Set2 Methyltransferase Facilitates DNA Replication and Promotes Genotoxic Stress Responses through MBF-Dependent Transcription

Highlights
- Set2 methyltransferase is required for efficient DNA replication
- Set2 loss reduces dNTP synthesis and alters replication origin firing
- Set2 promotes efficient MBF-dependent transcription
- Increasing dNTP synthesis restores replication following Set2 loss

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In Brief
Pai et al. find that the Set2 methyltransferase facilitates dNTP synthesis and DNA replication through promoting MBF-dependent transcription in fission yeast. Set2 loss results in reduced ribonucleotide reductase expression, reduced dNTP synthesis, altered replication origin firing, and checkpoint-dependent S-phase delay. These findings suggest how H3K36 methylation suppresses replication stress.

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SUMMARY

Chromatin modification through histone H3 lysine 36 methylation by the SETD2 tumor suppressor plays a key role in maintaining genome stability. Here, we describe a role for Set2-dependent H3K36 methylation in facilitating DNA replication and the transcriptional responses to both replication stress and DNA damage through promoting MluI cell-cycle box (MCB) binding factor (MBF)-complex-dependent transcription in fission yeast. Set2 loss leads to reduced MBF-dependent ribonucleotide reductase (RNR) expression, reduced deoxyribonucleoside triphosphate (dNTP) synthesis, altered replication origin firing, and a checkpoint-dependent S-phase delay. Accordingly, prolonged S phase in the absence of Set2 is suppressed by increasing dNTP synthesis. Furthermore, H3K36 is di- and tri-methylated at these MBF gene promoters, and Set2 loss leads to reduced MBF binding and transcription in response to genotoxic stress. Together, these findings provide new insights into how H3K36 methylation facilitates DNA replication and promotes genotoxic stress responses in fission yeast.

INTRODUCTION

DNA replication is a highly regulated process, and its fidelity plays a primary role in maintaining genome stability (Aguilera and Gómez-González, 2008). DNA synthesis can be divided into the stages of pre-replication complex (pre-RC) formation, replication initiation, elongation, and termination. Licensing of replication origins begins with chromatin binding of the origin recognition complex (ORC) and Cdc6/Cdc18. These factors facilitate the Cdt1-dependent loading of the mini-chromosome maintenance (MCM) complex, and subsequently multiple additional replication factors associate with the replication origin following activation of cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK). Pol ε is recruited to origins as part of the initiation complex and effects leading strand synthesis following helicase activation and priming by Pol α, whereas Pol δ is thought to replicate the lagging strand (Larrea et al., 2010; Pursell et al., 2007; Tanaka and Araki, 2013).

Efficient and accurate DNA replication elongation requires a balanced supply of deoxyribonucleoside triphosphates (dNTPs), generated in eukaryotes through the reduction of ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNPs), which is catalyzed by ribonucleotide reductase (RNR). RNR is a heterotetrameric or possibly higher-order complex (Mathews, 2016) that in the fission yeast Schizosaccharomyces pombe is minimally formed from two catalytic Cdc22 subunits and two small Suc22 regulatory subunits. RNR activity is highly regulated through a number of mechanisms (Guarino et al., 2014). In S. pombe, RNR is transcriptionally regulated by the MluI cell-cycle box (MCB) binding factor (MBF) complex (Liu et al., 2005). The MBF complex consists of essential core subunits including the product of the START gene cdc10*, together with Res1 and Res2, which constitute a heterodimeric DNA binding domain, and the co-activators Rep1 and Rep2 (Aves et al., 1985; Caligiuri and Beach, 1993; Miyamoto Purcell et al., 2007; Tanaka et al., 1992; Zhu et al., 1997). The MBF complex, which is functionally analogous to the E2F complex in humans induces transcription of a set of genes, including cdc22* (encoding the catalytic RNR subunit) cdt1* and cdc18* (encoding the replication licensing factors), at the G1/S transition of the cell cycle to facilitate DNA synthesis. In addition, RNR is subject to MBF-dependent transcriptional regulation by DNA integrity checkpoints in response to DNA damage and replication stresses. Following replication stress caused by RNR inhibition by hydroxyurea (HU), MBF-dependent transcription of the cdc22* gene is activated by the S-phase checkpoint kinase Cds1 through phosphorylation of Cdc10 (Dutta et al., 2008) and by inhibition of the MBF-associated transcriptional co-repressors Nrm1 and Yox1, thus facilitating resumption of DNA replication (Caetano et al., 2011; de Bruin et al., 2006, 2008; Gómez-Escoda et al., 2011). Activation of the DNA damage checkpoint displays a more complex regulation of Cdc10-dependent transcription.
Although DNA damage in G2 cells significantly upregulates a number of Miwi-box transcripts, such as cdc22, in a Rad3 and Chk1-dependent manner (Watson et al., 2004), it is clear that Chk1 activation can also inhibit MBF-dependent transcription of certain genes and phosphorylation of Cdc10 can result in its release from MBF targets (Ivanova et al., 2013). RNR activity is also post-translationally regulated through direct inhibition by Spd1 both during the cell cycle and following stress conditions (Håkansson et al., 2006; Liu et al., 2003; Moss et al., 2010). Moreover, RNR is also under allosteric control by dNTPs and NTPs. A mutation in the R1 subunit of RNR cdc22-D57N alleviates allosteric feedback inhibition by dATP and causes elevated dNTP pools (Cardas and Martin, 1988; Chabes et al., 2003; Fleck et al., 2013).

DNA replication origin usage can be positively or negatively regulated by the chromatin environment (Aparicio et al., 2004; Knott et al., 2009; Ménchali et al., 2013; Yoshida et al., 2014). Previous studies have described links between Set2-dependent histone H3 lysine 36 (H3K36) methylation and the timing of DNA replication (Biswas et al., 2008; Pryde et al., 2009), although the mechanism is unclear. Furthermore, loss of the tumor suppressor SETD2, in human cells, is associated with slower replication fork progression and with DNA replication stress (Kanu et al., 2019; Pfister et al., 2019). Moreover, studies in both fission yeast and humans showed histone H3K36 methylation to be cell-cycle regulated, with H3K36me3 over, studies in both fission yeast and humans showed histone H3K36 methylation to be cell-cycle regulated, with H3K36me3 levels peaking at the G1-S transition (Li et al., 2013; Pai et al., 2014). Together, these findings led us to study the function of histone H3K36 methylation in DNA replication.

Here, we establish a role for Set2 in efficient DNA replication and in facilitating efficient dNTP synthesis through promoting MBF gene transcription under normal conditions and in response to genotoxic stress.

RESULTS

set2Δ Cells Show Perturbed Progression through S Phase

We wanted to investigate a possible role for Set2 in DNA replication. Both wild-type and set2Δ cells were grown to log phase in YES medium and processed for flow cytometry analysis. Asynchronous set2Δ cells do not exhibit significant S-phase delay compared to wild-type cells (Figure S1A). This result was determined by flow cytometry analysis, which may not be sufficiently sensitive to detect subtle effects on the length of S phase. We therefore performed nucleotide incorporation experiments in wild-type and set2Δ cells in asynchronous cultures. Efficient incorporation of 5-ethyl-2'-deoxyuridine (EdU) into fission yeast requires expression of both thymidine kinase (TK) and a nucleoside transporter (hENT1) (Hua and Kearsey, 2011). Here, we showed that wild-type cells, or set2Δ cells expressing Drosophila melanogaster deoxyribonucleoside kinase (DmdNK) under the control of the fission yeast adh promoter, together with the human equilibrative nucleoside transporter (hENT1) (adhl-Dm-dNK-adh-hENT1) (Fleck et al., 2017) are sensitive to 5 μM EdU, whereas wild-type or set2Δ cells are not sensitive to EdU (Figure 1A), consistent with previous studies showing that EdU is toxic to incorporating strains (Hua and Kearsey, 2011).

With medium containing 100 μM EdU for 15 min, no significant cell-cycle delay was observed in wild-type or set2Δ cells expressing adhl-Dm-dNK-adh-hENT1 (Figure 1B). However, during a short pulse, significantly more cells incorporating EdU were detected using fluorescent microscopy in the absence of Set2 (Figures 1C and 1D), suggesting a prolonged S phase in set2Δ cells.

To explore this further, we investigated a role for Set2 in DNA replication using a synchronous G1 block and release experiment. Both wild-type and set2Δ cells were synchronized in G1 by nitrogen starvation and released from the block at 32°C. Samples were then taken at the indicated time points and analyzed by flow cytometry. In wild-type (WT) cells, DNA replication started 2 hr after release from the G1 arrest and was finished by 5 hr (Figure 1E, WT). In contrast, we found that set2Δ cells displayed a significant delay in S-phase progression (Figure 1E, set2Δ), as was also observed in a methyltransferase-dead set2-R255G mutant cells (Figure 1E, set2-R255G). This indicates that methyltransferase activity of Set2 is required for efficient DNA replication. Similar conclusions were drawn from results obtained from cells synchronized using a cdc25-22 G2-M block and release protocol (Figure 1F; Figures S1B and S1C). Collectively, these findings identify a role for Set2 in efficient DNA replication initiation and/or elongation in synchronous cells.

set2Δ Does Not Alter the Efficiency of Replication Origins

Given that histone modifications have been reported to affect the activity of origin firing, we examined the possible role of Set2 in regulating the pattern of replication origin usage. We performed a polymerase usage sequence (Pu-seq) method to map genome-wide origin usage as previously described (Dai et al., 2015; Keszthelyi et al., 2015). In wild-type cells, we identified 1,386 initiation sites at 18°C and 1,207 at 34°C including efficient (>50% usage per cell cycle), moderately efficient (50–25%), and inefficient (<25%) origins (Figure 2A, wild-type). In the set2Δ background, we mapped 1,444 initiation sites at 18°C and 1,303 at 34°C (Figure 2A, set2Δ), suggesting a modest increase in the number of replicating origins in set2Δ compared to wild-type cells. Interestingly, this increase is mostly caused by increased use of inefficient origins (Figure 2B) and the overall efficiency of the vast majority of origins was not changed (Figure 2C) —for example, the average efficiency of the top 10% of efficient origins (Figure S2). Analysis of the distribution of origin usage in set2Δ cells revealed that these new inefficient origins are randomly located throughout the yeast genome. However, there is no significant difference of the number of origins identified at sub-telomeric regions of set2Δ compared to wild-type cells (Figure S2). In conclusion, we found that set2Δ subtly alters genome-wide replication origin usage but does not significantly alter the efficiency of the vast majority of origins. The main effect is to increase the total number of inefficient (previously dormant) origins used in the fission yeast genome, suggestive of a general slowdown in replication after origins have fired (Anglana et al., 2003).

MBF-Regulated Gene Expression Is Downregulated in set2Δ Cells

We have previously investigated gene expression levels in response to DNA damage in a set2Δ background (Pai et al.,
We found a number of genes to be upregulated and downregulated in response to DNA damage compared to wild-type in a set2Δ background. Notably, a cluster of four transcripts: cdc18+, cdt1+, cdc22+, and tos4+, involved in cell-cycle regulation, were consistently downregulated in set2Δ cells following treatment with bleomycin for 30 min when compared to wild-type (Figure 3A). The products of two of these genes, Cdt1 and Cdc18, are required for Mcm2-7 association with ORC in pre-RC formation (Nishitani et al., 2000). The third, Cdc22, is the large subunit of RNR whose interaction with Suc22 (the small subunit) is required for dNTP production (Fernandez Sarabia et al., 1993). The fourth, Tos4, is a putative G1-S transcription factor containing a forkhead domain, and is an effector of the DNA replication checkpoint (Bastos de Oliveira et al., 2012; Horak et al., 2002).

Analysis of the promoters of these genes indicated that they are all cell-cycle regulated and under the transcriptional control of the MBF complex, strongly suggesting that Set2 has a function in MBF transcription activation. Accordingly, following qPCR validation of the above results, we found set2Δ-dependent downregulation of cdc18+, cdt1+, cdc22+, and tos4+, both in the absence and presence of bleomycin, suggesting a role for Set2 in facilitating basal, as well as damage-induced MBF transcription (Figure 3B). Furthermore, we found one other MBF-dependent gene that also showed reduced transcription in
set2Δ cells compared to wild-type following bleomycin treatment: nrm1+ (Figure 3B). However, other MBF-dependent genes such as ctp1+ and mrc1+ showed no alteration in transcription based on microarray analysis (data not shown). In accordance with a role for Set2 in MBF activation following DNA damage, we found that protein levels of Cdc18, Cdc22, and Cdt1 were downregulated in response to bleomycin treatment in a set2Δ background compared to wild-type cells (Figures 3C–3E).

**Licensing Factors Cdt1 and Cdc18 Are Misregulated in set2Δ Cells**

Given the replication delay seen in set2Δ cells and the involvement of three members of the cluster in efficient DNA replication, we analyzed mRNA and protein levels of these replication factors, Cdc18, Cdt1, and Cdc22, in the absence of genotoxic stress. In asynchronous cell cultures where most cells are in G2, we found that these replication factors were all downregulated in a set2Δ background compared to wild-type cells (Figures 3C–3E), further supporting a role for Set2 in DNA replication.

Next, we investigated protein levels of the licensing factors Cdc18 and Cdt1 using a synchronous G1 block and release experiment. Both licensing factors showed oscillating protein levels in wild-type cells (Figures 4D and 4F, wild-type), in accordance with normal S-phase progression (Figures 4C and 4E). In contrast, set2Δ cells showed delayed expression of Cdc18 and Cdt1 after release from G1 arrest (Figures 4C–4F, set2Δ), and the final levels achieved were lower than seen in wild-type cells. Similar conclusions were drawn from results obtained from cells synchronized using a cdc25-22 G2-M block and release protocol (Figures S3A and S3B). In accordance with protein levels, set2Δ cells also showed delayed mRNA expression of cdc18+ and cdt1+ after release from G1 arrest (Figures S4A and S4B), supporting the idea that changes are transcriptional, although there may also be post-translational changes. This result suggests that a delay in pre-RC formation or origin binding of initiating factors is contributing to the slow S phase observed for set2Δ cells (Figure 1). Consistent with this, previous studies in budding yeast showed that chromatin association of initiation factor Cdc45 was delayed in a set2Δ strain (Pryde et al., 2009), presumably due to delayed expression of licensing factors.

To see whether the delayed S-phase entry in set2Δ cells could be rescued by increasing the expression of Cdt1 and Cdc18, the genes were ectopically expressed from a REP81X plasmid in cdc25-22 and cdc25-22 set2Δ cells. However, in a cdc25-22 set2Δ background, cells grown in the absence of thiamine had a similar profile to those grown in its presence, indicating overexpression of Cdc18 and Cdt1 did not suppress the replication defect observed in set2Δ cells (Figure S5). This suggests that other DNA replication steps were affected and account for the slow growth phenotype.

Of the cluster of four genes that have significantly decreased transcript levels in response to bleomycin in set2Δ cells, Tos4 has been identified in S. cerevisiae as a G1-S transcription
factor (Horak et al., 2002). Given the replication delay seen in set2\(\Delta\) cells and the involvement of the other three members of the cluster in efficient DNA replication, the cell-cycle progression of tos4\(\Delta\) cells was analyzed. However, in contrast to the defective S-phase progression seen in set2\(\Delta\) cells, a cdc25-22 tos4\(\Delta\) double mutant did not exhibit significant delay in cell-cycle progression following G2-M block and release (Figure S6).

**Set2 Is Required for Balanced dNTP Synthesis**

The four dNTP precursors for DNA synthesis are potentially limiting for initiation and elongation if levels are too low (Yekezare et al., 2013). Importantly, imbalanced dNTP pools caused by altered expression and/or activity of RNR can affect cell-cycle progression and lead to mutagenesis (Chabes and Stillman, 2007; Fleck et al., 2013). Because our earlier results showed that Cdc22 levels were also downregulated in a set2\(\Delta\) mutant background, we monitored the levels of the catalytic subunit of RNR, Cdc22, in wild-type and set2\(\Delta\) cells during a G1 block and release cell cycle. In contrast to wild-type, RNR protein and mRNA levels did not rise around the time of S phase, consistent with the slow S-phase progression seen in set2\(\Delta\) cells (Figures 4G and 4H; Figure S4C). This result indicates that the replication defect in a set2\(\Delta\) background could have arisen from low or imbalanced levels of dNTPs, which could possibly cause replication progression defects in a set2\(\Delta\) background. To verify this, we measured dNTP levels in asynchronous set2\(\Delta\) cells and found that dNTP levels were lower, with dCTP, dGTP, and dATP levels being significantly reduced (p < 0.05) compared to that of wild-type (Figure 5A), suggesting imbalanced dNTP levels could be limiting for DNA replication.

**Figure 3. A Cluster of G1-S Genes Are Downregulated following DNA Damage in the Absence of Set2**

(A) Left panel: Heatmap depicting cdc18\(+\), cdt1\(+\), cdc22\(+\), and tos4\(+\) transcript levels in wild-type and set2\(\Delta\) cells following 30 min of treatment with 5 \(\mu\)g/mL bleomycin. Blue depicts downregulated compared to before damage, and red depicts upregulated compared to before damage. Right panel: Quantification of cdc18\(+\), cdt1\(+\), cdc22\(+\), and tos4\(+\) transcript levels in wild-type (blue) and set2\(\Delta\) (red) cells following 30 min of treatment with 5 \(\mu\)g/mL bleomycin. Data represent mean of two experiments with independently derived RNA.

(B) Quantification of cdc18\(+\), cdt1\(+\), cdc22\(+\), nrm1\(+\), and tos4\(+\) transcript levels in wild-type and set2\(\Delta\) cells following 30 min of treatment with 5 \(\mu\)g/mL bleomycin. Error bars represent SD from three biological replicates. The asterisk (*) represents significant difference compared with wild-type and set2\(\Delta\) (p < 0.05, t test).

(C) The protein levels of Cdc18 were examined in wild-type and set2\(\Delta\) cells following 5 \(\mu\)g/mL bleomycin treatment. Samples of cells were taken at the indicated time points, and cell extracts were made using TCA method. Cdc18 was detected using an antibody against the TAP tag. \(\alpha\)-Tubulin is shown as a loading control.

(D) The protein levels of Cdc22 were examined in wild-type and set2\(\Delta\) cells following 5 \(\mu\)g/mL bleomycin addition. Total cell extracts were prepared at the indicated times and analyzed by western blotting. Cdc22 was detected using an anti-GFP antibody. \(\alpha\)-Tubulin is shown as a loading control.

(E) The protein levels of Cdt1 were examined in wild-type and set2\(\Delta\) cells following 5 \(\mu\)g/mL bleomycin addition. Samples of cells were taken at the indicated time points, and cell extracts were made using TCA method. Cdt1 was detected using an antibody against the TAP tag.
Elevated dNTP Pools Suppress Slow Replication in set2Δ Cells

To confirm whether the cause of replication delay in set2Δ cells was through Set2-dependent dNTP synthesis during the cell cycle, we tested whether it was possible to suppress the replication defects of set2Δ cells by increasing dNTP pools. To achieve this, the gene encoding Spd1, an inhibitor of RNR (Liu et al., 2003; Woollard et al., 1996), was deleted in a set2Δ background. We found that Spd1 depletion restored dNTP levels in set2Δ cells (Figure 5A). We repeated the G1 block and release experiment and found that deletion of spd1+ considerably rescued the DNA replication delay in set2Δ cells (Figure 5B), consistent with a Set2 function in promoting dNTP synthesis. However, we do not exclude the possibility that Spd1 may have other functions in regulating S-phase progression in fission yeast (Fleck et al., 2017).

S-Phase Delay in set2Δ Cells Is Checkpoint Dependent

We wanted to test whether delayed S-phase progression resulting from loss of H3K36 methylation resulted directly by limiting S-phase progression through nucleotide depletion or was exacerbated by checkpoint activation resulting from dNTP depletion. Indeed, it is well established that inhibition of DNA replication due to depletion or imbalanced of dNTP pools leads to activation of the S-phase checkpoint (Enoch and Nurse, 1990; Kumar et al., 2010; Murakami and Okayama, 1995). To test this idea, we deleted rad3+ encoding the checkpoint sensor Rad3 (ATR) in set2Δ cells and monitored cell-cycle progression by fluorescent-activated cell sorting (FACS) analysis. We found that deleting rad3+ suppressed the prolonged S-phase progression in set2Δ cells (Figure 6A). Consistent with this result, deleting cds1+ encoding the Cds1 replication checkpoint kinase also suppressed the slow S-phase progression in set2Δ cells (Figure 6B). This finding suggests that imbalanced nucleotide pools activate the intra-S checkpoint resulting in S-phase delay. Alternatively, DNA synthesis from low levels of pyrimidines (dCTP or dTTP) could cause DNA damage that activates the S-phase checkpoint.

Figure 4. Replication Factors Are Misregulated in set2Δ Cells

(A) Transcript levels of cdc18+, cdc22+, and cdt1+ were established in exponentially growing wild-type and set2Δ cells. RNA was extracted with Qiagen RNeasy kit and relative transcript levels of cdc18+, cdc22+, and cdt1+ were established by RT-qPCR. Error bars represent SD of three biological repeats. The asterisk (*) represents significant difference compared with wild-type and set2Δ.

(B) Cdc18, Cdt1, or Cdc22 protein levels of exponentially growing wild-type and set2Δ cells. Cell extracts were prepared from vegetative cells and processed for western blot. Immunoblots of total cell lysates were probed with PAP or GFP antibody. α-Tubulin is shown as a loading control.

(C) cdc18-TAP or cdc18-TAP set2Δ cells were arrested in G1 by nitrogen starvation and released, and samples were taken at time points indicated and subjected to FACS analysis. (D) In parallel, cdc18-TAP and cdc18-TAP set2Δ cells were processed for western blotting at indicated times.

(E) cdt1-TAP or cdt1-TAP set2Δ cells were arrested in G1 by nitrogen starvation, released, and samples taken at time points indicated and subjected to FACS analysis. (F) In parallel, cdt1-TAP and cdt1-TAP set2Δ cells were processed for western blotting at indicated times.

(G) A similar experiment to that described in (C) was carried out using cdc22-CFP or cdc22-CFP set2Δ cells.

(H) A similar experiment to that described in (D) was carried out using cdc22-CFP or cdc22-CFP set2Δ cells. Immunoblots of total cell extracts were probed with GFP antibody. α-Tubulin is shown as a loading control.
checkpoint, therefore arresting the cell-cycle progression (Kumar et al., 2010).

**DNA Integrity Checkpoint Activation Is Proficient in**
set2 Δ **Cells**

The above result implies that DNA replication checkpoint signaling is not affected by inactivation of Set2. To confirm this, we analyzed the function of Set2 in checkpoint signaling in response to replication stress. In contrast to deleting the checkpoint genes, set2Δ or H3K36R mutants exhibited only modest HU sensitivity (Figure 6C; Figure S7A). Treatment with HU was not found to result in an increased “cut” phenotype, in which incompletely replicated DNA is divided into two daughter cells, in a set2Δ background (Figure 6D), suggesting that mitosis was arrested in the presence of incomplete DNA replication and that the intra-S-phase checkpoint in set2Δ cells was intact. In fission yeast, Cds1 is the effector kinase of the DNA synthesis checkpoint pathway, stabilizing stalled forks and triggering transcriptional activation of G1-S-specific genes (Dutta et al., 2008). Moreover, Cds1 and Chk1 establish the redundant pathways of HU-induced checkpoint arrest and double mutants exhibit mitotic catastrophe in response to DNA damage (Lindsay et al., 1998; Zeng et al., 1998). We therefore further investigated the role of Set2 in HU-induced checkpoint arrest through examining the effect of inactivating Set2 in chk1Δ cells. We observed that, like wild-type cells, set2Δ chk1Δ cells exhibited an elongated phenotype in the presence of HU, confirming that Set2 is not required for proficient Cds1 activation (Figure 6E). Consistent with this result, HU-induced activation of Cds1 was not affected by the loss of Set2 (Figure S7B) as previously reported in fission yeast (Kim et al., 2008). We did not detect Cds1 phosphorylation in unperturbed set2Δ cells, perhaps due to low checkpoint activation reflecting a cell-cycle delay rather than an arrest. Taken together, these results suggest that Set2 is proficient in replication checkpoint activation.

**Set2 Facilitates Efficient DNA Replication through Promoting MBF-Induced dNTP Synthesis**

Our results suggest that Set2 is required for induced expression of a subset of MBF-dependent genes in response to bleomycin treatment. Previous studies have shown that MBF-dependent transcription is induced in response to replication stress (Bertoli et al., 2013), but can be both induced and inhibited in response to
Figure 6. The set2Δ Mutation Causes a Rad3 (ATR)-Dependent S-Phase Progression Defect

(A) Deletion of Rad3 (ATR) increases the speed of S-phase progression in set2Δ cells. Flow cytometry analysis of wild-type (WT), set2Δ and set2Δ rad3Δ strains after release from G1 into EMM+N at 32°C.

(B) Deletion of Cds1 (Chk2) increases the speed of S-phase progression in set2Δ cells. Flow cytometry analysis of wild-type (WT), set2Δ and set2Δ cds1Δ strains after release from G1 into EMM+N at 32°C.

(legend continued on next page)
DNA damage (Ivanova et al., 2013; Watson et al., 2004). Therefore, Set2 could either have a role in the induction of MBF-dependent transcription in response to replication stress or the prevention of DNA damage-induced inhibition of MBF target gene expression. To test this, we first performed RT-qPCR analysis of MBF-dependent genes following HU treatment to assess the role of Set2 in replication stress-induced transcription. Cells were treated with HU for an extended period of time to establish whether Set2 is required for the induction and/or maintenance of MBF-dependent gene expression in response to replication stress. We found that Set2 is required for rapid induction and sustained expression of MBF-dependent cdc22+ or cdc18+ under HU-induced replication stress conditions (Figures 7A and 7B).

To examine the possibility that Set2 depletion causes a higher degree of replication stress-induced DNA damage, which in turn indirectly activates Chk1 in response to replication stress and inactivates MBF at cdc22+ and cdc18+ promoters, we analyzed these MBF targets in set2Δ chk1Δ compared to set1Δ cells. The set2Δ and set1Δ chk1Δ cells show a similar pattern of MBF-dependent expression (Figure 7A), indicating the decrease in MBF-dependent transcription in HU is likely to be a direct effect of set2+ deletion, rather than DNA damage-dependent inhibition. This result supports the idea that the loss of G1-S transcription in a set2Δ background was due to MCB complex dysfunction. In support of our findings, deletion of yox1+, encoding the MBF transcription repressor Yox1, significantly elevated dNTP pools in set2Δ cells (Figure 7B), and further suppressed the prolonged-S phase in set2Δ cells, presumably due to elevated RNR levels (Figure 7C). These results together support a role for Set2-dependent H3K36 methylation in facilitating efficient DNA synthesis through regulation of MBF-dependent gene expression, primarily through regulation of RNR transcript levels.

To further investigate a role for Set2 in promoting MBF-dependent transcriptional regulation, chromatin immunoprecipitation (ChIP) analysis of MBF binding was assessed in wild-type and set2Δ cells. We found that binding of the MBF transcription factor subunit Res1 at cdc22+ and cdt1+ promoters is significantly reduced in a set2Δ background compared to wild-type in the presence of bleomycin (Figure 7D). Furthermore, we investigated whether Set2 was recruited to these gene promoters in a wild-type background following genotoxic stress. ChIP analysis indicated that the promoter regions of these MBF-dependent genes underwent H3K36 di- and tri-methylation (Figures 7E and 7F), demonstrating that Set2-dependent H3K36 methylation has the potential to regulate the binding of MBF. Together, these results suggest a role for Set2 in facilitating recruitment of the MBF transcription factor to MCB-containing promoters to facilitate their expression in response to genotoxic stress.

**DISCUSSION**

We have established important roles for Set2-dependent H3K36 methylation in facilitating efficient DNA replication, together with promoting the transcriptional responses to both replication and genotoxic stresses. Our data support a role for H3K36 methylation in facilitating these functions through promoting the basal and genotoxic stress-induced transcription of a subset of MBF-regulated genes.

We find Set2 facilitates efficient DNA replication through maintaining dNTP pools. Accordingly, the S-phase delay observed in a set2Δ background can be suppressed by increasing dNTP levels either through deleting the MBF repressor gene yox1+, or by deletion of spd1+ encoding an inhibitor of RNR in a set2Δ background. These data support an important role for Set2 in facilitating efficient DNA replication through MBF-dependent dNTP synthesis. Although replication origins fire with largely equivalent efficiency in wild-type and set2Δ cells, we observed that a number of additional low-efficiency origins (dormant origins) are utilized in set2Δ cells compared to wild-type cells. We found the S-phase delay in a set2Δ background could be largely suppressed by deleting Rad3 (ATR) or Cds1 (CHK2), strongly suggesting that the S-phase delay arose through activation of the intra-S checkpoint in a set2Δ background. These data are consistent with an insufficient supply of dNTPs in a set2Δ background leading to increased replication stress and/or DNA damage, and to subsequent checkpoint-dependent S-phase delay.

Analysis of DNA replication origins in a set2Δ background revealed an increase in the number of inefficient origins being fired, which are randomly distributed throughout the yeast genome as would be expected when replication is slowed (see above). Interestingly, a role for Set2 has recently been described in facilitating the recruitment of Shugoshin (Sgo2) to sub-telomeric regions and for the formation of highly condensed chromatin bodies or “knobs” flanking telomeric heterochromatin (Matsuda et al., 2015). Sgo2 regulates replication timing at the sub-telomeres by limiting Sld3 loading, and sub-telomeric regions were found to replicate early in sgo2Δ cells (Tashiro et al., 2016). Therefore, loss of Set2 could regulate early firing of sub-telomeric origins through facilitating increased Sld3 loading.

Our findings support a role for Set2 in facilitating the transcription of a subset of MBF-regulated genes, both at the basal level (without damage) and when activated in response to genotoxic stress. Consistent with this, Set2 loss resulted in significantly reduced mRNA and protein expression of a subset of MBF genes including cdc18+, cdt1+, and cdc22+ in asynchronous or synchronized conditions or in response to HU or bleomycin. How might Set2 facilitate MBF-dependent transcription? Our data are consistent with a role for Set2-dependent H3K36 methylation in promoting MBF-dependent

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(C) Serial dilution of a wild-type and cds1Δ, set2Δ, and H3K36R mutants were spotted onto YES medium containing 10 mM HU. Plates were incubated at 30°C for 2–3 days.

(D) Each fixed culture from wild-type, set2Δ chk1Δ, cdc18Δ, or rad3Δ strain was stained with 4′,6-diamidino-2-phenylindole (DAPI) and analyzed by fluorescence microscopy showing nuclear morphology for each strain.

(E) set2Δ chk1Δ cells were grown in YES medium containing 10 mM HU at indicated times and stained with DAPI followed by microscopy analysis for nuclear morphology.
transcription through regulating the binding and/or activity of the MBF complex to the promoters of MBF target genes. In this respect, we find significantly reduced levels of Res1 at MBF promoters in a set2Δ background following DNA damage. We note that genes whose expression is particularly sensitive to loss of Set2 exhibit a promoter pattern associated with two MCB elements (5'-AACGCG-3' and 5'-CGCGNCGCG-3'), which is present only in a subset of MBF-regulated transcripts.

Therefore, Set2 loss may affect expression of genes whose promoters contain similar MCB arrangements more robustly. However, the precise mechanism as to how H3K36 methylation promotes MBF binding to target genes in the absence or presence of genotoxic stress is currently unclear and will require further investigation. We previously showed that H3K36 modification was cell-cycle regulated with H3K36 tri-methylation being associated with an increased 1C DNA content (Pai et al., 2017).
incorporation and also separated the double-stranded DNA. The resulting ssDNA fragments were size selected on agarose gel (fragments between 300 and 500 bp were isolated). These fragments were then used for creating strand-specific next-generation sequencing libraries and sequenced on a Next-seq Illumina platform resulting in ~10M reads from each strain. Reads were aligned to the Schizosaccharomyces pombe reference sequence (http://www.pombase.org/downloads/genome-datasets), the reads were mapped using bowtie2 and the data were analyzed and origin positions and efficiencies were determined using the tools published and described in detail in Daigaku et al. (2015) and Keszthelyi et al. (2015) with default variables except that the “percentile threshold for origins” option was set to 0.2 = 20th percentile. Efficient origins were determined as origins with higher than 50% efficiency and inefficient origins had less than 25% efficiency. Because our previous work has indicated subtle changes to origin firing at different temperatures, we performed the analysis on cultures grown at either 18°C or 34°C.

ChIP

Yeast culture was grown in YES to OD_{595} = 0.3–0.5. 45 mL of culture was incubated with 1% of formaldehyde for 20 min for cross-linking. The reaction was quenched with 125 mM glycerol. Cells were lysed with lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM sodium chloride, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA) supplemented with protease inhibitors (complete Tablets Mini, EASYpack; Roche) by vortexing with glass beads (0.5 mm; Bio- spec Products). Chromatin was sheared to 500–1,000 bp by sonication (amplitude, 100%; process time, 5 min; ON time, 30 s; OFF time, 2 min). Chromatin lysate was incubated with specific antibodies (anti-H3K36me2 Active Motif 39255; anti-H3K36me3 Active Motif 61101; Myc-tag Santa Cruz 9E10: sc-40) at 4°C overnight rotating. Chromatin was pulled down with cold 50% protein A-Sepharose beads in lysis buffer (Sigma; P3391) for 3 hr and washed six times with washing buffer (50 mM Tris-HCl, 1% Triton X-100, 150 mM sodium chloride, 5 mM EDTA, 0.5% NP-40). De-cross-linking was performed with 10% Chelex in ultra-pure water and boiling for 10 min. DNA enrichment was established by qPCR with SYBR green according to the manufacturer’s recommendations and percentage of the whole-cell extract method. Primers used in this study are listed in Table S2.

**Protein Analysis**

Protein extracts were made by trichloroacetic acid (TCA) extraction and analyzed by western blotting as described previously (Pai et al., 2014). TAP-tagged proteins were detected with peroxidase-antiperoxidase-soluble complex (P1291; Sigma), Cdc22-GFP was detected using antibody 11814460001 (Roche), and α-tubulin was detected with antibody T5168 (Sigma). Phos-tag Acrylamide gel was used to detect Cds1-P (Wako).

**HPLC Analysis of dNTP**

dNTP analysis was carried out as previously described (Moss et al., 2010).

**qPCR Analysis**

qPCR analysis was carried out as previously described (Caetano et al., 2014). Total RNA was prepared using the RNeasy Plus Kit (Qiagen) as indicated in the manufacturer’s manual. Transcript levels were determined by RT-qPCR using the iScript One-Step RT-PCR kit with SYBR Green Supermix (Bio-Rad). RT-PCR were run on a Chromo-4 Real-Time PCR Detector (Bio-Rad) and obtained experimental values analyzed using MJ Opticon Analysis Software 3.0. Furthermore, data were normalized against actin and investigated using the C(t) method. Primers used in this study are listed in Table S2.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.058.

**AUTHOR CONTRIBUTIONS**

and analyzed data. C.-C.P. and T.C.H. wrote the manuscript with input from all authors. Experiments in Figure 1 were performed by C.-C.P., R.S.D., and S.E.K.; in Figure 2 were performed by A.K. and A.M.C.; in Figure 3 were performed by C.-C.P., A.K., and R.A.M.d.B.; in Figure 4 were performed by C.-C.P., A.K., and N.D.L.; in Figure 5 were performed by C.-C.P., L.F., and N.D.L.; in Figure 6 were performed by C.-C.P. and N.D.L.; in Figure 7 were performed by C.-C.P., A.K., and R.A.M.d.B.; and in Figure S7B were performed by I.S.

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