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The Hsk1(Cdc7) Replication Kinase Regulates Origin Efficiency

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Origins of DNA replication are generally inefficient, with most firing in fewer than half of cell cycles. However, neither the mechanism nor the importance of the regulation of origin efficiency is clear. In fission yeast, origin firing is stochastic, leading us to hypothesize that origin inefficiency and stochasticity are the result of a diffusible, rate-limiting activator. We show that the Hsk1-Dfp1 replication kinase (the fission yeast Cdc7-Dbf4 homologue) plays such a role. Increasing or decreasing Hsk1-Dfp1 levels correspondingly increases or decreases origin efficiency. Furthermore, tethering Hsk1-Dfp1 near an origin increases the efficiency of that origin, suggesting that the effective local concentration of Hsk1-Dfp1 regulates origin firing. Using photobleaching, we show that Hsk1-Dfp1 is freely diffusible in the nucleus. These results support a model in which the accessibility of replication origins to Hsk1-Dfp1 regulates origin efficiency and provides a potential mechanistic link between chromatin structure and replication timing. By manipulating Hsk1-Dfp1 levels, we show that increasing or decreasing origin firing rates leads to an increase in genomic instability, demonstrating the biological importance of appropriate origin efficiency.

INTRODUCTION

Eukaryotic DNA replication initiates at discrete replication origins. The mechanisms by which individual origins are established during G1 and fired during S phase are understood in some detail (Sclafani and Holzen, 2007). However, many questions about the regulation and coordination of origin firing remain (Gilbert, 2001). In particular, it is not understood why, of the many origins that are established during G1, only a subset fire in each S phase. In fission yeast and vertebrates, <50% of potential origins actually fire in each S phase (Dijkwel *et al*., 2002; Jeon *et al*., 2005; Feng *et al*., 2006; Heichinger *et al*., 2006; Lebofsky *et al*., 2006; Patel *et al*., 2006; Eshaghi *et al*., 2007; Mickle *et al*., 2007). This inefficient origin firing leads to a different subset of origins firing in each cell. Origins that do not fire are passively replicated by replication forks from neighboring origins, but they will fire if passive replication is prevented, suggesting that they are functional (Santocanale *et al*., 1999; Vujcic *et al*., 1999; Kim and Huberman, 2001). The excess origins may serve as backups to ensure efficient replication if origin firing is compromised or replication forks stall (Hyrien *et al*., 2003; Legouras *et al*., 2006; Woodward *et al*., 2006; Ge *et al*., 2007). The importance of maintaining normal levels of origin efficiency is suggested by the fact that both increasing and decreasing origin efficiency correlates with decreased genomic stability (Spruck *et al*., 1999; Lengronne and Schwob, 2002).

The origin activation cycle can be divided into four stages (Sclafani and Holzen, 2007). First, the origin recognition complex (ORC) binds to the origin DNA. In the second stage, referred to as licensing, ORC, in collaboration with Cdc6 and Cdt1, loads the minichromosome maintenance (MCM) complex to establish a prereplication complex (pre-RC). In the third stage, during S phase, pre-RCs are activated by cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) phosphorylation, which allows recruitment of additional factors, including Cdc45 and the GINS complex. Finally, the active origin is unwound, and the replication forks are established. Firing of an origin, or passive replication by a replication fork from a neighboring origin, disassembles the pre-RC and resets the cycle. The regulation of origin efficiency could occur at any of these steps in origin establishment and activation. However, because excess origins have established, functional pre-RCs, it seems that reg-

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Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; DDK, Dbf4-dependent kinase; HU, hydroxyurea; ORC, origin recognition complex; pre-RC, pre-replication complex; YES, yeast extract plus supplements.

ulation occurs after the licensing step (Shirahige *et al*., 1998; Santocanale *et al*., 1999; Vujcic *et al*., 1999; Kim and Huberman, 2001; Hayashi *et al*., 2007).

We have recently shown that origin firing in fission yeast is stochastic (Patel *et al*., 2006). That is, origins fire independently of their neighbors. We speculate that the same rate-limiting step underlies both the stochastic and inefficient qualities of origin firing. If the step that limits the efficiency of origin firing is randomly distributed among all potential origins, it will lead to the observed stochastic firing of origins. A plausible candidate for a rate-limiting step is origin phosphorylation by a freely diffusible kinase. Such a kinase would be expected to be required for origin firing throughout S phase, as is DDK (Bousset and Diffley, 1998; Donaldson *et al*., 1998). We show here that the Hsk1-Dfp1 kinase, the fission yeast DDK, is a freely diffusible, rate-limiting activator of origin firing.

MATERIALS AND METHODS

The strains used in this study are listed in Table 1. They were made and maintained using standard methods (Forsburg and Rhind, 2006). Unless otherwise stated, cells were grown at 25°C in yeast extract plus supplements (YES).

Strain and Plasmid Construction

The primers used for making strains and plasmids are listed in Supplemental Table 1. The *adh1:dfp1* allele was made in yFS240 by one-step promoter replacement using pFS255 and primers P96 and P97 (Sivakumar *et al*., 2004). The *GAL4-dfp1-3HA* allele was constructed by amplifying the *dfp1* open reading frame (ORF) from genomic DNA by using P112 and P113 and cloning in into SalI, XmaI-cut pFA6a-3HA-kanMX6 to create pFS295 (Wach *et al*., 1994). The coding sequence for the *GAL4* DNA binding domain (codons 1-147) was amplified from *Saccharomyces cerevisiae* genomic DNA by using P156 and P157 and cloned into BsiWI, AvrII-cut pFS295 to make pFS296. The fusion ORF was then amplified from pFS296 with P166 and P167 and transformed into yFS240 to replace genomic *dfp1* ORF. The 5x Gal4 binding site cassette was amplified from pFS294 and integrated into the Gal4-Dfp1 strain by using P127 and P128 for integration near AT3003 and P138 and P139 for integration near AT2103 to create yFS459 and yFS460, respectively. pFS294 was created by removing the 5x Gal4 DNA binding site cassette from pDZ43 with BglII and SalI and cloning it into a variant of pFA6a-kanMX6, which had been cut with HpaI and EcoRV and religated (Wach *et al*., 1994; Zappulla *et al*., 2002). The strain yFS449 expressing Dfp1–2xGFP was made by sequentially integrating green fluorescent protein (GFP) as a C-terminal tag of Dfp1 in yFS104, first from pFA6a-GFP(S65T)-hphMX6 by using P213 and P167 and then from pFA6a-GFP(S65T)-natMX6 by using P129 and P216 (Sato *et al*., 2005). pFS260 contains *ura4* and the *ars1* fragment from pFS253 cloned into pLIT28 (New England Biolabs, Ispwich, MA) (Sivakumar *et al*., 2004).

Statistical Analyses

For comparisons of origin efficiency and for correlation between the firing of neighboring origins, p is calculated from Fisher's exact test. In the former case, $p < 0.05$ indicates a significant difference between experiment and control; in the latter case, $p > 0.05$ indicates a lack of correlation. For curve fitting, R^2 is calculated from a linear fit on a semi-log plot; p is calculated from the χ^2 statistic for the fit, weighted with the SD of each point; $p > 0.05$ indicates a good fit.

Origin Efficiency Assays

Cell synchronization, labeling, DNA combing, and fluorescence in situ hybridization (FISH) were performed as described previously (Patel *et al*., 2006). Briefly, elutriation-synchronized, G2 cultures were grown in YES with 12 mM hydroxyurea (HU) and 500 nM 5-bromo-2′-deoxyuridine (BrdU) for 4 h to allow cells to complete their first G2, M, and G1 phases, and arrest in S phase. Cells were harvested and set in agarose plugs in which genomic DNA was prepared by SDS/protease-K digestion. The plugs were melted and digested with β-agarase to produce high-molecular-weight DNA in solution. The DNA was combed by dipping and slowly withdrawing a specially silanized glass coverslip, drawing out DNA molecules that have bound nonspecifically to the glass by an end, and laying the stretched DNA flat against the surface. The combed molecules were visualized by epifluorescence microscopy with anti-DNA and anti-BrdU antibodies. The cosmids C25H2, C3B8, and C2G2 were used for FISH probes in the *ars727* region.

Fluorescence Redistribution after Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP) Analysis

Dfp1-2xGFP (yFS449) cells were grown to mid-log at room temperature in YES, concentrated by centrifugation, and mounted in YES on a glass slide under a coverslip affixed by surface tension. Cells were imaged on a Leica SP2 laser scanning confocal microscope. Cells were imaged at 15% laser power to reduce bleaching during imaging. For FRAP, we used the FRAP application module and bleached a rectangular region of interest constituting the central one quarter of the nucleus at 100% laser power. For FLIP, we used the spot-bleach function at 100% laser power. For each experiment, we bleached one nucleus in a septated cell; the other was use as a control for bleaching during imaging. Images were saved as TIFF files and analyzed in ImageJ (National Institutes of Health, Bethesda, MD). All measurements were background corrected, and the change in signal from the bleach nucleus was normalized for bleaching during imaging. Images were uniformly adjusted for contrast, brightness, and pixel input range by using Canvas (Deneba Systems, Miami, FL). The graph of postbleach fluorescence versus bleach time was fit to the single exponential $y = 0.96 \times e^{\wedge}(-x/78)$, with $R^2 = 0.98$, $p = 0.37$ in Igor Pro6 (Wavemetrics, Lake Oswego, OR), yielding $\tau = 78 \pm 7$ ms. We approximated the effective diffusion coefficient D from the radius of the nucleus Rn (~1.1 μm), the radius of the bleach volume Rb (~0.4 μm), and τ (~78 ms), with the equation $D \approx (Rn - Rb)^2/\tau \approx 6\ \mu m^2/s$. We determined the geometry of the bleach volume by bleaching histone 2B-GFP cells fixed in 70% ethanol. The z-stack reconstructions of cells bleached for 50 ms showed the laser bleaches a roughly cylindrical volume, with a diameter of ~0.8 μm. Numerical modeling suggests that systematic errors in our measurements will affect our results by no >50%, giving a confidence interval for the effective diffusion coefficient from 4 to 10 $\mu m^2/s$.

Genome Stability Assays

Wild-type (yFS240), adh1-dfp1 (yFS458) and hsk1-1312 (yFS457) strains were transformed with either pFS260 or pDblet (Brun *et al*., 1995). The transformants were grown in a selective medium containing leucine, adenine and histidine (LAH) until mid-log phase (~OD 2), diluted 40-fold into nonselective YES, and repeatedly diluted whenever the cultures reached mid-log phase. At 0, 6, 16, 24, 48, 72, 96, and 124 h in YES, the cell density was counted, and equal numbers of cells were plated on YES and LAH. After 5–7 d, the numbers of colonies were counted on each plate. The experiment was performed three times. The rate of plasmid loss per generation, L, was calculated

Table 1. Strains used in this study

Strain	Genotype
yFS104	*h+ leu1-32 ura4-D18*
yFS199	*h+ leu1-32 ura4-D18 cds1::ura4*
yFS240	*h− leu1-32 ura4-D18 ade6-210 his7-366 leu1::pFS181 pJL218*
yFS449	*h+ leu1-32 ura4-D18 dfp1-2xGFP (natMX6)*
yFS457	*h− leu1-32 ura4-D18 ade6-216 his7-366? hsk1-1312 leu1::pFS181 pJL218*
yFS458	*h− leu1-32 ura4-D18 ade6-210 his7-366 adh1:dfp1 (kanMX6) leu1::pFS181 pJL218*
yFS459	*h− leu1-32 ura4-D18 ade6-210 his7-366 AT3003:5xGAL4 UAS (kanMX6) GAL4-dfp1-3HA (kanMX6) leu1::pFS181 pJL218*
yFS460	*h− leu1-32 ura4-D18 ade6-210 his7-366 AT2103:5xGAL4 UAS (kanMX6) GAL4-dfp1-3HA (kanMX6) leu1::pFS181 pJL218*
yFS461	*h− leu1-32? ura4-D18 ade6-210 his7-366? leu1::pFS181 pJL218*
yFS462	*h+ leu1-32 ura4-D18 ade6-210 his7-366? cds1::ura4 pJL218 Ch16*
yFS463	*h− leu1-32 ura4-D18 ade6-210 his7-366? adh1:dfp1 (kanMX6) pJL218 Ch16*
yFS464	*h+ leu1-32 ura4-D18 ade6-210 his7-366? cds1::ura4 adh1:dfp1 (kanMX6) pJL218 Ch16*

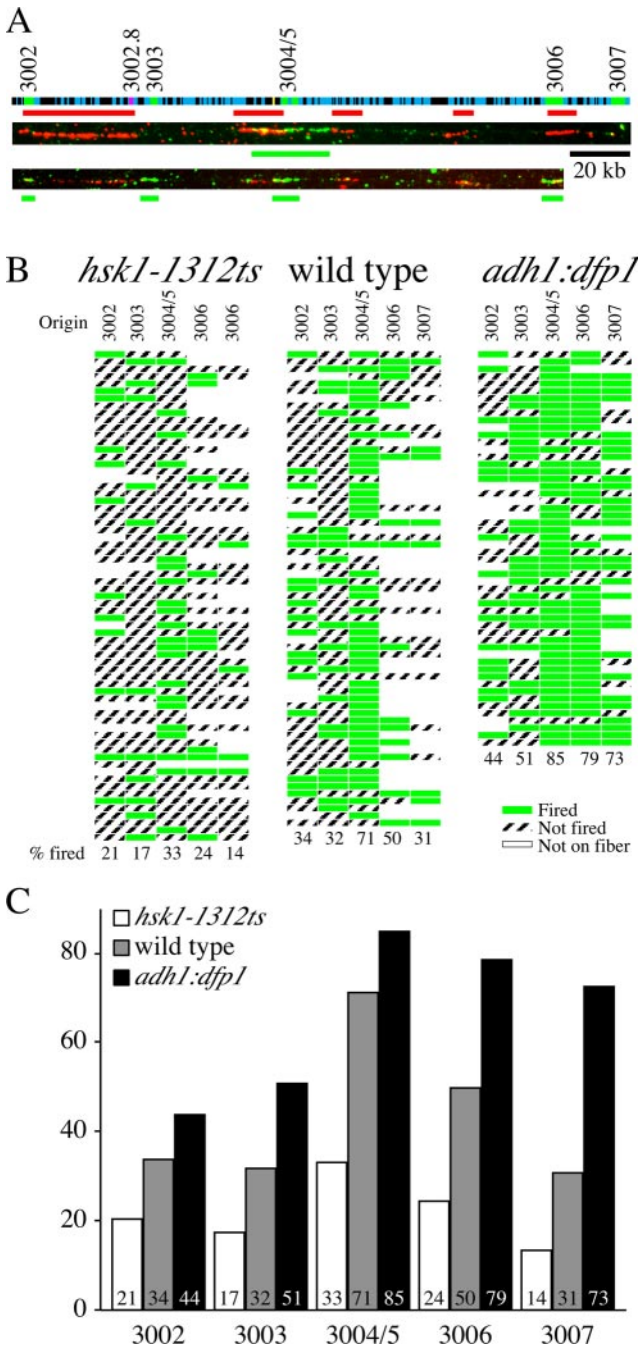

Figure 1. Hsk1-Dfp1 is rate determining for origin firing. (A) The *ura4* region of chromosome III and representative combed molecules. The graphic represents the left end of chromosome III. Blue represents coding sequences, black noncoding sequences, green noncoding sequences predicted to be origins by AT richness (Segurado *et al*., 2003), and yellow *ura4*. Predicted origins are labeled above with the systematic nomenclature of Segurado *et al*. (2003). An additional locus, labeled AT3002.8 and shown in pink, is just below the AT-threshold used for origin prediction. Origins AT3004 and AT3005 are too close together to be reliably distinguished by combing and are treated as one origin. The red bars represent the FISH probes used to identify molecules from this region. Two representative molecules labeled with BrdU and identified with the FISH probes are shown; the top molecule is from *hsk1-ts* (yFS457); the bottom molecule is from *adh1:dfp1* (yFS458). The BrdU signals scored as replication bubbles are indicated by green lines below the images. The greater length of the replication patch in the top image is presumably due to fewer origins firing in the *hsk1-ts* cell, so the replications forks go farther before depleting nucleotides and stalling. The length and continuity of all molecules were determined with anti-guanosine antibodies, which label DNA (data not shown). (B) A graphic table of origin use in the *ura4* region. *hsk1-ts* (yFS457), wild type (yFS240), and *adh1:dfp1* (yFS458) were analyzed for origin efficiency. The data for wild type (yFS240) is from Patel *et al*. (2006). Each row represents a DNA molecule; each column represents an origin in the region. A green box indicates the origin fired on that molecule; a hatched box indicates it did not; no box indicates that the fiber did not extend to include that origin. Below the table is the quantitation of the origin efficiencies. (C) Quantitation of the origin efficiencies in B.

from the ratio of the number of colonies on selective versus nonselective plates for a particular strain by using the equation $L = 100(1 - e^x)$, where $x = (\ln(R_n/R_0))/n$ (Huberman, 1999). R_0 is the proportion of cells retaining plasmid at the start in nonselective media; R_n is the proportion of cells retaining plasmid after n generations in nonselective media.

For minichromosome loss assays, yFS461, yFS462, yFS463, and yFS464 strains carrying the Ch16 minichromosome were grown in a defined medium containing leucine, uracil and histidine until mid-log phase and then diluted into YES with or without 1 mM HU. Similar to the plasmid loss assay described above, whenever the cells reached to mid-log phase they were diluted 40-fold with YES. After ~50 generations in YES, cells were counted and plated on YEA, which is low in adenine and causes Ade− colonies to become pink. After 5–7 d at 30°C, the total numbers of white and pink colonies were counted. Loss rates were calculated as described above.

RESULTS

Hsk1-Dfp1 Is Rate Determining for Origin Firing

If origin phosphorylation by the Hsk1-Dfp1 kinase is a stochastic event that leads to random origin activation, then raising or lowering the activity or abundance of Hsk1-Dfp1 should concomitantly increase or decrease the efficiency of origin firing. To test this prediction, we measured the efficiency of origin firing in early S phase after manipulating the activity of Hsk1-Dfp1.

To reduce the activity of the Hsk1-Dfp1 kinase, we used the temperature-sensitive *hsk1-1312ts* allele. Because *hsk1* is essential and because hypomorphic alleles have pleiotropic phenotypes, we examined *hsk1-1312ts* cells for a temperature at which Hsk1-Dfp1 activity would be only mildly decreased, so that we might see an effect on origin firing efficiency without disrupting replication altogether (Snaith *et al*., 2000). We found that 25°C is the highest temperature at which *hsk1-1312* cells grow well and show none of the previously reported pleiotropic phenotypes. To assay the rate of origin firing, we visualized active origins, which had been labeled in vivo with BrdU, on individual DNA molecules (Patel *et al*., 2006). Cells were elutriation synchronized in G2 and followed through mitosis and into S phase in the presence of the BrdU label and HU, which prevents deoxynucleotide synthesis and arrests cells in early S phase. This protocol allows origins to fire in early S phase and for BrdU to be incorporated in the newly synthesized DNA around those origins. Then, as nucleotides are depleted by replication, forks stall and the replication checkpoint inhibits further origin firing (Santocanale and Diffley, 1998). The labeled DNA molecules are prepared by DNA combing, in which individual, protein-free DNA fibers are stretched on a glass surface and visualized by epifluorescence microscopy (Bensimon *et al*., 1994). BrdU incorporation marks the origins on those fibers that fired before depletion of nucleotides caused replication to arrest.

We examined the effect of reducing Hsk1-Dfp1 activity on the efficiency of the well-studied origins in the *ura4* region of chromosome III (Dubey *et al*., 1994). To identify these ori-

gins, we combined DNA combing with fluorescent in situ hybridization (Figure 1A and Supplemental Figure S1A). This technique allows us to identify fibers from specific regions of the genome and to measure the efficiency of specific origins in those regions. On 67 fibers, we found that the efficiency of five origins from the *ura4* region was reduced by 49%, on average (the range is from 39 to 53%; Figure 1, B and C). By Fisher's exact test of the aggregate data, the difference is highly significant ($p = 7.3 \times 10^{-8}$).

To quantitate the effect of a reduction of Hsk1-Dfp1 activity on origin efficiency across the genome, we measured the distances between origins on unselected combed fibers. In 146 measurements, the average interorigin distance is 64% longer than the wild-type data set (149 vs. 91 kb). In addition, the size of the stalled replicons increase 2.4-fold (median size, 23.2 vs. 9.7 kb), as would be expected if there are fewer forks to deplete the available deoxynucleotides. These results suggest that fewer origins fire across the genome and corroborate the reduction in efficiency of the origins in the ura4 region.

To increase the activity of the Hsk1-Dfp1 kinase, we overexpressed Dfp1 from the constitutive *adh1* promoter, leading to an approximately threefold increase in Dfp1 protein and Hsk1-Dfp1 kinase activity relative to wild-type S phase levels (Supplemental Figure S2). The *adh1:dfp1* cells grow normally and have normal bulk replication kinetics by flow cytometry. However, the increase in Hsk1-Dfp1 kinase activity leads to an increase in origin firing efficiency. On 54 fibers, we found that the efficiency of five origins from the *ura4* region increased by 61%, on average (the range is from 20 to 136%; $p = 8.0 \times 10^{-7}$; Figure 1, B and C). In 443 whole-genome measurements, the average interorigin distance is 24% less than wild type (69 vs. 91 kb) and the replicon size was 20% smaller (median size, 7.8 vs. 9.7 kb), consistent with the increased efficiency seen in the *ura4* region.

In wild-type fission yeast, origin firing is stochastic (Patel *et al*., 2006). To determine whether it is still stochastic in these strains, we measured the correlation between the firing of neighboring origins in the *ura4* region. Similar to wild type, we see no significant positive or negative correlation between the firing of neighboring origins ($p > 0.1$).

Hsk1-Dfp1 Acts in cis to Regulate Origin Firing

Hsk1 and its homologues are known to bind to pre-RCs and phosphorylate the MCM complex (Pasero *et al*., 1999; Duncker *et al*., 2002; Masai *et al*., 2006; Sheu and Stillman, 2006). These observations, together with our results demonstrating that Hsk1-Dfp1 is a rate-determining factor for origin firing, suggest that Hsk1-Dfp1 phosphorylation of origins regulates their efficiency. However, it is also possible that Hsk1-Dfp1 indirectly regulates origin efficiency through other targets. For example, in budding yeast overexpression of Dbf4 interferes with the replication checkpoint, which could in turn affect origin efficiency. To test whether overexpression of Dfp1 interferes with or activities the replication checkpoint in fission yeast, we assayed HU sensitivity and Cds1 kinase activity in *adh1:dfp1* cell. We find no evidence of HU sensitivity, Cds1 inhibition or Cds1 activation, suggesting that the effects of Dfp1 overexpression are not due to indirect effects on the replication checkpoint (Supplemental Figure S3).

To directly test the hypothesis that Hsk1-Dfp1 acts directly on origins, we tethered it to a specific locus via a Gal4-Dfp1 fusion. We reasoned that if Hsk1-Dfp1 acts in *cis*, then increasing its local concentration at a locus should increase the efficiency of origins near that locus. We fused the DNA binding domain of Gal4 (amino acids 1–147) to the amino terminus of Dfp1 at its genomic locus; the strain expressing the Gal4-Dfp1 allele is wild type by standard growth and viability assays. We then integrated five Gal4 binding sites near the *ura4* locus, between the moderately efficient origin AT3003 and the inefficient origin AT3002.8. We examined 250 origin firings in the Gal4-Dfp1 strain—123 on 47 fibers with the Gal4 binding sites and 127 on 63 fibers without Gal4 binding sites—by using the HU-block/FISH approach described above. Consistent with our model, we see a 3.6-fold increase in efficiency of AT3002.8 (from 12 to 43%; $p = 2.8 \times 10^{-4}$ as measured by Fisher's exact test) and

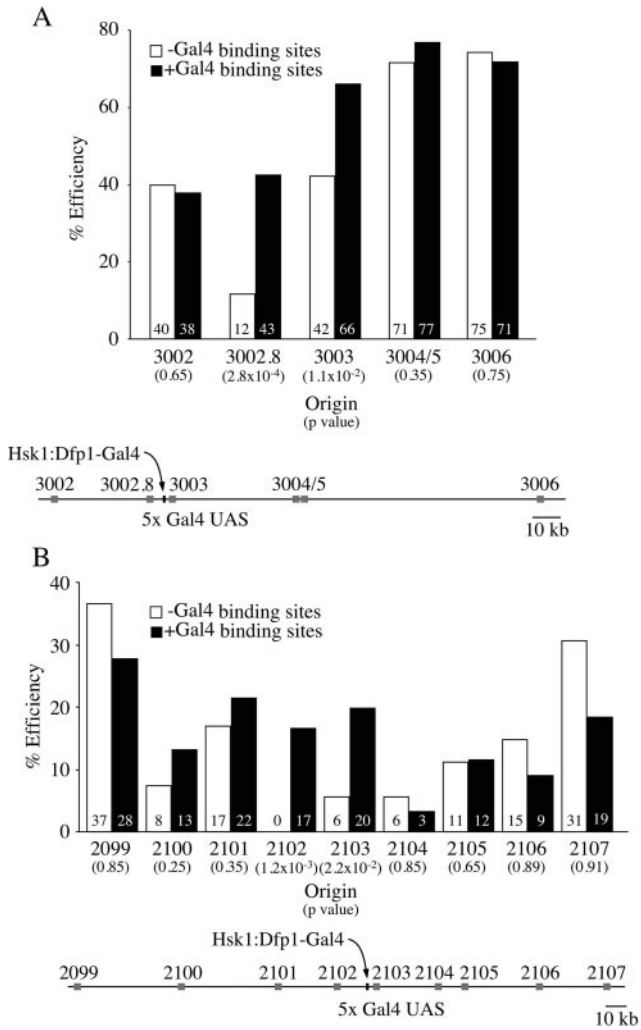

Figure 2. Hsk1-Dfp1 acts in *cis* to regulate origin firing. (A) 5 Gal4 DNA-binding sites were integrated adjacent to origin AT3003. The sites are ~3 kb from AT3003, 6 kb from 3002.8, and 40 kb from both AT3002 and AT3004. A map of the region is shown below the graph, with origins depicted as gray boxes and the Gal4 DNA-binding sites as a black box. The position, but not the size, of the boxes is to scale. Origin efficiency in a strain expressing Dfp1 fused to the DNA-binding domain of Gal4 (yFS459) was determined as in Figure 1. A strain with Gal4 sites integrated at *ars727* (yFS460) was used as the control. Forty-seven fibers were examined for yFS459 and 63 for yFS460. p values in parentheses for pairwise comparisons of efficiencies are from Fisher's exact test. (B) As in A, except the Gal4 DNA-binding sites were integrated adjacent to AT2103 (yFS460, in which the sites are ~3 kb from AT2103, 17 kb from AT2102, 32 kb from AT2104, and 45 kb from AT2101); a strain with Gal4 sites integrated at origin AT3003 (yFS459) was used as the control. Fifty-three fibers were examined for yFS460 and 60 for yFS459.

a 1.6-fold increase in the efficiency of AT3003 (from 42 to 66%; $p = 1.1 \times 10^{-2}$), but no significant effect on the flanking origins AT3002 and AT3004 (Figure 2A and Supplemental S1B). These results demonstrate that Hsk1-Dfp1 is rate determining in *cis* and suggest that the rate-determining step is direct phosphorylation of the origin by the Hsk1-Dfp1 kinase.

Most identified fission yeast origins fire with between 20 and 50% efficiency (Heichinger *et al*., 2006; Patel *et al*., 2006; Feng *et al*., 2006; Mickle *et al*., 2007). However, rare origins have been identified that fire very inefficiently, if at all, but do have assembled pre-RCs (Kim and Huberman, 2001; Feng *et al*., 2006; Hayashi *et al*., 2007). To test whether such cryptic origins are also limited by Hsk1-Dfp1 activity, we tethered Gal4-Dfp1 near the well-studied, cryptic origin *ars727*, which is called AT2103 in the systematic nomenclature of Segurado *et al*. (Kim and Huberman, 2001; Segurado *et al*., 2003; Yompakdee *et al*., 2004). We examined 125 origins firings—74 on 53 fibers with the Gal4 binding sites and 51 on 60 fibers without Gal4 binding sites—and we found that local tethering of Gal4-Dfp1 increases the efficiency of AT2103 3.3-fold (from 6 to 20%; $p = 2.2 \times 10^{-2}$) and increases the efficiency of the neighboring origin AT2102 from 0 to 17% ($p = 1.2 \times 10^{-3}$) without significantly affecting other origins in the region (Figure 2B and Supplemental S1B). These results show that even extremely inefficient origins can be activated by Hsk1-Dfp1, suggesting that Hsk1-Dfp1 is rate limiting for all origins, not just more efficient origins.

Although the simplest explanation for the effect of tethering Hsk1-Dfp1 on the efficiency of local origins in that Hsk1-Dfp1 is directly activating the origins by phosphorylating MCM, it is also possible that the local high concentration of Hsk1-Dfp1 affects local chromatin structure, which in turn indirectly affects local origin efficiency. We reasoned that any effect on local chromatin structure that would affect origin efficiency also would affect transcription. Therefore, to test for local chromatin affects, we used genome-wide transcriptional profiling to assay transcript levels in cells with and without Hsk1-Dfp1 tethered near the *ura4* locus (Oliva *et al*., 2005). We find no significant difference in transcript levels near *ura4* between wild-type cells and cells with Gal4-Dfp1 tethered at AT3003 (Supplemental Figure S4). Specifically, the change in transcript levels for the 24 genes within the 50 kb around AT3003 is 1.02-fold, compared with a genome-wide change of 1.01-fold ($p > 0.2$). These results suggest that the increased local concentration of Hsk1-Dfp1 is not affecting origin efficiency indirectly through local chromatin effects.

Hsk1-Dfp1 Is Freely Diffusible in the Nucleus

Our model that Hsk1-Dfp1 phosphorylation leads to the stochastic firing of origins predicts that Hsk1-Dfp1 should be freely diffusible in the nucleus and able to randomly interact with all potential origins. To test this prediction, we tagged Dfp1 with two copies of GFP and performed FRAP and FLIP experiments. The Dfp1-2xGFP strain grows normally, has wild-type replication kinetics by flow cytometry, and expresses approximately wild-type levels of the Dfp1 fusion protein (Supplemental Figure S2). As expected, Dfp1-GFP was localized primarily to the nucleus. For all of our photobleaching experiments, we use septated cells, which serve in fission yeast as a marker for S-phase cells. For FRAP experiments, we bleached a rectangular strip constituting the central quarter of the nucleus using the laser from a confocal microscope. In traditional FRAP analysis, one measures the kinetics of the redistribution of unbleached fluorescent molecules and from that data extracts the effective diffusion coefficient. However, in the 0.35 s that it took to image the nucleus after bleaching, the unbleached Dfp1-GFP uniformly redistributed throughout the nucleus. Because the fluorescence redistribution is faster than we can measure, FRAP only allows us to put a lower limit on the diffusibility of Hsk1-Dfp1. Using a standard FRAP model, we estimate that the effective diffusion coefficient for Dfp1-GFP is > 0.5 $\mu m^2/s$ (Axelrod *et al*., 1976; Carrero *et al*., 2003).

To estimate more accurately the effective diffusion coefficient, we developed a novel variant of the FLIP approach, which allows for the measurement of high effective diffusion coefficients without the need for fast data acquisition. In this technique, we bleach a small volume in the nucleus for varying times. When the bleach pulse is very short, only the molecules in the bleach volume are bleached, and the overall

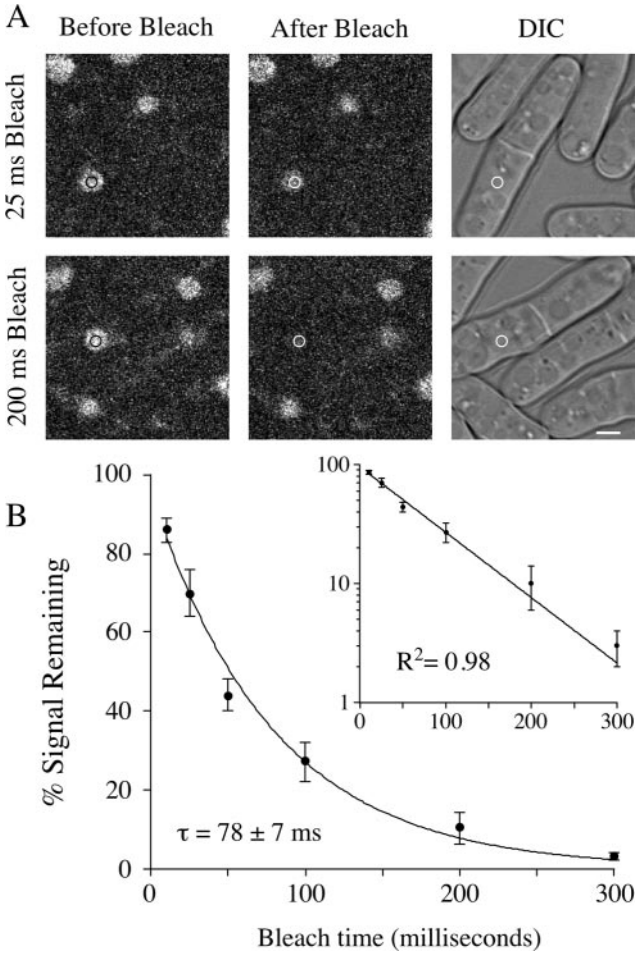

Figure 3. Hsk1-Dfp1 is freely diffusible in the nucleus. (A) *dfp1-GFP* cells (yFS449) were imaged before and after bleaching for the indicated time with an SP2 laser scanning confocal microscope (Leica, Wetzlar, Germany). Bleaching was preformed with the spot bleach function in the area indicated by the circle. Scale bars represent 2 μm. We see defuse nuclear localization of Dfp1 at all cell cycle stages, which is not inconsistent with the previous reports of punctate localization of Dfp1 homologues in G1 and S, because previous studies have used fixed cells that may well preferentially retain protein at sites of transient association (Pasero *et al*., 1999; Masai *et al*., 2006). (B) The total nuclear fluorescence was measured before and after bleaching. The data represent the average of three to seven experiments; the error bars represent the SE of the mean. The inset shows the same data on a semi-log plot.

fluorescence of the nucleus is reduced by an amount proportional to the volume of the bleach spot. However, when the bleach pulse is longer, molecules have time to diffuse in and out of the bleach volume. Thus, more molecules are bleached, and the overall fluorescence of the nucleus is further reduced. From the graph of bleach time versus loss of fluorescence, we can extract the effective diffusion constant. The major advantage of this approach is that it is limited by the duration of the bleach pulse, which can easily be on the millisecond time scale, not the speed of imaging the sample, which can take hundreds of milliseconds.

We bleached an ~0.8-μm-diameter spot—constituting about one fifth the volume of the 2.3-μm nucleus—for various times (Figure 3). When we bleached for 300 ms, 97% of the GFP signal was bleached, demonstrating that the bulk of the Dfp1-GFP moved through the bleached volume within 300 ms (Figure 3B). Shorter length pulses bleached less of the Dfp1-GFP fluorescence. By fitting an exponential curve to the graph of fluorescence versus bleach time, we estimated the average time until bleaching for any single GFP molecule to be 79 ms, and from that we estimated an effective diffusion coefficient of 6 $\mu m^2/s$ (see *Materials and Methods* for details). For comparison, the freely diffusible mammalian nuclear proteins HMG-17 and fibrillarin have effective diffusion coefficients ~0.5 $\mu m^2/s$ (Phair and Misteli, 2000). These diffusion coefficients are lower than the 20 $\mu m^2/s$ of free nuclear GFP, suggesting that the diffusion of these proteins is slowed by interaction with other proteins in the nucleus (Phair and Misteli, 2000; Essers *et al*., 2002). In Hsk1-Dfp1, effective diffusion could be slowed by its association with chromatin and, in particular, origins (Takeda *et al*., 1999; Weinreich and Stillman, 1999). However, the important conclusion is that Dfp1-GFP, and presumably Hsk1-Dfp1 complexes, diffuse rapidly in the nucleus on the 20-min time scale of replication. In particular, the average time it takes a molecule of Dfp1-GFP to bleach (~0.1 s) is approximately the time that it takes it to diffuse across the nucleus.

Hsk1-Dfp1 Levels Affect Plasmid and Chromosome Stability

Because the levels of Hsk1-Dfp1 activity regulate the efficiency of replication origins, such regulation is likely to benefit cells, perhaps by increasing genomic stability. For example, a reduction of origin efficiency could decrease genome stability by increasing the distance between active origins and thus increasing the chances of replication fork collapse, whereas an increase in origin efficiency could tax nucleotide pools or repair processes, making fork stalls more common or less efficiently repaired. This hypothesis predicts that either raising or lowering Hsk1-Dfp1 activity should increase genome instability. To test these predictions, we assayed the stability of two reporters: a plasmid that contains a single origin and a minichromosome that contains many origins.

The plasmid-stability assay is an indirect measure of origin efficiency. Because the plasmid has only one origin and that origin must fire for the plasmid to be inherited by both daughters, the stability of the plasmids is dominated by the efficiency of the plasmid origin. As expected, using *hsk1-ts* to reduce Hsk1-Dfp1 activity results in an increase in the rate of plasmid loss by almost twofold (Figure 4A). Conversely, overexpressing Dfp1 decreases plasmid loss by almost fivefold. As a control to demonstrate that the effect we measured was primarily due to origin efficiency, we repeated the experiments with pDblet, a plasmid carrying two copies of *ars3002* (Figure 4A) (Brun *et al*., 1995). The extra origin makes the plasmid almost fivefold more stable in wild-type cells, an effect comparable with the overexpression of Dfp1. Furthermore, overexpression of Dfp1 does not significantly increase the stability of pDblet, suggesting that pDblet stability is not limited by origin efficiency. However, pDblet is almost threefold less stable in *hsk1-ts* cells, suggesting that the plasmid is still sensitive to reductions in origin efficiency.

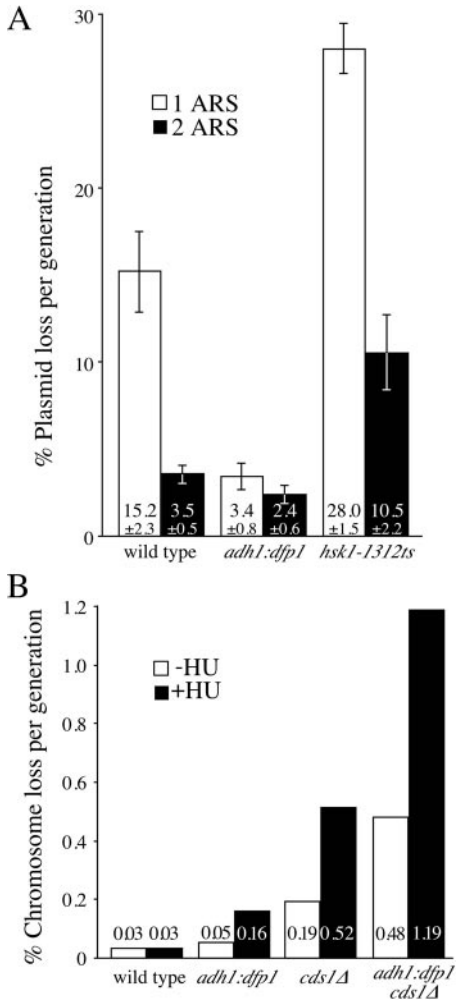

Figure 4. Hsk1-Dfp1 levels affect plasmid and chromosome stability. (A) Wild-type (yFS240), *hsk1-ts* (yFS457), and *adh1:dfp1* (yFS458) strains carrying plasmids with one copy (pFS118) or two copies of *ars3002* (pDblet) were analyzed for plasmid loss rates. The data represent the average and SD of three independent experiments. (B) Wild-type (yFS461), *adh1:dfp1* (yFS463), *cds1*Δ (yFS462), and *adh1:dfp1 cds1*Δ (yFS464) strains carrying the nonessential minichromosome Ch16 were analyzed for Ch16 loss rates in the presence or absence of 1 mM hydroxyurea.

The minichromosome assay measures global genome stability because it does not depend on the firing of any one origin. Instead, minichromosome Ch16 contains many origins, and the effect of origin efficiency on its loss is likely to be due to indirect effects on replication fork stability. We measured the loss of Ch16 in wild-type and *adh1:dfp1* cells and found no significant difference (Figure 4B). However, it is possible that any replication problems due to the increase in origin efficiency caused by overexpression of Dfp1 can be efficiently resolved by the replication checkpoint. We tested the importance of the replication checkpoint in maintaining the stability of Ch16 in *adh1:dfp1* cells by deleting the *cds1*

checkpoint gene. Ch16 stability is reduced over 10-fold in *adh1:dfp1 cds1*Δ cells, significantly lower than in either *adh1:dfp1* or *cds1*Δ cells alone, and lower even than the additive effect of the two genotypes (Figure 4B). This result demonstrates that the checkpoint is required to stabilize chromosomes in *adh1:dfp1* cells and suggests that abnormally high origin firing efficiencies lead to increased replication fork density, requiring the activity of the checkpoint to prevent increased irreparable fork collapse. However, because Hsk1-Dfp1 is thought to be directly involved it fork metabolism, it is also possible that the increase Hsk1-Dfp1 activity could directly affect fork stability.

The hypothesis that increased replication fork density in *adh1:dfp1* cells leads to increased requirement for the replication checkpoint predicts that chromosome stability in *adh1:dfp1* cells should be sensitive to mild replication stress caused by a moderate reduction in nucleotide levels. To test this hypothesis, we induced replication stress by growing the cells in a low level of hydroxyurea. The addition of 1 mM HU did not decrease the stability of Ch16 in wild-type cells, but it did decrease the stability of Ch16 in *adh1:dfp1* cells by more than threefold. This result suggests that the increased origin firing in *adh1:dfp1* cells interferes with the ability of cells to cope with the replication stress caused by nucleotide depletion. To test whether this ability requires the replication checkpoint, we examined chromosome stability in HU-treated *adh1:dfp1 cds1*Δ cells. Ch16 stability under these conditions is reduced more than sevenfold, significantly more than in either *adh1:dfp1* or *cds1*Δ cells alone, and more even than the additive effect of the two genotypes (Figure 4B). Together, the plasmid and minichromosome stability results show that either increasing or decreasing origin efficiency decreases genome stability and suggest that cells regulate the efficiency of their origins by setting the level of Hsk1-Dfp1 activity to maximize genome stability.

DISCUSSION

Replication origins in fission yeast fire inefficiently and stochastically (Dubey *et al*., 1994; Feng *et al*., 2006; Heichinger *et al*., 2006; Patel *et al*., 2006; Eshaghi *et al*., 2007; Hayashi *et al*., 2007; Mickle *et al*., 2007). We reasoned that both the inefficient and stochastic nature of origin firing could be explained if the rate of origin activation was regulated by a freely diffusible, rate-limiting factor. Such a factor would randomly interact with and activate licensed replication origins, leading to stochastic activation. But, because it is limiting, it would be able to activate only a subset of the licensed origins in any given S phase, leading to inefficient firing. We further reasoned that if such a factor was overexpressed, it should lead to increased origin firing. We therefore took a candidate protein approach, considering proteins known to be required for pre-RC formation and activation, including Cdc18, Mcm10, Cdc45, and the Hsk1-Dfp1 replication kinase. We obtained positive results with Hsk1-Dfp1 before obtaining conclusive results for other candidates and have thus focused on Hsk1-Dfp1.

Our results show that increasing or decreasing Hsk1-Dfp1 kinase activity leads to an increase or decrease in origin firing efficiency, respectively (Figure 1). We measured origin efficiency by labeling the nascent DNA at active origins in vivo with BrdU and visualizing the BrdU incorporation patches on individual combed DNA molecules. In combination with FISH to identify fibers from specific regions of the genome, this approach allows us to directly count how often a specific origin fires in a population of cells. Because we can count large numbers of fibers, we can measure changes in origin efficiency as small as 20% with high statistical confidence. To facilitate the unambiguous identification of origins that fire early in S phase and to prevent the fusion of BrdU incorporation patches from neighboring origins, we analyzed synchronized cells in early S phase and performed our BrdU labeling the presence of HU. HU prevents deoxynucleotide synthesis and blocks replication after ~10% of the genome is replicated (Patel *et al*., 2006). After origins have fired early in S phase, the early forks progress ~5 kb and in the process reduce deoxynucleotide levels to a point at which the replication checkpoint is triggered, forks stall, and further origin firing is prevented (Santocanale and Diffley, 1998; Kim and Huberman, 2001). Therefore, the checkpoint is not activated until after the firing of origins in early S phase, and thus the checkpoint is presumed not to affect the firing of those origins. Furthermore, origin efficiency estimates from this technique correlate well with estimates derived from microarray studies and a recent study in budding yeast showed that checkpoint activation does not disrupt the overall pattern of origin activation (Heichinger *et al*., 2006; Alvino *et al*., 2007). Therefore, we do not believe that HU treatment affects the efficiency of the early firing events that we are studying. We attempted to confirm our results by measuring origin efficiency using two-dimensional (2D) gels, but we found that 2D gels are not sufficiently quantitative to accurately measure the changes in efficiency that we see by combing.

Our results suggest that Hsk1-Dfp1 kinase activity, and presumably the phosphorylation of pre-RCs by Hsk1-Dfp1, is rate-determining in origin activation. As such, phosphorylation by Hsk1-Dfp1 is an excellent candidate for the stochastic step that leads to the random distribution of origin firing in fission yeast. Specifically, we propose that Hsk1-Dfp1 is limiting, but freely diffusible in the nucleus, and thus that it randomly interacts with origins during S phase; when these interactions lead to productive phosphorylation, the origin fires. This model accounts for the stochastic activation of origins and for the effects of increasing and decreasing Hsk1-Dfp1 activity on the probability of activation. It is possible that an earlier stochastic event determines the order of origin firing and that Hsk1-Dfp1 simply executes that order, but such a model seems unnecessarily complicated. Our results are consistent with recent work showing that Cdc7-Dbf4, the budding yeast DDK, is required for efficient origin firing (Hoang *et al*., 2007).

It should be noted that our observation that Hsk1-Dfp1 is freely diffusible in the nucleus does not contradict the observation that Hsk1-Dfp1 binds to chromatin (Takeda *et al*., 1999). All chromatin-binding proteins have a finite dissociation constant; their mobility in the nucleus is determined by the equilibrium between bound and unbound states. That the diffusion coefficient for Dfp1-GFP is approximately threefold lower than GFP alone suggests that Dfp1 spends ~70% of the time bound to fixed structures in the nucleus, presumably pre-RCs. Nevertheless, the time between chromatin binding allows Dfp1-GFP to rapidly sample the nucleus, diffusing across the nucleus in ~100 ms (Figure 3).

Phosphorylation of the MCM complex by Hsk1-Dfp1 is thought to be a critical trigger in origin activation (Sclafani and Holzen, 2007). Therefore, is seems likely the Hsk1-Dfp1 executes its rate-limiting function by directly phosphorylating pre-RCs. To test whether the Hsk1-Dfp1 role in regulating origin efficiency does in fact act locally at origins, we tethered Dfp1 via a Gal4 DNA binding fusion to a specific locus in the genome. We find that tethering of Hsk1-Dfp1

does increase the efficiency of local origins. We saw significant increase in efficiency for origins between 3 and 17 kb away from the tethered kinase (Figure 2, AT3002.8, AT3003, AT2102, and AT2103), but no significant effect on origins 32 kb or further away. In this type of experiment, with one protein (Hsk1-Dfp1) being tethered to DNA to increase its local concentration relative to another protein (an origin bound pre-RC) bound to the same region of DNA, the maximum increase in relative concentration is achieved if the two bound proteins are separated by ~3 or 4 times the persistence length of the chromatin—the persistence length being the length over which the chromatin is too stiff to bend easily (Rippe, 2001). The persistence length of yeast chromatin is ~3 kb, so the maximum concentration effect should be ~10 kb from the tethered Gal4-Dfp1 (Rippe, 2001; Dekker *et al*., 2002). The relative concentration effect falls off approximately exponentially with distance; a site 17 kb away from the tethered Hsk1-Dfp1 (such as AT2102) should have about one third the relative concentration of a site 10 kb away, whereas a site 32 kb away (such as AT2104) should have about 1/30th the concentration (Rippe, 2001). These calculations make it plausible that AT2102 would show a strong effect of Hsk1-Dfp1 tethering 17 kb away, but AT2104 would not show an effect of Hsk1-Dfp1 tethering 32 kb away. Therefore, we conclude that Hsk1-Dfp1 acts locally to regulate origin efficiency, presumably by phosphorylating MCM. However, Hsk1-Dfp1 could affect origin efficiency indirectly by phosphorylating other localized regulatory proteins, such as checkpoint kinases, which have been implicated in origin firing in other systems (Shechter and Gautier, 2005).

It is interesting to note that, although global and local increase of Hsk1-Dfp1 concentration increases origin efficiency, it does not increase efficiency to close to 100%. This outcome could result from not expressing enough Dfp1 to activate all licensed pre-RCs. Alternatively, it is possible that when Hsk1-Dfp1 is in excess some other factor becomes limiting for origin efficiency. For example, if ORC only binds to a specific origin in 60% of cells, that origin will never be >60% efficient. Origin efficiency in fission yeast roughly correlates with AT richness (Segurado *et al*., 2003; Dai *et al*., 2005). Because ORC is thought to bind AT tracts through the AT-hook motifs in Orc4, this result suggests that ORC binding is an important determinant in origin efficiency (Chuang and Kelly, 1999; Dai *et al*., 2005). Other limiting factors, such as ORC occupancy, may explain why some origins are more efficient than other (Feng *et al*., 2006; Heichinger *et al*., 2006; Eshaghi *et al*., 2007; Hayashi *et al*., 2007; Mickle *et al*., 2007). If Hsk1-Dfp1 interacts equivalently with most origins, those that are more likely to be licensed will be more likely to fire. However, other origins, such as the cryptic *ars727*, may be regulated by *cis*-acting motifs which could regulate Hsk1-Dfp1 accessibility (Yompakdee and Huberman, 2004).

Implications for the Regulation of Replication Timing

Our results show that the Hsk1-Dfp1 replication kinase is rate determining for origin firing in fission yeast, and they explain how both the stochastic and inefficient character of origin firing could result from the random activation of licensed origins by Hsk1-Dfp1 phosphorylation. This model also provides a plausible mechanism for differential regulation of origin efficiency within a cell, in which the efficiency of specific origins is regulated by their accessibility to Hsk1-Dfp1. A similar model has been proposed for origin regulation in budding yeast in which DDK "limited in amount and concentrated at origins would act locally to give the pace at which individual origins fire" (Nougarede *et al*., 2000). Regulation of DDK origin phosphorylation could be positive, by increasing the affinity of Hsk1-Dfp1 for certain origins, or negative, by restricting access of Hsk1-Dfp1, for example by sequestering origins within condensed chromatin (Goren *et al*., 2008). Regulating origin efficiency has been proposed as a mechanism for controlling origin timing (Hyrien *et al*., 2003; Rhind, 2006; Bechhoefer and Marshall, 2007; Lygeros *et al*., 2008). Therefore, the regulation of origin efficiency via the accessibility of DDK may provide a mechanistic link between chromosome structure and replication timing. Origins in transcriptionally active chromatin, which tend to replicate early in S phase, would fire earlier because they are more accessible to DDK, whereas origins in heterochromatic regions would be less accessible to DDK and thus be less efficient and later replicating. It should be noted that other diffusible, rate-limiting activators could be regulate origin firing in other organisms with the same general consequences for efficiency and timing. For example, both CDK activity and Cdc45 have been suggested to be limiting for origin firing in frog embryo extracts (Krasinska *et al*., 2008).

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