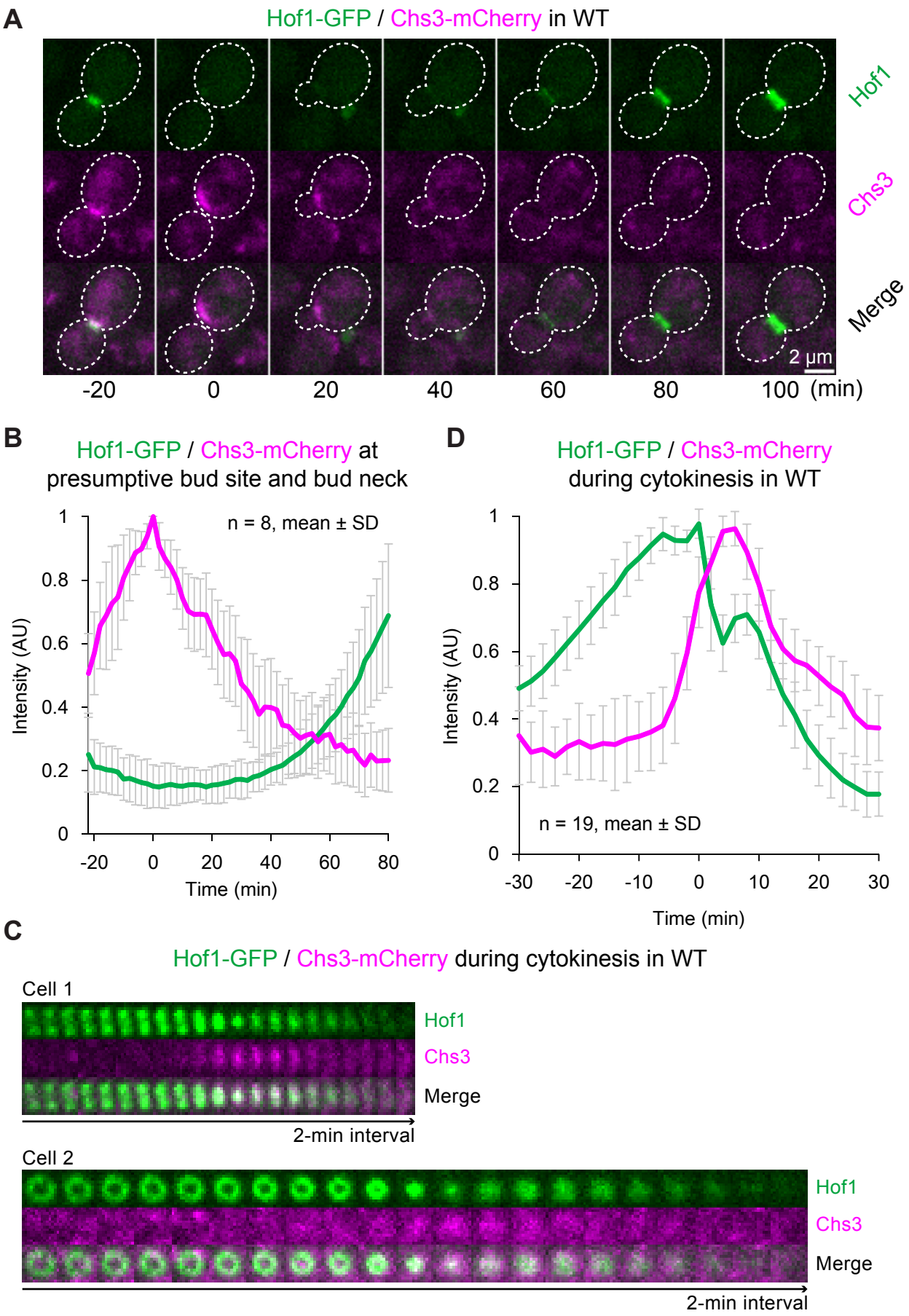
A
Hof1-GFP / Chs3-mCherry in WT
Hof1
Chs3
Merge
2 μm
-20
0
20
40
60
80
100 (min)
B
Hof1-GFP / Chs3-mCherry at
presumptive bud site and bud neck
n = 8, mean ± SD
Intensity (AU)
Time (min)
D
Hof1-GFP / Chs3-mCherry
during cytokinesis in WT
n = 19, mean ± SD
Intensity (AU)
Time (min)
C
Hof1-GFP / Chs3-mCherry during cytokinesis in WT
Cell 1
Hof1
Chs3
Merge
2-min interval
Cell 2
Hof1
Chs3
Merge
2-min interval

Figure S1. Localization of Hof1 in relation to Chs3 during the cell cycle. Related to Figure 1.

(A) Disappearance of Chs3 from the bud neck in G2/M correlates with the arrival of Hof1 to the bud neck. Cells of strain YEF5210 carrying Chs3-mCherry and Hof1-GFP were grown in SC media at 25°C and then imaged by spinning-disk microscopy. See also Movie S1A.

(B) Quantitative analysis of Chs3 and Hof1 localization dynamics before cytokinesis. Data generated in (A) were used for this analysis.

(C) The surge and fall of Hof1 at the division site during cytokinesis and cell separation precedes that of Chs3. Strain and imaging conditions were the same as described in (A). "Cell 1" provides a side view of the bud neck while "Cell 2" provides an en-face view of the bud neck. See also Movies S1B (side view) and S1C (en face view).

(D) Quantitative analysis of Chs3 and Hof1 localization dynamics during cytokinesis and cell separation. Data generated in (C) were used for this analysis.

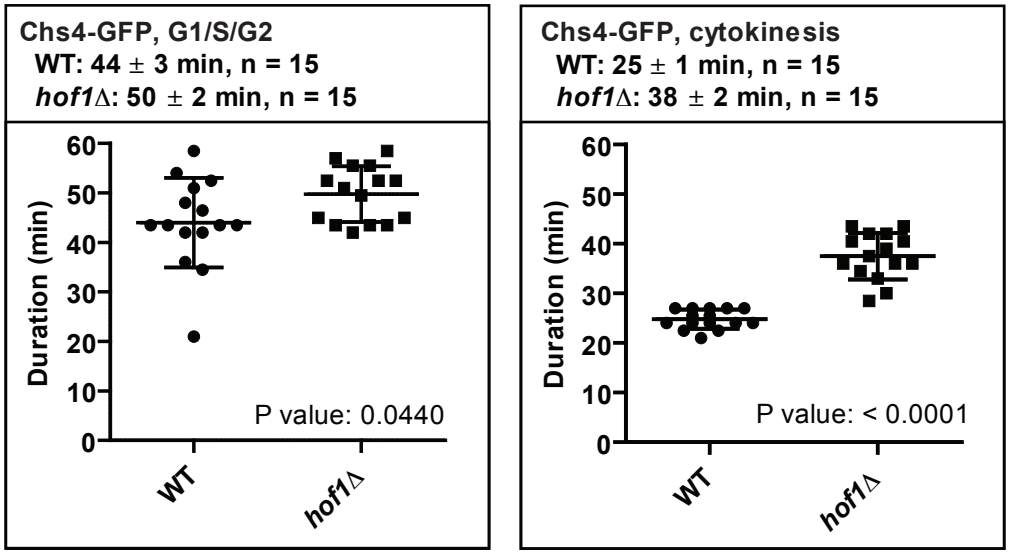
Chs4-GFP, G1/S/G2
WT: 44 ± 3 min, n = 15
hof1Δ: 50 ± 2 min, n = 15
Duration (min)
60
50
40
30
20
10
0
P value: 0.0440
WT
hof1Δ
Chs4-GFP, cytokinesis
WT: 25 ± 1 min, n = 15
hof1Δ: 38 ± 2 min, n = 15
Duration (min)
60
50
40
30
20
10
0
P value: < 0.0001
WT
hof1Δ

Figure S2. Quantitative analysis of Chs4 duration at the bud neck during the cell cycle in WT and *hof1Δ* cells. Related to Figure 4.

Imaging data as those presented in Figure 4A were analyzed for the duration time of Chs4 at the bud neck in WT (YEF5678) and *hof1Δ* (YEF5694) cells before G2/M (left) and during cytokinesis (right).

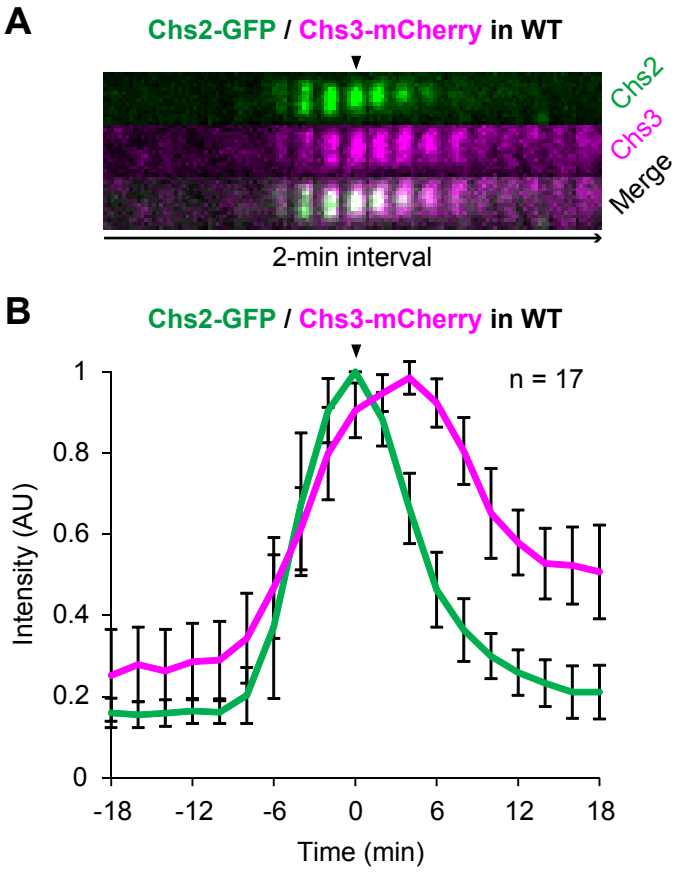
A
Chs2-GFP / Chs3-mCherry in WT
Chs2
Chs3
Merge
2-min interval
B
Chs2-GFP / Chs3-mCherry in WT
n = 17
Intensity (AU)
1
0.8
0.6
0.4
0.2
0
-18
-12
-6
0
6
12
18
Time (min)

Figure S3. Comparison of localization dynamics of Chs2 and Chs3 during cytokinesis in WT cells. Related to Figure 4.

(A) Chs2 and Chs3 arrive at the bud neck simultaneously, but peak at different times. Cells of strain YEF7967 carrying Chs2-GFP and Chs3-mCherry were grown in SC-His media at 25°C and then imaged by spinning-disk microscopy.

(B) Quantitative analysis of Chs2 and Chs3 localization dynamics. Data generated in (A) were used for this analysis.

Table S1. Yeast strains used in this study. Related to Figures 1-4, S1-3, and STAR METHODS.

Strain	Genotype	Source
Y860	α *his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 ura3-1::URA3-lexAop-ADE2*	C. Boone
Y1026	**a** *his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 ura3-1::URA3-lexAop-lacZ*	C. Boone
YEF473	**a**/α *his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	[S1]
YEF473A	**a** *his3 leu2 lys2 trp1 ura3*	[S1]
YEF473B	α *his3 leu2 lys2 trp1 ura3*	[S1]
YEF1951	**a** *hof1Δ::KanMX6*	[S2]
YEF2197	**a** *chs4Δ::TRP1*	[S3]
YEF2368	**a** *cyk3Δ::KanMX6*	This study
YEF2757	**a** *chs3Δ::HIS3 hof1Δ::KanMX6*	This study
YEF2769	**a** *bni4Δ::TRP1*	This study
YEF4559	**a** *chs3Δ::TRP1*	This study
YEF4600	**a** *hof1Δ::TRP1*	This study
YEF5210	**a** *HOF1-GFP-KanMX6 CHS3-mCherry-His3MX6*	This study
YEF5529	**a** *TRP1-pCET1-VN-CHS4*	This study
YEF5532	α *His3MX6-pCET1-VC-HOF1*	This study
YEF5535	**a**/α *TRP1- pCET1-VN-CHS4/CHS4 His3MX-pCET1-VC-HOF1/HOF1*	This study
YEF5678	**a** *SPC42-mCherry-His3MX6 GFP-Chs4-URA3*	This study
YEF5694	**a** *hof1Δ::TRP1 SPC42-mCherry-His3MX6 GFP-Chs4-URA3*	This study

YEF7965	**a** *CHS3-mCherry-His3MX6 GFP-CHS4-URA3*	This study
YEF7966	**a** *hof1Δ::TRP1 CHS3-mCherry-His3MX6 GFP-CHS4-URA3*	This study
YEF7967	**a** *CHS3-mCherry-His3MX6 CHS2-GFP-TRP1*	This study
YEF7980	**a**/α *TRP1-pCET1-VN-CHS4/CHS4 His3MX-pCET1-VC-HOF1/HOF1 TUB1::LEU2-pHIS3-mRuby2-TUB1/TUB1 MLC2-mApple-CaURA3 /MLC2*	This study

Table S2. Oligonucleotides used in this study. Related to STAR METHODS.

Sequence	Purpose
GCCAGATTATGCCTCTCCCGAATTCCCGGGGATCCGTCGAATG AGCTACAGTTATGAAGC (HOF1-FL-pJG4-5-F)	For making AD-HOF1 (1-669)
AAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGCGGCCGTC AAAGACCTTGATGCAGTA (HOF1-FL-pJG4-5-R)	For making AD-HOF1 (1-669)
AAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGCGGCCGTC ATTCAATGACTGGAAAACCTT (HOF1-SH3Δ-pJG4-5-R)	For making AD-HOF1-SH3Δ (1-601)
AAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGCGGCCGTC AGACTTCTGGAGATGGCAATG (HOF1-N-term-pJG4-5-R)	For making AD-HOF1-N-term (1-340)
GCCAGATTATGCCTCTCCCGAATTCCCGGGGATCCGTCGATCT TGCGACTCAATGATACT (HOF1-aa30-340-pJG4-5-F)	For making AD-HOF1-(30-340)
GCCAGATTATGCCTCTCCCGAATTCCCGGGGATCCGTCGAATC ACGGGAAAACTAGACAA (HOF1-aa55-340-pJG4-5-F)	For making AD-HOF1-(55-340)
GCCAGATTATGCCTCTCCCGAATTCCCGGGGATCCGTCGACTC AGTGTTGAGAAAGCTCG (HOF1-aa80-340-pJG4-5-F)	For making AD-HOF1-(80-340)
GCCAGATTATGCCTCTCCCGAATTCCCGGGGATCCGTCGAAAC TTGCAAGCAAGATATAC (HOF1-aa110-340-pJG4-5-F)	For making AD-HOF1-(110-340)
AAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGCGGCCGTC AGACTTCTGGAGATGGCAATG (HOF1-aa110-340-pJG4-5-R)	For making AD-HOF1-(110-340)
CGCGGATCCGATGAGCTACAGTTATGAAGCTTGT (HOF1-N-term-BamHI-F)	For making His6-tagged HOF1-N-term (1-340)

ACGCGTCGACTCAGACTTCTGGAGATGGCAATGG (HOF1-N-term-SalI-R)	For making His6-tagged HOF1-N-term (1-340)
CGCGGATCCGCGTTGGACTGTAGCTAGGA (CHS3-MID-BamHI-F)	For making His6-tagged CHS3-MID (477-1028)
ACGCGTCGACCTATTCAATACCAATCACAAATTGC (CHS3-MID-SalI-R)	For making His6-tagged CHS3-MID (477-1028)
CGCGGATCCATGCATAAGACTTCCAAAGGTGA (CHS4-SLR-BamH1-F)	For making MBP-CHS4-SLR (220-610)
ACGCGTCGACTCAGACTTCTGGAGATGGCAAT (CHS4-SLR-SalI-R)	For making MBP-CHS4-SLR (220-610)
CGAGGATGATGTAAGATTTAAGAGTAGAGACAAACTAGGTGA ATTCGAGCTCGTTTAAAC (VN-CHS4-BiFC-F4)	For making haploid strain carrying TRP1::pCET1-VN-CHS4
AATGCTTCTTATATGGATGTACCTGCGGTGAACTTGCCATAGT ACCACCAGAACCCTCGATGTTGTGGCGGATC (VN-CHS4-BiFC-R5)	For making haploid strain carrying TRP1::pCET1-VN-CHS4
AGTACACATTCACTGTATGAGGGAAAGAGGAAAGCCAGCTGA ATTCGAGCTCGTTTAAAC (VC-HOF1-BiFC-F4)	For making haploid strain carrying His3MX6::pCET1-VC-HOF1
CGTTTGGGTCCCAAAAACAAGCTTCATAACTGTAGCTCATAGT ACCACCAGAACCCTTGTACAGCTCGTCCATG (VC-HOF1-BiFC-R5)	For making haploid strain carrying

	His3MX6::pCET1-V C-HOF1

Supplemental references

[S1]. Bi, E., and Pringle, J.R. (1996). *ZDS1* and *ZDS2*, genes whose products may regulate Cdc42p in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *16*, 5264-5275.

[S2]. Vallen, E.A., Caviston, J., and Bi, E. (2000). Roles of Hof1p, Bni1p, Bnr1p, and Myo1p in cytokinesis in *Saccharomyces cerevisiae*. Mol. Biol. Cell *11*, 593-611.

[S3]. DeMarini, D.J., Adams, A.E.M., Fares, H., De Virgilio, C., Valle, G., Chuang, J.S., and Pringle, J.R. (1997). A septin-based hierarchy of proteins required for localized deposition of chitin in the *Saccharomyces cerevisiae* cell wall. J. Cell Biol. *139*, 75-93.