The yeast Dbf4 Zn\(^{2+}\) finger domain suppresses single-stranded DNA at replication forks initiated from a subset of origins

Jeff Bachant\(^1\) · Elizabeth A. Hoffman\(^2\) · Chris Caridi\(^3\) · Constance I. Nugent\(^1\) · Wenyi Feng\(^2\)

Received: 13 July 2021 / Revised: 23 December 2021 / Accepted: 6 January 2022 / Published online: 11 February 2022
© The Author(s) 2022

Abstract
Dbf4 is the cyclin-like subunit for the Dbf4-dependent protein kinase (DDK), required for activating the replicative helicase at DNA replication origin that fire during S phase. Dbf4 also functions as an adaptor, targeting the DDK to different groups of origins and substrates. Here we report a genome-wide analysis of origin firing in a budding yeast mutant, dbf4-\(\text{zn}\), lacking the Zn\(^{2+}\) finger domain within the C-terminus of Dbf4. At one group of origins, which we call dromedaries, we observe an unanticipated DNA replication phenotype: accumulation of single-stranded DNA spanning ± 5kbp from the center of the origins. A similar accumulation of single-stranded DNA at origins occurs more globally in pri1-m4 mutants defective for the catalytic subunit of DNA primase and rad53 mutants defective for the S phase checkpoint following DNA replication stress. We propose the Dbf4 Zn\(^{2+}\) finger suppresses single-stranded gaps at replication forks emanating from dromedary origins. Certain origins may impose an elevated requirement for the DDK to fully initiate DNA synthesis following origin activation. Alternatively, dbf4-\(\text{zn}\) may be defective for stabilizing/restarting replication forks emanating from dromedary origins during replication stress.

Keywords Dbf4 · DNA replication origin firing · DNA polymerase alpha · Primase · MCM

Introduction
Orderly genome duplication is achieved through a tightly regulated program of DNA replication origin (ORI) firing (Raghuraman and Brewer 2010; Marchal et al. 2019). ORIs in some nuclear regions fire early in S phase, while others fire late, leading to a defined spatiotemporal sequence for replicating chromosomes. An essential protein involved in controlling ORI firing is Dbf4, the activating subunit for Cdc7, the budding yeast Dbf4-dependent protein kinase (DDK; Jackson et al. 1993). Dbf4 is regulated through ubiquitin-mediated proteolysis in a cyclin-like manner, with Dbf4 accumulating in G\(_1\), peaking during the early S phase and declining at the metaphase to anaphase transition (Cheng et al. 1999; Weinreich and Stillman 1999; Godinho Ferreira et al. 2000). Activation of each ORI that fires during S phase requires cis-acting DDK phosphorylation of ORI-bound Mcm2-7 hexamers (Labib 2010). It has emerged that competition between ORIs for a limiting pool of active DDKs is a key determinant of when and how efficiently different ORIs fire (Boos and Ferreira 2019).

Both positive- and negative-acting mechanisms modulate DDK access and activity towards ORIs. One important mechanism in budding yeast is that ORIs adjacent to centromeres (CENs) gain preferential access to Dbf4, making CEN ORIs early and robustly firing ORIs (Raghuraman et al. 2001; Natsume et al. 2013). Dbf4 binds to the Ctf19/COMA kinetochore complex and is apparently off-loaded, increasing Dbf4 availability to proximal ORIs (Natsume et al. 2013). A second mechanism determining ORI early firing potential is controlled by the Forkhead transcription factors Fkh1 and Fkh2 (Knott et al. 2012). Fkh1/2 binds to specific chromatin regions, possibly forming chromosome domains that position ORIs to efficiently compete for...
Dbf4. Fkh1/2 also binds Dbf4, providing a mechanism for this competitive advantage (Fang et al. 2017). Conversely, ORIs can be delayed in firing through pathways acting in opposition to the DDK. One conserved mechanism involves Rif1 acting as a targeting factor for protein phosphatase 1 (PP1). Rif1/PP1 binds to particular ORIs and counteract Mcm2-7 phosphorylation, thereby conferring late firing timing (Hiraga et al. 2014; Dave et al. 2014; Mattarocci et al. 2014; Peace et al. 2014). Activation of the S phase checkpoint is an additional mechanism that delays ORI firing. In response to replication deterrents such as hydroxyurea (HU), the S phase checkpoint kinase Rad53 complexes with and extensively phosphorylates Dbf4, delaying activation of a large number of ORIs that fire later in the replication program (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010; Chen et al. 2013; Almawi et al. 2016). The Rad53 check on ORI firing minimizes the number of stressed forks, contributing to fork stability at early ORIs. (Feng et al. 2006; Poli et al. 2012; Zhong et al. 2013).

Current evidence indicates the DDK acts uniformly at all ORIs to initiate firing. During G1, paired Mcm2-7 hexamers are loaded at licensed ORIs in an inactive configuration (Remus et al. 2009). DDK phosphorylation of Mcm4 and Mcm6 induces Mcm2-7 conformational changes that melt ORI DNA and allow the hexamers to encircle template DNA single strands (ssDNA) in the necessary configuration for bidirectional DNA synthesis (Li and O’Donnell 2018). DDK phospho-targeting of Mcm2-7 also generates binding sites for the Cdc45, Sld3 and Sld7 proteins (Deegan et al. 2016). In parallel, Cdk1 bound to S phase cyclins phosphorylates Sld2 and Sld3, inducing additional protein interactions leading to the assembly of the Cdc45, MCM, GINS (CMG) replicative helicase (Tanaka et al. 2007; Muramatsu et al. 2010). Ablation of an auto-inhibitory activity within the N-terminus of Mcm4 or a gain-of-function mutation in Mcm5 bypass the essential requirement for Dbf4 and Cdc7 (Hardy et al. 1997; Sheu and Stillman 2010). Thus, the minimal essential role for the DDK in DNA replication is to activate Mcm2-7. DDK bypass mutations exhibit sensitivity to replication inhibitors (Sheu and Stillman 2010), indicating the DDK mediates additional functions that optimize DNA replication or allow cells to tolerate replication stress.

Structurally, Dbf4 and its homologues in other eukaryotes (e.g., Dbf4hs, Dbf4mm, and Chiffonmh) contain three conserved regions that mediate Dbf4 functions: motifs N, M and C (Masai and Arai 2000). Motif N contains a BRCT-like domain that binds Rad53 (Matthews et al. 2014), while motifs M and C are from separate interaction surfaces for Cdc7 (Dowell et al. 1994; Ogino et al. 2001). In particular, motif C contains a C2H2 Zn2+ finger domain that aligns motif C to contact Cdc7 (Hughes et al. 2012). In budding yeast, the entirety of motif C, including the Dbf4 Zn2+ finger, is not essential for cell growth, although the loss of the Zn finger leads to reduced DDK activity, temperature sensitivity, slow progression through S phase, and sensitivity to genotoxic stress (Harkins et al. 2009; Jones et al. 2010; Hughes et al. 2012). The basis for this spectrum of genome instability phenotypes is not well understood.

In a recent report, we analyzed genome-wide ssDNA replication intermediates in a dbf4-zn mutant lacking the Zn2+ finger domain (Julius et al. 2019). We found firing of CEN ORIs was strongly reliant on the Dbf4 Zn2+ finger. Here we extend our analysis of dbf4-zn to encompass other populations of ORIs. We identify a group of ORIs, which we call dromedary ORIs, that display an unanticipated replication defect in dbf4-zn in which ssDNA accumulates in the vicinity of ORIs. Based on the similarity of this phenotype to rad53 mutants and pri1-M4 mutants defective for the catalytic subunit of DNA primase, we suggest dromedary ORIs either impose an elevated requirement for the DDK to fully initiate DNA replication, or that the Dbf4 Zn2+ finger contributes to functions that maintain coupling between leading and lagging strand synthesis during replication stress.

Materials and methods

ssDNA mapping

Strain construction and methodology for generating genome-wide ssDNA datasets for wild type, dbf4-zn, rad53-21, rad53-21 dbf4-zn and rad53 dbf-D3 strains have been previously described (Julius et al. 2019). For PRI and pri1-M4 ssDNA mapping, isogenic PRII and pri1-M4 cells of BY4741 background were grown in synthetic complete medium at 25 °C to logarithmic phase before synchronization. Cells were arrested in G1 by incubating with 3 μM alpha-factor for approximately 1.5 generations until unbudded cells reach at least 95%. Cells were then released from G1 arrest by the addition of 0.3 mg/ml pronase and allowed to enter S phase at 37 °C, the restrictive temperature for pri1-M4 mutation. S phase samples collected at 30 min, along with G1 control samples collected before releasing into S phase, were embedded in agarose plugs and used for ssDNA mapping by microarrays as previously described (Feng et al. 2006, 2007). Briefly, agarose plugs containing the S phase samples from the PRII and pri1-M4 cells and their respective G1 controls were labeled for ssDNA via random-primed synthesis by Klenow (Exo-) at 37 °C for 1 h. Such a condition allows the labeling reaction to only occur on template DNA that contains single-stranded gaps, which are conducive to the incorporation of nucleotides without denaturation of the template DNA. The S phase DNA and G1 control were
differentially labeled with dNTP mixes containing Cy3- and Cy5-dUTP, respectively, before co-hybridization onto the Agilent Yeast Whole Genome ChIP-to-chip 4 × 44 K (G4493A) microarrays. Fluorescence data were extracted by the Agilent Feature Extraction Software (v9.5.1). The relative quantity of ssDNA at a given genomic locus was calculated as the ratio of the fluorescent signal from the S phase sample to that of the G1 control, followed by Loess-smoothing over a 6-kb window at a step size of 250 bp.

**Calculation of peak amplitude/base ratios and AUC values from composite ssDNA replication profiles**

Two methods were used to calculate profile peak to base ratios; results from both approaches are provided in Supplemental Table 2. In the first method, the base of the profile was defined as the distance between the minimum ssDNA values on either side of the ORI center. ssDNA maximum values on the left and right side of the profile (inclusive of the ORI center) were subtracted from the minimum values. Profile amplitude was defined as the average of the two differences.

The second approach utilized inflection points along the profile to determine amplitude to base ratios. For this approach, a one kbp sliding window was first used to smooth the profile data. The percent change between profile values at 250–300 bp intervals was then calculated. Using the positions of ssDNA maximum values (inclusive of the ORI center) as starting points, positions where the percent change of on either side of the profile flattened to 0.5% (or, for low amplitude profiles, 0.25%) were used to delimit the left and right sides of the major ssDNA feature within the profiles. Peak amplitude was defined as the average of ssDNA maximum values minus ssDNA inflection point values on either side of the ORI. Deconvolving the profiles in this fashion proved useful in defining the boundaries of profile features reflecting the accumulation of ORI ssDNA. Low amplitude profiles where 0.25% change, rather than 0.5% change, was used to identify major inflection points included: rad53 dbf4-4 1 and 2 unchecked and checked ORIs, WT checked ORIs, dbf4-4n checked ORIs, prl1-M4 checked ORIs.

AUC values were calculated as the sum of ssDNA values at each point within the meta-profile, extending between the two minimum values on either side of the ORI center.

**Results**

**Meta-analyses of ssDNA replication profiles in rad53 dbf4 double mutants**

We recently reported genome datasets for ssDNA replication intermediates in dbf4 strains containing mutations affecting the C-terminus of the Dbf4 protein (Julius et al. 2019). Strains for these datasets were generated by transforming dbf4-Δ or rad53-21 dbf4-Δ cells with low copy plasmids expressing either DBF4, dbf4-4n or dbf4-D3 under control of the endogenous promoter, referred to here as wild type (WT), dbf4-4n, rad53-21, rad53-21 dbf4-4n and rad53 dbf4-D3 strains. dbf4-4n is an internal deletion of amino acids 660–688 that form the Zn2+ finger motif C. dbf4-D3 contains a R701G mutation in a 26 amino acid C-terminal extension beyond the Zn2+ finger. dbf4-4n and dbf4-D3 behave as recessive loss of function mutations, with dbf4-4n exhibiting more severe phenotypes than dbf4-D3. To generate ssDNA datasets, strains were arrested in G1 and released into media containing 200 mM HU at 30 °C. After 60 min ssDNA was isolated and hybridized to genome microarrays. ssDNA values from HU samples were normalized to the signal from G1 arrested cells, providing S/G1 ssDNA ratios for each position on the array.

The purpose of this current study was to perform a meta-analysis of ORI firing in the dbf4-4n mutant. A meta-analysis means ssDNA replication profiles for user-defined cohorts of ORIs are averaged, producing a composite meta-profile that can reveal emergent features of the data. Figure 1 provides information regarding the interpretation of meta-profiles and terminology. The pronounced accumulation of ssDNA at ORIs in rad53 + HU (Fig. 1B) reflects: (1) formation of gapped replication bubbles due to uncoupling of leading and/or lagging strand synthesis in HU (Lopes et al. 2001; Sogo et al. 2002; Bermejo et al. 2011; Gan et al. 2017); (2) expansion of ssDNA gaps through exonuclease resection (Cotta-Ramusino et al. 2005); (3) defects in initiating lagging strand synthesis due to limiting Pol α/primase activity (Sogo et al. 2002); (4) reduced fork advance as a consequence of these defects. A schematic depicting abnormal rad53 + HU fork structures that have been proposed in the literature is shown in Supplemental Fig. 1.

As a first comparison, we examined whether the presence of dbf4-4n or dbf4-D3 reduced ORI firing in HU-treated rad53 mutants. We hypothesized such a reduction might occur if loss of Dbf4 compensated for the failure of Rad53 to inhibit Dbf4 in restraint of ORI firing. Figure 2A shows an alignment comparing meta-profiles for 146 unchecked ORIs in WT + HU, rad53 + HU, and dbf4-4n + HU; WT + HU and rad53 + HU meta-profiles are the same as shown in Fig. 1A and Fig. 1B for illustrative purposes. The checked ORI meta-profiles comprise a cohort of 186 additional ORIs activated in rad53 + HU as previously defined in Julius et al. (2019). Several observations arise from these comparisons. First, reflecting the pronounced accumulation of ORI ssDNA in rad53 + HU, the AUC of rad53 + HU meta-profiles for unchecked and checked ORIs increased relative to WT (Fig. 2A, Supplemental Table 2). Second, the AUC for the
rad53 + HU unchecked ORI meta-profile is greater than the AUC for rad53 + HU at checked ORIs, suggesting checked ORIs in rad53 + HU are activated less efficiently (Fig. 2A, Supplemental Table 2). Third, rad53 + HU ssDNA/base ratios are similar between unchecked and checked ORI meta-profiles. This suggests forks from unchecked and checked ORIs in rad53 + HU experience similar forms of deregulation leading to accumulation of ssDNA. This is notable since unchecked and checked ORIs in rad53 + HU fire before and after nucleotide depletion, respectively.

Figure 2B shows the corresponding unchecked and checked ORI meta-profiles for HU treated rad53, rad53 dbf4-D3 and duplicate rad53 dbf4-zn strains. AUC measurements for both unchecked and checked ORI meta-profiles were decreased in rad53 dbf4-D3 + HU and rad53 dbf4-zn + HU compared to rad53 + HU controls, dramatically so for rad53 dbf4-zn (20X and 24X reductions; Fig. 2B, Supplemental Table 2). 71 of the 186 checked ORIs that fire in rad53 + HU failed to fire in rad53 dbf4-zn + HU. A notable feature of rad53 dbf4 + HU unchecked ORI meta-profiles was that reduced firing corresponded with a displacement of ssDNA further away from the ORI (Fig. 2B). This was most apparent for rad53 dbf4-D3 + HU but could also be visualized with rad53 dbf4-zn + HU. The bottom graphs in Fig. 2B show an expanded y-axis of the same rad53 dbf4-zn + HU meta-profiles to better resolve this change in profile. In keeping with the displacement of the ssDNA signal away from ORIs, ssDNA/base ratios for rad53 dbf4-D3 + HU and rad53 dbf4-zn + HU unchecked ORI meta-profiles were reduced 1.9X and 13.2X, respectively, compared to rad53 + HU, while checked ORI ssDNA/base ratios were reduced 1.6X and 6.2X (Fig. 2C, Supplemental Table 2). This suggests
reduced ORI utilization in rad53 dbf4 mutants corresponds with a partial amelioration of rad53 generating excess ssDNA over ORIs. Minimizing competition between forks may allow forks in rad53 dbf4 mutants to progress further before they experience catastrophes.

**Phenotypic categories of ORIs affected by dbf4-zn**

Several additional observations became apparent during our meta-analysis of dbf4-zn + HU. We previously found that the Dbf4 Zn$^{2+}$ finger is required for robust firing of CEN ORIs (Julius et al. 2019). A meta-analysis of a ± 50 kbp window centered around all 16 CENs provided a striking visualization of this phenotype (Fig. 3B). Four data series are shown in Fig. 3B. The first series (open circles) shows all unchecked ORIs within a ± 50 kbp CEN window, plotted at the top of the graph relative to x-axis kbp coordinates. This series reveals the high density of ORIs flanking CENs throughout the genome. The second series is a CEN-centered ssDNA meta-profile for WT + HU (blue line, plotted relative to the left y-axis). This series reveals a broad accumulation of ssDNA replication intermediates associated with forks converging on CENs. The third series is the corresponding ssDNA meta-profile for dbf4-zn + HU (orange line, plotted relative to the left y-axis). This shows that ssDNA accumulation at CENs is largely eliminated in dbf4-zn. For the fourth series, WT + HU and dbf4-zn + HU datasets were inspected for ORIs with clearly delineated ORI profiles, and AUC values were determined. The ratios of individual dbf4-zn/WT AUCs were plotted (red X’s) relative to the right y-axis. 39 ORIs are depicted, revealing a prominent reduction in ORI utilization ± 20 kbp from the CEN in dbf4-zn + HU.

Unexpectedly, the unchecked ORI meta-profile for dbf4-zn + HU exhibited features of both the WT + HU and rad53 + HU profiles. While there were split ssDNA peaks as in the WT + HU meta-profile, there was also an indication of a ssDNA peak centered over the ORI, reminiscent of rad53 + HU (Fig. 2A). This led us to suspect that the dbf4-zn + HU composite was a combination of these two profiles. Inspection of individual ORIs in WT + HU and dbf4-zn + HU revealed this was indeed the case. Figure 3A shows a region of chromosome 16 where, for dbf4-zn + HU, two ORIs (ARS1623 and ARS1626.5) exhibit split peak profiles, one ORI (ARS1627) exhibits the unanticipated single ssDNA peak, one CEN ORI (XVI-559) were firing is strongly reduced, and one ORI (ARS1624) that is not readily assignable to split peak or single peak profiles. Since both split peak and single peak profiles were present in dbf4-zn + HU, we needed a convenient way to refer to them, and adopted the terms camel ORIs and dromedary ORIs, respectively, for this purpose. Camels are simply ORIs demonstrating the split peak ssDNA profile characteristic of normal fork advance in HU, while the form of dromedary ORIs suggests a novel component to the dbf4-zn + HU phenotype. To evaluate this possibility, we scored all 146 unchecked ORIs in dbf4-zn + HU and found ~75% of them could be assigned as either camel ORIs, dromedary ORIs, or CEN ORIs exhibiting reduced firing potential (Supplemental Table 1). 29 camel ORIs (Supplemental Fig. 2, Supplemental Table 1) and 49 dromedary ORIs (Supplemental Fig. 3, Supplemental Table 1) were selected as cohorts for meta-profile comparisons between dbf4-zn + HU, WT + HU and rad53 + HU (Fig. 3C); these cohorts will be called camel ORIs and dromedary ORIs when referring to all three datasets. For dbf4-zn + HU, camel and dromedary ORI cohorts resolved with clearly distinct split peak and or single peak meta-profiles, respectively. WT + HU camel and dromedary cohorts both resolved with split peak meta-profiles, while rad53 + HU camel and dromedary cohorts both resolved with single-peaked meta-profiles (Fig. 3C). Thus, the dbf4-zn + HU dromedary cohort represents a subset of unchecked ORIs that accumulate aberrant ssDNA like rad53 + HU.

**Features of dbf4-zn + HU dromedary ORIs**

We next asked what features distinguish the dromedary ORI cohort. As a first observation, it was apparent that forks from dbf4-zn + HU camel ORIs progressed further, on average than forks from WT + HU camel ORIs (Fig. 4A). One explanation is that reduced CEN ORI firing in dbf4-zn + HU increases dNTP availability to forks from other ORIs (Poli et al. 2012; Zhong et al. 2013). As a second observation, the average AUC for WT + HU camel ORIs was ~3 time greater than the average AUC for WT + HU dromedary ORIs (Fig. 4B, p < 0.001; Student’s t test). The AUC distribution for WT + HU dromedary ORIs contained four high-end outliers, all of which were CEN ORIs greatly reduced in firing potential in dbf4-zn + HU. This suggests that camel ORIs tend to fire more efficiently than dromedary ORIs in WT + HU cells.

We asked if local genome elements are known to alter ORI activity, including tRNAs, transposable elements, RNA: DNA hybrids and relative orientation of replication and transcription (Voytas and Boeke 1993; Hoffman et al. 2015; Costantino and Koshland 2018) differentiated camel and dromedary ORIs, but no significant difference in the
association was observed (Supplemental Table 3). However, we did observe a higher nucleosome accessibility in camel ORIs compared to dromedaries based on nucleoATAC scores (Schep et al. 2015), average score 2.62 vs. 0.56, $p = 0.14$, Student’s $t$ Test, one-tailed distribution, equal variance). This suggests camels reside in a more open chromatin environment conducive to ORI activation.

We also examined whether camel and dromedary ORI differed in their reliance on the Fkh1/2 pathway for early firing potential. To do this, we utilized the
dataset published by Knott et al. (2012) to calculate a ratio between the AUC for BRDU incorporation in a Δakh1 Δfkh2 + pFKH2ΔC mutant and the AUC for BRDU incorporation in their WT control for 143 ORIs described in their study. In Fig. 4C the set of Δakh1 Δfkh2 + pFKH2ΔC/WT ratios are plotted as a function of the corresponding WT BRDU AUC. ORIs that, by the criteria of Knott et al. (2012), were evaluated as Fkh1/2-dependent (red circles) or Fkh1/2-independent (blue circles) are indicated. CEN ORIs are also plotted as a separate Fkh1/2-independent category (green circles). The identity of camel and dromedary ORIs was then superimposed (Fig. 4D), revealing camel ORIs are almost uniformly Fkh1/2-dependent ORIs. Of the 29 ORIs selected as camels (filled circles), 27 (93%) were Fkh1/2-dependent. In contrast, dromedary ORIs (open circles) tended to be Fkh1/2-independent, although this correlation was not as predictive (30 of 49 dromedaries were Fkh1/2-independent). To summarize, camel ORIs are characterized by Fkh1/2-dependent recruitment of Dbf4, an open chromatin environment, and split peak ssDNA replication profiles in dbf4- ΔH + HU. Dromedary ORIs, in contrast, tend to lack CEN- or Fkh1/2-specified DDK recruitment, corresponding with reduced firing potential and accumulation of ORI ssDNA in dbf4- ΔH + HU.

dbf4- ΔH and pri1- M4 mutants show a similar ssDNA profile at dromedary ORIs

Finally, we considered the nature of the dbf4- ΔH + HU defect leading to ssDNA accumulation at dromedary ORIs. Previously, defective replication fork structures in rad53 + ΔH have been compared to pri1-M4 mutants defective for the catalytic subunit of DNA primase (Marini et al. 1997; Sogo et al. 2002). Remarkably similar single-stranded replication bubbles were visualized in both mutants, leading to the suggestion that rad53 + ΔH and pri1-M4 might share a common defect in lagging strand synthesis (Sogo et al. 2002). Accordingly, we generated a new ssDNA dataset for pri1-M4, releasing cells from G1 at a pri1-M4 non-permissive temperature of 37 °C. To parallel the conditions of Sogo et al. 2002 as closely as possible, we note the pri1-M4 strain was not treated with HU. Meta-analysis of the pri1-M4 camel cohort of ORIs showed a dramatic accumulation of ORI-centered ssDNA (Fig. 5A), with a ssDNA/base ratio 2.3 × higher than rad53 + ΔH (Fig. 5B and 5C, Supplemental Table 2). pri1-M4 mutants also accumulated a smaller peak of ORI ssDNA in the meta-profile for the dromedary cohort of ORIs (Fig. 5A). Remarkably, the pri1-M4 dromedary meta-profile was largely superimposable upon the dromedary meta-profile of dbf4- ΔH + HU (Fig. 5B), with AUC and ssDNA peak/base ratios that were quite similar between the two strains (Fig. 5C). Thus, qualitatively and quantitatively, the dbf4- ΔH + HU ssDNA profile at dromedary ORIs closely resembles the profile observed following a reduction in DNA primase activity.

Discussion

In this report, we performed a genome-wide analysis of ORI firing in a dbf4- ΔH mutant lacking the C-terminal Zn finger domain, focusing on 146 unchecked ORIs that fire in S phase checkpoint proficient cells. A principal new finding is that 49 of these ORIs, which we call dromedary ORIs, display an aberrant ssDNA replication profile in dbf4- ΔH + HU reminiscent of rad53 + ΔH and pri1-M4 mutants. Both rad53 + ΔH and pri1-M4 form DNA replication bubbles containing extensive single-stranded gaps (Sogo et al. 2002), which likely determine their ssDNA replication profiles. Thus, rather than an “all or none” firing defect, the effect of dbf4- ΔH at dromedary ORIs would appear to be a perturbation to DNA synthesis that leads to single-stranded gaps in replication forks. To our knowledge, this is a novel phenotype for a budding yeast dbf4 mutant. Defective DNA replication forks may underlie genome instability phenotypes previously associated with loss of the Dbf4 Zn finger domain in yeast, including slow progression through S phase, accumulation of DNA damage and sensitivity to forms of genotoxic stress including HU (Harkins et al. 2009; Jones et al. 2010; Hughes et al. 2012; Julius et al. 2019). The questions become what causes ssDNA gaps to accumulate at forks in dbf4- ΔH + HU, why is this
defect specific to particular ORIs and what insights does the dromedary phenotype provide into Dbf4 function?

A consideration of aberrant replication fork structures in rad53 + HU and pri1-M4 mutants is likely to be informative regarding the dbf4-zn + HU dromedary
phenotype. An early study examined replication bubbles in rad53 + HU and pri1-M4 mutants by electron microscopy, revealing extensively gapped forks and hemi-replicated bubbles (i.e. one side of the bubble completely double-stranded and the other side completely single-stranded) in both strains (Sogo et al. 2002). To account for this, it was proposed firing of unchecked ORIs in rad53 + HU elevated the catalytic demand on Polα primase, leading to a failure to initiate or maintain lagging strand synthesis (Supplemental Fig. 1B, D, F). More recent evidence suggests an additional role for Rad53 in maintaining a functional coupling between CMG and Polε at the site of leading strand synthesis, with failure to restrain CMG/Pole in rad53 + HU exposing unwound ssDNA on the leading strand template (Gan et al. 2017; Devbhandari and Remus 2020) (Supplemental Fig. 1C). Notably, exonuclease resection of nascent leading or lagging strands arising from initiation of elongation defects in rad53 + HU or pri1-M4 mutants could extend ssDNA gaps towards ORIs, potentially generating hemi-replicated bubbles (Sogo et al. 2002; Cotta-Ramusino et al. 2005; Feng et al. 2006) (Supplemental Fig. 1E). Hemi-replicated bubbles could also arise directly through defective initiation of DNA synthesis, for example, initiation defects producing unidirectional forks (Sogo et al. 2002) (Supplemental Fig. 1F).

Thus, while defective initiation of lagging strand synthesis likely accounts for the ssDNA replication profile associated with pri1-M4, rad53 + HU may experience a broader range of fork defects, potentially differentially affecting ORIs that fire before or after nucleotide depletion. Thus, the dbf4-zn dromedary phenotype seems likely to arise through initiation or elongation defects in fork structure.

The molecular defect leading to the dbf4-zn dromedary phenotype is not resolved by our study. As laid out in the Introduction, however, the best-understood role for the DDK is to activate paired Mcm2-7 hexamers to encircle melted template strands in a configuration supporting bi-directional fork movement. DDK phosphorylation of Mcm2-7 is also necessary to recruit initiation factors for CMG assembly. Recent evidence suggests the multiplicity of DDK phosphorylation on Mcm2-7 corresponds with the extent of initiation factor recruitment, with more robustly phosphorylated subunits potentiating a later acting, rate limiting, step in CMG assembly (De Jesús-Kim et al. 2021). Other DDK regulatory circuits, such as a role for Mcm10 in DDK phosphorylation of Mcm2, have also been proposed, potentially stimulating RPA and Polo loading at ORIs (Walter and Newport 2000; Zhu et al. 2007; Perez-Arnaiz et al. 2017). Our analysis suggests a distinguishing feature of dromedary ORIs is that they lack known (CEN- or Fkh1/2-directed) Dbf4 enrichment mechanisms. Dromedary ORIs in dbf4-zn + HU may therefore fire with comparatively reduced Mcm2-7 phosphorylation, allowing hexamers to pass on opposing strands but not completely supporting subsequent events in CMG/replicosome assembly or initiation of DNA synthesis.

With respect to post-initiation forms of regulation by the DDK, accumulating evidence, mostly in animal cells, suggests continued DDK activity is necessary for fork advance during replication stress (Dolson et al. 2021). Roles for the DDK appear to include regulation of fork reversals, nuclelease processing, and gap filling as a means to restart stalled forks (Sasi et al. 2018; Rainey et al. 2020; Jones et al. 2021; Cabello-Lobato et al. 2021). Additionally, continued DDK phosphorylation of Mcm2-7 (Bastia et al. 2016; Alver et al.
2017), and potentially phosphorylation of fork pausing factors (Murakami and Keeney 2014), may enforce polymerase coupling at stalled forks. We, therefore, speculate incomplete DDK phosphorylation of Mcm2-7 or other substrates in dbf4-zn could predispose forks from dromedary ORIs to become destabilized in HU. Another factor that may function with the DDK is the Stn1 protein, a component of the CST telomere binding complex (Grandin et al. 1997). Previous work in yeast and human cells indicates Stn1 stimulates ORI firing under conditions of replication stress and physically interacts with Polα/primase, Mcm2 and Mcm7 (Gasparyan et al. 2009; Wang et al. 2019). In an accompanying paper, we present evidence that yeast Stn1 may stimulate DDK towards Mcm2-7 and that Stn1 abrogation leads to the accumulation of ssDNA at non-telomeric chromosomal regions. We are currently investigating the possibility that the fkh1/2 mutant AUC to the WT AUC was calculated as a measurement of Fkh1/2-dependence. This ratio was then plotted as a function of the WT BRDU AUC for all 143 ORIs. Green circles are CEN ORIs, which are largely Fkh1/2-independent in their firing potential. Red circles are ORIs that by the criteria of Knott et al. (2012) were identified as Fkh1/2-dependent ORIs. Blue circles are the remaining Fkh1/2-independent group of ORIs; these tend to fire less robustly in WT. Note that some Fkh1/2-independent ORIs show increased utilization in the fkh mutant (fkh/WT ratios > 1).

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00294-022-01230-6.

**Acknowledgements** We would like to thank Charles Boone for the PRI1 and pri1-M4 strains in the BY4741 background.

**Funding** No funding was secured for this manuscript.

**Declaration**

**Conflict of interest** The authors declare that they have no conflict of interest.
Fig. 5 Similarities between dbf4-zn and pri1-M4 mutants at dromedary ORIs. A Meta-analysis of camel and dromedary ORIs in pri1-M4 mutants. Genome-wide ssDNA replication profiles were generated for pri1-M4 mutants and an isogenic WT control (PRI1) at a pri1-M4 non-permissive temperature of 37 °C. Graphs display ssDNA meta-profiles (solid lines) and profiles for individual ORIs (lighter data points) for 29 camel and 49 dromedary ORIs from the pri1-M4 dataset. pri1-M4 cells display accumulation of ssDNA over both camel and dromedary ORIs. PRI1 controls in this experiment have largely completed S phase at the time of sampling, producing meta-profiles that are close to baseline values. This is because HU was not included in the experiment to reproduce the conditions under which pri1-M4 and rad53+HU were initially compared in the work of Sogo et al. (2002). B Overlays comparing dbf4-zn+HU, rad53+HU and pri1-M4 meta-profiles for camel and dromedary ORIs. Note the superimposition of the dbf4-zn+HU and pri1-M4 dromedary meta-profiles. C Comparison of AUC and ssDNA accumulation (ssDNA/kbp) over camel (red) and dromedary (blue) ORI meta-profiles in WT+HU, rad53+HU, dbf4-zn+HU and pri1-M4 datasets. rad53+HU dbf4-zn+HU and pri1-M4 cells show a quantitatively similar tendency to accumulate ssDNA at ORIs.

References

Almawi AW, Matthew LA, Larasati et al (2016) ‘AND’ logic gates at work: crystal structure of Rad53 bound to Dbf4 and Cdc7. Sci Rep. https://doi.org/10.1038/srep34237

Alver RC, Chadha GS, Gillespie PJ, Blow JJ (2017) Reversal of DDK-mediated MCM phosphorylation by Rif1-PP1 regulates replication initiation and replisome stability independently of ATR/Chk1. Cell Rep 18:2508–2520. https://doi.org/10.1016/j.celrep.2017.02.042
Bastia D, Srivastava P, Zaman S et al (2016) Phosphorylation of CMG helicase and Top1 is required for programmed fork arrest. Proc Natl Acad Sci USA 113:E3639–3648. https://doi.org/10.1073/pnas.1607552113
Bermejo R, Capra T, Jossen R et al (2011) The replication checkpoint protects fork stability by releasing transcribed genes from nuclear pores. Cell 146:233–246. https://doi.org/10.1016/j.cell.2011.06.033
Boo D, Ferreira P (2019) Origin firing regulations to control genome replication timing. Genes (basel). https://doi.org/10.3390 GENETICS.10030199
Cabanillas-Lobo MJ, González-Garrido C, Cano-Linares MI et al (2021) Physical interactions between MCM and Rad51 facilitate replication fork lesion bypass and ssDNA gap filling by non-recombinogenic functions. Cell Rep. https://doi.org/10.1016/j.celrep.2021.109440
Chen Y-C, Kenworthy J, Gabrielse C et al (2013) DNA replication checkpoint signaling depends on a Rad53-Dbf4 N-terminal interaction in Saccharomyces cerevisiae. Genetics 194:389–401. https://doi.org/10.1534/gene.113.149740
Cheng L, Collyer T, Hardy CFJ (1999) Cell cycle regulation of DNA replication initiator factor Dbf4p. Mol Cell Biol 19:4270–4277.
Costantino L, Koshland D (2018) Genome-wide map of R-loop- Chelator (2022) Current Genetics 68:253–265
De Jesús-Kim L, Friedman LJ, Lõoke M et al (2021) DDK regulates checkpoint kinase Rad53 limits CMG helicase uncoupling from DNA synthesis at replication forks. Mol Cell 17:153–159. https://doi.org/10.1016/j.molcel.2004.11.032
Davies A, Cooley C, Garg M, Bianchi A (2014) Protein phosphatase 1 recruitment by Rif1 regulates DNA replication ori- gin firing by counteracting DDK activity. Cell Rep 7:53–61. https://doi.org/10.1016/j.celrep.2014.02.019
De Jesús-Kim L, Frieden LJ, Lõoke M et al (2021) DDK replicates replication initiation by controlling the multiplicity of Cdc45- GINS binding to Mcm2-7. Elife 10:e65471. https://doi.org/10.7554/eLife.65471
Deegan TD, Yeeles JT, Diffley JF (2016) Phosphopeptide binding by Sld3 links Dbf4-dependent kinase to MCM replicative helicase activation. EMBO J 35:961–973. https://doi.org/10.15252/emboj.201593552
Debnathri S, Remus D (2020) Rad53 limits CMG helicase uncoupling from DNA synthesis at replication forks. Nat Struct Mol Biol 27:461–471. https://doi.org/10.1038/s41594-020-0407-7
Dolson A, Sauty SM, Shaban K, Yankulov K (2021) Dbf4-dependent kinase: DKC-activated to post-initiation events in DNA replication. Cell Cycle. https://doi.org/10.1080/15384101.2021.1986999
Dowell SJ, Romanowski P, Diffley JF (1994) Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science 265:1243–1246. https://doi.org/10.1126/science.8066465
Fang D, Lengronne A, Shi D et al (2017) Dbf4 recruitment by forkhead transcription factors defines an upstream rate-limiting step in determining origin firing timing. Genes Dev 31:2405–2415. https://doi.org/10.1101/gad.306571.117
Fang W, Collingwood D, Boeck ME et al (2006) Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. Nat Cell Biol 8:148–155. https://doi.org/10.1038/ncb1358
Fang W, Raghuraman MK, Brewer BJ (2007) Mapping yeast origins of replication via single-stranded DNA detection. Methods 41:151–157. https://doi.org/10.1016/j.ymeth.2006.07.023
Feng W, Bachant J, Collingwood D et al (2009) Centromere replication timing determines different forms of genomic instability in saccharomyces cerevisiae checkpoint mutants during replication stress. Genetics 183:1249–1260. https://doi.org/10.1534/genetics.109.107508
Feng W, Di Rienzi SC, Raghuraman MK, Brewer BJ (2011) Replication stress-induced chromosome breakage is correlated with replication fork progression and is preceded by single-stranded DNA formation. G3 (Bethesda) 1:327–335. https://doi.org/10.1534/g3.111.005554
Gan H, Yu C, Devbhandari S et al (2017) Checkpoint kinase Rad53 couples leading- and lagging-strand DNA synthesis under replication stress. Mol Cell 68:446–455.e3. https://doi.org/10.1016/j.molcel.2017.09.018
Gasparian HI, Xu L, Petreca RC et al (2009) Yeast telomere capping protein Stn1 overrides DNA replication control through the S phase checkpoint. Proc Natl Acad Sci USA 106:2206–2211. https://doi.org/10.1073/pnas.0812605106
Godinho Ferreira M, Santocanale C, Drury LS, Diffley JFX (2000) Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. Mol Cell Biol 20:242–248
Grandin N, Reed SI, Charbonneau M (1997) Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13. Genes Dev 11:512–527
Hardy CFJ, Dryga O, Seematter S et al (1997) ccm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci USA 94:3151–3155
Harkins V, Gabrielse C, Haste L, Weinreich M (2009) Budding yeast Dbf4 sequences required for Cdc7 kinase activation and identification of a functional relationship between the Dbf4 and Rev1 BRCT domains. Genetics 183:1260–1282. https://doi.org/10.1534/GENETICS.109.110155
Hiraga S-I, Alvirino GM, Chang F et al (2014) Rif1 controls DNA replication by directing protein phosphatase 1 to reverse Cdc7-mediated phosphorylation of the MCM complex. Genes Dev 28:372–383. https://doi.org/10.1101/gad.231258.113
Hoffman EA, McCulley A, Haefer B et al (2015) Break-seq reveals hydroxyurea-induced chromosome fragility as a result of unscheduled conflict between DNA replication and transcription. Genome Res 25:402–412. https://doi.org/10.1101/gr.180497.114
Hughes S, Elustondo F, Di Fonzo A et al (2012) Crystal structure of human CDC7 kinase in complex with its activator DBF4. Nat Struct Mol Biol 19:1101–1107. https://doi.org/10.1038/nsmb.2404
Jackson AL, Pahl PM, Harrison K et al (1993) Cell cycle regulation of the yeast Cdc7p kinase by association with the Dbf4 protein. Mol Cell Biol 13:2899–2908
Jones DR, Prasad AA, Chan PK, Duncker BP (2010) The Dbf4 motif C zinc finger promotes DNA replication and mediates resistance to genotoxic stress. Cell Cycle. 9:2018–2026. https://doi.org/10.4161/cc.9.10.11752
Jones MJK, Gelot C, Munk S et al (2021) Human DDK rescues stalled forks and counteracts checkpoint inhibition at unfired origins to complete DNA replication. Mol Cell 81:426–441.e8. https://doi.org/10.1016/j.molcel.2021.01.004
Julius J, Peng J, McCulley A et al (2019) Inhibition of spindle extension through the yeast S phase checkpoint is coupled to replication fork stability and the integrity of centromeric DNA. Mol Biol Cell 30:2771–2789. https://doi.org/10.1091/mbc.E19-03-0156
Knott SRV, Peace JM, Ostrow AZ et al (2012) Forkhead transcription factors establish origin timing and long-range clustering in S. cerevisiae. Cell 148:99–111. https://doi.org/10.1016/j.cell.2011.12.012
Labib K (2010) How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells? Genes Dev 24:1208–1219. https://doi.org/10.1101/gad.1933010

Li H, O’Donnell M (2018) The eukaryotic CMG helicase at the replication fork: emerging architecture reveals an unexpected mechanism. Bio Essays. https://doi.org/10.1002/bies.201700208

Lopes M, Cotta-Ramusino C, Pellicioli A et al (2001) The DNA replication checkpoint response stabilizes stalled replication forks. Nature 412:557–561. https://doi.org/10.1038/35087613

Lopez-Mosqueda J, Maas NL, Jonsson ZO et al (2010) Damage-induced phosphorylation of Sld3 is important to block late origin firing. Nature 467:479–483. https://doi.org/10.1038/nature09377

Marchal C, Sima J, Gilbert DM (2019) Control of DNA replication timing in the 3D genome. Nat Rev Mol Cell Biol 20:721–737. https://doi.org/10.1038/s41580-019-0162-y

Marini F, Pellicioli A, Paciotti V et al (1997) A role for DNA primase in coupling DNA replication to DNA damage response. EMBO J 16:639–650. https://doi.org/10.1093/emboj/16.6.639

Masai H, Arai K (2000) Dbf4 motifs: conserved motifs in activation subunits for Cdc7 kinases essential for S-phase. Biochem Biophys Res Commun 275:228–232. https://doi.org/10.1006/bbrc.2000.3281

Matarocchi S, Shayin M, Lemmens L et al (2014) Rif1 controls DNA replication timing in yeast through the PP1 phosphatase Gcl7. Cell Rep 7:62–69. https://doi.org/10.1016/j.celrep.2014.03.010

Matthews LA, Selvaratnam R, Jones DR et al (2012) dNTP pools determine fork progression and origin usage under replication stress. EMBO J 31:883–894. https://doi.org/10.1038/emboj.2011.470

Perez-Arnaiz P, Bruck I, Colbert MK, Kaplan DL (2017) An intact Mcm10 coiled-coil interaction surface is important for origin melting, helicase assembly and the recruitment of Pol-α to Mcm2-7. Nucleic Acids Res 45:7261–7275. https://doi.org/10.1093/nar/gkx438

Poli J, Tsaponina O, Crabbé L et al (2012) dNTP pools determine fork progression and origin usage under replication stress. EMBO J 31:883–894. https://doi.org/10.1038/emboj.2011.470

Raghuraman MK, Brewer BJ (2010) Molecular analysis of the replication program in unicellular model organisms. Chromosome Res 18:19–34. https://doi.org/10.1007/s10577-009-9099-x

Raghuraman MK, Winzeler EA, Collingswood D et al (2001) Replication dynamics of the yeast genome. Science 294:115–121. https://doi.org/10.1126/science.294.5540.115

Rainey MD, Quinlan A, Cazzaniga C et al (2020) CDC7 kinase promotes MRE11 fork processing, modulating fork speed and chromosomal breakage. EMBO Rep 21:e48920. https://doi.org/10.15252/embr.201948920

Remus D, Beuron F, Tolun G et al (2009) Concerted Loading of Mcm2-7 Double Hexamers Around DNA during DNA Replication Origin Licensing. Cell 139:719–730. https://doi.org/10.1016/j.cell.2009.10.015

Sasi NK, Coquel F, Lin Y-L et al (2018) Dbf4 has a primary role in processing stalled replication forks to initiate downstream checkpoint signaling. Neoplasia 20:985–995. https://doi.org/10.1016/j.neo.2018.08.001

Schepps AN, Buenrostro JD, Denny SK et al (2015) Structured nucleosome fingerprints enable high-resolution mapping of chromatin architecture within regulatory regions. Genome Res 25:1757–1770. https://doi.org/10.1101/gr.192294.115

Sheu Y-J, Stillman B (2010) The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4. Nature 463:1113–1117. https://doi.org/10.1038/nature08647

Sogo JM, Lopes M, Foiani M (2002) Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science 297:599–602. https://doi.org/10.1126/science.1074023

Tanaka S, Unemori T, Hirai K et al (2007) Cdk-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature 445:328–332. https://doi.org/10.1038/nature05465

Voytas DF, Boeke JD (1993) Yeast retrotransposons and RNAs. Trends Genet 9:421–427. https://doi.org/10.1016/0168-9525(93)90105-q

Walter J, Newport J (2000) Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, Rpa, and DNA polymerase alpha. Mol Cell 5:617–627. https://doi.org/10.1016/s1097-2765(00)00241-5

Wang Y, Brady KS, Caiello BP et al (2019) Human CST suppresses origin licensing and promotes AND-1/Ctf4 chromatin association. Life Sci Alliance. https://doi.org/10.26508/lsa.201800270

Weinreich M, Stillman B (1999) Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. EMBO J 18:5334–5346. https://doi.org/10.1093/emboj/18.19.5334

Zegerman P, Diffley JFX (2010) Checkpoint dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. Nature 467:474–478. https://doi.org/10.1038/nature09373

Zhang Y, Nellimoottil T, Peace JM et al (2013) The level of origin firing inversely affects the rate of replication fork progression. J Cell Biol 201:373–383. https://doi.org/10.1083/jcb.201208060

Zhu W, Ukomadu C, Jha S et al (2007) Mcm10 and And-1/CTF4 mediate the interaction between Rad53 and Dbf4 proteins. J Biol Chem 282:2589–2599. https://doi.org/10.1074/jbc.M111.317060

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.