A mammalian-like DNA damage response of fission yeast to nucleoside analogues

Sarah A. Sabatinos*, Tara L. Mastro*, Marc D. Green* and Susan L. Forsburg*  

*Department of Molecular and Computational Biology, University of Southern California, Los Angeles, CA 90089 USA
Running title: DNA damage response to BrdU and EdU

Key words/phrases:

- dNTP metabolism
- DNA damage response
- replication checkpoint
- thymidine arrest
- thymidine salvage pathway

Corresponding Author: Susan L. Forsburg, Department of Molecular and Computational Biology, University of Southern California, Los Angeles, CA 90089 USA. Telephone: (213) 740-7342. Email: forsburg@usc.edu
ABSTRACT

Nucleoside analogues are frequently used to label newly synthesized DNA. These analogues are toxic in many cells, with the exception of the budding yeast. We show that Schizosaccharomyces pombe behaves similarly to metazoans in response to analogues 5-bromo-2’-deoxyuridine (BrdU) and 5-ethynyl-2’-deoxyuridine (EdU). Incorporation causes DNA damage that activates the damage checkpoint kinase Chk1, and sensitizes cells to UV light and other DNA damaging drugs. Replication checkpoint mutant cds1Δ shows increased DNA damage response after exposure. Finally, we demonstrate that the response to BrdU is influenced by the ribonucleotide reductase inhibitor, Spd1, suggesting that BrdU causes dNTP pool imbalance in fission yeast, as in metazoans. Consistent with this, we show that excess thymidine induces G1-arrest in wild-type fission yeast expressing thymidine kinase. Thus, fission yeast responds to nucleoside analogues similarly to mammalian cells, which has implications for their use in replication and damage research, as well as dNTP metabolism.
INTRODUCTION

Understanding mechanisms that maintain DNA replication fidelity are key to understanding cancer (Barlogie et al. 1976; Christov and Vassilev 1988; Johnson et al. 1981). Replication must be controlled to avoid mutations, promote DNA repair, and restrain re-replication. Predictably, abnormally replicating and proliferating cells are a hallmark of tumorigenesis (Barlogie et al. 1976; Christov and Vassilev 1988; Johnson et al. 1981). Thus, accurate analysis of replication states is essential to understanding the response of a cell population under study.

Studies of replication dynamics rely on manipulations of DNA nucleotide metabolism. For example, the drug hydroxyurea (HU) is commonly used to inhibit nucleotide synthesis. This causes replication fork stalling and checkpoint activation until dNTP levels are re-established (Alvino et al. 2007; Kim and Huberman 2001; Lopes et al. 2001; Poli et al. 2012; Santocanale and Diffley 1998). Dysregulation of nucleotide levels is associated with disruptions in cell cycle and checkpoint control (Davidson et al. 2012; Kumar et al. 2011; Kumar et al. 2010; Poli et al. 2012). The regulatory response to nucleotide levels is different between the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. Budding yeast S. cerevisiae is highly resistant to HU, and its checkpoint proteins directly regulate nucleotide biosynthesis during the normal cell cycle (Chabes et al. 2003; Poli et al. 2012). In contrast, fission yeast S. pombe is sensitive to much lower levels of HU, and uses checkpoint-
independent mechanisms to control nucleotide levels during normal cell growth (HAKANSSON et al. 2006; NESTORAS et al. 2010).

Exposure to exogenous nucleosides alters metazoan dNTP metabolism and cell cycle dynamics (MEUTH and GREEN 1974a; MEUTH and GREEN 1974b), which is a potential problem in assays that rely on incorporation of modified or antigenic nucleoside analogues. By measuring incorporation of analogue, the replicative capacity of a culture is inferred (BOHMER and ELLWART 1981; CRISSMAN and STEINKAMP 1987; FRUM and DEB 2003), providing a direct metric of DNA synthesis. Thymidine analogues 5-bromo-2'-deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU) (DIERMEIER-DAUCHER et al. 2009) are commonly used nucleosides. BrdU is detected using antibodies (HODSON et al. 2003), while EdU is covalently labeled by bio-orthogonal, copper-based chemistry (BUCK et al. 2008). In yeasts, which lack a thymidine salvage pathway, analogue incorporation requires an exogenous thymidine kinase (e.g. Herpes Simplex Virus, hsv-tk+) (HODSON et al. 2003; SCLAFAI and FANGMAN 1986; SIVAKUMAR et al. 2004; VIGGIANI and APARICIO 2006).

A disadvantage to this method is the inherent toxicity of nucleoside analogues such as BrdU, FdU and IdU (SIVAKUMAR et al. 2004). Observations in bacteria and mammals indicate that BrdU is both a mutagen and a teratogen (DAVIDSON and KAUFMAN 1978; KAUFMAN and DAVIDSON 1978; LASKEN and GOODMAN 1984), able to cause T-C transition mutations (GOODMAN et al. 1985). However, up to 400 µg/mL BrdU may be added to a culture of Saccharomyces cerevisiae before toxic effects are observed (LENGRONNE et al. 2001). In fission
yeast, both EdU and BrdU analogues show toxicity at much lower doses and may activate a Rad3 (ATR/Mec1)-dependent damage response pathway (Hodson et al. 2003; Hua and Kearsey 2011; Sivakumar et al. 2004). Differences in dosage sensitivity may reflect the differences in nucleotide metabolism between these different yeast species.

We examine the range of toxicity and the induction of DNA damage in S. pombe cells that incorporate either BrdU or EdU. Checkpoint mutants rad3Δ, chk1Δ and cds1Δ show hypersensitivity to BrdU and EdU, which is exacerbated in minimal media. Cells exposed to low doses of BrdU are sensitized to additional DNA damaging agents and show increased mutation rates. Even in low-dose analogue, we observe induction of damage markers including phosphorylated histone H2A, and Rad52 foci. Chk1 and Cdc2 phosphorylation indicates the DNA damage checkpoint is activated, showing that analogue incorporation generates DNA damage.

Consistent with this, we observe that completion of S phase and entry to the next cell cycle is delayed with increased analogue dose. This effect is not limited to analogues, however, because fission yeast cells exposed to thymidine undergo G1-arrest, similar to thymidine synchronization in human cells. Finally, the ribonucleotide reductase (RNR) inhibitor Spd1 modulates cell response to BrdU and EdU. While spd1Δ cells are less sensitive to chronic exposure, acute viability is decreased and surviving spd1Δ cells frequently carry mutations. Thus, the power of using analogue incorporation to detect new DNA synthesis must be
tempered by appropriate cautions to maximize relevant conclusions and minimize disruptions to normal nucleotide metabolism.

MATERIALS AND METHODS

Yeast strains, analogue addition, growth and mutagenesis

Fission yeast strains are described in Table 1. Cells were grown as described in (SABATINOS and FORSBURG 2010), and BrdU (5 mg/mL in water) or EdU (10 mM stock, Invitrogen) added as required to liquid cultures or plates. Media were yeast extract with supplements (YE5S, hereafter “YES”), Edinburgh minimal medium (EMM- ammonium chloride nitrogen source), or S. pombe minimal glutamate medium (PMG- sodium glutamate nitrogen source) (SABATINOS and FORSBURG 2010). Physiology experiments including Chk1-protein, Rad52-foci, mutagenesis and flow cytometry were all performed in liquid EMM cultures. Protein extracts were prepared from 0.3 M NaOH treated cells, and lysed in 2x SDS-PAGE sample buffer by boiling 5 minutes, as described in (SABATINOS and FORSBURG 2010). Choice of HA monoclonal produced a different non-specific background band location on Chk1-HA blots (Roche 12CA5, or Covance 16B12).

Mutation rate was determined by splitting cultures (+/−BrdU), treating with 32.6 μM BrdU for 2h, and then plating on YES and PMG+canavanine. Canavanine plates (70 μg/mL in PMG+ supplements) were scored after 8 days growth (32°C) and numbers of can1- colonies were compared to the concentration derived from titre plates (YES). Grouped experiments for Table 2
and Figure 7 were performed independently and rates calculated using FALCOR (http://www.keshavsingh.org/protocols/FALCOR.html) and MSS-MLE or Lea-Coulson method of the median. MSS-MLE results were analyzed by 2-tailed t-test, and Mann-Whitney U-tests were used for Lea-Coulson significance testing (http://vassarstats.net/utest.html). Frequency of hsv-tk\(^+\) loss was scored as the number of FUdR-resistant or sectored colonies per total, and the proportions assessed with Z-tests (http://vassarstats.net/propdiff_ind.html).

**Microscopy**

Cells were fixed in cold 70% ethanol for cell cycle analysis or microscopy. Cells were rehydrated in water, and incubated for 10 minutes in 1mg/mL of aniline blue (Sigma M6900). Cells in mount (50% glycerol, 1 µg/mL DAPI and 1 µg/mL p-Phenylenediamine) were photographed on a Leica DMR wide-field epifluorescent microscope using a 63X objective lens (NA 1.4 Plan Apo), 100W Hg arc lamp for excitation, and 12-bit Hamamatsu ORCA-100 CCD camera. OpenLab v3.1.7 (Improvision, Lexington, MA) software was used at acquisition and ImageJ (SCHNEIDER et al. 2012) for analysis. DAPI counterstaining did not significantly affect BrdU or EdU signal intensity.

Live-cell Rad52-YFP foci were imaged on a DeltaVision Core microscope (with softWoRx v4.1, Applied Precision, Issaquah, WA) using a 60x (NA1.4 PlanApo) lens, solid-state illuminator and 12-bit Roper CoolSnap HQII CCD camera. The system x-y pixel size was 0.1092 µm. YFP fluorescence for single timepoints was acquired as 18 0.3µm z-sections. Long-term time-lapse movies used 9 z-steps of 0.5µm. Rad52-YFP images were deconvolved and Maximum
Intensity Projected (softWoRx). Movies were performed in CellAsics (Hayward, CA) microfluidics plates (Y04C series), with supplemented EMM medium. Transmitted light images were fused with DAPI or Rad52-projected images. Images were contrast adjusted using a histogram stretch and equivalent scale in all samples. A threshold of 2x over the average nuclear YFP signal was used for focus discrimination. Rad52 foci are presented as the proportion of nuclei with Rad52 foci ±95% confidence interval (CI). Significance was assessed with Chi-squared tests.

**Flow Cytometry**

Whole cell SytoxGreen flow cytometry (FACS) was performed as described in (SABATINOS and FORSBURG 2009). Whole cell FACS for EdU was performed using Click-iT (Invitrogen) with AlexaFluor 488 on rehydrated cells. FACS for BrdU was performed on “ghosts” (CARLSON et al. 1997), prepared by spheroplasting cells in 0.1 M KCl with 5 mg/mL Zymolyase 20T and then 1% TritonX-100 before sonicating to release nuclei. Nuclei were blocked (10% FCS, 1% BSA) for 30 min, and then incubated with mouse anti-BrdU (Becton Dickinson, B44). Secondary antibody was Alexa-488 conjugated anti-mouse (Invitrogen), and nuclei were counterstained with propidium iodide for total DNA content.

**RESULTS**

**Cell signal and viability vary with analogue dose**
Acute nucleoside analogue toxicity is proportional to dose in human cells (Popescu 1999). To compare this with fission yeast we used BrdU and EdU concentrations typically used in replication studies. Our strains contained hsv-tk+ and the human equilibrative nucleoside transporter (hENT), which allowed us to use lower analogue doses and achieve effective transport into cells (Hodson et al. 2003; Sivakumar et al. 2004). Since BrdU dose is frequently presented in µg/mL, we began with a starting BrdU dose of 100 µg/mL (326 µM), half the dose used in fission yeast genome labeling work without hENT (e.g. (Hayano et al. 2011)), and less than commonly used in budding yeast (e.g. (Lengronne et al. 2001)). However, to compare between BrdU (typically mg/mL) and EdU (typically µM), we will report all doses in molarity. The starting dose for each (326 µM BrdU and 10 µM EdU) was determined by previous protocols.

We compared BrdU signals using flow cytometry on HU-blocked cells released into BrdU (32.6 or 326 µM), or EdU (1 or 10 mM), to determine whether there was a corresponding decrease in the resulting signal (Fig 1). Low-dose BrdU (32.5 µM) produced similar signal to the full dose of 326 µM, but with better viability (Fig 1A). EdU toxicity appeared at a dose of 10 µM (Fig 1D) consistent with (Hua and Kearsey 2011), yet a 10-fold decrease in EdU dose to 1 µM significantly decreased EdU signal intensity (Fig 1C). Thus, minimum doses of 32.6 µM BrdU and 10 µM EdU are required for optimal short-term labeling in hsv-tk+ hENT+ strains. Higher doses of nucleoside analogue may be required to detect small incorporation differences during short incubations, but enhanced signal comes at a cost to cell viability.
Disruptions in DNA replication and repair activate checkpoint pathways (reviewed in (SABATINOS and FORSBURG 2012)). The master regulator in fission yeast is the Rad3^{ATR} kinase, which activates Chk1 kinase at DNA double strand breaks (DSBs). During replication fork stalling, Cds1 kinase is activated by Rad3 via the fork processivity factor Mrc1. Mutations in these checkpoint proteins cause sensitivity to drugs that activate the replication checkpoint. Previous work indicated that Rad3 is required for viability in EdU, but did not investigate the downstream pathways required (HUA and KEARSEY 2011).

We examined cell viability in replication checkpoint mutant cds1Δ and DNA damage checkpoint mutant chk1Δ, compared to wild-type and rad3Δ incorporating strains (Fig 1B, 1D). We found chk1Δ and rad3Δ are strikingly sensitive to BrdU and EdU treatment. While cds1Δ behaved similarly to wild-type cells during the first 4h of incubation, prolonged BrdU exposure (8h) caused decreased cds1Δ viability (similar results for mrc1Δ, data not shown). Lower BrdU and EdU doses improved viability in all genotypes (Fig S1), yet rad3Δ and chk1Δ cells were still most EdU-sensitive.

Wild-type cells without the incorporation cassette continued to divide normally during exposure (Fig 1E). Wild-type hsv-tk^+ hENT^+ cells had reduced division during analogue exposure, as did cds1Δ, rad3Δ and mrc1Δ (data not shown). In contrast, the chk1Δ incorporating strain continued to divide in BrdU and EdU, suggesting that Chk1 activation is required to inhibit cell division during exposure.
Checkpoint and cell cycle phenotypes can be sensitive to media composition. We examined sensitivity to BrdU and EdU in spot tests on three media types: rich YES, defined EMM, and lower-nitrogen PMG (Fig 2, S1C). On YES, \( \text{chk1} \Delta \) incorporating cells show modest sensitivity at 16.3 \( \mu M \), while growth inhibition in \( \text{rad3} \Delta \) and \( \text{cds1} \Delta \) began to show at 32.6 \( \mu M \) (Fig 2A). EdU sensitivity was highest in \( \text{chk1} \Delta \) on YES (5 \( \mu M \)) followed by \( \text{cds1} \Delta \) (10 \( \mu M \)). Unexpectedly, \( \text{rad3} \Delta \) sensitivity was similar to wild-type at 10 \( \mu M \) EdU in YES (Fig 2A).

On EMM, which contains high-level nitrogen, we found that wild-type, \( \text{chk1} \Delta \) and \( \text{rad3} \Delta \text{ hsv-tk}^+ \text{ hENT}^+ \) cells were all BrdU sensitive (Fig 2B). Yet, \( \text{mrc1} \Delta \) and \( \text{cds1} \Delta \) were less sensitive. Wild-type, \( \text{chk1} \Delta \) and \( \text{rad3} \Delta \text{ hsv-tk}^+ \text{ hENT}^+ \) cells were most sensitive at 10 \( \mu M \) EdU in EMM, followed by \( \text{cds1} \Delta \) and \( \text{mrc1} \Delta \). Non-incorporating cells were not sensitive to EdU or BrdU on any media.

PMG medium contains low levels of nitrogen. All incorporator strains were sensitive to 16.3 \( \mu M \) BrdU in PMG (Fig S1C), above which there was little growth. Similarly, EdU sensitivity was higher in PMG and \( \text{chk1} \Delta \) cells were most sensitive to EdU in PMG, followed by \( \text{cds1} \Delta \). Thus, cells are less sensitive to analogues on rich medium than minimal medium, while \( \text{chk1} \Delta \) restricts growth in all cases.

**S-phase progression is slowed by analogue incorporation**

We next tested whether analogue incorporation has an effect on DNA synthesis. We blocked cells in early S-phase with HU and released into medium with low- or high-dose BrdU or EdU to monitor replication. Non-incorporating cells completed S-phase by 1h post-release, with the appearance of a 2C DNA
peak. A second S-phase was detected 1.5-2h post-release by 4C DNA content and septation index (Fig 3A, 3B, S2A). We detected a shift in forward scatter (FSC) to small cells (Fig S2B), correlating with cell division at 2h post-release.

BrdU exposure at both doses delayed S-phase completion in incorporating strains. The DNA peak moved slowly to a 2C position, completing by 2h post-release. Septation was highest at 3h, coincident with a small 4C peak observed by FACS (Fig 3A, S2A). FSC confirmed the BrdU-incorporated cells were elongated (Fig S2B), consistent with incomplete septation.

Cells treated with 1µM EdU completed the first S-phase by 1h and entered the second S-phase at 2h post-release with increased septa and 4C DNA (Fig 3B, S2A). Cells in 10 µM EdU completed a first S-phase within 2h but had much slower transit through the second S-phase, which started at 2 h and was not resolved by 3h (Fig S2A). Thus, EdU causes a dose-dependent inhibition of cell cycle progression in incorporating cells.

Thymidine is commonly used in human cell cultures to arrest cells in G1 (HARPER 2005), but does not affect yeast since S. pombe does not have a thymidine salvage pathway (non-incorporating cells). We asked whether thymidine changes cell cycle in hENT⁺ hsv-tk⁺ strains (Fig 3C). We treated cells with 2 mM thymidine (HARPER 2005) and saw no effect in non-incorporating cells, or in cells with hENT⁺ but no hsv-tk. However, an incorporating strain treated with 2 mM thymidine arrested with a 1C DNA content. After thymidine removal, cells shifted toward 2C DNA content. Thus, thymidine reversibly arrests hENT⁺ hsv-tk⁺ S. pombe cells with a 1C DNA content, as in mammals.
Nucleoside analogues induce DNA damage response

Reduced viability of checkpoint mutants and delayed S-phase progression during analogue incorporation suggested that DNA damage was generated. We examined molecular markers of DNA damage after BrdU treatment. First, we detected histone H2A phosphorylated at S129 (p-H2A), and BrdU. Rad3 and Tel1 kinases phosphorylate p-H2A in response to DNA double strand breaks (DSBs) and replication stress (Ballis et al. 2008; Nakamura et al. 2004). Non-incorporating cells had few p-H2A foci (Fig 3D), while BrdU-labeled cells frequently co-stained with p-H2A. These results suggest that BrdU incorporation activates a DNA damage response, which is enhanced in cds1Δ cells that cannot properly respond to S-phase stress.

Next, we looked for activation of checkpoint components. We examined HA-tagged Chk1 protein (Fig 4A), the G2-M kinase of the S. pombe DNA damage response (DDR). We observed that Chk1HA shows a characteristic phospho-shift (Capasso et al. 2002) after 1h exposure to 326 μM BrdU in hENT+ hsv-tk+ cells (Fig 4A), and phospho-Chk1HA after 3h in 32.6 μM. Thus, BrdU dose influences G2-DDR activation. However, Cds1-myc kinase, required for response to replication stress, was not significantly modified relative to overall protein level in BrdU (Fig 4A).

We observed a phospho-shift in the upstream G2-DDR checkpoint mediator Crb2 in BrdU (Fig 4A), again consistent with G2 checkpoint activation. Activated Chk1 leads to Cdc2 phosphorylation at tyrosine 15 to maintain G2
checkpoint arrest (O'CONNELL et al. 1997), and we saw phospho-Cdc2 accumulated in BrdU-treated incorporating cells (Fig 4A). A dose of 326 μM BrdU was lethal to most cells during 3h exposure (Fig 4B), suggesting the damage is catastrophic.

Similar results were seen in EdU, causing Chk1HA and Crb2 phospho-shifts (Fig 4C) and increased phospho-Cdc2 (Fig 4D), which was proportional to EdU dose (Fig 4E). These data indicate that the DNA damage checkpoint is fully activated during analogue exposure.

**Loss of the replication checkpoint influences damage response**

Our data suggest that the Chk1-DNA damage checkpoint pathway is a primary response to analogues. However, the modest sensitivity caused by \textit{cds1Δ} indicated a role for the replication checkpoint during treatment. Cds1 is particularly important for replication fork stalling and restart (e.g. (KIM and HUBERMAN 2001; LINDSAY et al. 1998; MIYABE et al. 2009)). The homologous recombination protein Rad52 forms nuclear foci in response to a variety of lesions including double strand breaks and collapsed or restarting replication forks (BAILIS et al. 2008; MEISTER et al. 2005). We monitored Rad52-YFP focus formation in wild-type and \textit{cds1Δ} incorporating strains after 3h in 32.6 or 326 μM BrdU (Fig 5A). Incorporating cells formed more Rad52 foci than without BrdU treatment, consistent with increased damage (Fig 5B). This effect was dose-dependent, and more foci were detected at 326 μM BrdU (wild-type) and at all BrdU doses (\textit{cds1Δ}).
Live cell analysis in a microfluidics chamber allowed us to track individual cells during and after analogue exposure (online Movies). We monitored Rad52 foci during 3h exposure to 32.6 μM (Movies 1,2) or 10 μM EdU (Movies 3,4) in wild-type and cds1Δ hsv-tk+ hENT+ cells. We used a dose of 32.6 μM BrdU because this was the lowest dose that produced a difference in Rad52 foci (Fig 5B), and close to the 10 μM EdU dose required to effectively label cells for replication studies. BrdU movies recapitulated our static timepoint data (Fig 5A, 5B), confirming that cds1Δ cells generated more Rad52 foci during analogue exposure. We also monitored recovery during 3h after analogue was removed, observing that multiple Rad52 foci (2+) frequently resolved into 1 focus. Further, cells which septated and presumably entered the next cell cycle during BrdU (Movies 1,2) or EdU exposure (Movies 3,4) promptly formed Rad52 foci, suggesting an immediate response is mounted during S-phase. However, cds1Δ cells that entered S-phase during analogue exposure frequently lysed during recovery (Fig 5C, 5D).

We next asked whether differences in Rad52 foci levels in different BrdU doses could be attributed to different levels of BrdU incorporation in DNA. We extracted total DNA from cells after 3h exposure to 32.6 or 326 μM BrdU and blotted several amounts of heat-denatured DNA (heat-denatured) to probe with BrdU antibody (Fig 5E). Surprisingly, we found that the amount of BrdU incorporated at either 32.6 or 326 μM did not change. Thus, BrdU incorporation is saturated at 32.6 μM, consistent with our earlier FACS results (Fig 1A). However, wild-type cell viability was higher in 32.6 μM Brdu than at 326 μM (Fig 4B). This
suggests that additional stress occurs at the higher dose of BrdU that does not involve BrdU-base substitution. Interestingly, we detected less BrdU incorporation in $cds1\Delta$ compared to wild-type in the samples with the higher levels of DNA. Thus, enhanced analogue sensitivity in $cds1\Delta$ is accompanied by less efficient BrdU-incorporation. This could reflect replisome disruption in $cds1\Delta$, or Cds1-mediated interactions with dNTP metabolism.

**Enhanced sensitivity to “second-hit” damage after BrdU exposure**

Since $hsv-tk^+\ hENT^+$ cells acquired DNA damage and G2 arrest signals, we investigated whether BrdU incorporation changes cell sensitivity to other DNA damage, as reported for human cells (e.g. [ACKLAND et al. 1988; CECCHINI et al. 2005]). UV treatment on BrdU-substituted human cells induces DSBs, demonstrating that BrdU-DNA is sensitized to additional lesions. To test this in *S. pombe*, we treated cells with 3h of low-dose (32.6$\mu$M) BrdU for minimal toxicity with saturated incorporation, and examined viability with UV treatment (Fig 5F). Non-incorporating cells experienced a slight viability decrease, to 88 ± 3%, after UV dose of 100 J/m$^2$. However, BrdU-incorporation significantly decreased UV survival and further, was dose-dependent: cells treated with 3.2 $\mu$M BrdU were slightly more UV sensitive (58% viability), while saturating doses of 32.6 or 326 $\mu$M had the same effect on UV-survival to less than 20% viability.

Next, we incubated cells with 32.6 $\mu$M BrdU for 2h, and then examined their sensitivity to a panel of DNA damaging drugs by spot test (Fig 6A). First, we examined sensitivity to phleomycin, a radio-mimetic (Fig 6B). All cells showed
increased sensitivity to phleomycin after BrdU pre-treatment. DDR pathway mutants rad3Δ and chk1Δ were most severely affected at lower doses, followed by mrc1Δ and cds1Δ, and then wild-type hsv-tk+ hENT+ cells.

BrdU-substitution also enhanced sensitivity to camptothecin (CPT) in chk1Δ (5 μM) and cds1Δ (10 μM) (Fig 6C). CPT causes single-strand DNA breaks by covalently linking topoisomerase I to DNA, damage that is later converted to a DSB (HSIANG et al. 1989). Although rad3Δ and mrc1Δ are highly sensitive to CPT, we detected no increased CPT sensitivity following BrdU exposure in rad3Δ and mrc1Δ cells.

The alkylating agent methanemethyl sulfonate (MMS) methylates DNA bases and stalls replication forks. The rad3Δ cells were already highly sensitive to MMS regardless of BrdU pre-treatment (Fig S3B). However, the remaining hsv-tk+ hENT+ strains were all more MMS-sensitive following BrdU-incorporation, with the highest sensitivity in chk1Δ (Fig S3B). Hydroxyurea (HU) stalls replication forks, but acts through dNTP depletion as opposed to DNA base damage (Fig 6D). BrdU pre-treatment enhanced HU sensitivity only in the chk1Δ hsv-tk+ hENT+ strain. UV lesions stall replication and transcription, so we looked at different doses of UV post-BrdU. All strains except rad3Δ showed increased UV sensitivity after BrdU, with chk1Δ being the most affected (Fig S3A).

We then determined spontaneous mutation frequencies after 32.6 μM BrdU treatment. We used the can1+ gene, which encodes an arginine transporter that imports the toxic precursor canavanine; can1− mutants are resistant to canavanine (FANTES and CREANOR 1984). Increased can1 mutation occurred
after BrdU exposure in wild-type and \( cds1\Delta \) \( hsv-tk^+ \) \( hENT^+ \) cells (Fig 6E, Table 2), but not \( chk1\Delta \) and non-incorporating cells. Intriguingly, \( rad3\Delta \) and \( mrc1\Delta \) \( hsv-tk^+ \) \( hENT^+ \) cells had a significantly lower mutation rate after BrdU treatment.

We amplified the \( can1 \) locus from \( can1^+ \) colonies in all genotypes (with/without BrdU) to determine whether \( can1 \) mutation occurred by gross chromosom al rearrangement. Instead, we saw a 3.8kb \( can1 \) band in 91% of BrdU-treated \( hsv-tk^+ \) \( hENT^+ \) \( can1^- \) isolates (Fig S3C). We checked for smaller deletions by restriction fragment length polymorphism (RFLP) analysis, and did not see differences between fragments. Thus, we infer that BrdU-induced mutation in wild-type or \( cds1\Delta \) cells at \( can1 \) is largely due to point mutations, as in metazoans (GOODMAN et al. 1985).

**dNTP pools influence BrdU toxicity and mutagenesis**

We next asked whether BrdU affects dNTP pools, which would cause toxicity or mutation. Fission yeast Spd1 inhibits ribonucleotide reductase (RNR) to regulate RNR activity throughout the cell cycle and in response to DNA damage and repair. Previous work has shown that \( spd1\Delta \) cells have higher endogenous dNTP pools (HOLMBERG et al. 2005). We reasoned that increased pools of normal dNTPs might dampen the sensitivity to exogenous nucleotides.

Consistent with this prediction, we found that \( spd1\Delta \) \( hENT \) \( hsv-tk^+ \) cells were less sensitive to thymidine, BrdU or EdU than wild-type (Fig 7A, S4). \( spd1\Delta \) cells were minimally sensitive to BrdU or EdU in YES (Fig S4A). In EMM with BrdU or EdU, we observed two colony sizes in \( spd1\Delta \) strains: large colonies, similar to other
incorporating cells, amidst a high background population of small colonies (Fig S4B). We found that spd1Δ cells incorporate BrdU similarly to wild-type, and produce p-H2A foci in BrdU-labeled nuclei (Fig S4C).

Because spd1Δ hsv-tk+ hENT+ cells were less sensitive to analogues than wild-type incorporating cells in chronic exposure, we were surprised to find that spd1Δ viability was lower than wild-type during acute BrdU treatment in liquid culture (Fig 7B). However, spd1Δ hsv-tk+ hENT+ cells continued to divide in 32.6 μM BrdU, while wild-type and rad3Δ cells delayed division (Fig 7C). We examined can1 reversion, in a direct comparison between wild-type and spd1Δ after 32.6 μM BrdU for 2h at 32°C (Fig 7D). Consistent with Figure 6E, wild-type hsv-tk+ hENT+ cells experienced an increase in can1 mutation with 10 μg/mL BrdU treatment. Notably, spd1Δ cells already had a higher rate of mutation before BrdU, which increased a further 10-fold after BrdU incorporation.

We reasoned that enhanced spd1Δ survival on plates (despite poor viability and enhanced mutagenesis in liquid) might be attributed to genetic selection combined with the increased rate of mutation, leading to loss of the hsv-tk+ cassette. If this were to happen, a sub-population of cells would accumulate that are insensitive to further analogue incorporation, which could explain the background small cells on streaked plates (Fig S4B). Cells expressing hsv-tk+ are sensitive to FudR, so loss of the cassette can be scored by assessing the frequency of FudR resistant colonies or sectors (KIELY et al. 2000). We observed that untreated spd1Δ cells give rise to FudR resistant colonies infrequently, at a rate similar to wild-type (Fig 7E and Table 3).
However, BrdU treatment caused a significant increase in FudR resistance in \textit{spd1Δ} compared to wild-type cells. Significantly, higher colony sectoring (Fig 7F) is also consistent with an increase in mutation frequency, and suggests that \textit{spd1Δ} has higher genomic plasticity even within single colonies.

**DISCUSSION**

Previous studies in budding yeast indicated that BrdU incorporation does not perturb yeast cell growth (Lengronne \textit{et al.} 2001), although specific mutants may be more sensitive to BrdU during replication (Hodgson \textit{et al.} 2007). We propose that BrdU, EdU and thymidine all skew dNTP pools in \textit{hsv-tk⁺ hENT⁺} fission yeast. Our data show that \textit{S. cerevisiae} and \textit{S. pombe} are different in their response to nucleotide analogues and to dNTP imbalance. This is consistent with a 10-fold difference in HU sensitivities between the yeasts; HU also acts by depleting dNTPs.

Studies in metazoans found that BrdU treatment inhibits ribonucleotide reductase (RNR) and causes a “dCTP-less” state (Meuth and Green 1974b) (Fig 7G, part 1). BrdU-induced mutagenesis is attributed to low levels of dCTP (Hopkins and Goodman 1980; Meuth 1989), which is suppressed by co-culture with exogenous cytidine (Davidson and Kaufman 1978; Meuth and Green 1974b). Thymidine arrest in mammalian cells (Harper 2005) is also caused by decreased dCTP levels, which are restored once excess thymidine is removed from medium (Kunz and Kohalmi 1991; Meuth 1989). Unlike BrdU, thymidine is minimally toxic over short time periods of time (Lockshin \textit{et al.} 1985), although
high doses (>2 mM) induce mutation (PHEAR and MEUTH 1989), polyploidy, and chromosomal aberrations (POTTER 1971). Interestingly, lower BrdU concentrations (e.g. 32.6 μM) skew dNTP pools similar to much higher thymidine doses (2 mM) (MEUTH et al. 1976; MEUTH and GREEN 1974a).

We hypothesize that exposure to exogenous nucleosides creates dNTP pool imbalance in S. pombe. During thymidine incubation, hsv-tk hENT+ cells arrest with 1C DNA content and release into S-phase within 45 minutes. Previous work by Mitchison and Creanor (1971) determined that thymidine had no effect on S. pombe, consistent with the absence of a thymidine salvage pathway in wild-type cells. However, our incorporating strains have a reconstituted thymidine salvage pathway and are able to convert exogenous thymidine, BrdU or EdU into nucleotides.

Mitchson and Creanor (1971) also demonstrated that deoxyadenosine treatment induces a short-term G1 arrest in wild-type cells (MITCHISON and CREANOR 1971). This is consistent with our data, and suggests that dNTP pools are skewed by exogenous nucleosides in S. pombe if they are converted into useable nucleotides, to create a transient G1 arrest. We propose that Br- and ethynyl-dUTP (BrdU and EdU respectively) or thymidine, in a hsv-tk+ strain, causes an apparent increase in dTTP. Excess thymidine or analogue may also inhibit RNR as in human cells (MEUTH and GREEN 1974a; MEUTH and GREEN 1974b), depleting the dCTP pool (Fig 7G parts 1,2). If so, BrdU and cytidine co-treatment will suppress toxicity and mutagenesis as in metazoans (DAVIDSON and KAUFMAN 1978; POPESCU 1999).
Future work will determine how dNTP pools are altered with these nucleosides, but we predict that thymidine, BrdU or EdU all cause a dCTP-less state as in human cells. Interestingly, the fission yeast dCMP deaminase mutant (dcd1Δ) has an opposite effect: CTP pools become high while dTTP is low. The effects on cell fitness are similar to what we observed. Consistent with our model, dcd1Δ cells expressing hsv-tk+ increase dTTP pools, relieving growth defects and dcd1Δ sensitivities to UV, HU and bleomycin (SANCHEZ et al. 2012).

A second observation supports dNTP pool imbalance as a cause of observed phenotypes in S. pombe hsv-tk* hENT+ cells: while BrdU above 32.6 µM does not increase BrdU substitution in DNA, cell viability strikingly decreases above this dose. We observe no evidence for additional BrdU substitution, which excludes further T^C point mutations as the source of high-dose sensitivity. Instead, we propose that this dosage sensitivity reflects increased changes in cellular dNTP pools, perhaps via negative allosteric inhibition of RNR by analogues/thymidine (MEUTH and GREEN 1974b). To confirm this hypothesis, measurements of cellular dNTPs and RNR activity during BrdU and EdU are required.

Changes in dNTP pools cause point mutations in metazoans (MEUTH 1989; PHEAR and MEUTH 1989), by substituting the nucleotide in excess during replication (PHEAR and MEUTH 1989). We find that BrdU treatment increases can1 forward mutations in wild-type, spd1Δ and cds1Δ hsv-tk* hENT+ cells. However, the rate of mutagenesis is not significantly changed in chk1Δ, while rad3Δ and mrc1Δ experience decreased mutation. We suggest that the
replication checkpoint does not actively prevent BrdU-dependent mutation, while an active G2/M checkpoint \((\textit{chk1}^+)\) promotes mutagenesis. This may reflect the increased survival of checkpoint-intact cells, or checkpoint effects on Spd1 and nucleotide metabolism (see below). We assume that \(\textit{can1}^-\) mutations are G•C^A•T transitions caused by Br-dUTP substitution for the lowered dCTP analogue, or A•T ^G•C transitions via enhanced Br-dUTP pairing with guanine in template DNA (\textit{GOODMAN et al. 1985; HOPKINS and GOODMAN 1980; LASKEN and GOODMAN 1984}) (Fig 7G part 3). This is confirmed by our RFLP analysis of \(\textit{can1}\) mutants, which did not show any gross structural changes.

Fission yeast dNTP metabolism is controlled by RNR, which is inhibited by Spd1 (\textit{HAKANSSON et al. 2006; HOLMBERG et al. 2005; NESTORAS et al. 2010}). Spd1 is ubiquitinated during stress so that the dNTP pool is expanded, which ties nucleotide metabolism to the cell cycle and DDR (\textit{MOSS et al. 2010; NESTORAS et al. 2010}). We found that \(\textit{spd1}\Delta\) cells are less sensitive to chronic nucleoside exposure, but their relative viability in acute BrdU is worse. Intriguingly, \(\textit{spd1}\Delta\) cells showed a 10x-increase in mutations of \(\textit{can1}^+\) or \(\textit{hsv-tk}^+\) and frequent sectoring, indicating that \(\textit{spd1}\Delta\) cells are unstable and prone to mutation. Previous work showed \(\textit{spd1}\Delta\) suppresses mutation in \(\textit{ddb1}\Delta\) cells that are incapable of activating RNR (\textit{HOLMBERG et al. 2005}). However, we show that \(\textit{spd1}\Delta\) cells are intrinsic mutators, an effect worsened after BrdU treatment.

Increased basal dNTP pools in \(\textit{spd1}\Delta\) (\textit{HOLMBERG et al. 2005}) could promote this mutagenesis by facilitating replication during exogenous thymidine/dUTP treatment, and allowing additional Br-dUTP incorporation.
opposite G (LASKEN and GOODMAN 1984; LASKEN and GOODMAN 1985).

Alternatively, transient changes in one dNTP during spd1Δ replication may not prompt arrest. Our data are consistent with models linking dNTP regulation to genome stability in S. pombe (HOLMBERG et al. 2005; MOSS et al. 2010; NESTORAS et al. 2010). While chk1Δ hsv-tk+ hENT+ cells do not show increased mutation post-BrdU, we hypothesize that a spd1Δ chk1Δ double mutant may have a very high mutation rate due to extra damage and larger dNTP pools.

Rad3 is required to survive BrdU as reported in (HUA and KEARSEY 2011). We show this occurs through the Chk1 G2-DDR path downstream of Rad3, resulting in Cdc2-phosphorylation and Rad52 focus formation. BrdU also increases phospho-histone H2A, a signal of DSBs and/or replication fork stress (e.g., BAILIS et al. 2008; ROZENZHAK et al. 2010)). Our model suggests that halogenated-dUTP causes DNA damage (Fig 7G, part 4), perhaps via removal of substituted bases by base excision repair (BER) (KRYCH et al. 1979; MORGAN et al. 2007; SZYSZKO et al. 1983). BER under dNTP depletion could additionally cause single strand DNA breaks (SSBs) that stall replication forks and/or convert to DSBs during S-phase. This would promote cell accumulation in S-phase and a modest requirement for the Cds1 pathway.

Alternatively, topoisomerase I has RNaseH activity on a double strand DNA template with only one dUTP substitution (SEKIGUCHI and SHUMAN 1997) and could contribute to SSB and DSB accumulation. While BrdU-induced DNA damage in human cells is documented, the cause is not known (ACKLAND et al. 2011).

As in mammals, we observe that a second DNA damaging agent is more dangerous after BrdU-substitution. Sensitivity of chk1Δ cells to other drugs following BrdU incorporation suggests that BrdU substitution increases the DNA damage “load” in treated cells. In mammalian cells, UV exposure following BrdU-substitution causes DSBs and interstrand crosslinks (Cecchini et al. 2005; Murray and Martin 1989), and enhanced sensitivity to bleomycin (Ackland et al. 1988) and cisplatin (Russo et al. 1986). Budding yeast is also UV-sensitive after BrdU exposure (Sclafani and Fangman 1986). Some part of this sensitivity may result from S-phase slowing in BrdU, a more vulnerable time for DNA damage. We show increased sensitivity in cds1Δ strains, implying that replication fork stability is diminished, perhaps from imperfect BrdU-base pairing (Lasken and Goodman 1985). We find that rad3Δ response to CPT, UV, MMS or HU is unchanged with BrdU pre-treatment, probably because of catastrophic failure of rad3Δ in these drugs.

Our results point to challenges in the use of nucleoside analogues to analyze DNA replication. While we do not directly compare between the two analogues, our results indicate that fission yeast is extremely sensitive to nucleotide analogues BrdU and EdU, when treated at doses similar to those used in human cells. Consistent with replication stability problems, the increased toxicity of EdU, and cell cycle effects at lower doses, may reflect a larger ethynyl side group and thus greater steric interference during replication. The long-term
effects associated with BrdU and EdU exposure means that these analogues are most useful for analysis in a single cell cycle. Further, thymidine may be a potential reversible blocking agent in *S. pombe*, yet its effects must be more clearly described. In all cases, appropriate care must be taken to mitigate analogue effects, lest disruption of nucleotide levels interfere with the very process under study.

ACKNOWLEDGMENTS

We thank Antony Carr for the *spd1Δ* strain, Myron Goodman for helpful discussions, Oscar Aparicio for flow cytometer access, members of the Forsburg lab for comments, and anonymous reviewers for suggestions. This work is supported by NIH R01 GM59321 and NIH R01 GM081418 to SLF.

REFERENCES


MEUTH, M., 1989 The molecular basis of mutations induced by deoxyribonucleoside triphosphate pool imbalances in mammalian cells. Experimental cell research 181: 305-316.


MIYABE, I., T. MORISHITA, H. SHINAGAWA and A. M. CARR, 2009 Schizosaccharomyces pombe Cds1Chk2 regulates homologous


MURRAY, V., and R. F. MARTIN, 1989 The degree of ultraviolet light damage to DNA containing iododeoxyuridine or bromodeoxyuridine is dependent on the DNA sequence. Nucleic acids research 17: 2675-2691.


O'CONNELL, M. J., J. M. RALEIGH, H. M. VERKADE and P. NURSE, 1997 Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. The EMBO journal 16: 545-554.


ROZENZHAK, S., E. MEJIA-RAMIREZ, J. S. WILLIAMS, L. SCHAFFER, J. A. HAMMOND et al., 2010 Rad3 decorates critical chromosomal domains with gammaH2A to protect genome integrity during S-Phase in fission yeast. PLoS genetics 6: e1001032.


SZYSZKO, J., I. PIETRZYKOWSKA, T. TWARDOWSKI and D. SHUGAR, 1983 Identification of uracil as a major lesion in E. coli DNA following the incorporation of 5-bromouracil, and some of the accompanying effects. Mutation research **108**: 13-27.

### TABLE 1: FISSION YEAST STRAINS USED IN THIS STUDY

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY2317</td>
<td>h+ leu1-32::hENT1-leu1+(pJAH29) his7-366::hsv-tk-his7+(pJAH31) ura4-D18 ade6-M210</td>
<td>(HODSON et al. 2003)</td>
</tr>
<tr>
<td>FY3179</td>
<td>h+ mrc1::ura4+ leu1-32::hENT1-leu1+(pJAH29) his7-366::hsv-tk-his7+(pJAH31) ura4-D18 ade6-M210</td>
<td>this study</td>
</tr>
<tr>
<td>FY3454</td>
<td>h+ ura4-D18 ade6-M210</td>
<td>this study</td>
</tr>
<tr>
<td>FY5148</td>
<td>h+ cds1::ura4 leu1-32::hENT1-leu1+(pJAH29) his7-366::hsv-tk-his7+(pJAH31) ura4-D18 ade6-M210</td>
<td>this study</td>
</tr>
<tr>
<td>FY5149</td>
<td>h+ chk1::ura4+ leu1-32::hENT1-leu1+(pJAH29) his7-366::hsv-tk-his7+(pJAH31) ura4-D18 ade6-M210</td>
<td>this study</td>
</tr>
<tr>
<td>FY5150</td>
<td>h+ rad3::ura4+ leu1-32::hENT1-leu1+(pJAH29) his7-366::hsv-tk-his7+(pJAH31) ura4-D18 ade6-M210</td>
<td>this study</td>
</tr>
<tr>
<td>FY5155</td>
<td>h- cds1::ura4+ pola-FLAG::ura4+ rad11-myc::KanMX6 rad22-YFP::natMX leu1-32::[hENT-leu1+] his7-366::[hsv-tk his7+] ura4-D18 ade6-M210</td>
<td>this study</td>
</tr>
<tr>
<td>FY5159</td>
<td>h- pola-FLAG::ura4+ rad11-myc::KanMX6 rad22-YFP::natMX leu1-32::[hENT-leu1+] his7-366::[hsv-tk his7+] ura4-D18 ade6-M210</td>
<td>this study</td>
</tr>
<tr>
<td>FY5030</td>
<td>h- cds1-13myc::KanMX leu1-32::[hENT leu1+] his7-366::[his7+] ade6-M210 ura4-D18</td>
<td>this study</td>
</tr>
<tr>
<td>FY5031</td>
<td>h+ cds1-13myc::KanMX chk1HA leu1-32::[hENT leu1+] his7-366::[hsv-tk his7+] ade6-M216 ura4-D18</td>
<td>this study</td>
</tr>
<tr>
<td>FY6247</td>
<td>h+ spd1::ura4+ ura4-D18 leu1-32::hENT1-leu1+(pJAH29) his7-366::hsv-tk-his7+(pJAH31) ade6-?</td>
<td>this study</td>
</tr>
</tbody>
</table>
### TABLE 2: MUTATION RATES FOR SPONTANEOUS AND BRDU-INDUCED CANAVANINE FORWARD MUTATION ANALYSIS.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment^a</th>
<th>Number of mutations (m)^b</th>
<th>can'T Rate per 10^7 generations^b</th>
<th>95% CI^b</th>
<th>t-test^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type, non-inc (FY3454)</td>
<td>untreated (n=8)</td>
<td>4.669</td>
<td>5.59</td>
<td>3.02 / 8.70</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=8)</td>
<td>4.77</td>
<td>6.82</td>
<td>3.70 / 10.60</td>
<td></td>
</tr>
<tr>
<td>wild type (FY2317)</td>
<td>untreated (n=8)</td>
<td>5.601</td>
<td>9.56</td>
<td>5.37 / 14.59</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=7)</td>
<td>9.079</td>
<td>14.19</td>
<td>8.41 / 21.00</td>
<td></td>
</tr>
<tr>
<td>cdslΔ (FY5148)</td>
<td>untreated (n=8)</td>
<td>5.617</td>
<td>9.84</td>
<td>5.53 / 15.01</td>
<td>p&lt;0.025</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=8)</td>
<td>7.350</td>
<td>14.21</td>
<td>8.43 / 21.05</td>
<td></td>
</tr>
<tr>
<td>chk1Δ (FY5149)</td>
<td>untreated (n=8)</td>
<td>8.079</td>
<td>15.82</td>
<td>9.55 / 23.19</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=8)</td>
<td>8.714</td>
<td>16.80</td>
<td>10.28 / 24.45</td>
<td></td>
</tr>
<tr>
<td>rad3Δ (FY5149)</td>
<td>untreated (n=8)</td>
<td>4.272</td>
<td>9.68</td>
<td>5.12 / 15.24</td>
<td>p&lt;0.025</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=8)</td>
<td>5.868</td>
<td>9.56</td>
<td>5.43 / 14.52</td>
<td></td>
</tr>
<tr>
<td>mrc1Δ (FY6247)</td>
<td>untreated (n=8)</td>
<td>6.525</td>
<td>11.36</td>
<td>6.59 / 17.04</td>
<td>p&lt;0.025</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=8)</td>
<td>8.393</td>
<td>9.23</td>
<td>5.61 / 13.48</td>
<td></td>
</tr>
</tbody>
</table>

^a experiments were plated in duplicate and results summed for analysis. Total number of biological replicate experiments is indicated ("n") for each sample.

^b number of mutations (m), mutation rate and 95% confidence interval (CI) calculated using the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method FALCOR calculator (http://www.keshavsingh.org/protocols/FALCOR.html). Data from 8 independent assays. Citation, Hall, B.M., Ma, C., Liang, P. & Singh, K.K. (2009) Fluctuation AnaLysis CalculatOR (FALCOR): a web tool for the determination of mutation rate using Luria-Delbruck fluctuation analysis. Bioinformatics, 25(12): 1564-1565

^b Pair-wise 2-tailed t-test calculated within genotypes with/without BrdU from MSS-MLE results using mutation number (m) and calculated variance, with (n_withBrdU+n_withoutBrdU-2) degrees of freedom. asterix- not significant, p>0.05.
### TABLE 3: FREQUENCY OF *hsv-tk*+ LOSS OR SECTORING IN INCORPORATING WILD TYPE AND *SPD1Δ* CULTURES, WITH OR WITHOUT BRDU TREATMENT.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>FUdR resistance</th>
<th>Sectored colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type (FY2317)</td>
<td>untreated (n=8422)</td>
<td>0.226% (±0.101)</td>
<td>1.35% (±0.25)</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=5474)</td>
<td>0.274% (±0.138)</td>
<td>1.41% (±0.31)</td>
</tr>
<tr>
<td>spd1Δ (FY6247)</td>
<td>untreated (n=6968)</td>
<td>0.172% (±0.097)</td>
<td>2.65% (±0.38)</td>
</tr>
<tr>
<td></td>
<td>+BrdU (n=612)</td>
<td>2.78% (±1.30)</td>
<td>33.50% (±3.74)</td>
</tr>
</tbody>
</table>

*a* FUdR resistance and sectoring frequencies presented with 95% confidence intervals (CI). Data from 2 (wt) or 3 (*spd1Δ*) independent experiments.
FIGURE LEGENDS

FIGURE 1: BrdU and EdU doses affect signal, viability and cell division.

A. Time course of incorporation at 32.6 or 326 µM BrdU in HU-synchronized cells after release. Asynchronous (AS) cultures were blocked for 4h in HU (HU timepoint) before release at 32°C in medium with BrdU at indicated concentration. BrdU signal was detected in isolated nuclei.

B. Relative viability during 32.6 µM BrdU incubation, comparing non-incorporating (wt), or hsv-tk+ hENT+ cells both wild-type (wt-inc) and checkpoint mutants. Means ±SEM of 3 experiments.

C. As in A, time course of EdU incorporation at 1 and 10 µM doses in HU-synchronized cells post-release. Whole cells were treated with ClickIt reaction before flow cytometry.

D. Relative viability in 10 µM EdU treatment over time for non-incorporating (wt) or incorporating wild-type (wt-inc) or checkpoint-mutant incorporating cells. Means ±SEM of 3 experiments.

E. Cells were counted during BrdU or EdU treatment to determine proliferation in non-incorporating (wt) or hsv-tk+ hENT+ wild-type (wt-inc) and checkpoint mutant cells. Cell concentrations were normalized to the 0h sample for each cell line/condition, and are shown as means ±SEM (n=3).

FIGURE 2: Media formulation alters BrdU and EdU sensitivity.
A. Serial dilution assay in YES medium of non-incorporating wild-type (WT) and $hsv$-$tk^+$ $hENT^+$ wild-type (WT) and checkpoint mutant strains $cds1\Delta$, $chk1\Delta$, $rad3\Delta$ and $mrc1\Delta$. Plates containing BrdU, thymidine (Thy) control or EdU were compared using 1/5 dilutions of cells, grown at 32°C for 3 days.

B. As in A, spot tests on defined nitrogen-rich EMM medium. Refer to Supplemental Data (Fig S1) for PMG media effects.

**FIGURE 3: BrdU and EdU cause prolonged DNA synthesis, cell cycle slowing and DNA damage.**

A. DNA synthesis profiles of wild-type non-incorporating (non-inc) and incorporating (Inc) cells, out of hydroxyurea arrest (HU), released into medium with 32.6 or 326 µM BrdU to detect DNA replication. Left, whole cell DNA content (SytoxGreen) FACS profiles. Right, septation index for non-inc (NI) and Inc (I) cells stained with aniline blue and DAPI, at different BrdU doses (µM).

B. As in A, cells released from HU were released into medium with 1 or 10 µM EdU. Left, FACS profiles of whole cell DNA content. Right, septation index at different EdU doses (µM).

C. Asynchronous (AS) cells were treated with 2 mM thymidine (+Thy) or DMSO (vehicle control) for 3h (32°C), and then released for 0.75 h. DNA content (SytoxGreen FACS) for each time point, analyzed by FACS, is shown at each point. DMSO control was to test response to DMSO only, and was not released. Similar results seen with aqueous thymidine solution (not shown).
D. Cells were exposed to 32.6 μM BrdU for 2h (32°C) and then processed for BrdU and phospho-histone H2A (p-H2A) immunofluorescence. DNA was counterstained with DAPI. Merged image is BrdU and p-H2A signals. Scale 10 μm.

FIGURE 4: BrdU exposure triggers the DNA damage response.

A. Cells were treated with the indicated dose of BrdU and harvested for protein extraction hourly. Chk1-HA was detected with anti-HA (16B12), asterix indicates non-specific background signal and open arrow indicates phospho-Chk1HA. Crb2 modification indicated by an open arrow. Cds1-myc was detected with anti-myc (solid arrow). Phospho-Cdc2 (p-Cdc2, open arrow) and Cdc2 also detected. PCNA and beta-tubulin (β-tubulin) are loading controls. The black line indicates a split between 2 independent gels, identical lysates.

B. Incorporating strain viability (FY5031) proportional to BrdU dose is shown as means of 3 independent experiments ±SEM.

C. Chk-HA and Crb2 phosphorylation after 3h EdU (µM), in wild-type hsv-tk⁺ or non-incorporating cells. BrdU (326 µM) included as a control. Chk1-HA and Crb2 band shifts are indicated with open arrows. Asterix indicates non-specific background band (above Chk1HA) detected using a different antibody from A (αHA, 12CA5).
D. Phospho-Cdc2 (p-Cdc2) after 3h BrdU or EdU exposure (doses µM). Below bands are the quantified band intensities of p-Cdc2, normalized to total Cdc2 (below).

E. Quantification of p-Cdc2, relative to total Cdc2 levels, from 3 independent experiments. Mean values ±SEM.

**FIGURE 5: BrdU and EdU induce a DNA damage response.**

A. Rad52-YFP foci were monitored in untreated cells (untrt), or after 3h BrdU at 32°C. Rad52 foci (left) or DAPI stained nuclei (right) on transmitted light background, scale 10 µm.

B. Quantification of 3 independent experiments in A. Shown are proportions of nuclei with 2+ Rad52-YFP foci after 3h BrdU ± 95% confidence interval (CI).

C, D. Timepoints selected from movies of wild-type (C) or cds1Δ (D) cells treated with 10 µM EdU. Arrow (in D) indicates cell that forms foci and lyses. Scale 10 µm.

E. BrdU incorporated is similar at 32.6 and 326 µM doses. Shown is the mean BrdU signal per dot (±SEM, 3 independent experiments) at 2.5, 0.25 or 0.025 µg of heat-denatured total DNA, blotted and detected with BrdU antibody. Below, example of BrdU detection on DNA spots.

F. Wild-type non-incorporating or hsv-tk+ hENT+ cells were treated BrdU doses for 3h, plated on YES, and then irradiated with 100 J/m² UV light. Comparison plates were not treated with BrdU, to calculate %viability after BrdU+UV
treatment. Shown is the mean viability after BrdU+UV relative to BrdU only, for 3 independent experiments ± SEM.

**FIGURE 6: BrdU pre-treatment changes sensitivity to DNA damaging drugs.**

A to C. Cells were either untreated (untrt) or pre-treated (+BrdU) with 32.6 µM BrdU for 2h at 32°C, and then spotted onto drug plates in a 1/5 serial dilution. All plates are YES medium. Arrows, far right, indicate strains that were more sensitive to drug following BrdU pre-treatment. Strains FY3454, 2317, 3179, 5148, 5149, 5150. Also refer to Supplemental Data.

A. YES control for plating efficiency.

B. Sensitivity to Phleomycin.

C. Camptothecin (CPT) sensitivity.

D. Hydroxyurea (HU) sensitivity.

E. Forward mutation analysis for loss of Can1 wild-type status. Cells were incubated with 32.6 µM BrdU for 2h (32°C), and then plated to assess colony number on titre dishes. Remaining culture was plated on PMG+Canavanine and incubated 7 days at 32°C. Mutation rate, per 10^7 generations, was calculated comparing can1 mutants that grew on canavanine plates to the total number plated. Refer to Table 2 for significance results.

**FIGURE 7: Spd1 protects cells from division and mutation during dNTP imbalance.**
A. Comparison between wild-type and \( spd1\Delta \) \( hsv-tk^+ \) \( hENT^+ \) strains by spot test on EMM plates containing BrdU, EdU or thymidine. DMSO is a vehicle control for EdU. Shown is the minimal dose where wild-type cells began to show sensitivity to analogues. Strains FY 2317, 3454, 6247.

B. Cultures were treated with 32.6 \( \mu \)M BrdU and plated to calculate viability relative to 0h. Wild-type (wt) and \( spd1\Delta \) strains express \( hsv-tk^+ \) \( hENT^+ \) (FY2317, 6247), while the non-incorporating control (non-inc, FY3454) does not. Shown are mean viability values from 3 independent experiments ± SEM.

C. Proliferation was monitored by counting cell concentration during BrdU treatment for cultures as in A, in addition to \( rad3\Delta \) \( hsv-tk^+ \) \( hENT^+ \) (FY5150).

D. Canavanine mutation was scored for incorporating wild-type and \( spd1\Delta \) strains (FY2317, 6247), with or without 32.6 \( \mu \)M BrdU treatment (2h, 32°C). Lea and Coulson fluctuation analysis was used to calculate the rate of \( can1^+ \) forward mutation (per \( 10^7 \) generations) in independent cultures over 3 experiments (wt \( n=12 \), \( spd1\Delta \) \( n=15 \)). Shown are median mutation rates with quartile bounding boxes, and 95% CI error whiskers. Significance was assessed by 2-tailed pairwise Mann-Whitney U-tests, * \( p=0.0001 \), **\( p<0.0001 \).

E. Colonies of wild-type or \( spd1\Delta \) cells, untreated (no drug) or following 2h in 32.6 \( \mu \)M BrdU, were grown on YES and then replicated onto medium with FUdR to score for \( hsv-tk^+ \) loss. Significance was assessed by 2-tailed Z-test (**\( p<0.0002 \)
F. Enhanced sectoring of colonies on FUdR was noted for spd1Δ cells either untreated or following BrdU exposure as in E (2-tailed Z-test ** p<0.0002), compared to BrdU-treated wild-type cells. Inset, example of spd1Δ colony with hsv-tk+ loss (*), or sectored area (arrow). Frequencies were calculated from independent experiments, presented with 95% CI.

G. Model for the effect of exogenous thymidine (Thy) and nucleoside analogues in fission yeast cells expressing a reconstituted thymidine salvage pathway (hsv-tk+). Details in Discussion.
FIGURE 4  Sabatinos et al

A

dose (µM)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>time + BrdU (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chk1-HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crb2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>βtubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cds1-myc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Cdc2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

% viable relative to t=0

Dose (µM)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>time + BrdU (h)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>32.6</td>
<td>326</td>
<td></td>
</tr>
</tbody>
</table>

C

hsv-tk

hENT

EdU / BrdU dose (µM)

<table>
<thead>
<tr>
<th></th>
<th>E</th>
<th>B</th>
<th>E</th>
<th>E</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsv-tk</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hENT</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EdU</td>
<td></td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>BrdU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dose (µM)</td>
<td>10</td>
<td>326</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

p-Cdc2

<table>
<thead>
<tr>
<th></th>
<th>1.0</th>
<th>0.8</th>
<th>1.1</th>
<th>2.5</th>
<th>0.3</th>
<th>1.3</th>
<th>2.0</th>
<th>3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cdc2

D

E

Normalized p-Cdc2

<table>
<thead>
<tr>
<th></th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AS

EdU (µM)

1

10