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## Cdc14 targets the Holliday Junction resolvase Yen1 to the nucleus in early anaphase.

Comentario [Félix Mac1]: 96 caracteres con espacios (máximo es 100)

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Running title: **Cdc14 targets Yen1 to the nucleus.**

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## Abstract

The only canonical Holliday Junction (HJ) resolvase identified in eukaryotes thus far is Yen1/GEN1. Nevertheless, Yen1/GEN1 appears to have a minor role in HJ resolution and, instead, other structure-specific endonucleases (SSE) that recognize branched DNA play the leading roles. Among all SSEs, Mus81-Mms4/EME1 is the most important in budding yeast. Interestingly, cells tightly regulate the activity of each HJ resolvase during the yeast cell cycle. Thus, Mms4-Mus81 is activated in G2/M while Yen1 gets activated shortly afterwards [1]. Nevertheless, cytological studies have shown that Yen1 is sequestered out of the nucleus when CDK activity is high; i.e. all cell cycle but G1 [2]. We here show that the mitotic master phosphatase Cdc14 targets Yen1 to the nucleus in early anaphase. This nuclear localization is rather transient and no Yen1 signal is found in late anaphase. We will further show that this Cdc14-driven event back-ups Mms4-Mus81 in removing branched DNA structures which are especially found in the long chromosome arms upon replication stress. Our results highlight the essential role that early-activated Cdc14, i.e. FEAR network, has in removing all kind of non-proteinaceous linkages that might preclude faithful sister chromatid segregation in anaphase [3].

## Introduction

Joint molecules (JMs) comprise different branched DNA junctions that link chromatids through base-pairing during the cell cycle. JMs are formed as intermediate products during the repair of DNA double strand breaks (DSBs) through the homologous recombination pathway (HR). HR-dependent JM are also formed during the bypass of stalled replication forks (SRF) and postreplicative gaps, which may arise due to DNA base damage, interstrand crosslink, etc. According to the physical nature of the branched junction, the most important JM is believed to be the Holliday junction (HJ) [1]. Several combinations of this basic four-way structure give rise to other JMs with special features related to their processing. This is particularly important for three variants: the uninterrupted single and double HJ (sHJ and dHJ respectively), and the nicked HJ (nHJ). Central to HJs processing, eukaryotic cells possess one helicase-topoisomerase complex and three conserved structure-specific endonucleases (SSEs) [Schwartz & Heyer, 2011; Krejci, 2012; //Sgs1?]. The helicase-topoisomerase complex (hereafter refer to as STR) comprises the budding yeast helicase Sgs1 (Bloom's syndrome BLM in humans), the type I topoisomerase Top3 (TOPOIII $\alpha$ ) and the cofactor Rmi1 (RMI1-RMI2); whereas the three SSEs are Slx1-Slx4 (SLX1-SLX4), Mms4-Mus81 (EME1-MUS81) and Yen1 (GEN1). *In vitro*, STR can eliminate model dHJ by branch-migrating each sHJ towards each other in a process termed "dHJ dissolution" [2]. By contrast, SSEs cut HJs, albeit with different specificities. Thus, Yen1 and Slx1-Slx4 are mostly active against sHJs and Mms4-Mus81 prefers nHJs [Kaliraman, 2001; Fricke and Brill, 2003; Fricke et al., 2005; Ip et al., 2008; Matos, 2011]. *In vivo*, STR appears as the cell first and best choice in mitosis because dHJ dissolution always results in sequence exchange between chromatids (i.e., non-crossovers) [Ira, 2003; Wu & Hickson, 2003; Mimitou & Symington, 2009?]. As for the SSEs, cells choose them to promote crossovers when needing genetic variation in meiosis or when STR is presumed to fail in mitosis (e.g., sHJs and nHJs) [3]. Whereas a

bunch of studies have shown that disruption of different SSEs and the STR synergistically enhance the presence of JMs *in vivo* [///; Ho, 2010; Ashton, 2011; Mazon et al, 2012; Szakal, 2013], the question of why eukaryotes have three different SSE to process HJs remains open. This is more puzzling when just one SSE, the nHJ-resolvase Mus81-Mms4, appears to be sufficient to accomplish all HJ resolution []. Even more intriguing is the fact that the actual equivalent to the bacterial HJ resolvase RuvC, i.e. Yen1, has only a minor role in eukaryotes. In this context, several recent reports have defined the cell cycle regulation of STR, Mus81-Mms4 and Yen1 activities and found that, while STR is active all throughout, Mus81-Mms4 becomes active once cells are in G2/M, and Yen1 appears active shortly afterwards []. Mus81-Mms4 activity depends on both high cyclin-dependent kinase (CDK) activity and the activation of the Polo-like kinase Cdc5, which in turns takes place at /// []. Less is known about the Yen1 activity. Aside from being active when purified from cells transiting in the M-phase [], Yen1 contains a nuclear location signal (NLS) which is masked by phosphorylation through Cyclin-dependent kinase activity (CDK) []. Thus, Yen1 is nuclear when CDK is low, i.e. G1, and cytoplasmic when CDK is high, i.e. S/G2/M. This implies an intriguing paradox since Yen1 appears active against HJ *in vitro* when it is actually sequestered out of the nucleus *in vivo*.

CDK-mediated phosphorylation can be reverted in targeted proteins through three different, though not necessarily exclusive, manners. First, when all CDK drops at the very end of mitosis *de novo* CDK-targeted proteins would remain unphosphorylated. This is particularly effective in those proteins with a rapid turnover. Secondly, the pool of different cyclins which substitute one another to maintain high CDK from G1 to telophase can also change the specific substrates for CDK-mediated phosphorylation. Lastly, specific phosphatases can revert CDK-dependent phosphorylation. When coupled to the lowering of CDK, these phosphatases accomplish that even stable proteins remain unphosphorylated. The master phosphatase that removes the phosphate

from the CDK consensus site in *S. cerevisiae* is Cdc14. Sequestered through most of the cell cycle within the nucleolus, Cdc14 is released in two waves at the end of mitosis. The first wave takes place shortly after the anaphase onset and mediates dephosphorylation of selected CDK-targeted proteins. This wave is also known as FEAR, and stands for Cdc14 early anaphase release. The FEAR network co-ordinates precisely critical events that take place during anaphase; i.e., lengthening of the mitotic spindle, topological unlinkage of sister chromatids, control of the forces that pull apart sister chromatids, correct positioning of the Spindle Pole Bodies (SPBs) in each daughter cell, etc. The second wave triggers the Mitotic Exit Network (MEN), which is responsible for switching off CDK, cytokinesis and the transition to a new G1.

The nuclear location of Yen1 is a prerequisite for its HJ activity since budding yeast carried out a close mitosis, i.e. the nuclear membrane is not dismantled in mitosis. Hence, in this work, we aimed to determine if Yen1 was ever targeted to the nucleus out of G1, particularly in anaphase; and if so, for how long and whether Cdc14 was important for this targeting *in vivo*. We indeed found that Yen1 transiently shuttles from the cytoplasm to the nucleus in early anaphase and that this is carried out through the activation of Cdc14. We further show that Cdc14 is epistatic to Yen1 in terms of JM resolution in the absence of Mus81-Mms4 activity.

### **///to result///**

Correspondingly, the *cdc14-1* thermosensitive allele and the *yen1* null mutant show similar defects in terms of resolution of DNA-mediated linkages at the restrictive temperature.

The ~2C DNA content of the *cdc15-2* and *cdc14-1* arrest for these mutants suggests that if stalled RFs were responsible for the linkages they should be in very close convergent proximity.

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### **Results and Discussion.**

We tried to address the issue of Yen1 subcellular localization by fluorescence microscopy through tagging the YEN1 gene with the GFP. In order to follow the dynamics of such localization, we performed time-course experiments during one synchronous G1-to-telophase cell cycle. To block cells in telophase, before the CDK drops, we made use of a thermosensitive conditional allele for the critical Mitotic Exit Network (MEN) component Cdc15 (i.e., *cdc15-2*). To precisely follow up anaphase we further tagged the SPB component Spc42 with RedStar fluorescent protein. We first tagged the GFP gene at the 3' end of YEN1 gene, which thus was still under the control of its native promoter. However, we were unable to see any fluorescence under the microscope, or was difficult to subtract the specific signal from that of the background ([data not shown](#)). This is likely due to the fact that Yen1 seems to be present at low concentrations within the cell [ ]. We next placed YEN1 under the control of the strong GAL promoter and tagged GFP at the N-terminal. This same strategy was used to demonstrate G1-specific nuclear location of Yen1 previously [ ]. When we induced GAL-GFP:YEN1 in an asynchronous culture we found the same previously reported location pattern; i.e. nuclear in unbudded cells and cytosolic (or absent) in budded cells. Strikingly though, when we tried to grow this strain on galactose plates, we found that GFP-Yen1 was non-functional; i.e., a GFP:YEN1 *mms4D* strain grew as bad as a *yen1D mms4D* double mutant in the presence of exogenously induced DNA damage by MMS ([data not shown](#)). Finally we tried to construct the GAL-YEN1:GFP strain, with YEN1 under the control of the GAL promoter and GFP tagged at the C-terminal. Unlike the two other previous constructs, we managed to easily see Yen1-GFP under the microscope and this chimera appears functional in the growth assay with MMS ([Figure 1](#)). As for the location pattern, galactose induction in the asynchronous culture yields

also a strong nuclear signal in unbudded cells and a much weaker and diffuse signal in budded cells. In order to better follow up the dynamics of Yen1-GFP we synchronized cells in G1 before inducing the tagged gene, then induced the gene in galactose while keeping the cells in G1, and finally released all cells into a synchronous cell cycle. In order to avoid interference with de novo synthesis of Yen1-GFP out of G1, we switched the gene off at the time of the G1 release. Finally, the culture temperature was also switched to 37°C in order to inactivate Cdc15-2 and allow a final block in telophase; i.e., a G1-to-telophase synchronous cell cycle. Thus, we observed that Yen1-GFP was nuclear at the G1 arrest but rapidly disappeared from the nucleus upon S-phase entry (Figure 2A). This pattern is reminiscent of what has been previously reported for the GAL-driven GFP-Yen1 [1], which appeared non-functional in our background. Interestingly, there was a short window at about 100-120 minutes after the G1 release where we observed Yen1-GFP coming back to the nucleus when this was stretched between the mother and the bud (Figure 2A). This new nuclear location peaked when the distance between the SPBs was between 3-6  $\mu\text{m}$ , which roughly corresponds to early anaphase (Figure 2B). When cells reached late anaphase/telophase; SPB distances  $> 8 \mu\text{m}$ , this nuclear Yen1-GFP signal faded away greatly. As mentioned above, Yen1 is a HJ resolvase and steady-state levels of HJ can be increased by making cells to use HR more often. Thus, we also follow a synchronous cell cycle upon continuous low levels of MMS (0.004% v/v). This low MMS concentration interfered little with the cell cycle progression in the *cdc15-2*, *cdc15-2 yen1D*, and *cdc15-2 GAL-YEN1:GFP* strains (data not shown). Remarkably, the same relocalization pattern was observed upon MMS treatment, although the Yen1-GFP nuclear signal appeared more granulated or even forming distinct foci (Figure 2A).

The nuclear relocalization signal of Yen1 in early anaphase prompted us to check whether Cdc14 might be targeting Yen1 to the nucleus through the FEAR network. As described above, Yen1 bears a CDK consensus site within its NLS region.

Phosphorylation of such site masks the NLS and keeps Yen1 out of the nucleus []. Besides, Yen1 gets widely dephosphorylated around G2/M [Matos, 2011 (Cell)], and Cdc14 is the master anti-CDK phosphatase that acts in that period of the cell cycle []. In order to test the Cdc14-driven relocalization hypothesis, we performed the same cell cycle-dependent Yen1-GFP localization in a *cdc14-1* background at 37°C. Upon Yen1-GFP overexpression in G1, its nuclear localization was equivalent to that of *cdc15-2*, and this location was lost in a similar way after entering S-phase. However, no nuclear relocalization was seen throughout, not even when MMS was present (Figure 2). This indicates that Cdc14 plays a key role in targeting Yen1 to the nucleus in early anaphase. Our results also point out that Cdc14-driven Yen1 relocalization occurs always in every cell cycle (i.e., this relocalization does not necessarily depend on induced DNA damage).

In order to see whether the regulation of Cdc14 over Yen1 was important to prevent and/or resolve HJs, we looked at the presence of JMs in the *cdc14-1* background by PFGE. Sister chromatids with DNA-mediated linkages (i.e., JMs or persistent SRFs) are known to not enter a PFGE [///, Ho, 2010 (Mol Cell)]. Thus, we observed that large chromosomes such as chromosome XII (cXII) and IV (cIV) were less visible in the *cdc15-2 mms4Δ yen1Δ* strain upon MMS treatment than in any other mutant combinations for *cdc15-2* (Figure 3A). A Southern blot with a probe against the longest chromosome, cXII, showed that most of it got trapped in the well. Like in the *cdc15-2* reference, most chromosomes were visible in a *cdc14-1* telophase block, even when DNA damage was induced with MMS (Figure 3A). Strikingly, cXII was barely visible in the *cdc14-1 mms4Δ* strain upon DNA damage, in a pattern very similar to *cdc15-2 mms4Δ yen1Δ*. However, *cdc14-1 yen1Δ* was not different to just *cdc14-1*, nor was *cdc14-1 mms4Δ yen1Δ* to *cdc14-1 mms4Δ*. Lastly, to make sure that this PFGE well retention was not due to stalled replication in the *cdc14-2 mms4D* mutant, we followed bulk DNA replication in all mutants and found no differences (Figure 3B). In



all cases, replication was completed by 90 minutes after G1 release (i.e., 2.5h before we took the samples for PFGE analysis). Therefore, all these findings fit well with Cdc14 having a role in JM resolution through Yen1. Besides, this Yen1 activity is complementary to that of Mms4-Mus81, which in turn is independent of Cdc14.

The data we present herein showed that Yen1 is transiently targeted to the nucleus in early anaphase and this event depends on Cdc14. Taking into account that Mms4-Mus81 has been reported to mainly act in G2/M [1], Yen1 may therefore serve as a last-resource backup JM-resolving pathway that operates in early anaphase. Another interesting possibility is that, by restricting Yen1 to anaphase, the spindle pulling forces (or the lack of cohesin) may give directionality to the resolution of HJ towards a less toxic or genetic-compromising outcome. The observed regulation of Cdc14 over Yen1 extends the key role of the former in preventing all types of non-proteinaceous sister chromatid linkages (i.e., catenations and DNA-mediated) that might form a bridge between segregating chromosomes in anaphase [2].

## **Material and Methods.**

### **Yeast strains, growth and experimental conditions.**

All yeast strains used in this work come from the S288C background and are listed in [Table 1](#). N- and C-terminal tagging with yeGFP or RedStar, gene deletions, marker swap, gene deletions and allele/promotor replacements were engineered through PCR strategies [[Janke et al., 2004](#)]. All strains were grown overnight in air orbital incubators at 25°C in YP raffinose media (///) supplemented with adenine /// unless stated otherwise. G1 blocks were performed by incubating cultures at  $OD_{660}/ml=0.5$  with 50 ng/ml of alpha-factor (all tested strains were *bar1Δ*) for 3h. Yen1-GFP was then induced for 2h by adding Galactose 2% (w/v). The induction was monitored by fluorescence microscopy. The G1-to-telophase synchronous cell cycle was performed as follows: G1-blocked cells with induced Yen1-GFP were released from the block by first washing twice with YPD (glucose 2%, stops Yen1-GFP production); resuspending in freshed YPD supplemented with adenine /// and pronase E ///; and incubating at 37°C for 4h. In the instances where DNA damage was exogenously induced, 0.004 % (v/v) MMS was added at the time of the G1 release. MMS was kept in the media until the telophase arrest. Throughout the G1-to-telophase cell cycle, samples were taken at different time points for further analysis. Error bars in graphs represent the standard error of the mean (SEM).

### **Fluorescence microscopy.**

Fluorescent proteins were analyzed by wide-field fluorescence microscopy as previously reported [Quevedo, 2012]. Synchrony was determined by cell morphology and segregation of the nucleus. In order to follow up segregation of the nucleus, an aliquot was frozen at -20°C for 48h before DNA was stained using DAPI at 4 µg/ml final concentration after short cell treatment with 1% v/v Triton X-100. Yen1-GFP and Spc42-RedStar were determined directly in cells freshly harvested. Scale bars in micrographs depict 5 µm. ///

#### **Pulse Field Gel Electrophoresis (PFGE), Southern blots and FACS analysis.**

PFGE to see all yeast chromosomes was performed using a CHEF DR-III system (Bio-Rad) in a 0.8% agarose gel in 0.5× TBE buffer and run at 12°C for 20 h at 6 V/cm with an initial switching time of 80 seconds, a final of 150 seconds, and an angle of 120°. Yeast chromosomes were photographed after staining with ethidium bromide. Southern blot was carried out by a saline downwards transference onto positively-charge membranes (Roche) followed by hybridization with a fluorescein-labelled probe targeted to the NTS2 region within the rDNA unit.

Flow cytometry analysis was carried out as previously described in order to follow up bulk DNA replication [Quevedo, 2012].

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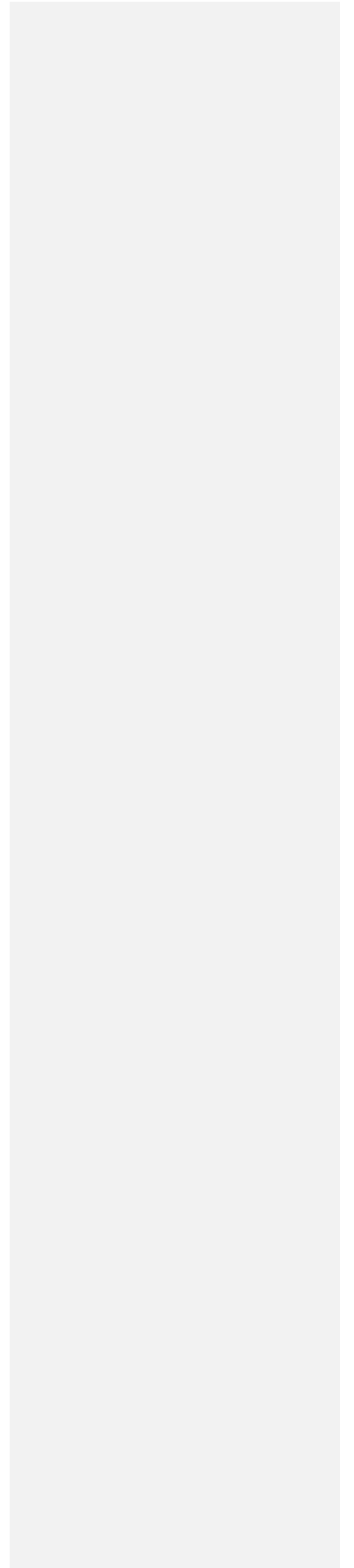
We thank Steven West for sharing some unpublished data with us. We also thank other members of both labs for help and discussion. Authors contributed as follows: JGL has performed most of the experimental work. ACB ///. FM, LA and JGL has planned and supervised this work. FM has written the paper. LA hosted JGL in his lab for learning methods related to this work.

Authors declare no competing financial interests

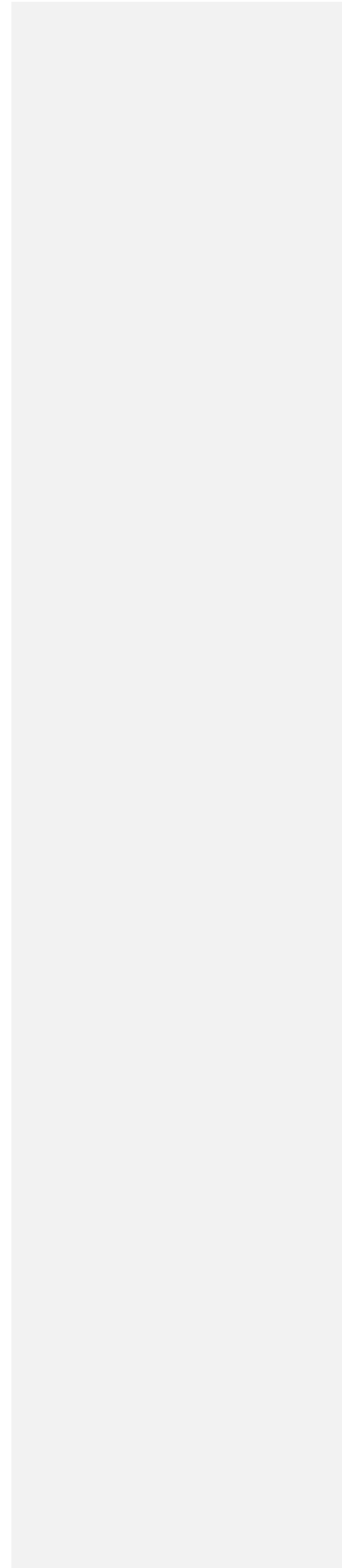
Correspondence and requests for materials should be addressed to F.M.

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**References.**



**Figure 1. Overexpressed Yen1-GFP suppresses MMS hypersensitivity of the *mms4Δ yen1Δ* strain.** Overnight cultures of the strain FM1292 (*cdc15-2*) plus derivatives of it carrying *mms4Δ*, *mms4Δ yen1Δ*, *GAL:YEN1:GFP* and *mms4Δ GAL:YEN1:GFP* were serial diluted and plated onto four different plates; YPD, YPGal, and either media further supplemented with MMS 0.004% (v/v). Pictures to determine ability to grow were taken after /// days of incubation at 25°C.



**Figure 2. Yen1 transiently re-localizes back to the nucleus in early anaphase in a Cdc14-dependent manner.** Overnight YP raffinose cultures of the strains FM1/// (*cdc15-2 GAL:YEN1:GFP SPC42:RedStar*) and FM1/// (*cdc14-1 GAL:YEN1:GFP SPC42:RedStar*) were first arrested in G1 for 3h in the grown media. Next, galactose 2% (w/v) was added and the culture was left in the G1-blocked for another 2h. They then were split into two fresh YPD media, one of them containing MMS 0.004% (v/v), and finally released into a synchronous cell cycle at 37°C for 4h. **A.** Representative micrographs of z-stack maximum projections from the G1 blocks after 2h of galactose addition (G1), and about /// minutes after the G1 release at 37°C with or without MMS. (m) points to an S/G2 cell with an unaligned ~2 μm spindle (i.e., distance between SPBs) and no nuclear Yen1-GFP signal. (a) points to examples of cells in early anaphase according to spindle orientation and length, all with a visible nuclear Yen1. (t) points to a cell already blocked in telophase with no nuclear Yen1 signal. **B.** Percentage of cells with nuclear Yen1-GFP relative to the distance between SPBs (mean ± SEM, n=3). Only budded cells with two SPBs were counted.

**Figure 3. Yen1 and Cdc14 are epistatic in resolving Joint Molecules in the absence of Mms4-Mus81.** Overnight YPD cultures of the strains FM1292 (*cdc15-2*) and FM1292 (*cdc14-1*), knocked out single mutant derivatives of them for *MMS4* and *YEN1*, and the double mutants *mms4* $\Delta$  *yen1* $\Delta$  were first arrested in G1 at 25°C. They were then split into two media, one of them containing MMS 0.004% (v/v), and finally released into a synchronous cell cycle at 37°C for 4 h. **A.** Pulse Field Gel Electrophoresis of all yeast chromosomes at the telophase blocks. Upper pictures: gel after ethidium bromide staining. Lower pictures: Southern blot of that gel against a chromosome XII probe. **B.** Flow cytometry analysis of the DNA content of the G1-to-telophase time-course for the *cdc14-1* strains.



**Table 1. Strains used in this work. Parental strain is also included.**

Strain name	Relevant genotype	Reference
AS499	<i>MATa bar1Δ leu2-3,112 ura3-52 his3-Δ200 trp1-Δ63 ade2-1 lys2-801 pep4</i>	A. Strunnikov
FM1292	AS499; <i>cdc15-2:9myc:Hph</i>	This work
FM//	FM1292; <i>mms4Δ:://</i>	This work
FM//	FM1292; <i>mms4Δ:://; yen1Δ:://</i>	This work
FM//	FM1292; <i>NatNT2:GAL1:YEN1:://; SPC42:RedStar:KanMX</i>	This work
FM//	FM1292; <i>mms4Δ:://; NatNT2:GAL1:YEN1:://; SPC42:RedStar:KanMX</i>	This work
FM//	AS499; <i>cdc14-1:9myc:TRP1</i>	This work
FM//	FM1272; <i>mms4Δ:://</i>	This work
FM//	FM1272; <i>mms4Δ:://; yen1Δ:://</i>	This work
FM//	FM1272; <i>NatNT2:GAL1:YEN1:://; SPC42:RedStar:KanMX</i>	This work
FM//	FM1272; <i>mms4Δ:://; NatNT2:GAL1:YEN1:://; SPC42:RedStar:KanMX</i>	This work

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