Checkpoint Regulation of Nuclear Tos4 Defines S Phase Arrest in Fission Yeast

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ABSTRACT From yeast to humans, the cell cycle is tightly controlled by regulatory networks that regulate cell proliferation and can be monitored by dynamic visual markers in living cells. We have observed S phase progression by monitoring nuclear accumulation of the FHA-containing DNA binding protein Tos4, which is expressed in the G1/S phase transition. We use Tos4 localization to distinguish three classes of DNA replication mutants: those that arrest with an apparent 1C DNA content and accumulate Tos4 at the restrictive temperature; those that arrest with an apparent 2C DNA content, that do not accumulate Tos4; and those that proceed into mitosis despite a 1C DNA content, again without Tos4 accumulation. Our data indicate that Tos4 localization in these conditions is responsive to checkpoint kinases, with activation of the Cds1 checkpoint kinase promoting Tos4 retention in the nucleus, and activation of the Chk1 damage checkpoint promoting its turnover. Tos4 localization therefore allows us to monitor checkpoint-dependent activation that responds to replication failure in early vs. late S phase.

KEYWORDS Checkpoint Cell cycle Tos4 fission yeast S phase

The cell cycle proceeds through a rhythmic pattern of oscillators driven by cell-cycle specific transcription, patterns of protein modification, and protein degradation (reviewed in (Bertoli et al. 2013; Malumbres 2014; Alber et al. 2019)). Fission yeast is an important model system for studying cell cycle dynamics and genome stability. The rod-shaped cells are divided by medial fission with distinct cell morphologies (Piel and Tran 2009). Typically, mitosis is completed and S phase begins when cells are in a binucleate stage, prior to septation (Gomez and Forsburg 2004; Peng et al. 2005; Piel and Tran 2009). Thus, new-born cells are considered to be in late S to G2 phase, while S phase begins in binucleates (MacNeill and Fantet 1997). Distinguishing early from late S phase is typically done by monitoring nuclear DNA content by methods such as FACS or BrdU (Hodson et al. 2003; Sabatinos and Forsburg 2015b). Isotopic labeling methods suggest that the bulk of DNA synthesis is complete in a short time, leading to the conclusion that S phase is quite short and G2 phase extended (Nasmyth et al. 1979).

However, many replication mutants in fission yeast show an approximately 2C DNA content upon cell cycle arrest; based on genetic studies, this has been proposed to be late S phase (e.g., (Nurse et al. 1976; Nasmyth and Nurse 1981; Coxon et al. 1992; Forsburg and Nurse 1994)). Whether this arrest represents failure to duplicate specific late regions remains to be seen. Generally, late-replicating genome regions show increased prevalence of mutations and fragile sites (Le Beau et al. 1998; Stamatoianopoulos et al. 2009; Lang and Murray 2011). Very late DNA replication has been observed, even into M phase for repair synthesis (Widrow et al. 1998; Bergoglio et al. 2013; Minocherhomji et al. 2015). Indeed, models of replication stress increasingly suggest the issue is not within early S phase but disruptions of chromosome segregation during mitosis (Zeman and Cimprich 2014; Minocherhomji et al. 2015; Zhang et al. 2019).

We are interested in identifying early S phase cells and distinguishing them from late S phase or G2. Recent advances in live cell imaging have been accompanied by developing markers that are specific to particular cell cycle compartments. For example, the FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator) system has been deployed using tagged, ubiquitylated proteins that are specific to G1/S or G2 cells (Sakaue-Sawano et al. 2008) and further refined by additional markers specific to G0 (Oki et al. 2014) or to multiple cell cycle phases (Bajar et al. 2016). These proteins vary temporally and spatially, giving a snapshot of cells in a particular cell cycle phase. There are excellent markers for mitotic landmarks including fluoresceinly tagged spindle...
pole body protein Sad1 (King and Drivas 2008) or tubulin (Savin and Tran 2006), and septation is easily examined under light microscopy (Tran 2006), and septation is easily examined under light microscopy and fluorescent tagged RPA and Rad52 proteins (Sabatinos et al. 2012; Smolka et al. 2012). It is known to be localized to the spindle pole body protein Sad1 (King and Drivas 2008) or tubulin (Sawin and Drivas 2008; King and Drivas 2008).

The forkhead-associated domain (FHA)-containing DNA binding protein Tos4 is conserved in budding and fission yeast (Y. P. Jang et al. 2010; Hayles et al. 2013) which is phosphorylated during the G1/S phase (Swaffer et al., 2016). Overproduction leads to cell cycle delay (Vachon et al., 2013). Of particularly interest to us, Tos4-GFP shows periodic accumulation in the nucleus coincident with S phase, consistent with its known regulation and the maturation timing of GFP (Kiang et al. 2009; Bastos de Oliveira et al. 2012). In fission yeast, Tos4 is a non-essential protein with relatively minor phenotypes (Kim et al., 2010; Hayles et al. 2013) which is phosphorylated during the G1/S phase (Swaffer et al., 2016). Overproduction leads to cell cycle delay (Vachon et al., 2013). Of particularly interest to us, Tos4-GFP shows periodic accumulation in the nucleus coincident with S phase, consistent with its known regulation and the maturation timing of GFP (Kiang et al. 2009; Bastos de Oliveira et al. 2012; Escorcia et al. 2019; Shen and Forsburg 2019). In fission yeast, this has been exploited in studies of cyclical re-replication induced by cyclin inhibition (Kiang et al. 2010).

In this study, we characterize Tos4-GFP as a dynamic marker for S phase and determine its response to a variety of replication stresses, using both fluorescence microscopy and flow cytometry. We observe consistent timing of Tos4 accumulation relative to SPB duplication and septation in wild type cells. Tos4 persists in the nucleus of cells arrested in S phase by hydroxyurea (HU) or cell cycle mutant cdc22-M45, treatments which activate the replication checkpoint kinase Cds1. Consistent with this, accumulation of nuclear Tos4 requires Cds1, kinase activity, and the FHA domain. Surprisingly, however, replication mutants that show presumed late S phase arrest lack nuclear Tos4. This suggests that Tos4 specifically delineates an early stage of S phase and leads to the possibility that “late S phase” defined by replication mutants overlaps with what we commonly call G2 phase in which low yet detectable levels of DNA synthesis is occurring (Kelly and Callegari 2019).

### MATERIALS AND METHODS

#### Yeast strains and media

*S. pombe* strains (Table 1) were grown in supplemented Edinburgh minimal medium (EMM) for live cell imaging, Western blot, and flow cytometry. Cells were treated with 12 mM hydroxyurea (HU, Sigma), incubated at 36°C for 4 h, or pre-treated with 12 mM HU for 2 h at 25°C and then incubated at 36°C for 4 h.

#### Live-cell microscopy

Cells cultured in supplemented EMM media were placed on 2% agarose pads sealed with VaLaP (1/1/1 [wt/wt/wt] Vaseline/lanolin/parafin) for live-cell imaging. Images were acquired using a DeltaVision fluorescence microscope (with softWoRx version 4.1; GE, Issaquah, WA) using a 60x (NA 1.4 PlanApo) lens, solid-state illuminator, and 12-bit CCD camera. Images were deconvolved and maximum intensity projected for fluorescence images (softWoRX) and transmitted light images were inverted and added for outline of the cells (Image) (Schindelin et al. 2012).

#### Western blot

Proteins extracts were prepared from equal number of Tos4-GFP cells in asynchronous culture grown in supplemented EMM media, after treatment with 12 mM hydroxyurea (HU), and after washing twice with media for release from HU. Cells in mid-log phase were harvested and whole-cell protein extract was prepared by vortexing...
acid-washed glass beads in 20% trichloroacetic acid (TCA) and washing beads with 5% TCA. Lysates were boiled for 5 min in Laemmli Sample buffer (4% SDS, 60 mM Tris-HCl, pH 6.8, 5% glycerol, 4% 2-mercaptoethanol, 0.01% bromophenol blue) and analyzed by 4–12% SDS-PAGE (Expedeon), followed by immunoblotting with rabbit anti-GFP (Abcam 290; 1:1000) and rabbit anti-cdc2 (gift from Nurse lab; 1:4000) as loading control. After secondary antibody (anti-rabbit Alexa Flour 488; 1:4000) incubation, blots were developed using Amersham Typhoon biomolecular imager.

Flow cytometry
Cells were fixed in cold 70% ethanol and processed in 50 mM sodium citrate, 100 μg/ml RNase A, and 8 μg/ml propidium iodide (PI). Samples were sonicated and then run on the flow cytometer (BD Accuri C6 Plus) for GFP and PI.

Statistical Analysis
A two-tailed Student’s t-test was used to determine significance: * P < 0.05, ** P < 0.01, *** P < 0.001, n.s. not significant. Error bars represent Standard Error (SE).

Data availability
Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at figshare: https://doi.org/10.25387/g3.10257524.

RESULTS
Dynamics of Tos4 in cycling wild type cells
To observe the dynamics of Tos4 accumulation in relation to the cell cycle, we imaged cells with Tos4-GFP and the spindle pole marker Sad1-DsRed. Tos4 accumulated in the nucleus of dividing cells, correlating to cells early S phase (Figure 1A and Supplemental Material, Movie 1). Nuclear Tos4-GFP was present largely in binucleate cells, corresponding to early S phase, and was also observed in some short mononucleate cells (newborns) following completion of separation, but was absent as the cells elongated, suggesting that nuclear Tos4 is lost in late S or G2. The duration of the presence of nuclear Tos4-GFP was about 60 min (Figure 1B) which was about 18% of the time of cell cycle (Figure 1C). Timing of nuclear Tos4-GFP relative to spindle duplication varied little between individual cells.
We next examined the use of Tos4 as a dynamic marker to characterize S phase.

**Tos4 accumulation in cell cycle arrest defines early S phase**

Treatment with hydroxyurea (HU) or temperature sensitive mutation of the ribonucleotide reductase component cdc22 leads to depletion of nucleotide pools, and arrest of cells in early S phase, with a largely unreplicated DNA content (Timson 1975; Sarabia et al. 1993). We observed Tos4-GFP accumulation both in HU-treated or temperature-sensitive cdc22-M45 at the restrictive temperature (Figure 2A,B). Tos4 was depleted when cdc22-M45 cells were released back to the permissive temperature (25°C), consistent with return to the cell cycle (Figure 2B). Cells arrested in G1 by mutation of the MBF transcription factor that regulates tos4+ expression (cdc10-V50) or in G2 by the mitotic inducing phosphatase (cdc25-22) showed no nuclear accumulation of Tos4 at 36°C but gained nuclear Tos4 upon release to 25°C (Figure 2B). Similarly, cells arrested at mitosis (nda3-KM311) had no nuclear Tos4 at 17°C but gained nuclear Tos4 when released to 32°C (Figure 2C).

| A | Asynchronous | HU | Release from HU |
|---|-------------|----|----------------|
| Tos4-GFP | WT | | |

| B | Asynchronous | 36°C | Release to 25°C |
|---|-------------|-----|----------------|
| Tos4-GFP | cdc22-M45 | | |
| | cdc10-V50 | | |
| | cdc25-22 | | |

| C | Asynchronous | 17°C | 32°C |
|---|-------------|-----|-----|
| Tos4-GFP | nda3-KM311 | | |

Figure 2. Tos4-GFP accumulate in the nuclei of cells arrested in early S phase. (A) WT cells were imaged for Tos4-GFP in asynchronous culture, after treatment with 12 mM HU for 4 h, and 1 h after release from HU. (B) Temperature-sensitive cell cycle mutants, cdc22-M45 (S phase arrest), cdc10-V50 (G1 phase arrest), cdc25-22 (G2 phase arrest) were imaged for Tos4-GFP in asynchronous culture at 25°C, after 4 h at 36°C, or after 1 h-1.5 h after release to 25°C. (C) Cold-sensitive nda3-KM311 (M phase arrest) was imaged for Tos4-GFP in asynchronous culture at 32°C, after 4 h at 17°C, or after 0.5 h after release to 32°C.
Next, we examined Tos4 accumulation in a variety of S phase mutants. During replication, the MCM helicase, which comprises six subunits, unwinds the DNA duplex and promote replication initiation and progression (Forsburg 2004). The canonical temperature-sensitive mutant mcm4-M68 (mcm4-ts) synthesizes a near 2C DNA content at restrictive temperature (36°C) but shows low viability when released to permissive temperature (25°C) (Nasmyth and Nurse 1981; Coxon et al. 1992; Liang et al. 1999; Sabatinos et al. 2015). A large C-terminal truncation mutant mcm4-c106, also shows 2C DNA content at 36°C but much higher viability upon release than mcm4-M68 (Nitani et al. 2008; Ranatunga and Forsburg 2016). A different temperature allele mcm4-dg that has a degron cassette added to mcm4-M68, undergoes rapid protein turnover at 36°C with limited DNA synthesis and a 1C DNA content (Lindner et al. 2002; Sabatinos et al. 2015) although it

Figure 3 Replication mutants lack nuclear Tos4-GFP. Temperature-sensitive Mcm4 helicase mutants (mcm4-M68, mcm4-c106, mcm4-dg) (A), MCM loader mutant (cdc18-K46), ligase mutants (cdc17-M45 and cdc17-K42) (B), and polymerase delta mutants (cdc6-23, cdc6-ts2, and cdc27-K3) (C) were imaged for Tos4-GFP in asynchronous culture at 25°C or after 4 h at 36°C.
fails to arrest divisions (Sabatinos et al. 2015). Interestingly, all three mcm4 mutants lacked nuclear Tos4 when placed at 36°C, even though they have different DNA contents and phenotypes (Figure 3A). We also tested temperature-sensitive mutants affecting the MCM loader cdc18-K46, DNA ligase mutants (cdc17-M45 and cdc17-K42), and mutants affecting DNA polymerase delta subunits (cdc6-23, cdc6-ts2, and cdc27-K3). All of these arrest with a near 2C DNA content (Nasmyth and Nurse 1981). None maintained nuclear Tos4 (Figure 3B,C). Thus, Tos4 accumulation is different in early S phase (HU, cdc22) compared to late S phase mutants, and its accumulation is not limited by DNA content.

The Anaphase-Promoting Complex (APC) is an ubiquitin ligase that targets various proteins for proteasome-mediated degradation (Harper et al. 2002; Sivakumar and Gorbsky 2015). APC mutants have mitotic arrest with mostly 2C DNA content (Weinert and Hartwell 1993; Yuan et al. 2014). In budding yeast, Tos4 interacts with Cdh1, a WD40-repeat-containing activator of APC complex that recognizes degradation motifs in substrates (Ostapenko et al. 2012). Cdh1Sc deletion results in partial stabilization of Tos4Sc (Ostapenko et al. 2012) but not the temperature sensitive APC mutant cdc23-1Sc, suggesting Tos4Sc protein turnover depends on multiple pathways. Fission yeast Tos4 does have potential APC recognition motifs (destruction box and KEN box) although the prediction score is not as high as Tos4 in budding yeast (Liu et al. 2012). We observed Tos4-GFP in three different temperature sensitive APC mutants: cut9-665, cut4-533, and nuc2-663. At 36°C, Tos4 did not accumulate in any of these APC mutants (Figure 4A), consistent with a cell cycle arrest in mitosis. We pretreated APC mutants with HU and then released to 36°C. If Tos4 protein is a target for APC-mediated degradation, we reasoned Tos4 would remain nuclear. HU-treated APC mutant cells accumulated nuclear Tos4 at both 25° and 36°C but lost the signal when released from HU to 36°C (Figure 4B and Figure S1A). Moreover, Ste9 (homologous to Cdh1Sc) deletion did not result in Tos4 accumulation (Figure S1B). Together these findings suggest that Tos4 is unlikely to be an APC target in fission yeast.

**Tos4 localization correlated with protein levels**

We determined whether observed accumulation of nuclear Tos4 is due to nuclear localization of Tos4 alone or whether it correlates with protein levels changes during S phase, using western blot analysis and flow cytometry (FACS) analysis. Lysates were collected from cells arrested in S phase with HU and released. Tos4 protein level increased in cells of different temperature-sensitive APC mutants at 36°C.
arrested in S phase compared to cells in asynchronous culture (Figure 5A). Tos4 protein level decreased to basal levels 30-60 min after release from HU. We also used FACS analysis to detect the GFP signal. This showed similar results, with the GFP peak increased in cells arrested in S phase and decreased back as cells were released from HU (Figure 5B). These results demonstrate that both Tos4 localization and protein turnover are regulated during S phase.

**Tos4 accumulation in early S phase is Cds1-dependent**

HU blocks DNA synthesis by depleting deoxynucleoside triphosphate (dNTP) pools (Reichard 1988), which results in activation of the replication checkpoint kinase Cds1 (Lopes et al. 2001; Kai et al. 2005). Cds1 stabilizes replication forks and prevents cell division during replication arrest (Lindsay et al. 1998; Kai and Wang 2003). Previously, we showed that cds1Δ mutants fail to stop DNA synthesis during HU treatment, with lethal consequences (Sabatinos et al. 2012). Activation of Cds1 has been shown to upregulate the MBF transcription factor (de Bruin et al. 2008; Dutta et al. 2008; Chu et al. 2009; Bastos de Oliveira et al. 2012) (reviewed in (Smolka et al. 2012; Bertoli et al. 2013)). Consistent with this, we observed that nuclear Tos4 accumulation during HU treatment or in cdc22-M45 arrest is Cds1-dependent (Figure 6A,B). We also observed that this requires the forkhead-associated domain (FHA) of Cds1, a phospho-peptide-binding module that mediates association with proteins such as Mrc1 and Mus81 (Boddy et al. 2000; Tanaka and Russell 2004). The cds1-fha" allele has mutations at two highly conserved residues (S79A and H82A) in the FHA domain and decreases DNA damage tolerance (Boddy et al. 2000). Similar to cds1Δ, the cds1-fha" cells treated with HU did not show nuclear Tos4 (Figure 6A). Kinase activity of Cds1 is also required for nuclear retention of Tos4 as kinase-dead Cds1 expression in cds1Δ did not retain nuclear Tos4 in HU (Figure S1C).

We next examined whether activating Cds1 with HU first would be sufficient to maintain nuclear Tos4 in cells with temperature-sensitive mutations in replication mutants that normally do not accumulate Tos4. Temperature-sensitive mcm4 mutants, mcm4-M68 and mcm4-dg, treated with HU accumulated nuclear Tos4 as expected, but this was lost upon release from HU to 36°C (Figure 7A,B), demonstrating that transient hyperactivation of Cds1 by HU is not sufficient to maintain nuclear Tos4. We next asked whether there was a difference if we maintained HU treatment at the restrictive temperature, so we shifted mcm4-M68 and mcm4-dg from HU at 25°C to HU at 36°C. The mcm4-M68 cells maintain nuclear Tos4 under both temperature conditions, and this depends upon Cds1 (Figure 7A,B). Other replication mutants cdc45/sna41, cdc18, cdc6, cdc17, and cdc27 also maintain nuclear Tos4 in the continued presence of HU at 36°C (Figure S2). Surprisingly, however, mcm4-dg cells do not maintain nuclear Tos4 in HU at 36°C (Figure 7A,B). Additionally, we showed that this loss of Tos4 in HU at 36°C in

Figure 5  Tos4 protein level is increased in S phase cells arrested by HU. (A) WT cells with Tos4-GFP in asynchronous culture, after treatment with 12 mM HU, or after release from HU were lysed and immunoblotted for GFP and cdc2 (loading control). (B) WT cells used in (A) were fixed in 70% ethanol, and FACS analyzed for GFP and propidium iodide (PI). Green in scatter plot represents population with high GFP and low PI while red represents population with low GFP and high PI.
mcm4-dg is rescued by deletion of the damage checkpoint kinase Chk1 (Figure 7A,B).

The mcm4-dg allele is unusual as it bypasses normal cell cycle arrest and continues into mitosis despite the absence of substantial DNA synthesis (Sabatinos et al. 2015). We looked at two additional temperature sensitive replication mutants. The hsk1-1312 mutation affects, the catalytic subunit of the fission yeast Dbf4-dependent kinase (DDK) that regulates initiation of DNA replication via MCM, and rad4-116 (cut5) is also required for initiation, yet both proceed into mitosis at the restrictive temperature (Saka et al. 1997; Ostapenko et al. 2012). Similar to mcm4-dg, both these mutants lose nuclear Tos4 at 36°C and fail to maintain nuclear Tos4 in continued presence of HU at 36°C (Figure 8A).

DISCUSSION

Using an imaging-based approach, we demonstrate that nuclear Tos4 accumulation marks early S phase stage independent of DNA content, and allows us to identify three distinctive types of temperature sensitive S phase mutants (Figure 8B): Class 1 mutants arrest replication with a 1C DNA content at the restrictive temperature in the continued presence of HU, consistent with spontaneous cell cycle progression. Consistent with this, we do not observe Tos4 accumulating in chk1Δ mcm4-dg at the restrictive temperature. which we conclude may reflect their ongoing cell cycle progression. Consistent with this, we do not observe Tos4 accumulation in chk1Δ mcm4-dg at the restrictive temperature.

Interestingly, however, these class 3 mutants also fail to retain Tos4 if shifted to the restrictive temperature in the continued presence of HU, despite the absence of DNA synthesis (Figure 7A, 8A). Our previous data provide some insight into this difference. We showed that mcm4-dg cells at the restrictive temperature do not activate Chk1 (Sabatinos et al. 2015), and do not show evidence for double strand breaks as measure.
by accumulation of H2A(X) phosphorylation (Bailis et al. 2008). However, if we shift mcm4-dg mutants to 36°C in the ongoing presence of HU, they do accumulate H2A(X) phosphorylation (Bailis et al. 2008), which suggests they have a different form of disruption or fork collapse than observed in the absence of HU. DSBs activate the Chk1 damage kinase, which we predict should repress the MBF and result in the loss of nuclear Tos4. Consistent with this, we observe that a chk1Δ mcm4-dg double mutant maintains nuclear Tos4 in HU at the restrictive temperature. (We were unable to determine the effect in hsk1-1312 and rad4-116 double mutants because chk1Δ has synthetic lethality or a growth defect in these backgrounds (Walworth et al. 1993; McFarlane et al. 1997; Snaithe et al. 2000; Taricani and Wang 2006). Thus, we conclude that the failure to maintain Tos4 in HU at 36°C for mcm4-dg reflects activation of Chk1, which is not the case in the absence of HU. Whether this accounts for the response of rad4Δ, which is required for Chk1 activation (Furuya et al. 2004) remains to be determined.

Temperature-sensitive APC mutants (cut9-665, cut4-533, and nuc2-663) did not stabilize nuclear Tos4 when released from HU to the restrictive temperature, suggesting Tos4 may not be an APC target in fission yeast. Ste9, a WD-repeat protein homologous to budding yeast Cdh1, activates APC and promotes degradation of mitotic cyclins (Kitamura et al. 1998; Blanco et al. 2000). Cds1 phosphorylates and inhibits Ste9 to protect the MBF activator Rep2 from degradation (Chu et al. 2009), but we see no effect of ste9Δ on Tos4 localization. The presence of Tos4 in mutant backgrounds at restrictive conditions may be more precisely a measure for different pathways of checkpoint activation rather than position within S phase. Importantly, cells apparently blocked nominally in late S phase may actually be in G2 phase, consistent with recent evidence (Kelly and Callegari 2019). This raises the possibility that the distinction between S phase and G2 actually depends upon the ability to activate the damage checkpoint. Tos4 is

Figure 7 Continued Cds1 activation induces S phase arrest in mcm4-M68 but not mcm4-dg. (A) mcm4-M68, cds1Δ mcm4-M68, mcm4-dg, chk1Δ mcm4-dg were imaged for Tos4-GFP after treatment with 12 mM HU at 25°C, after release from HU to 36°C, or after pre-treatment with HU at 25°C then transfer to 36°C. (B) Quantification of % cells with nuclear Tos4-GFP in (A). N> 300 cells analyzed for each strain. A two-tailed Student’s t-test was used to determine significance: * P < 0.05, ** P < 0.01, *** P < 0.001, Error bars represent Standard Error (SE).
known to be a phosphoprotein (Swaffer et al., 2018), so it is possible it is directly regulated by checkpoint kinases. Using various fluorescently-tagged cell cycle dependent proteins in combination with other cell cycle and checkpoint mutants will help elucidate whether or not there are distinctive changes that distinguish late S phase and G2 phase.

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