Specific replication origins promote DNA amplification in fission yeast

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Summary

To ensure equal replication of the genome in every eukaryotic cell cycle, replication origins fire only once each S phase and do not fire after passive replication. Failure in these controls can lead to local amplification, contributing to genome instability and the development of cancer. To identify features of replication origins important for such amplification, we have investigated origin firing and local genome amplification in the presence of excess helicase loaders Cdc18 and Cdt1 in fission yeast. We find that S phase controls are attenuated and coordination of origin firing is lost, resulting in local amplification. Specific origins are necessary for amplification but act only within a permissive chromosomal context. Origins associated with amplification are highly AT-rich, fire efficiently and early during mitotic S phase, and are located in large intergenic regions. We propose that these features predispose replication origins to re-fire within a single S phase, or to remain active after passive replication.

Key words: Cell cycle, DNA amplification, DNA replication, Fission yeast, Replication origin

Introduction

For stable genome inheritance, S phase occurs once per cell cycle, and within S phase the genome is completely and evenly replicated. Unscheduled DNA synthesis can lead to local amplification (Arias and Walter, 2007; Varshavsky, 1981) and genome instability (Schimke et al., 1986; Seo et al., 2005; Varshavsky, 1981), and can be induced by two types of perturbation. First, depletion of G2–M cyclin-dependent kinase (CDK) activity in yeast and fruit fly, and mouse and human cell lines leads to repeated DNA doublings without mitosis (Arias and Walter, 2007; Kiang et al., 2009; Mihaylov et al., 2002). In fission yeast *Schizosaccharomyces pombe* this occurs by a largely normal S phase program; given that normal S phase origins are used, periodic rounds of DNA synthesis are correlated with normal G1–S gene expression and cell mass doubling, and there is even replication of the genome (Kiang et al., 2009). Second, dysregulation of the replication factors Cdc6 and Cdt1 brings about increased DNA content in a range of organisms (Arias and Walter, 2007; Gonzalez et al., 2006; Melixetian et al., 2004; Mihaylov et al., 2002; Vaziri et al., 2003). Cdc6 and Cdt1 are components of the pre-replicative complex (Pre-RC) (Bell and Dutta, 2002; Kelly et al., 1993) that bind at replication origins and recruit the mini chromosome maintenance (MCM) complex, the likely replicative helicase (Bell and Dutta, 2002). In fission yeast, overexpression of the *CDC6* homologue *cdc18* in G2 induces re-initiation of DNA synthesis up to a DNA content of about 8C–16C (Nishitani and Nurse, 1995); during co-overexpression with *cdt1* (*cdc18 cdt1* co-oe), DNA content both increases more rapidly and attains a higher ploidy level (~32C) (Gopalakrishnan et al., 2001; Nishitani et al., 2000; Yanow et al., 2001) (see supplementary material Table S1 for genotypes). In these cells, the S phase controls, ensuring that an origin fires no more than once per round of replication, may be abrogated, because overexpression of a *cdc18* phosphorylation-site mutant brings about some local amplification, particularly at the telomeres (Mickle et al., 2007). Therefore, we asked whether genome-wide coordination of origin firing is lost in *cdc18 cdt1* co-oe, leading to local amplification, and if so, what features of replication origins might be responsible for that amplification.

Results and Discussion

We analyzed the pattern of DNA synthesis and G1–S gene expression in the presence of excess Cdc18 and Cdt1. By pulse-labeling *S. pombe* cells with BrdU (Fig. 1A,B; supplementary material Fig. S1A,B) (Sivakumar et al., 2004) or observing the G1–S gene expression marker Tos4-GFP (supplementary material Fig. S1C) (Kiang et al., 2009) as cells increased their DNA content from 2C to 32C, we found that *cdc18 cdt1* co-oe does not show characteristics of repeated, periodic S phases that require G1–S gene expression. Rather, it induces an extended period of continued DNA synthesis, resulting in approximately four doublings in DNA content. Since this pattern of DNA synthesis did not resemble a normal S phase, we asked whether normal S phase origins of replication were being used to replicate the genome and whether they fired in a coordinated fashion. First, we mapped the origins that become activated, using the DNA synthesis inhibitor hydroxyurea (HU) to inhibit fork progression and estimating the DNA content in the vicinity of fired origins by using whole genome DNA microarrays (Heichinger et al., 2006) (Fig. 1D,E). We identified 796 origins that fired in *cdc18 cdt1* co-oe (supplementary material Table S2 and Table S3A). In comparing origins activated in *cdc18 cdt1* co-oe versus normal mitotic S phase, we distinguished three classes: origins that fire in both (683 of 904 S phase origins),

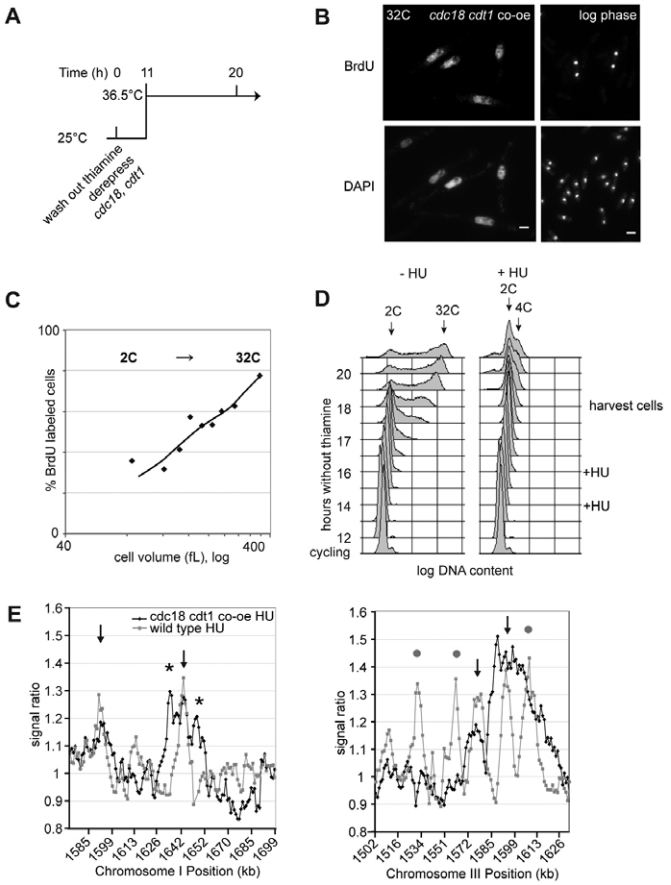

Fig. 1. BrdU pulse labeling and mapping origins in re-replication of a *cdc18 cdt1* co-oe strain. (**A**) Depletion of thiamine allows co-overexpression of *cdc18* and *cdt1*, which are under control of the strong thiamine-repressible promoter *nmt1*. Temperature-sensitive conditional mutant *cdc25-22* cells arrest in G2 when put at restrictive temperature. DNA synthesis does not normally occur during G2 in the cell cycle, but is induced by co-overexpression of *cdc18* and *cdt1*. (**B**) Left panels: DNA synthesis was induced in *cdc25-22 cdc18 cdt1 co-oe tk hENT* cells. Ten-minute pulses of 300 µM BrdU were performed every 30 minutes from 2.5 hours onwards after temperature shift. DNA was visualized by DAPI staining and BrdU was detected by indirect immunofluorescence. Cells with a DNA content of ~32C are shown. Right panels: cells in log phase were pulsed with 300 µM BrdU for 10 minutes. BrdU incorporation is seen in binucleate and recently divided cells. Most cells in a log phase culture have a DNA content of 2C because G2 is the longest phase in the cell cycle. Scale bars: 5 µm. (**C**) Cell volume (fL) was calculated by estimating the shape of the cell as a cylinder with a half-sphere at each end. Cells from all time points were binned according to their fixed cell volumes, and the percentage of cells with incorporated BrdU in each bin was plotted as a function of fixed cell volume (Kiang et al., 2009). (**D**) Fluorescence-activated cell sorter (FACS) analysis. To map origins in the *cdc25-22 cdc18 cdt1* co-oe strain, a protocol as described for A was followed, and HU was added prior to the onset of re-replication at 14 and 16 hours. Samples for origin mapping were collected at 18 hours when the culture without HU had replicated to a DNA content of ~8C. (**E**) Comparison of wild type (Heichinger et al., 2006) and *cdc18 cdt1* co-oe replication profiles. ↓, Origins used in both normal S phase and in *cdc18 cdt1* co-oe; ●, normal S phase origins not used in *cdc18 cdt1* co-oe; *, new origins used only in *cdc18 cdt1* co-oe. Regions were selected to illustrate all three types of origin.

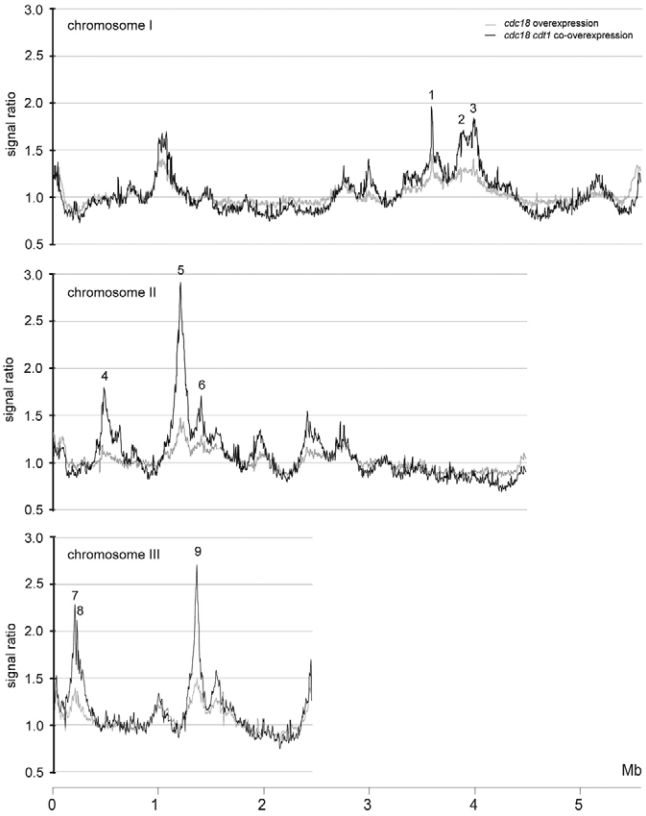

Fig. 2. Uneven replication across the genome after *cdc18 cdt1* co-overexpression. Genomic DNA from *cdc18* oe or *cdc18 cdt1* co-oe cells that re-replicated to a DNA content of ~32C was hybridized against reference DNA to ORF arrays. The signal was normalized to the rest of the genome. Each profile is the moving average of duplicate ORF array experiments. Peak amplitudes can vary among experiments; the nine regions that were distinct peaks with an amplitude consistently greater than 1.6 relative signal ratio over all experiments are numbered. Regions that were not consistently clear peaks >1.6 in all experiments, for example, the peak around 1 Mb from the left end of chromosome *I*, were not further analyzed.

origins that fire in *cdc18 cdt1* co-oe but not S phase (113) and origins that fire in S phase but not *cdc18 cdt1* co-oe (221) (supplementary material Table S3A,B). The three classes of origin were similar with regard to mean AT content and intergenic size, features that are correlated with the ability to act as an autonomously replicating sequence on a plasmid (Dai et al., 2005), mean efficiency of origin usage in a normal S phase and colocalization with sites of Pre-RC assembly in G1 (Hayashi et al., 2007). The only difference was that the 221 origins not activated were about half as efficient as the average origin in a normal S phase (supplementary material Table S3B). We found that the majority of origins activated in *cdc18 cdt1* co-oe are also active or potential origins in a normal S phase, although some less-efficient S phase origins are not activated.

Next, we investigated whether controls ensuring that origins fire only once per round of DNA replication are still operative. We analysed genomic DNA from cells at a DNA content of ~32C (equivalent to four rounds of replication) using microarrays, normalizing to the median signal ratio (Fig. 2). If replication were equal across the genome, as in the absence of G2–M CDK (Kiang et al., 2009), the relative signal ratio would be uniform across the genome with no significant peaks above the baseline signal ratio of one. In *cdc18 cdt1* co-oe, there were nine distinct regions that were consistently amplified as peaks reaching 1.6- to 4-fold over

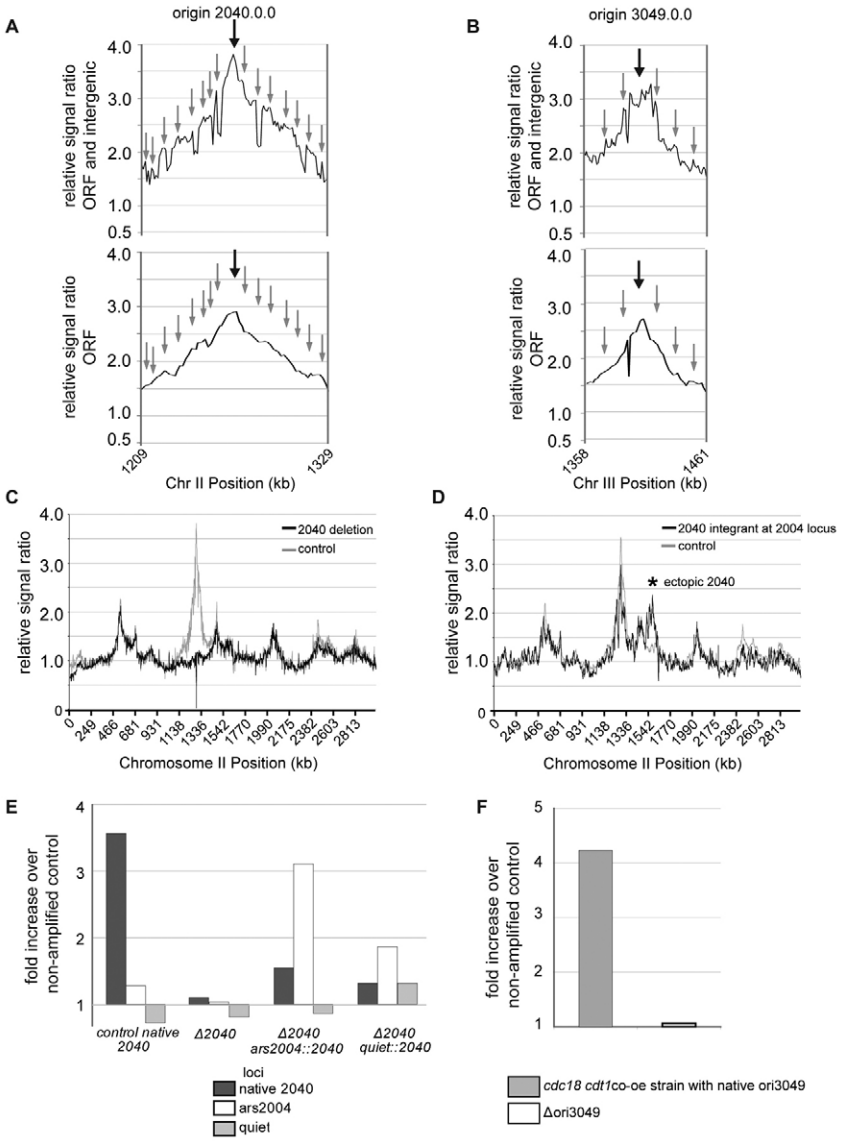

Fig. 3. Origin 2040.0.0 is necessary but not sufficient for local amplification. (**A–D**) Microarray analysis of genomic DNA from cells at a DNA content of ~32C. The signal is normalized to the rest of the genome. Black arrows, origins centered within amplification peaks are S phase origins active in rereplication; gray arrows, neighbouring origins active in co-overexpression. (A) S phase origins within *cdc18 cdt1* co-oe amplification peak 5, chromosome *II*. (Top) *cdc18 cdt1* co-oe Δorigin 3048.0.0 experiment done at high resolution with ORF and intergenic tiling microarrays. (Bottom) Same peak in *cdc18 cdt1* co-oe strain using ORF arrays only; whole-genome ORF profile is shown in supplementary material Fig. S4. (B) S phase origins within *cdc18 cdt1* co-oe amplification peak 9, chromosome *III*. (Top) *cdc18 cdt1* co-oe Δorigin 2040.0.0 experiment using ORF and intergenic arrays; (bottom) same peak in *cdc18 cdt1* co-oe using ORF arrays. (C,D) Gray, normal unperturbed amplification in *cdc18 cdt1* co-oe Δorigin 3048.0.0 strain. (C) Δorigin 2040.0.0 in *cdc18 cdt1* co-oe. (D) An extra copy of origin 2040.0.0 was integrated by replacement of ars2004 (origin 2050.0.0). *, Integration site. (**E**,**F**) Genomic DNA from cells at a DNA content of ~32C was analyzed by qPCR. The fold increase over a non-amplified control region was determined. (E) Fold amplification at native and ectopic loci in four strains. Dark gray, signal near native origin 2040.0.0 locus; white, signal at ars2004 locus; light gray, signal at C19F5.02. (F) Light gray, fold amplification near native origin 3049.0.0; white, origin 3049.0.0 deleted.

baseline, and were amplified to a lesser extent when *cdc18* was overexpressed alone (Fig. 2). Two of these peaks are close to centromeres. The remaining 92% of the genome fell between a signal ratio of 0.5:1 and 1.5:1, indicating that the majority of the genome was roughly equally replicated during the four doublings in DNA content. During the extended DNA synthesis induced by *cdc18 cdt1* co-oe, the majority of origins do not re-fire within a round of replication, but the nine amplified regions must contain origins that escape this control.

To determine whether the amplified regions are dependent upon specific origins, we mapped origins of replication located at the center of the two most highly amplified regions (Fig. 3A,B). Deletion of the central origin sequence in both amplification peaks abolished amplification in these regions [origins 2040.0.0 (Fig. 3C,E) and 3049.0.0 (Fig. 3F), equivalent to ‘strong’ origins 2040 and 3049, respectively (Heichinger et al., 2006), see supplementary material Table S2 for nomenclature]. By contrast, deletion of neighboring origins under the peaks had no effect on amplification (data not shown). These data indicate that, in each region, a single specific origin drives local amplification. To test whether such an origin is sufficient to induce amplification, a 3.65 kb sequence including the AT-rich island of origin 2040.0.0 was integrated into a region of chromosome *II* that undergoes only limited amplification. The 3.65 kb sequence induced significantly increased amplification at this ectopic locus in a strain deleted for origin 2040.0.0 (Fig. 3E), and in a strain containing both the endogenous origin 2040.0.0 and the ectopic origin 2040.0.0 (Fig. 3D). Therefore, origin 2040.0.0 induces amplification outside of its normal chromosomal context. However, when this origin was inserted into a region of chromosome *II* that normally shows no amplification, the ectopic origin was unable to induce amplification (Fig. 3E). In the absence of *cdc18 cdt1* co-overexpression, the origin does fire in this context, albeit at about half its efficiency when located in its native position (signal ratio ~0.2:1 versus ~0.44:1) (supplementary material Fig. S2). We conclude that specific origins are likely to be necessary for amplification within each amplified region, and also that these origins can only induce local amplification in specific chromosomal contexts.

We next identified parameters that characterize the origins that drive amplification. Origins centered within the nine amplification peaks (Fig. 3A,B; supplementary material Fig. S3) were found to share three characteristics: high AT content, efficient firing in early

S phase and chromosomal location within long intergenic stretches (Fig. 4). When all S phase origins were assessed according to efficiency versus AT content, they formed a wedge-shaped distribution with a minimum AT content of ~69% required for origin activity (Fig. 4A). Efficient origins are AT-rich, but AT-rich origins are not necessarily efficient. The nine origins centrally located in the local amplification regions are highly AT-rich, with a mean AT content of 81.0% compared with the mean for all origins of 73.8%. Their mean efficiency in S phase is 2.7-fold greater than the average for all origins and they fire early during the first quarter of a normal S phase, at 74 minutes on average. They are embedded in intergenic regions that are on average threefold longer than the mean for all S phase origins (supplementary material Table S3A,B), and 2.5-fold longer than origins of similar AT content and efficiency not associated with amplification. They also all colocalize with established sites of Pre-RC assembly (Hayashi et al., 2007) (supplementary material Table S3B). Among these criteria, the most predictive parameter for a central origin at an amplification peak was AT content, followed by efficiency in mitotic S phase. Therefore, there was no single predictor of origin amplification; rather, the combination of these key parameters – together with position in the chromosome – dictates amplification behaviour.

We have demonstrated that in the presence of excess helicase loaders, cell cycle controls over replication are attenuated: local amplification occurs and DNA synthesis is continuous, unaccompanied by Mlu1-binding factor (MBF)-mediated G1–S gene expression, and lacks correlation between re-initiation and increase of cell volume. Nine S phase origins have been identified that are likely to bring about this local amplification and possess specific features that allow them to escape the once-per-S-phase firing controls. We identified these features as high AT richness, early and efficient firing in a normal S phase, and location within extended intergenic regions. Our data are in general agreement with an earlier study (Mickle et al., 2007), which related re-replication to broad genomic regions that contained more active S phase origins. We present here a more detailed analysis with fine origin mapping and test whether individual origins are responsible for amplification. We found that the origin 2040.0.0 is necessary but not sufficient for local amplification and that the ability to induce amplification is sensitive to chromosomal context. Higher-order chromosome structure has a role in the ability to amplify, as has been observed in *Drosophila melanogaster* (de Cicco and Spradling, 1984). Amplifying origins might represent highly efficient S phase replication origins in a privileged chromosome context. Replication factor binding and access to replication machinery may be selectively permitted or restricted within distinct chromosomal regions, with potential influences including chromatin and subnuclear organization. In support of this model, modeling and in vivo data show that the highly efficient origins described by Heichinger and colleagues (Heichinger et al., 2006) are nucleosome-depleted with an open chromatin architecture, whereas less-efficient origins have higher nucleosome occupancy and relatively closed chromatin (Field et al., 2008; Lantermann et al., 2010).

The origin features that are responsible for local amplification may have relevance for genome stability in other species. In support of this possibility, the chromosomal regions that over-replicate upon *cdc6 cdt1* co-overexpression in mammalian cells are also the regions replicated earliest in S phase, as is the case for efficient fission yeast origins (Vaziri et al., 2003). Our results might also be relevant for the development of cancer because

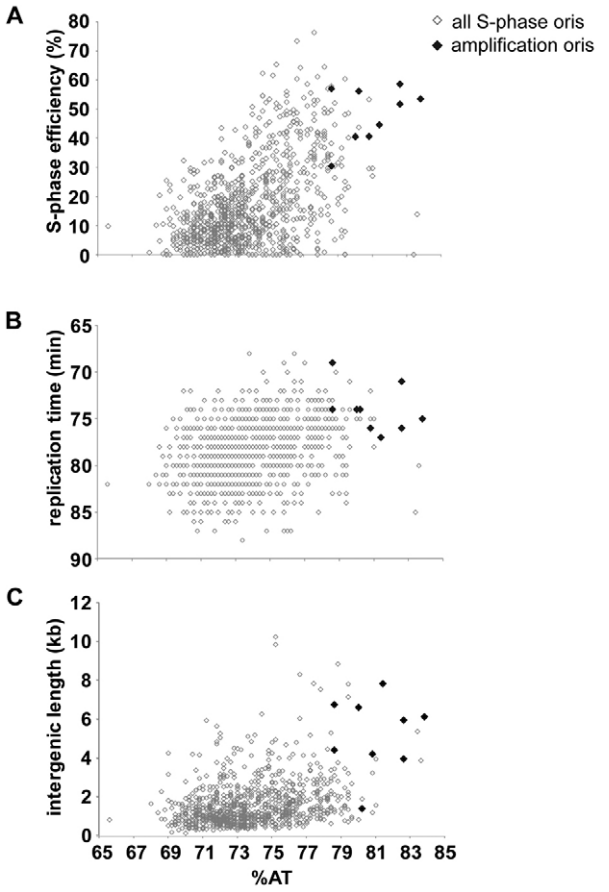

Fig. 4. Origins associated with amplification peaks have extreme features among S phase origins. (**A**) Correlation between %AT content and origin efficiency in a wild-type mitotic S phase. Origin efficiency for all 904 origins is plotted as a function of %AT content. (**B**) Origin replication time in mitotic S phase as a function of %AT content (**C**) Length of the intergene containing each origin as a function of %AT content. Plots are coded according to origin behaviour in *cdc18 cdt1* co-oe; ◆, mitotic S phase origins centered under local peaks amplified >1.6- to 4-fold over the genome average. Oris, origins.

NIH3T3 cells that overexpress *Cdt1* accumulate structural chromosomal abnormalities (Seo et al., 2005) and form tumors in mice (Arentson et al., 2001), while overexpression of *Cdt1* in T-cells of *p53*-null mice leads to lymphoblastic lymphoma (Seo et al., 2005). Origins that escape genome-wide coordination of firing may initiate such alterations to the genome. Repeated refiring of origins leads to onion-skin amplification structures (Claycomb and Orr-Weaver, 2005). These might be resolved via recombination to multiple chromosomal copies of a given gene – especially under selective pressure (Dunham et al., 2002; Koszul et al., 2004) – which could eventually evolve to confer new functions (Conrad and Antonarakis, 2007; Ohno, 1970). Repeated firing of origins with these features therefore could potentiate genome instability driving evolution, and the onset of cancer (Conrad and Antonarakis, 2007; Schimke et al., 1986).

Materials and Methods

Strains and growth conditions

Standard growth conditions and methods were used (Moreno et al., 1991). Experiments were performed in filtered Edinburgh Minimal Medium with supplements unless otherwise stated. The *tos4-GFP S. pombe* strain was grown with

750 mg/l adenine to reduce autofluorescence. *Cdc18 cdt1* co-oe (Yanow et al., 2001) and *cdc18* oe (Nishitani and Nurse, 1995) strains were thiamine-depleted to induce rereplication. After 11 hours, the culture was shifted to restrictive temperature. Strains for pulse labeling contained *tk* and *hENT* (Sivakumar et al., 2004); *tos4-GFP* strains were as previously described (Kiang et al., 2009). Origins were deleted and integrated according to Bähler and colleagues (Bähler et al., 1998).

DNA microarray experiments

Microarray design, DNA preparation, hybridizations, data acquisition and analysis, and origin mapping were as described for HU experiments (Heichinger et al., 2006). Data analysis was on the basis of the genome sequence of June 2006, available at ftp://ftp.sanger.ac.uk/pub/yeast/pombe/Chromosome_contigs/. Microarray data are available at www.ebi.ac.uk/arrayexpress, accession number E-MTAB-139. Reference DNA was from *cdc25-22* cells blocked at the restrictive temperature of 36.5°C for 4 hours, which have a DNA content of 2C. Each origin mapping profile depicts the average of two experiments using open reading frame (ORF) and intergenic tiling microarrays unless otherwise noted. Control amplification profiles in all other figures depict the clearest representative profile: for chromosomes *I* and *II*, the origin 3048.0.0 deletion strain, because deletion has no effect on amplification and the profile is unaltered (see supplementary material Fig. S4); for chromosome *III*, the origin 2040.0.0 deletion. The AT content in percent (%AT) was defined as the highest %AT in a 500 bp window (50 bp step) in the intergenic region with at least 65% AT richness that maps to or close to the origin. Intergenic length refers to the number of base pairs in the intergenic region to which the AT-rich island of the origin maps. Pre-RC colocalization with origins was analyzed as previously described (Kiang et al., 2009).

Quantitative PCR

For quantitative PCR (qPCR), the starting quantity of DNA was estimated from the number of cycles (C_t value) required to reach the threshold. Primers specific to the centers of origin 2040.0.0, ars2004 (also contained within the integrated fragments) and 3049.0.0, and adjacent and control regions were designed (available upon request). Representative data are shown.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/18/3047/DC1

References

Arentson, E., Faloon, P., Seo, J., Moon, E., Studt, J. M., Fremont, D. H. and Choi, K. (2001). Oncogenic potential of the DNA replication licensing protein CDT1. *Oncogene* **21**, 1150-1158.

Arias, E. E. and Walter, J. C. (2007). Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. *Genes Dev.* **21**, 497-518.

Bähler, J., Wu, J.-Q., Longtine, M. S., Shah, N. G., McKenzie, A., III, Steever, A. B., Wach, A., Philippsen, P. and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast* **14**, 943-951.

Bell, S. P. and Dutta, A. (2002). DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**, 333.

Claycomb, J. M. and Orr-Weaver, T. (2005). Developmental gene amplification: insights into DNA replication and gene expression. *Trends Genet.* **21**, 149-162.

Conrad, B. and Antonarakis, S. E. (2007). Gene duplication: a drive for phenotypic diversity and cause of human disease. *Annu. Rev. Genomics Hum. Genet.* **8**, 17.

Dai, J., Chuang, R.-Y. and Kelly, T. J. (2005). DNA replication origins in the Schizosaccharomyces pombe genome. *Proc. Natl. Acad. Sci. USA* **102**, 337-342.

de Cicco, D. V. and Spradling, A. C. (1984). Localization of a cis-acting element responsible for the developmentally regulated amplification of drosophila chorion genes. *Cell* **38**, 45-54.

Dunham, M. J., Badrane, H., Ferea, T., Adams, J., Brown, P. O., Rosenzweig, F. and Botstein, D. (2002). Characteristic genome rearrangements in experimental evolution of Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA* **99**, 16144-16149.

Field, Y., Kaplan, N., Fondufe-Mittendorf, Y., Moore, I. K., Sharon, E., Lubling, Y., Widom, J. and Segal, E. (2008). Distinct modes of regulation by chromatin encoded through nucleosome positioning signals. *PLoS Comput. Biol.* **4**, e1000216.

Gonzalez, M. A., Tachibana, K. E., Adams, D. J., van der Weyden, L., Hemberger, M., Coleman, N., Bradley, A. and Laskey, R. A. (2006). Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. *Genes Dev.* **20**, 1880-1884.

Gopalakrishnan, V., Simancek, P., Houchens, C., Snaith, H. A., Frattini, M. G., Sazer, S. and Kelly, T. J. (2001). Redundant control of rereplication in fission yeast. *Proc. Natl. Acad. Sci. USA* **98**, 13114-13119.

Hayashi, M., Katou, Y., Itoh, T., Tazumi, A., Yamada, Y., Takahashi, T., Nakagawa, T., Shirahige, K. and Masukata, H. (2007). Genome-wide localization of pre-RC sites and identification of replication origins in fission yeast. *EMBO J.* **26**, 1327-1339.

Heichinger, C., Penkett, C. J., Bähler, J. and Nurse, P. (2006). Genome-wide characterization of fission yeast DNA replication origins. *EMBO J.* **25**, 5171-5179.

Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A. and Nurse, P. (1993). The fission yeast cdc18+ gene product couples S phase to START and mitosis. *Cell* **74**, 371-382.

Kiang, L., Heichinger, C., Watt, S., Bahler, J. and Nurse, P. (2009). Cyclin-dependent kinase inhibits reinitiation of a normal S-phase program during G2 in fission yeast. *Mol. Cell. Biol.* **29**, 4025-4032.

Koszul, R., Caburet, S., Dujon, B. and Fischer, G. (2004). Eucaryotic genome evolution through the spontaneous duplication of large chromosomal segments. *EMBO J.* **23**, 234-243.

Lantermann, A. B., Straub, T., Stralfors, A., Yuan, G.-C., Ekwall, K. and Korber, P. (2010). Schizosaccharomyces pombe genome-wide nucleosome mapping reveals positioning mechanisms distinct from those of Saccharomyces cerevisiae. *Nat. Struct. Mol. Biol.* **17**, 251-257.

Melixetian, M., Ballabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., Lukas, J. and Helin, K. (2004). Loss of Geminin induces rereplication in the presence of functional p53. *J. Cell Biol.* **165**, 473-482.

Mickle, K., Oliva, A., Huberman, J. and Leatherwood, J. (2007). Checkpoint effects and telomere amplification during DNA re-replication in fission yeast. *BMC Mol. Biol.* **8**, 119.

Mihaylov, I. S., Kondo, T., Jones, L., Ryzhikov, S., Tanaka, J., Zheng, J., Higa, L. A., Minamino, N., Cooley, L. and Zhang, H. (2002). Control of DNA replication and chromosome ploidy by geminin and cyclin A. *Mol. Cell. Biol.* **22**, 1868-1880.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. *Methods Enzymol.* **194**, 795-823.

Nishitani, H. and Nurse, P. (1995). p65cdc18 plays a major role controlling the initiation of DNA replication in fission yeast. *Cell* **83**, 397-405.

Nishitani, H., Lygerou, Z., Nishimoto, T. and Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature* **404**, 625-628.

Ohno, S. (1970). *Evolution by Gene Duplication.* Berlin: Springer-Verlag.

Schimke, R. T., Sherwood, S. W., Hill, A. B. and Johnston, R. N. (1986). Overreplication and recombination of DNA in higher eukaryotes: potential consequences and biological implications. *Proc. Natl. Acad. Sci. USA* **83**, 2157-2161.

Segurado, M., de Luis, A. and Antequera, F. (2003). Genome-wide distribution of DNA replication origins at A+T islands in *Schizosaccharomyces pombe*. *EMBO rep.* **4**, 1048-1053.

Seo, J., Chung, Y. S., Sharma, G. G., Moon, E., Burack, W. R., Pandita, T. K. and Choi, K. (2005). Cdt1 transgenic mice develop lymphoblastic lymphoma in the absence of p53. *Oncogene* **24**, 8176-8186.

Sivakumar, S., Porter-Goff, M., Patel, P. K., Benoit, K. and Rhind, N. (2004). In vivo labeling of fission yeast DNA with thymidine and thymidine analogs. *Methods* **33**, 213-219.

Varshavsky, A. (1981). On the possibility of metabolic control of replicon "misfiring": relationship to emergence of malignant phenotypes in mammalian cell lineages. *Proc. Natl. Acad. Sci. USA* **78**, 3673-3677.

Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S. and Dutta, A. (2003). A p53-dependent checkpoint pathway prevents rereplication. *Mol. Cell* **11**, 997-1008.

Yanow, S. K., Lygerou, Z. and Nurse, P. (2001). Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication. *EMBO J.* **20**, 4648-4656.

Supplementary Table S1. Strains used in this study

Genotype	Additional names/ shorthand	Nurse Lab collection No.
ade6-M210 tos4-GFP-kanMX6 h-		PN10633
cdc25-22 rep3x-cdc18+ leu1-32 ura4-D18 h-	*cdc18* oe	PN1803
cdc25-22 rep3x-cdc18+ pREP4-cdt1+ leu1-32 ura4- h-	*cdc18 cdt1* co-oe	PN3613
cdc25-22 rep3x-cdc18+ pREP4-cdt1+ adh1-tk-his7+ adh1-hENT1-ade6+ leu1-32 ura4-D18 his7-366 ade6-210 h+		PN10642
cdc25-22 rep3x-cdc18 pREP4-cdt1 leu1-32 ura4-D18 tos4-GFP-kanMX6		PN10643
_ori2040.0.0:: kanMX6 cdc25-22 rep3X-cdc18+ pREP4-cdt1::ura4 ade6-D1 ura4-D18 leu1-32 h-		PN10644
_ori2040.0.0:: kanMX6 ars2004::2040.0.0-ade6+ cdc25-22 rep3X-cdc18+ pREP4-cdt1::ura4 ade6-D1 ura4-D18 leu1-32		PN10645
ars2004::2040.0.0-ade6+ cdc25-22 rep3X-cdc18+ pREP4-cdt1::ura4 ade6-D1 ura4-D18 leu1-32		PN10646
_ori2040.0.0:: kanMX6 C19F5.02::2040.0.0-ade6+ cdc25-22 rep3X-cdc18+ pREP4-cdt1::ura4 ade6-D1 ura4-D18 leu1-32		PN10647

Supplementary Table S3

(A) Origins used in mitotic S phase and in *cdc18 cdt1* co-overexpression (co-oe)

[a] not including the additional origins which do not fire in the mitotic cell cycle

(B) Categories of origins according to usage in re-replication

[b] these origins do not fire in mitotic S phase, so there is no mitosis origin efficiency

[c] subset of 683 mitotic origins used in *cdc18 cdt1* co-oe

[d] refers to all intergenes, genome-wide (Dai et al., 2005; Segurado et al., 2003)

[e] refers to all intergenic regions in the genome, regardless of origin activity

For definitions of all parameters, see Materials and Methods

A.

Type of DNA synthesis	Number of origins	Mean AT content (%)	Mean mitotic efficiency (%)	Mean intergenic size (bp)
Mitotic S phase	904	73.8	18	1678
cdc18 cdt1 co-oe	796	73.8	19.7[a]	1684

B.

	Total origins in group	Mean AT-content (%)	Mean mitotic efficiency (%)	Mean intergenic size (bp)	Co-localized with Pre-RC (Hayashi et al., 2007) (%)
Mitotic origins used in *cdc18 cdt1* co-oe	683	74.1	19.7	1732	82
Non-mitotic origins used in *cdc18 cdt1* co-oe	113	72.2	n/a[b]	1397	73
Mitotic origins not used in *cdc18 cdt1* co-oe	221	73.0	10.9	1522	79
Mitotic origins centered under amplification peaks [c]	9	81.0	48.1	5269	100
Genome average		70 [d]		960 [e]	