Precocious activation of APC/C-Cdh1 at pre-anaphase causes genome instability

Kazuhiro Toda, Asuka Suzuki, Takanori Ohyoshi, Masaru Ueno*, Masahiro Uritani, Ayumu Yamamoto and Takashi Ushimaru#

Faculty of Science, Shizuoka University, Shizuoka University, Shizuoka 422-8529, Japan,

#Corresponding author:
Takashi Ushimaru
Department of Biological Science, Faculty of Science, Shizuoka University
Shizuoka 422-8529, Japan
FAX +81-54-238-0986
E-mail sbtushi@ipc.shizuoka.ac.jp

*Present address: Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan

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Including four Figures, Supplementary Table 1

Faithful chromosome segregation and thereby accurate gene transmission are crucial for all organisms. Until proper attachment of the mitotic spindle to the kinetochore is established, the ubiquitin ligase (E3) Cdc20-activated APC/C (anaphase promoting complex/cyclosome) is repressed by the spindle assembly checkpoint (SAC) and sister chromatin cohesion is protected [1, 2]. Mutants defective in SAC fail to arrest at metaphase even in the presence of damaged microtubules. Interestingly, a similar phenomenon occurs in yeast cells defective in Bub2, a negative factor of the mitotic exit network (MEN), which is required for telophase onset [2-5], although its precise molecular mechanism is unknown. Here, we show that chromosome missegregation occurs frequently in *bub2Δ* cells in the presence of damaged microtubules. The loss of Bub2 caused precocious activation of APC/C-Cdh1/Hct1 at pre-anaphase, leading to securin degradation and then separase-mediated cohesin cleavage. Overexpression of *CDH1* and *CDC14*, encoding Cdc14 phosphatase, at pre-anaphase similarly caused chromosome missegregation. Thus, sequential activation of APC/C-Cdc20 and then APC/C-Cdh1 is critical for precise chromosome segregation and precocious activation of APC/C-Cdh1 at pre-anaphase causes genomic instability. Since degradation of human securin is also mediated by APC/C-Cdc20 and APC/C-Cdh1, this study predicts that precocious activation APC/C-Cdh1 in human cells similarly causes genomic instability, and thereby cell death or tumorigenesis.

In the case of SAC mutants (*mad1-3*, *bub1*, and *bub3*), chromosome missegregation occurs frequently, causing genomic instability potentially leading to cell death or tumorigenesis [6, 7]. *BUB2* (*budding uninhibited by benzimidazole*) was originally identified by screening cells defective in metaphase arrest in the presence of microtubule damage, together with *BUB1* [8]. Although Bub2 negatively regulates MEN, *bub2*-deficient cells are hypersensitive to microtubule poisons, as also seen in SAC mutants [8]. Therefore, we first examined whether the *bub2* defect causes genomic instability. We monitored sister chromatid separation using the LacO/LacI-GFP (green

fluorescent protein) system [9], together with bulk chromosome segregation by 4',6-diamidino-2-phenylindole (DAPI) staining. Chromosome separation and segregation, as well as re-budding (index of mitotic exit), gradually increased in nocodazole-treated *bub2Δ* cells (Fig. 1a-c, e), as described previously [9-11]. Importantly, chromosome missegregation progressed simultaneously (Fig. 1a, d). This indicates that chromosome separation occurs with aberrant kinetochore-microtubule attachment, for example, mono-polar attachment, under these conditions. Overexpression of the MEN inhibitor Bfa1 completely suppressed these defects (Fig. 1a-e). This demonstrates that chromosome (mis)segregation in nocodazole-treated *bub2Δ* is due to MEN activation. This conclusion is supported by a finding that chromosome separation in nocodazole-treated *bub2Δ* cells is suppressed by depletion of the MEN factor Tem1 [11].

After DNA replication, sister chromosomes are physically linked by the cohesin complex. In the case of metazoa, the cohesin complex is dissociated from the chromosome via two distinct pathways: the bulk cohesin complexes on the chromosome arms dissociate at prophase in a separase-independent manner, and a residual cohesin complex at the centromere dissociates by separase-dependent Scc1 cleavage at anaphase onset [12, 13]. Separase activation requires APC/C-Cdc20-dependent securin (Pds1 in yeast) degradation. Although separase-independent cohesin dissociation has not been reported in yeasts, we suspected a possibility that Bub2 dysfunction may promote separase-independent cohesin dissociation, because deletion of *BUB2* overcomes the defect of sister chromatid separation in *cdc20-3* mutant cells [14]. To test this, we constructed *bub2Δ* cells carrying *GAL-SCC1-RRDD* conditionally expressing non-cleavable Scc1 [15]. Scc1-RRDD remarkably suppressed sister chromatid separation and segregation, but not re-budding, in *bub2Δ* (Fig. 1f, g). This demonstrates that *bub2Δ*-mediated sister chromatid separation is dependent on separase-mediated Scc1 cleavage.

The protein level of Pds1 decreased in nocodazole-treated *bub2Δ* cells [10] (data not shown). This suggests that Esp1 is released from Pds1 and activated to cleave Scc1 in the nocodazole-treated *bub2Δ* cells. However, Cdc20 depletion failed to suppress sister

chromatid separation (Fig. 2a, b) in the same manner as the *cdc20-3* mutation [14]. Consistently, the level of Pds1 was lower in the *bub2Δ* mutant, even in combination with *cdc20-3* [16]. Moreover, ectopic expression of *pds1-db* (*pds1* with mutated destruction/D box), which is not recognized by APC/C-Cdc20, did not effectively suppress chromosome separation and segregation in nocodazole-treated *bub2Δ* cells (Fig. 2a, b). Thus, sister chromatid separation in nocodazole-treated *bub2Δ* cells is not caused by APC/C-Cdc20-mediated Pds1 degradation.

Yeast cells have another APC/C complex, APC/C-Cdh1/Hct1, which is active from telophase to G1 phase [17, 18]. The activity of APC/C-Cdh1-dependent Pds1 degradation at G1 phase was detected [19]. APC/C-Cdh1 has a higher ubiquitination activity as compared to APC/C-Cdc20 towards Pds1 in vitro [20]. These findings prompted us to suspect a possibility that aberrant activation of APC/C-Cdh1 at pre-anaphase causes Pds1 degradation in nocodazole-treated *bub2Δ* cells, leading to cohesin cleavage-dependent sister chromatid separation. This possibility is feasible because these cells would causes MEN activation, leading to activation of protein phosphatase Cdc14 and in turn Cdh1. This was indeed the case: the loss of *CDH1* markedly repressed sister chromatid separation and bulk chromosome segregation in nocodazole-treated *bub2Δ* cells (Fig. 2a, b). Furthermore, ectopic expression of *pds1-dkb* (*pds1* with mutated D and KEN boxes) [20, 21], which is not ubiquitinated by APC/C-Cdh1, effectively repressed chromatin separation and segregation (Fig. 2a, b). These findings clearly indicate that APC/C-Cdh1-mediated Pds1 degradation is required for sister chromatid separation and bulk chromosome segregation in nocodazole-treated *bub2Δ* cells. Because APC/C-Cdh1 can still ubiquitinate Pds1-db via the KEN box, it is reasonable that expression of *pds1-db* partially suppressed sister chromatid separation and chromosome segregation. Thus, when Bub2 function is lost, cells cannot arrest in pre-anaphase even if the proper kinetochore-microtubule attachment is not established, because of APC/C-Cdh1 activation caused by aberrant activation of the MEN.

One would expect that SAC is still active in nocodazole-treated *bub2Δ* cells even after APC/C-Cdh1-promoted anaphase onset. We observed clear kinetochore

localization of Mad2 (index of SAC activation) in *bub2Δ* cells, as well as in wild-type cells (Fig. 2c, d). Importantly, the *bub2Δ*, but not the wild-type, strain showed two dots clearly separated from one another in a single cell. This indicates that Mad2 is still localized on the kinetochore after chromosome separation at anaphase. Thus, SAC appears to be proficient in the absence of Bub2 and sister chromatids were abnormally segregated despite SAC activation. The rate of two dots of Mad2 kinetochore localization (10%) was low, as compared to that of sister chromosome separation (41% in Fig. 1b). However, this observation is not so surprising as SAC becomes inactive after anaphase onset because the SAC component Mps1 kinase is degraded in a D-box-dependent manner [22].

Anaphase and telophase progressions are initiated by degradation of the securin Pds1 and the M-phase cyclin Clb2, respectively. In the nocodazole-treated *bub2Δ* cells, anaphase and telophase could start simultaneously because degradation of Pds1 and Clb2 starts upon APC/C-Cdh1 activation. One would suspect that this feature might cause anaphase to be skipped. However, the rates of chromosome separation and segregation were nearly the same as that of rebudding (Fig. 2b). This indicates that anaphase was completed before telophase progression. We confirmed this conclusion by another experiment. Intense chromosome compaction (condensation) of a large repetitive rDNA (encoding rRNA) array on chromosome XII is essential for separation of this region at early anaphase [23-25]. We monitored rDNA separation using the nucleolar protein Nop1, as the nucleolus is formed around the rDNA region. In the nocodazole-treated wild-type cells, the nucleolus was not segregated into sister cells, like bulk DNA (Fig. 4c). By contrast, the nucleolus segregated completely into both mother and daughter cells, together with the chromosomes in *bub2Δ* cells. This would be reasonable because Esp1 and Cdc14 were activated in nocodazole-treated *bub2Δ* cells and because rDNA compaction and separation requires the FEAR (Cdc fourteen early release) network, including Esp1 and Cdc14. Thus, APC/C-Cdh1 seems to be substituted for APC/C-Cdc20 as for anaphase progression (although the chromosomes are missegregated).

Why is anaphase not skipped in nocodazole-treated *bub2Δ* cells? We suspected that this resulted from the difference in degradation rates of Pds1 and Clb2. Indeed, when Pds1 degradation was repressed by ectopic expression of the non-degradable Pds1-dkb, sister chromatid separation and segregation, but not rebudding, were repressed; namely, some cells skipping anaphase appeared (Fig. 3a, b). Similar but inefficient effects were obtained when the degradation rate of Pds1 was retarded by expression of Pds1-db. Thus, probably because APC/C-Cdh1-mediated degradation of Pds1 is faster than that of Clb2, anaphase progresses before telophase in the nocodazole-treated *bub2Δ* cells, even if degradation of Pds1 and Clb2 start simultaneously (Fig. 3).

The idea that precocious activation of APC/C-Cdh1 at pre-anaphase promotes anaphase prompted us to anticipate that unscheduled activation of Cdc14 or Cdh1 at pre-anaphase would also lead to sister chromatid segregation accompanied by chromosome missegregation. This was indeed the case, where overexpression of *CDC14* or *CDH1* in pre-anaphase cells treated with nocodazole brought about chromosome separation and segregation and chromosome missegregation (Fig. 4). Furthermore, these *CDC14* overexpression-induced defects were cancelled by the loss of Cdh1 (Fig. 4c, d). These findings reveal the physiological meaning of Cdc14 sequestration to the nucleolus as an inactive form by the nucleolar protein Net1 until metaphase and could account for the fact that frequent chromosome loss occurs in *TAB6-1* (a dominant active allele of *CDC14*) and *tab2-1* (*net1-1*) mutants [26], in which affinity between Cdc14 and Net1 is decreased.

In this study, we showed that precocious activation of Cdh1 at pre-anaphase brings about genome instability. Importantly, microtubule damage can repress APC/C-Cdc20, but not APC/C-Cdh1, via SAC activation. Therefore, the MEN inhibitors (Bub2, Bfa1, and Ibd2) [27, 28] are also critical for the repression of securin degradation, as well as the SAC factors. This study strongly demonstrates that sequential activation of APC/C-Cdc20 and then APC/C-Cdh1 is critical for proper chromosome segregation. In the case of human cells, securin PTTG is similarly

degraded in a manner dependent on the D- and KEN-boxes via APC/C-Cdc20 and APC/C-Cdh1 [29, 30]. Furthermore, securin degradation was also observed in nocodazole-arrested human cells when *CDH1* was overexpressed [29]. Thus, it is likely that APC/C-Cdh1 dependent securin degradation is conserved in human cells. This predicts that precocious activation APC/C-Cdh1 may cause genomic instability and tumorigenesis in human cells.

Methods Summary

Strains, plasmids, and Media

S. cerevisiae strains and plasmids used are listed in Supplementary Table 1. Plasmids expressing *pds1-db* and *pds1-dkb* in bacteria (gifts from David Morgan) [21] were used as PCR templates to produce p416GAL1-pds1-db and p416GAL1-pds1-dkb. The composition of synthetic minimal medium (SD) complemented with appropriate nutrients for plasmid maintenance. SGalR and SRGly are identical to SD except that they contain 1% galactose plus 1% raffinose, and 2% galactose plus 3% glycerol instead of 2% glucose, respectively.

Microscope observations

Logarithmically growing cells expressing GFP-tagged proteins were fixed with 70% ethanol for 30 sec. After washing with distilled water, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) at 1 μg/ml for 15 min. Washed cells were viewed using an Olympus IX71-23FL/S microscope (100x objective) and a cooled charge-couple device (CCD) camera (ORCA-ER-1, Hamamatsu Photonics) connected to a Scanalytics Image Processor LuminaVision (Mitani Corp., Tokyo, Japan). For data shown in Fig. 2E, a Carl Zeiss Axio Imager M1 microscope (100x objective) and a cooled CCD camera (Carl Zeiss AxioCam MRm) were used.

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Author information

Correspondence and requests for materials should be addressed to T.U. (sbtushi@ipc.shizuoka.ac.jp).

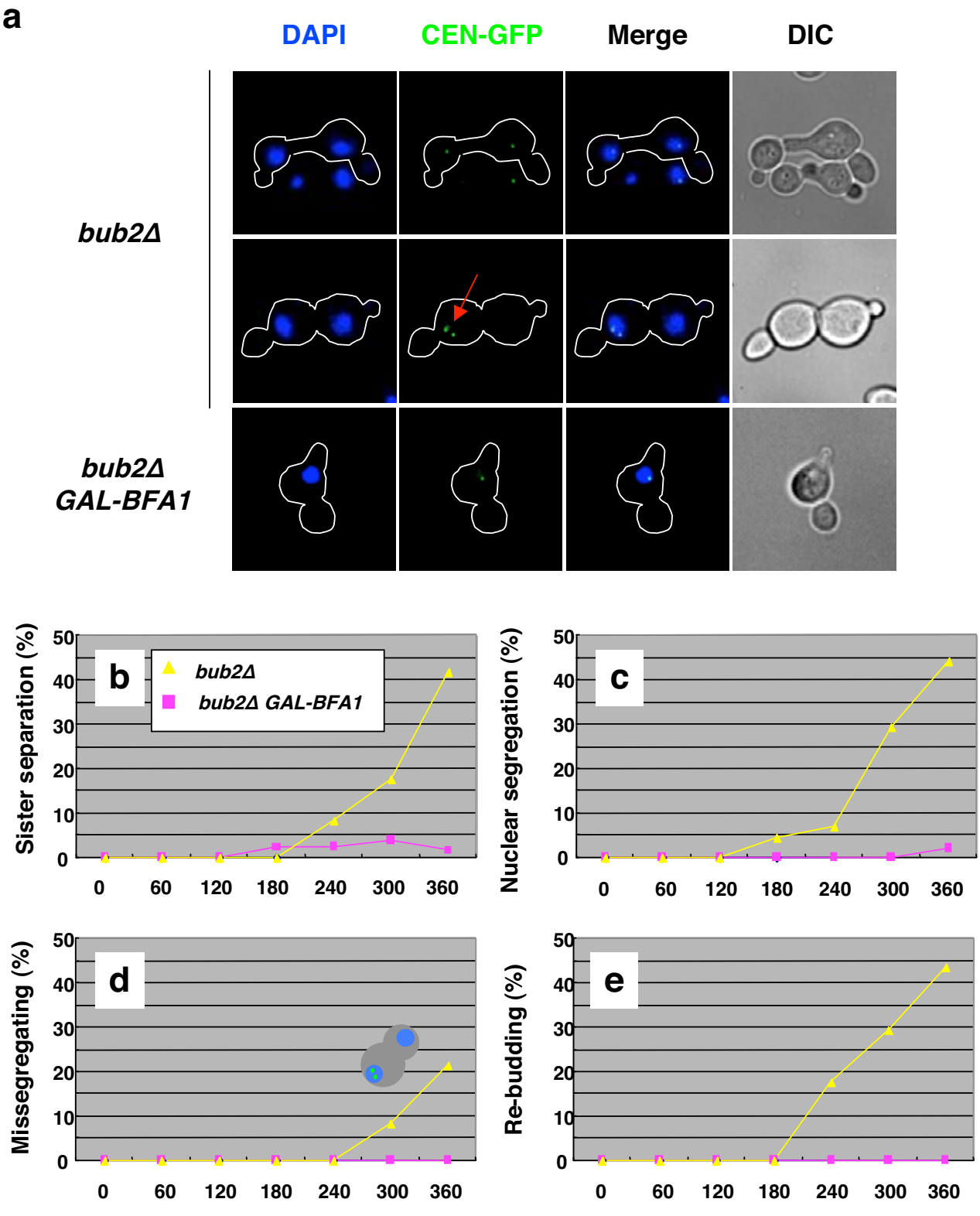
Figure legends

Figure 1. Chromosome missegregation occurs in nocodazole-treated *bub2Δ* cells in a manner dependent on MEN activation and separase-mediated Scc1 cleavage. (a-e) Cells of strains, *bub2Δ CEN4-GFP* (SCU397) and *bub2Δ CEN4-GFP GAL-BFA1* (SCU397 with plasmid pSCU896), were released from α-factor into nocodazole (15 μg/ml)-containing SGalR medium (time 0). The centromeres of chromosome IX in the cells were marked with green fluorescence protein (CEN4-GFP) and were monitored as an index of sister chromatid separation (b) and missegregation (d). Mass chromosome segregation was observed by staining with 4',6-diamidino-2-phenylindole (DAPI) (c). Re-budding, as a marker of MEN activation, was counted (e). Representative cells at the 6 h time point are shown in panel A. Cells with chromosome missegregation (Red arrow). (f, g) Scc1 cleavage by separase is required for sister chromatid separation and chromosome segregation in the nocodazole-treated *bub2Δ* cells. Cells of strain *bub2Δ CEN-GFP SCC1-RRDD* (SCU400) were released from α-factor into nocodazole-containing SGalR medium for 6 h. The data for *bub2Δ* cells was taken from (b), (c) and (e).

Figure 2. APC/C-Cdh1-dependent anaphase progression of nocodazole-treated *bub2Δ* cells. (a, b) Cells of strains *bub2Δ CEN-GFP MET3-CDC20* (SCU410), *bub2Δ CEN-GFP cdh1Δ* (SCU1336), *bub2Δ CEN-GFP GAL1-pds1-db* (SCU371 with plasmid pSCU1212), and *bub2Δ CEN-GFP GAL1-pds1-dkb* (SCU371 with plasmid pSCU1214) were released from α-factor into nocodazole-containing SGalR medium for 6 h. (c, d) Spindle assembly checkpoint (SAC) is active in the nocodazole-treated *bub2Δ* cells. Cells of strains *MAD2-GFP* (SCU1337) and *bub2Δ MAD2-GFP* (SCU1338) were released from α-factor into nocodazole-containing medium for 6 h. Dot-like kinetochore localization of GFP-tagged Mad2 was monitored and counted. (e) The nucleolus segregates in the nocodazole-treated *bub2Δ* cells. The nucleolus was observed using GFP-tagged Nop1.

Figure 3. A model for precocious activation of APC/C-Cdh1 in *bub2* mutant cells. For details, see text.

Figure 4. Overexpression of *CDH1* and *CDC14* at pre-anaphase causes chromosome missegregation. (a, b) Cells of strain *CEN4-GFP* (SCU396) harboring plasmid pGAL-CDH1 (pSCU878) or an empty vector (pSCU145) were released from a-factor into nocodazole-containing medium for 6 h. (c, d) Cells of strains *CEN4-GFP* (SCU396) and *cdh1 CEN4-GFP* (SCU1700) harboring plasmid pGAL-CDC14 (pSCU802) or an empty vector (pSCU134) were arrested in the nocodazole-containing SRGly medium and galactose was added for overexpression of *CDC14* for 3 h. When *CDC14* was overexpressed at G1 phase, cell cycles were arrested at G1 phase (data not shown).



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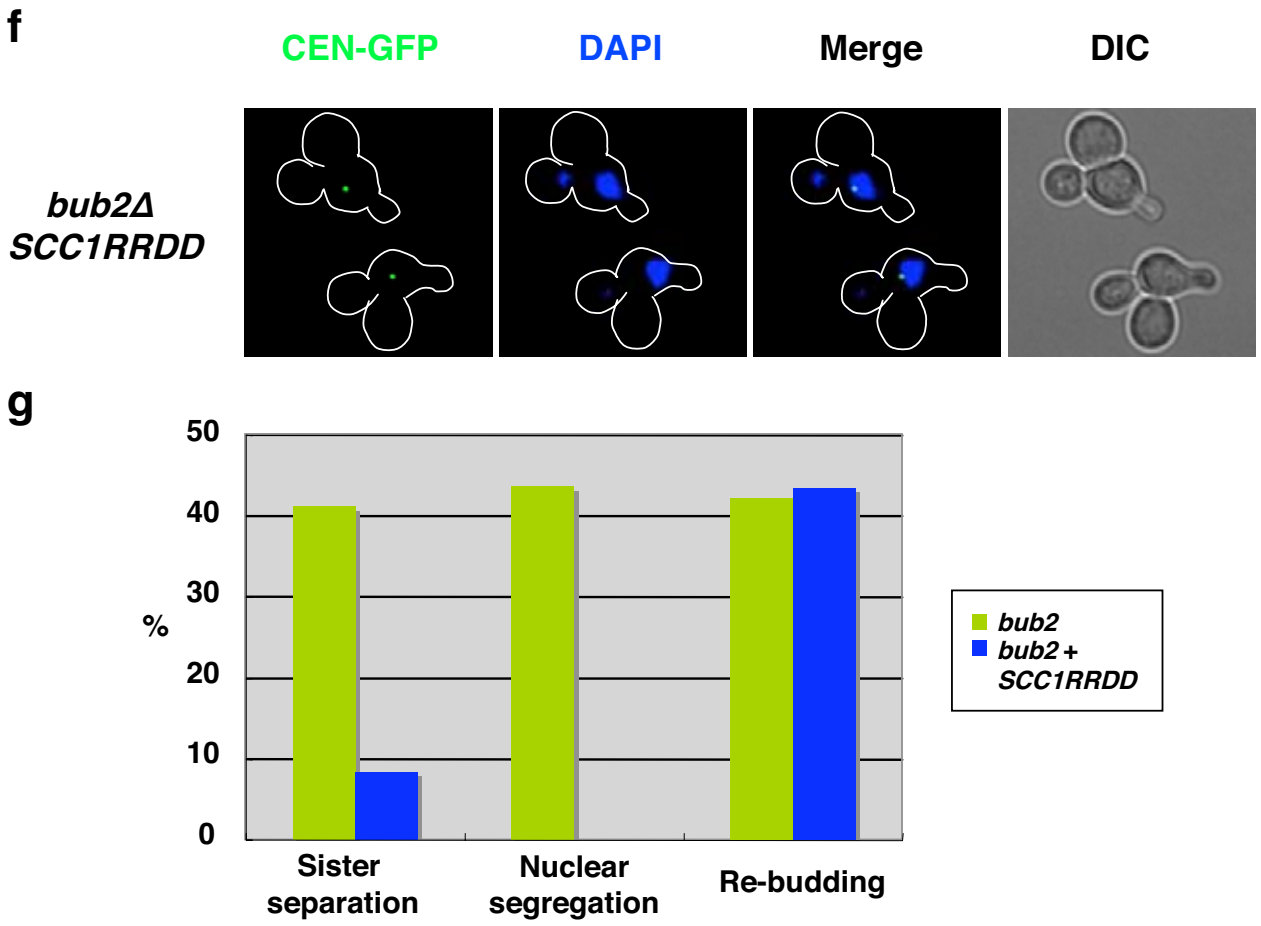

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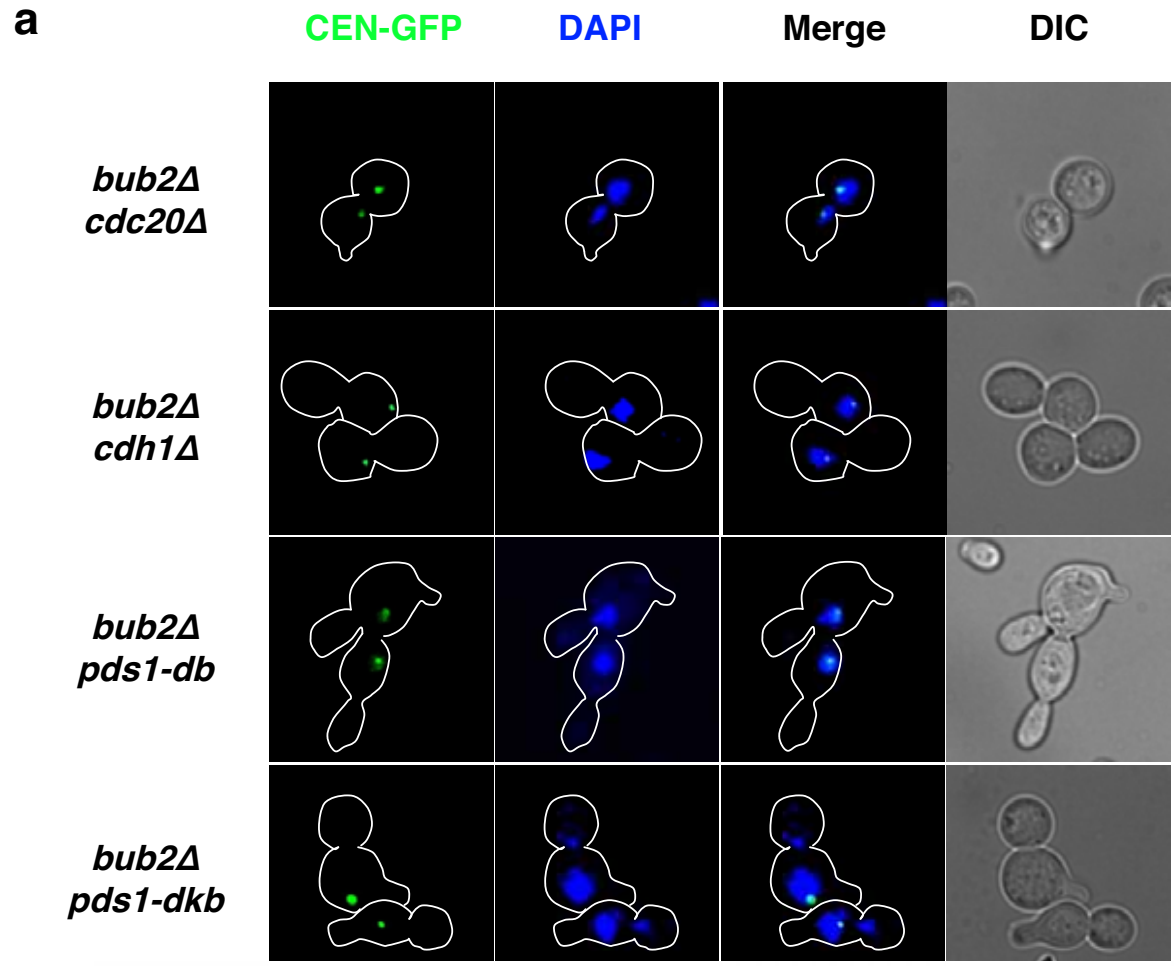

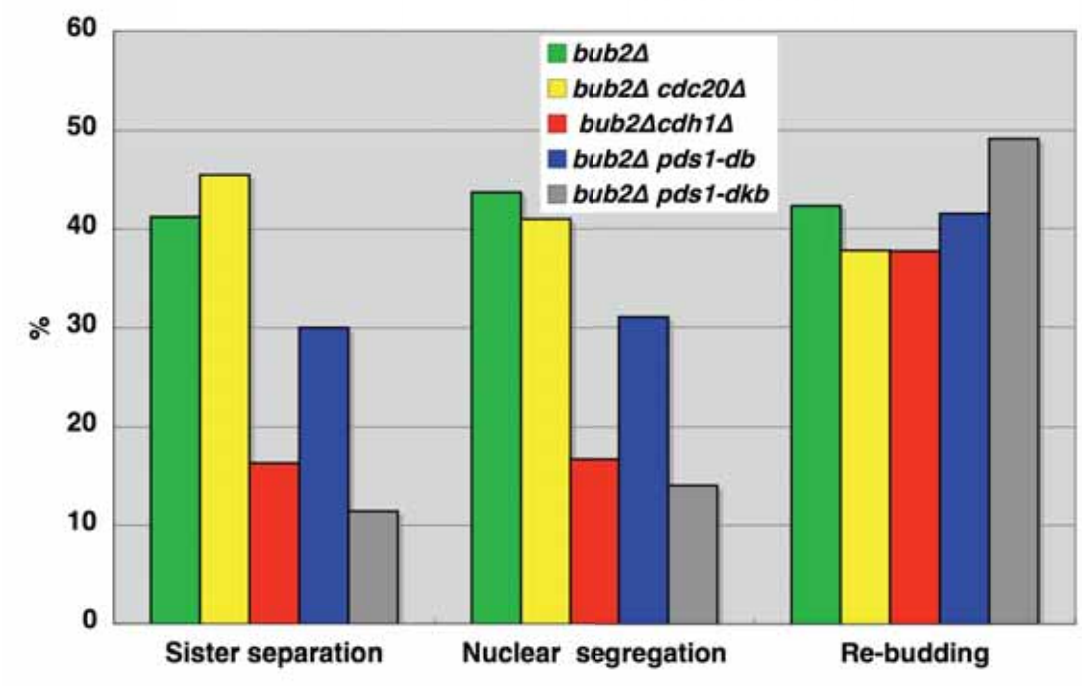

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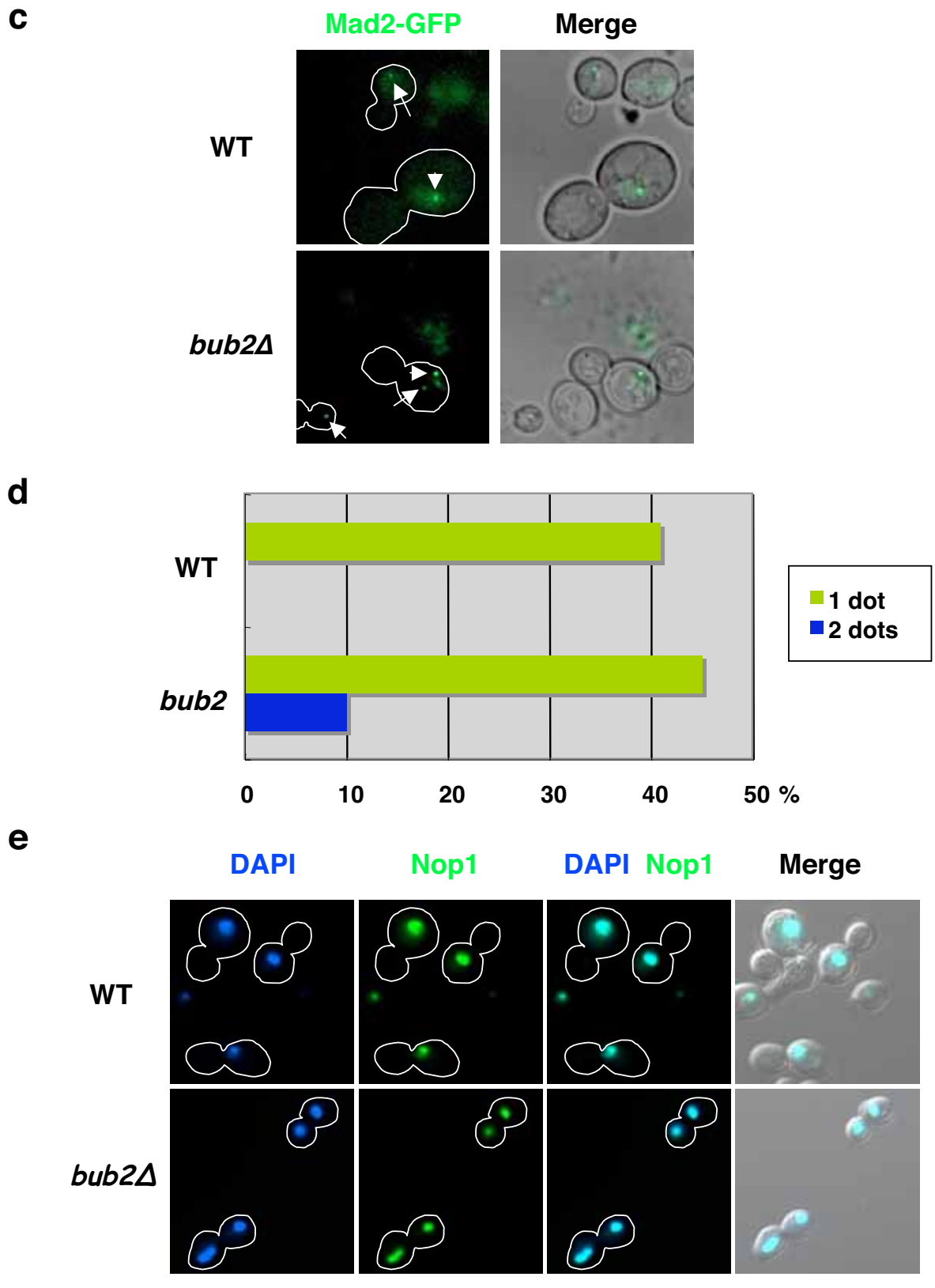

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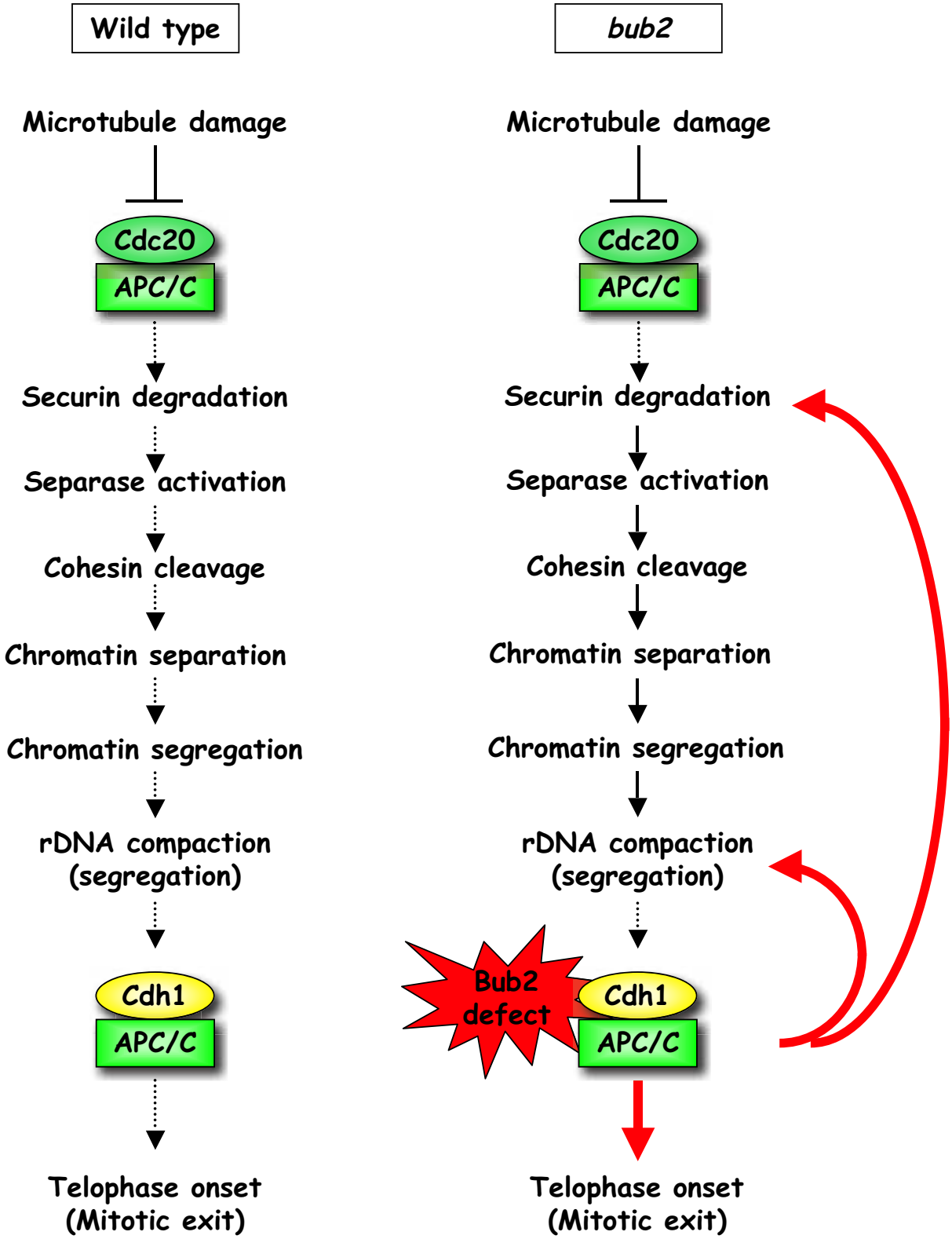

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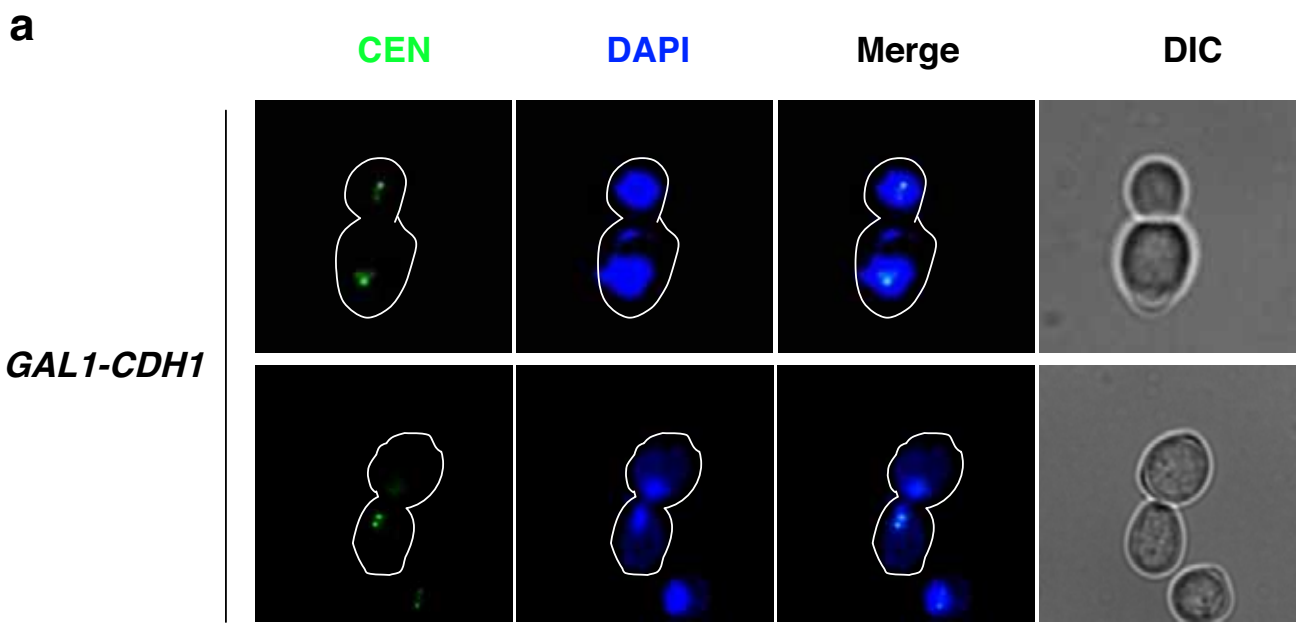

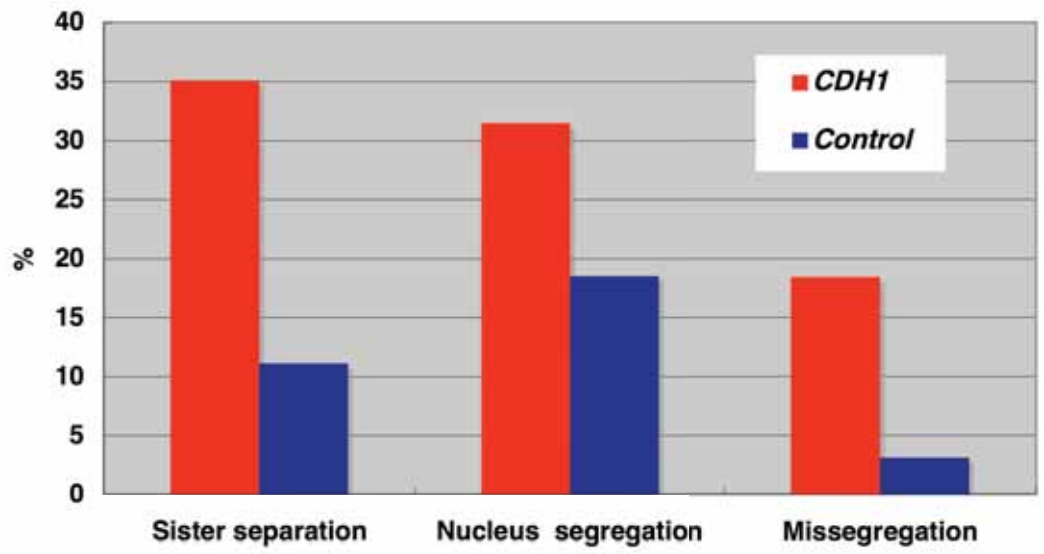

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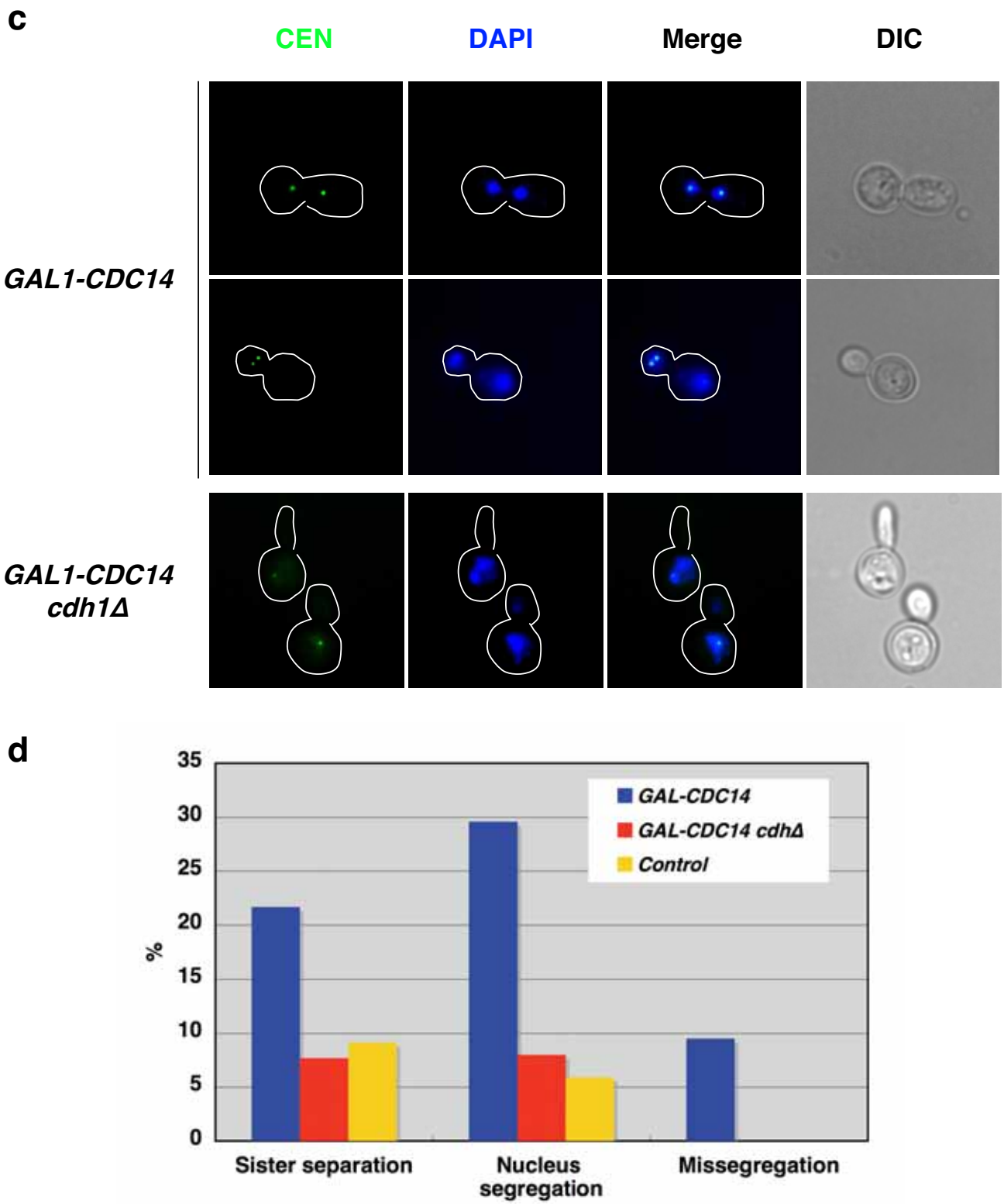

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Supplementary Table 1 Yeast strains and Plasmids

Name (Arias)	Description (Source)
Strains	
SCU15 (W303a)	*Mat**a** ura3 his3 leu2 trp1 ade2 can1* (Lab stock)
SCU893 (US356)	W303a *bar1:: hisG* (U. Surana)
SCU151 (*bub2Δ*)	SCU893 *bub2::hphMX4* (This study)
SCU396 (*CEN4-GFP*)	SCU893 *his3::GFP12-LacI12-NLS::HIS3 trp1::LacO-TRP1* (This study)
SCU397 (*bub2Δ CEN4-GFP*)	SCU396 *bub2::loxP* (This study)
SCU398 (*CEN3-GFP*)	SCU893 *ura3::tetO::URA3 leu2::tetR-GFP-NLS::LEU2* (This study)
SCU399 (*bub2Δ CEN3-GFP*)	SCU398 *bub2::hphMX* (This study)
SCU400 (*CEN4-GFP GAL-SCC1-RRDD*)	SCU396 *GAL1-SCC1(R180D/R268D)-HA3::LEU2* (This study)
SCU401(*bub2Δ CEN4-GFP GAL-SCC1-RRDD*)	SCU400 *bub2::loxP* (This study)
SCU407 (*PDS1-HA3*)	SCU893 *PDS1-HA3::URA3* (This study)
SCU408 (*bub2Δ PDS1-HA3*)	SCU151 *PDS1-HA3::URA3* (This study)
SCU409 (*MET3-CDC20 CEN3-GFP*)	SCU398 *MET3-CDC20::TRP1* (This study)
SCU410 (*bub2Δ MET3-CDC20 CEN3-GFP*)	SCU399 *MET3-CDC20::TRP1* (This study)
SCU1226 (*cdh1Δ CEN3-GFP*)	W303a *ura3::tetO::URA3 leu2::tetR-GFP-NLS::LEU2 cdh1::HIS3* [1]
SCU1228 (*cdh1Δ*)	SCU15 *MATa cdh1::kanR* [1]
SCU1700 (*cdh1Δ CEN4-GFP*)	SCU1228 *trp1::LacO:TRP1 his3::HIS3p-GFP13-LacI12NLS::HIS3* (This study)
SCU1336 (*bub2Δ cdh1Δ CEN3-GFP*)	SCU1226 *bub2::kanMX* (This study)
SCU1337 (*MAD2-GFP*)	SCU893 *mad2::kanM*X [pMAD2-GFP::URA3] (This study)
SCU1338 (*bub2Δ MAD2-GFP*)	SCU151 *mad2::kanMX* [pMAD2-GFP::URA3] (This study)
Plasmids	
pSCU896（pGAL1-BFA1）	*GAL1-BFA1 URA3 CEN* [2]
pSCU134 (p416GAL1)	*GAL1 URA3 CEN* [3]
pSCU145 (pRS414)	*TRP1 CEN* [4]
pSCU1212（p416GAL1-pds1-db）	*GAL1-PDS1* with mutated D-box *URA3 CEN* (This study)
pSCU1214（p416GAL1-pds1-dkb）	*GAL1-PDS1* with mutated D/KEN-box *URA3 CEN* (This study)
pSCU802（pGAL1-CDC14-His6）	*GAL-CDC14-His6 URA3 CEN* [5]
pSCU878 (pGAL-CDH1-GFP)	GAL-CDH1-GFP TRP1 CEN [6]
pSCU973 (pMAD2-GFP)	*MAD2-GFP URA3 CEN* [7]

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