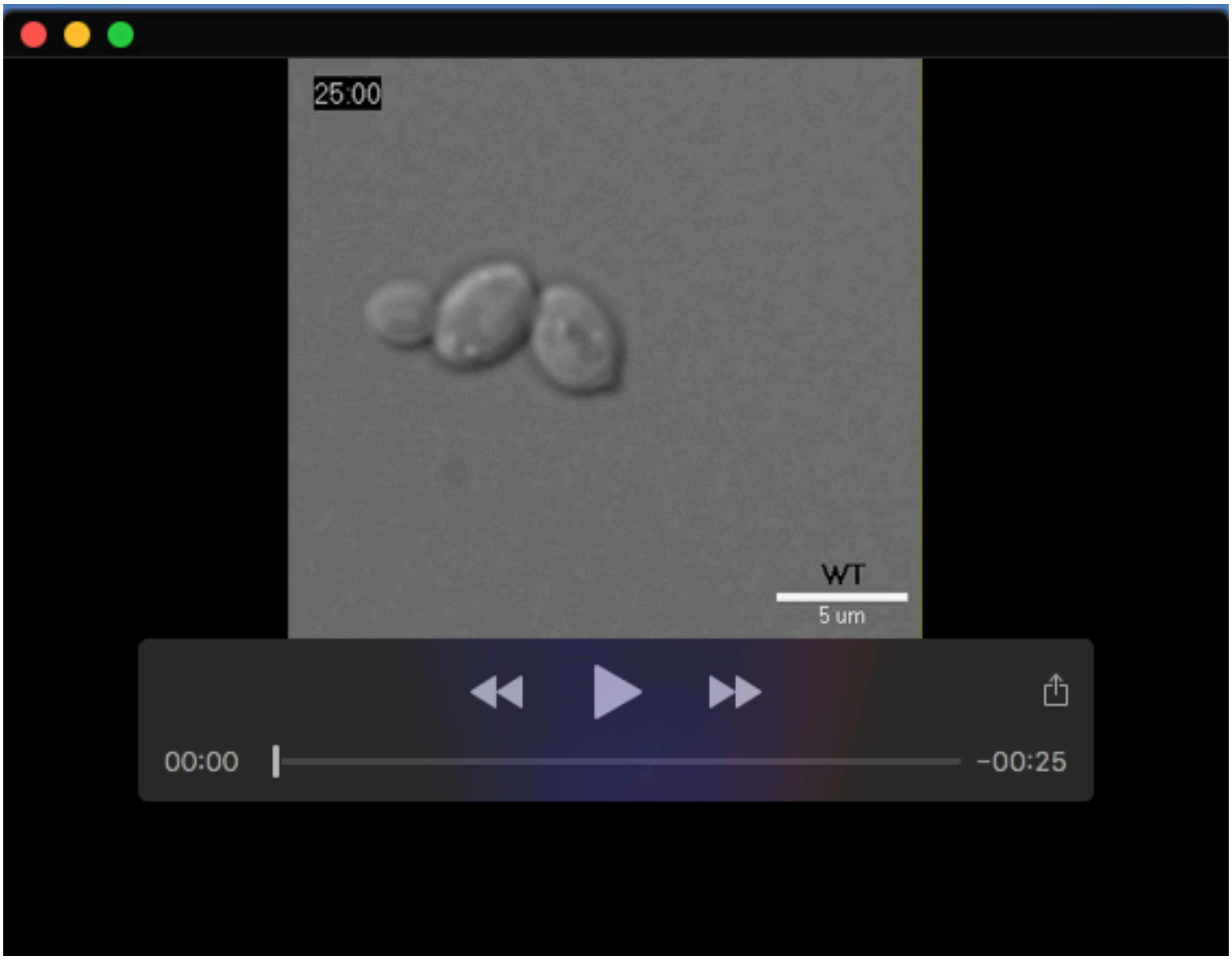
* Complete sequences of all plasmids listed are avaiblable upon request.

Table S3. Deletions tested for synthetic lethality with *Klrho5*

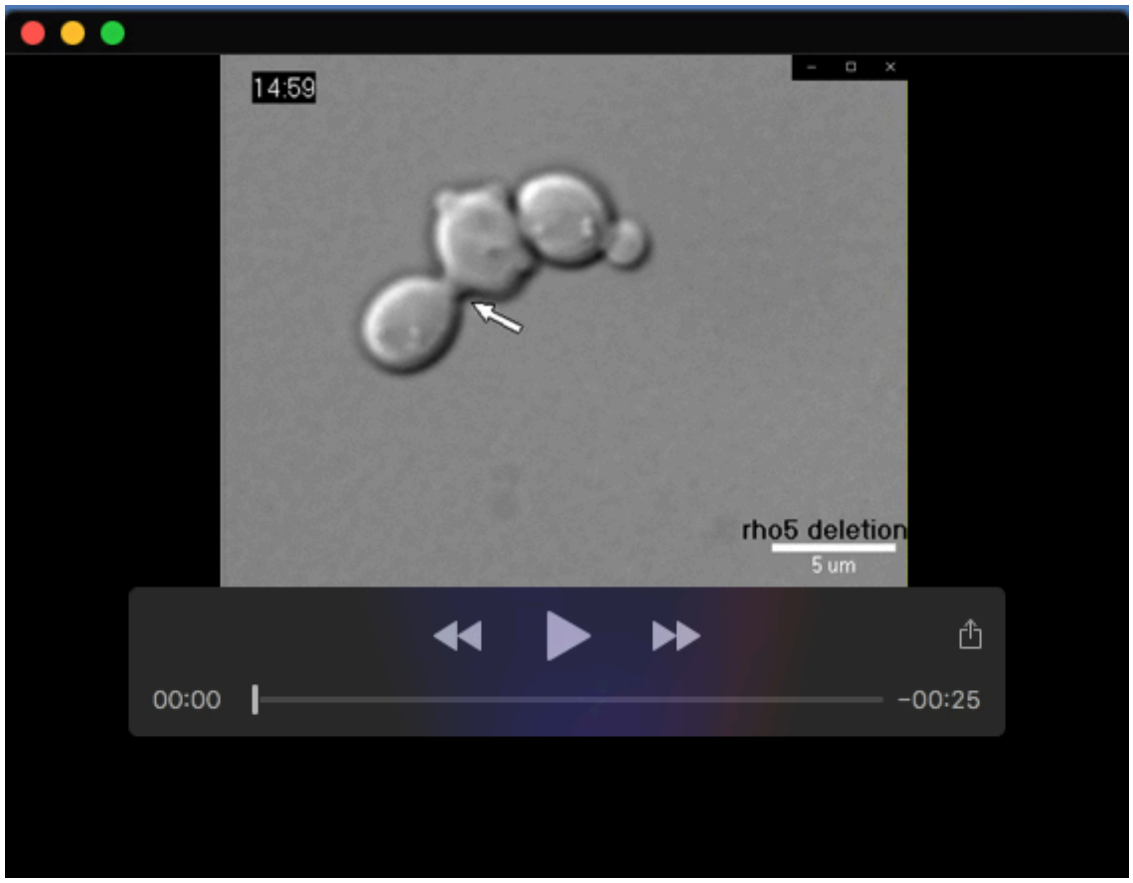
Strain [1]	Deleted gene ::marker	Function of wild-type protein [2]	Double deletion with *Klrho5* [3]
KHO341	*Klsnf1::kanMX*	carbohydrate signaling	viable
KHO293	*Klsch9::ScLEU2*	nutrient signaling	viable
KHO291	*Klgpr1::ScLEU2*	nutrient signaling	viable
KHO354	*Klzwf1::ScLEU2*	pentose phosphate pathway	viable
KHO344	*Klrpe1::SpHIS5*	pentose phosphate pathway	viable
KMO24	*Kltal1::ScLEU2*	pentose phosphate pathway	viable
KHO381	*Klpor1::SkHIS3*	mitochondrial ion channel (VDAC)	viable
KHO316	*Klsod1::ScLEU2*	oxidative stress response	viable
KHO314	*Klsod2::SpHIS5*	oxidative stress response	viable
KHO377	*Klmpk1::ScLEU2*	cell wall integrity signaling	viable
KHO379	*Klbck1::kanMX*	cell wall integrity signaling	viable
KHO378	*Klrlm1::ScURA3*	cell wall integrity signaling	viable
KHO276	*Klpil1::ScHIS3*	eisosome formation	viable
KHO277	*Kllsp1::ScLEU2*	eisosome formation	viable
KHO338	*Klmyo1::kanMX*	cytokinesis	viable
KMO17	*Klhof1::ScURA3*	regulation of cytokinesis	viable
KHO368	*Kltdp1::ScLEU2*	DNA repair	viable

For all listed deletions, the entire open reading frames were substituted for the indicated heterologous markers by homologous recombination with the respective cassettes flanked by target sequences primarily added by PCR. All strain manipulations were performed exclusively in the congenic strain series used in this work and first described in (Heinisch *et al.*, 2010), taking advantage of the *ku80::loxP* deletion. Most deletions were constructed in the course of this study. Published work is available for *Klsnf1* (Rippert *et al.*, 2017), *Klzwf1* (Heinisch *et al.*, 2020), *Kltal1* (Jacoby *et al.*, 1993), *Klmpk1* (Kirchrath *et al.*, 2000), *Klbck1* (Jacoby *et al.*, 1999), *Klmyo1* and *Klhof1* (Rippert *et al.*, 2014). For works published prior to 2010 with other *K. lactis* strains, deletions were reconstructed in the congenic strain series, herein.

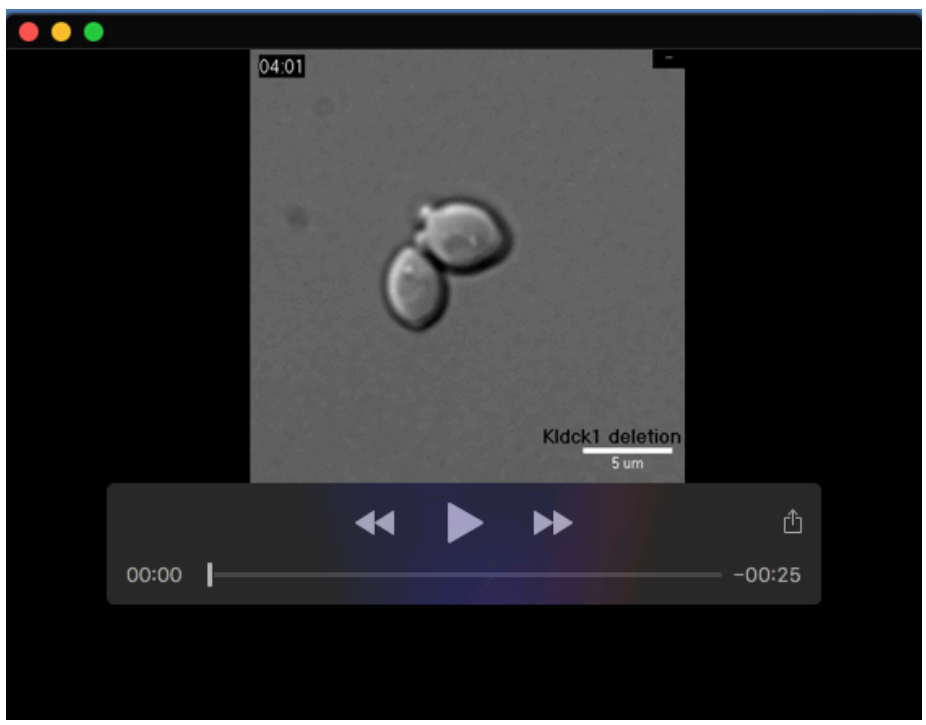
[1] Listed are the diploid strains heterozygous for both the respective deletion and a *Klrho5* allele, which were sporulated to test for synthetic lethality by tetrad analyses. All spores were allowed to germinate on rich medium (YEPD) for at least three days at 30°C.
[2] Functions of the proteins encoded by the respective wild-type genes were either studied in the works cited above or deduced from those of their *S. cerevisiae* homologs.
[3] A *Klrho5::kanMX* deletion allele was used throughout to test for synthetic lethality, except for the crosses with *Klsnf1::kanMX* and *Klbck1::kanMX*, for which a *Klrho5::SkHIS3* allele was employed.



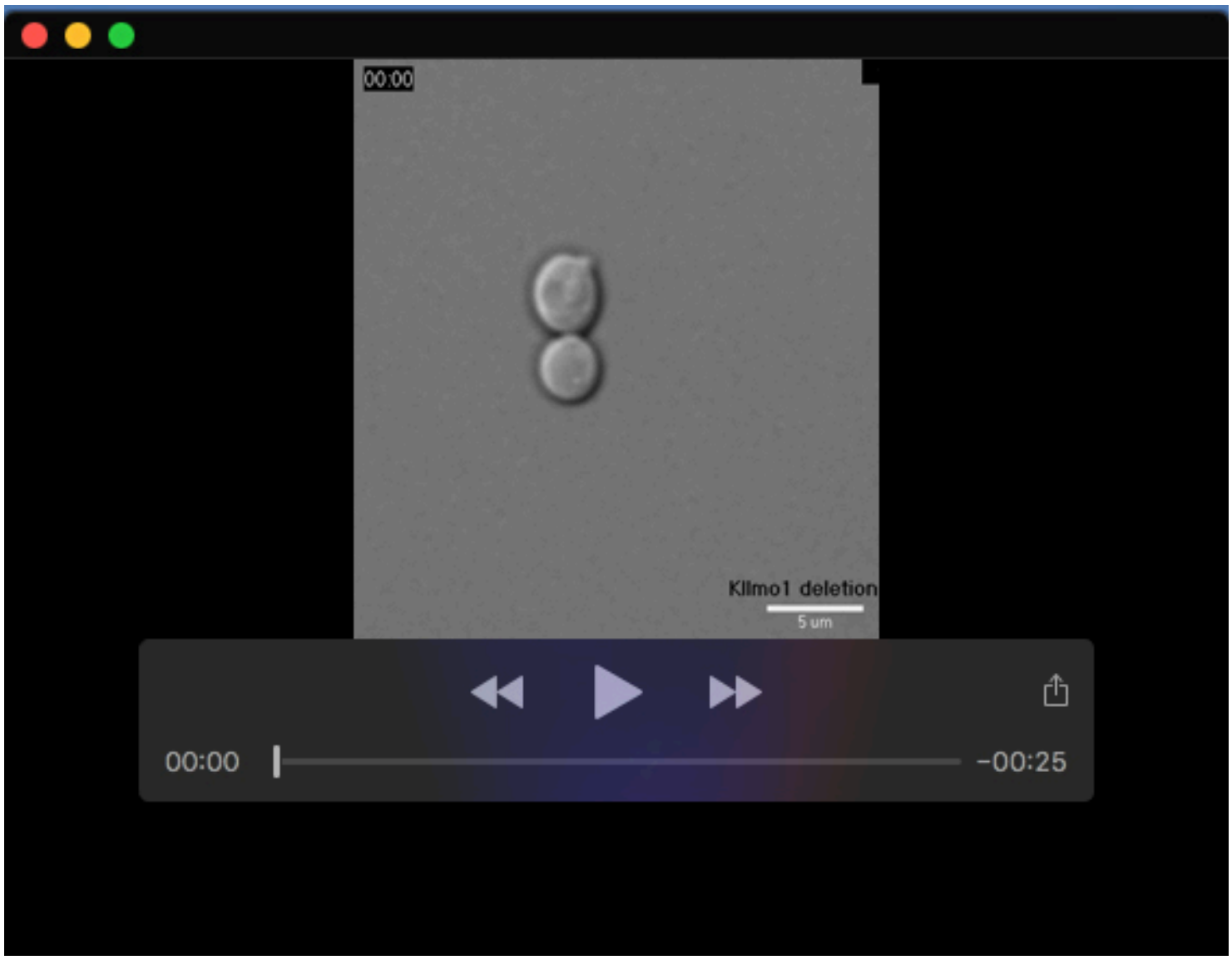
Movie 1. Time-lapse images following budding of wild-type (CBS2359) cells for 12.5 hours. Pictures were taken every five minutes with the 100fold magnification lens. In the movie, the time between the pictures was set to 1 second. The scale bar is 5 μm.



Movie 2. Time-lapse images following budding of *Klrho5* (KHO208-8B) cells for 8 hours. Positions of budding resulting in protruding bud scars are indicated by arrows. Magnification, compression for time lapse, and scale bar are as described in the legend to Movie 1.



Movie 3. Time-lapse images following budding of *Kldck1* (KHO.01-14C) cells for 8 hours and pictures were taken every two minutes. Positions of budding resulting in protruding bud scars are indicated by arrows. Magnification and scale bar are as described in the legend to Movie 1.



Movie 4. Time-lapse images following budding of *Kllmo1* (KHO.03-1D) cells for 4.5 hours and pictures were taken every five minutes. Positions of budding resulting in protruding bud scars are indicated by arrows. Magnification, compression for time lapse, and scale bar are as described in the legend to Movie 1.

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