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The *RAD6* DNA Damage Tolerance Pathway Operates Uncoupled from the Replication Fork and Is Functional Beyond S Phase

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SUMMARY

Damaged DNA templates provide an obstacle to the replication fork and can cause genome instability. In eukaryotes, tolerance to damaged DNA is mediated largely by the *RAD6* pathway involving ubiquitylation of the DNA polymerase processivity factor PCNA. Whereas monoubiquitylation of PCNA mediates error-prone translesion synthesis (TLS), polyubiquitylation triggers an error-free pathway. Both branches of this pathway are believed to occur in S phase in order to ensure replication completion. However, we found that limiting TLS or the error-free pathway to the G2/M phase of the cell-cycle efficiently promote lesion tolerance. Thus, our findings indicate that both branches of the DNA damage tolerance pathway operate effectively after chromosomal replication, outside S phase. We therefore propose that the *RAD6* pathway acts on single-stranded gaps left behind newly restarted replication forks.

INTRODUCTION

DNA lesions that remain unrepaired before entering S phase pose a serious problem during replication. Besides a discontinuity of chromosomal replication, stalled replication forks are dangerous as they can collapse, causing chromosome breaks and genomic instability (Cox et al., 2000; Osborn et al., 2002). To cope with this problem, all organisms possess so-called DNA damage tolerance (DDT) pathways, which ensure cell survival in the presence of DNA polymerase-blocking lesions (Friedberg, 2005). Notably different from conventional DNA repair pathways, DDT does not result in repair of the primary DNA lesion but rather cures their symptoms that manifest during replication. DDT usually becomes activated as a result of a replication block-induced temporal uncoupling of DNA unwinding and synthesis (Chang and Cimprich, 2009). This leads to the formation of single-stranded DNA (ssDNA), a key trigger of DDT (Higgins et al., 1976; Little and Mount, 1982).

In bacteria, DDT appears to promote restart of stalled replication forks, which frequently involves repriming at the damaged template (Courcelle and Hanawalt, 2003). Interestingly, both pro- and eukaryotes utilize two distinct DDT modes: an error-prone mechanism, involving translesion polymerases that can bypass bulky DNA lesions by catalyzing DNA synthesis across the damaged template, and an error-free pathway that engages recombination proteins (Friedberg, 2005). As polymerases involved in translesion synthesis (TLS) can also incorporate an incorrect nucleotide across the damaged site, DDT is largely accountable for mutagenesis (Friedberg, 2005).

Distinctly different from the prokaryotic system, eukaryotic DDT requires the ubiquitin protein modification pathway, which does not exist in bacteria. Indeed, a large number of genes involved in eukaryotic DDT (called the *RAD6* pathway) encode enzymes of this protein modification system (Broomfield et al., 1998; Jentsch et al., 1987; Ulrich and Jentsch, 2000). The crucial substrate of this pathway is PCNA (Hoege et al., 2002), a homotrimeric, DNA-encircling protein, which functions as a DNA polymerase processivity factor and platform for replication-linked factors (Moldovan et al., 2007). Different types of ubiquitin modifications that become induced upon DNA damage dictate whether DDT proceeds via the error-prone or the error-free branch. Error-prone DDT is triggered by conjugation of a single ubiquitin moiety (monoubiquitylation) to PCNA at lysine-164 (K164), which involves the Rad6 ubiquitin-conjugating (E2) enzyme and Rad18, a RING-finger ubiquitin ligase (E3) that binds PCNA (Hoege et al., 2002; Stelter and Ulrich, 2003). Monoubiquitylated PCNA in turn promotes TLS possibly through direct recruitment of TLS polymerases that possess ubiquitin-binding motifs (Bienko et al., 2005; Kannouche et al., 2004; Lehmann et al., 2007; Watanabe et al., 2004). By contrast, error-free DDT requires modification of the same residue of PCNA by a polyubiquitin chain that is linked via K63 of ubiquitin (Hoege et al., 2002). Synthesis of this polyubiquitin chain requires in addition to Rad6 and Rad18 the heterodimeric E2 Ubc13-Mms2, and the RING-finger E3 ubiquitin ligase Rad5, which binds PCNA and Rad18 (Hoege et al., 2002; Ulrich and Jentsch, 2000). Once modified by this polyubiquitin chain, PCNA triggers by an unknown mechanism lesion bypass involving the undamaged template (template switching) and specific repair proteins (Branzei et al., 2008; Giot et al., 1997; Zhang and Lawrence, 2005). Furthermore, K164 of PCNA can alternatively be modified by the ubiquitin-related modifier SUMO (Hoege et al., 2002). In *S. cerevisiae*, this leads to the recruitment of Srs2, an antirecombinogenic helicase, which helps to keep at check an alternative error-free DDT mode that utilizes the Rad51 recombinase (Papouli et al., 2005; Pfander et al., 2005).

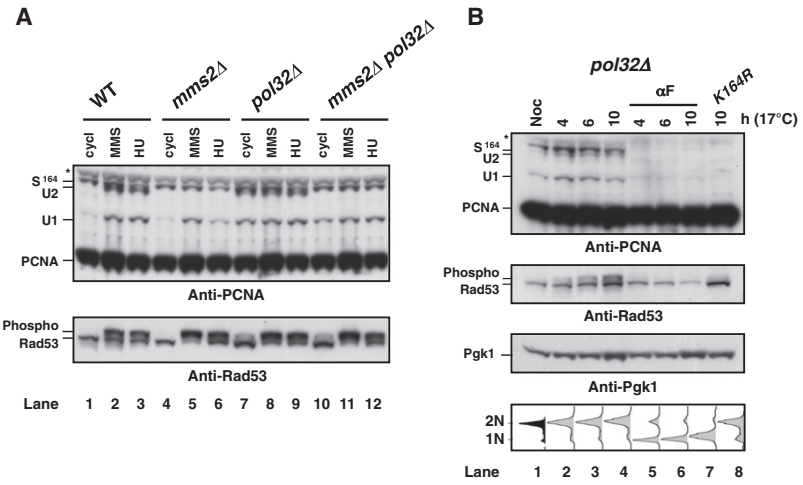

Figure 1. PCNA Ubiquitylation and Checkpoint Activation In *pol32*Δ Mutants

(A) Increased PCNA ubiquitylation and checkpoint activation in *pol32*Δ cells grown at permissive conditions. Cycling cultures (cycl; 30°C) were treated with 0.02% MMS (MMS) or 200 mM hydroxyurea (HU) for 2 hr, and whole-cell extracts were analyzed by western blot against PCNA and Rad53.

(B) Increased checkpoint activation (Rad53 phosphorylation) in *pol32*Δ at restrictive temperatures requires passage through S phase. Cells arrested in G2/M by nocodazole treatment were rapidly released and grown at 17°C in the absence (lanes 2–4) or presence of 10 μM α factor (lanes 5–7). Samples were withdrawn after 4, 6, or 10 hr and analyzed by western blot (Pgk1 used for loading control) and FACS (lower panel).

See also Figure S1.

Although DDT was initially coined "post-replicative DNA repair" (Howard-Flanders, 1968), the prevailing view today is that DDT acts directly at the replication fork in S phase (Andersen et al., 2008; Barbour and Xiao, 2003; Chang and Cimprich, 2009; Lee and Myung, 2008; Prakash et al., 2005; Ulrich, 2009). PCNA ubiquitylation is also believed to be physically coupled to stalled forks (Davies et al., 2008; Ulrich, 2009; Yang and Zou, 2009) and to promote fork progression (Bi et al., 2006; Leach and Michael, 2005). These and several other studies led to the broadly accepted model that TLS promotes "bypass replication" across the lesion at the replication fork, and that the error-free template-switching mode—either by sister chromatid junctions (SCJs) or fork regression leading to a DNA structure called "chicken foot"—acts near the replication fork, and promotes replication restart similar to bacterial DDT.

On the other hand, growing evidence has shown that a fraction of TLS can occur in the rear of the fork (Edmunds et al., 2008; Jansen et al., 2009a; Jansen et al., 2009b; Lopes et al., 2006; Waters and Walker, 2006). However, this issue still remains unsettled, as it was thus far not tested when and in which phase of the cell cycle the *RAD6* DDT pathway has to operate. This question is not only central from a mechanistic point of view, but also of general importance as DDT is crucial for cell survival upon DNA damage, genome stability, and tumor biology.

In this report, we test the present models directly by expressing key components of the error-prone and the error-free pathway specifically in the G2/M phase of the cell cycle. Surprisingly, they fully supported DDT virtually identical to wild-type (WT) cells. We also found that replication of damaged DNA continues and stalled replication restarts even in the absence of DDT. These findings strongly suggest that both branches of DDT in eukaryotes operate post chromosomal replication.

RESULTS

Replication Stress in *pol32*Δ Cells Activates the *RAD6* Pathway

Polymerase δ (Pol δ) plays an essential role in replication by catalyzing lagging-strand synthesis (Nick McElhinny et al., 2008). In budding yeast, it consists of two essential subunits, Pol3 and Pol31 (Hys2), and a small, nonessential subunit, Pol32 (Burgers and Gerik, 1998; Gerik et al., 1998). Although yeast cells lacking Pol32 (*pol32*Δ) proliferate well, they exhibit a delay at the G2/M phase of the cell cycle, which develops into a terminal G2/M arrest at low temperatures (Gerik et al., 1998; Huang et al., 1997; Huang et al., 1999). Notably, this phenotype is accompanied with phosphorylated checkpoint kinase Rad53 (Figure 1A, lane 7, and Figure S1A available online, lower panel) and the presence of nonsegregated chromosomes (Gerik et al., 1998; Huang et al., 2000), indicative of a DNA damage checkpoint-induced anaphase arrest (Sanchez et al., 1999). Importantly, similar to the temperature sensitivity of a subset of Pol δ mutants (Branzei et al., 2002; Giot et al., 1997; Vijeh Motlagh et al., 2006), the cold-sensitivity of *pol32*Δ cells can be efficiently suppressed by mutants in the *RAD6* pathway or by mutants expressing modification-deficient PCNA (*pol30*^{K164R}) (Figure S1B). We therefore conclude that this phenotype of *pol32*Δ cells largely depends on PCNA ubiquitylation.

PCNA ubiquitylation is barely detectable in unchallenged WT cells (Hoege et al., 2002) (Figure 1A and Figure S1A). However, we noticed that PCNA mono- and polyubiquitylation was strongly induced in *pol32*Δ mutants in S phase (Figures S1C and S1D). Interestingly, the level of ubiquitylated PCNA in *pol32*Δ cells was as high as in WT cells that have been exposed to methyl methanesulfonate (MMS) or hydroxyurea (HU) (Figure 1A). One possible explanation for this phenotype is that Pol32 may function as an enzymatic inhibitor of PCNA ubiquitylation. However, cells expressing a functionally compromised catalytic subunit of Pol δ (Pol3; *cdc2-2*) induced PCNA mono- and polyubiquitylation as well (Figure S1E). This indicates that faulty replication, rather than the absence of a specific polymerase subunit, is the cause that led to the induction of PCNA ubiquitylation in *pol32*Δ cells. Importantly, we also observed that *pol32*Δ cells at its restrictive temperature would not fully activate a DNA damage checkpoint response unless they were allowed to pass through S phase (Figure 1B). Thus, the primary defect of *pol32*Δ mutants originates during S phase, strongly suggesting that they experience replication stress. In fact, because the induced ubiquitylation of PCNA in *pol32*Δ cells could not be further enhanced by DNA-damaging agents