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RESEARCH ARTICLE

DNA polymerase α interacts with PrSet7 and mediates H4K20 monomethylation in *Drosophila*

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ABSTRACT

In human cells, appropriate monomethylation of histone H4 lysine 20 by PrSet7 (also known as SET8 and SETD7) is important for the correct transcription of specific genes and timely progression through the cell cycle. Over-methylation appears to be prevented through the interaction of PrSet7 with proliferating cell nuclear antigen (PCNA), which targets PrSet7 for destruction through the pathway mediated by $CRL4^{Cdt2}$ (the cullin ring finger ligase-4 complex containing Cdt2). However, the factors involved in positive regulation of PrSet7 histone methylation remain undefined. Here, we present biochemical and genetic evidence for a previously undocumented interaction between *Drosophila* PrSet7 (dPrSet7) and DNA polymerase α in *Drosophila*. Depletion of the polymerase reduces H4K20 monomethylation suggesting that it is required for dPrSet7 histone methylation activity. We also show that the interaction between PCNA and PrSet7 is conserved in *Drosophila*, but is only detectable in chromatin fractions. Consistent with this, S2 cells show a significant loss of chromatin-bound dPrSet7 protein as S phase progresses. Based on these data we suggest that interaction with the DNA polymerase represents an important route for stimulation of PrSet7 histone methylase activity that is mediated by allowing loading of dPrSet7 onto chromatin or its subsequent activation.

KEY WORDS: PrSet7, DNA polymerase, *Drosophila*, PCNA, Histone methylation

INTRODUCTION

The methylation of histones in chromatin has been shown to have profound effects on the ability of the associated DNA to participate in its metabolic activity. To date, more than 20 methylation sites on arginine and lysine residues have been identified, and these have been suggested to have various effects on DNA transcription, replication and repair. The effect of the modification seems to depend on the site modified, the extent of the modification (mono-, di- and tri-methylation often have different effects), and the context of surrounding modifications.

Histone H4K20 monomethylation is carried out by the PrSet7 (also known as SET8 and SETD7 in mammals) methyltransferase. This was first identified in human cells by fractionation of extracts (Nishioka et al., 2002; Fang et al., 2002; Rice et al., 2002) and subsequent studies identified orthologues in all other eukaryotes with the exception of *S. cerevisiae*. Analysis of the cellular function of PrSet7 protein suggests a complex picture, with some possibility that it might show species specific differences.

Several studies have suggested that there is a transcriptional role for human PrSet7 (hPrSet7). Immunofluorescence staining experiments in interphase cells show that the staining for monomethylated H4K20 almost completely overlaps the staining for RNA polymerase II (RNApolII) suggesting an involvement in active chromatin (Talasz et al., 2005), and it has been suggested to serve as a co-activator for Wnt target gene expression through a direct interaction with TCF4 and LEF1 (Li et al., 2011). Conversely, many genes are repressed by overexpressing hPrSet7, with particularly notable effects on E2F1-controlled genes and histone-encoding genes (Abbas et al., 2010). Other specific examples of repressed genes are *RUNX1*, where hPrSet7 seems to act by stimulating increased binding of the L3MBTL1 repressor (Sims and Rice, 2008; Kalakonda et al., 2008), E- and N-cadherin (Yang et al., 2012) where it serves as a corepressor with TWIST and a subset of p53-controlled genes, where it is thought to act via methylation of p53 (Shi et al., 2007; Driskell et al., 2012). Finally, in some cases no correlation with transcriptional activity is observed (Houston et al., 2008; Fang et al., 2002).

A role for PrSet7 in DNA repair was proposed based on the observation that the closest *S. pombe* orthologue (SET9) has no effect on transcription but functions in DNA repair by recruitment of tudor-domain-containing proteins (Sanders et al., 2004). However, SET9 can catalyse mono-, di- and tri-methylation reactions, and structural studies have shown that the dimethylated form of H4K20 is the active form for recruitment of tudor domain proteins (Botuyan et al., 2006). In addition, if chromatin is confined to a monomethylated state by deletion of SUV4-20 (also known as Hmt4-20) (Schotta et al., 2008) it seems to become prone to double-strand breaks (DSBs) and chromosomal aberrations.

More recently several studies have shown a role for hPrSet7 in DNA replication. Depletion of hPrSet7 causes cells to accumulate in S phase, with accompanying decreases in the rate of fork movement and increased DNA damage (Tardat et al., 2007; Jørgensen et al., 2007). Inappropriate expression of PrSet7 also causes S phase defects and increased DNA damage. These are accompanied by changes in chromatin compaction, suggesting that aberrant chromatin structure provides one mechanism for the observed effects. PrSet7 also appears to show specific effects at origins of replication, related to the binding of pre-replication complex (preRC) components (Tardat et al., 2010), that might also require the activity of the SUV4-20 H1 and H2 methylases, and di- and tri-methylation of H4K20 (Beck et al., 2012). Human PrSet7 directly interacts with the DNA replication factor PCNA

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through its PIP box motif (Huen et al., 2008), and this interaction is needed for controlled degradation of PrSet7 through the pathway mediated by $CRL4^{Cdt2}$ (the cullin ring finger ligase-4 complex containing Cdt2) (Abbas et al., 2010; Centore et al., 2010; Oda et al., 2010; Jørgensen et al., 2011; Tardat et al., 2010). PCNA-independent degradation of PrSet7 also occurs through the anaphase-promoting complex (APC)–Cdh1-mediated pathway (Wu et al., 2010).

Drosophila PrSet7 (dPrSet7) is much larger than its human orthologue owing to a 30-kDa extension at the N-terminus of the protein. On polytene chromosomes dPrSet7 is mainly associated with facultative and constitutive heterochromatin. It can also suppress position-effect variegation (PEV) (Karachentsev et al., 2005), and therefore the transcriptional role of dPrSet7 has been suggested to be mainly repressive. This is also consistent with the association of methylated H4K20 with regions of low gene expression (Karachentsev et al., 2005; Nishioka et al., 2002; Fang et al., 2002). Imaginal disc cells in mutant larvae appear to replicate their DNA but not to undergo mitosis (Karachentsev et al., 2005), and mutant larval neuroblasts show a mei-41 (ATM)-dependent block at M/G1 with low levels of cyclin B (Sakaguchi and Steward, 2007) and increased DNA damage. Depletion of dPrSet7 in S2 cells has also been seen to cause cells to accumulate in S and early M phases (Sakaguchi et al., 2012).

DNA polymerase α is a heterotetrameric enzyme. It was the first replicative polymerase isolated from eukaryotic cells, and extensive studies have been carried out with this enzyme both structurally and functionally (Muzi-Falconi et al., 2003). Its main function, based on its high abundance and the fact that it possesses both polymerase and primase activities, is thought to be in the initiation of DNA replication at origins of replication and of production of Okazaki fragments on the lagging strand. In addition to this, it has been proposed to function in control of telomere elongation (Chen et al., 2013) and in the epigenetic control of transcriptional silencing in fission yeast through Swi6 (Nakayama et al., 2001). In *Arabidopsis*, genetic analysis suggests that there is a role for the polymerase in ensuring correct maintenance of histone methylation (Hyun et al., 2013), but the mechanisms by which this is done are unclear.

Here, we characterise a previously undocumented interaction between dPrSet7 and DNA polymerase α in S2 cells and flies. We present data related to a possible role for this interaction in DNA replication and dPrSet7 activity. We further show that the interaction between PrSet7 and PCNA, previously reported in mammals, is conserved in *Drosophila*, and characterise this interaction with respect to the interaction in mammals and the polymerase interaction. Based on these results, we suggest a possible role for the DNA polymerase α in the loading or activation of PrSet7 on chromatin.

RESULTS

Drosophila PrSet7 interacts with DNA polymerase α

We have previously reported results from a two-hybrid screen using the large subunit of the DNA polymerase α as bait (Máthé et al., 2000; Donaghue et al., 2001; Crevel et al., 2001). Among the proteins identified was a 300-amino-acid region at the C-terminus of the dPrSet7 protein. This suggested that dPrSet7 interacts with the large subunit of DNA polymerase α.

To confirm this interaction, we carried out immunoprecipitation experiments in soluble extracts from *Drosophila* embryos (Fig. 1A). We also constructed S2 cell lines expressing full-length dPrSet7 containing a V5 tag, and showed that the

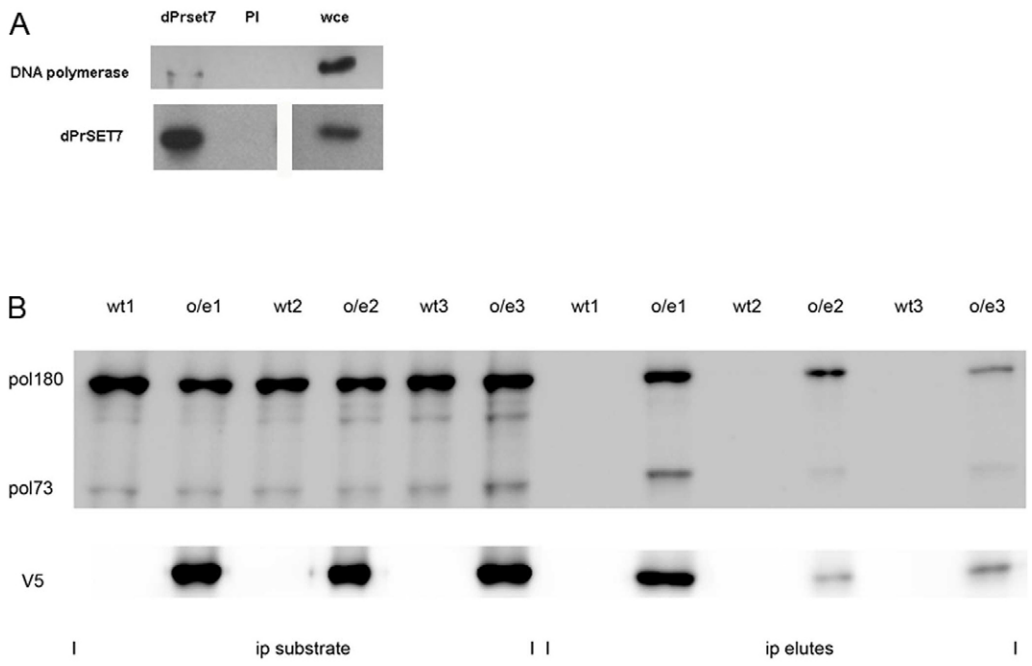

Fig. 1. dPrSet7 shows a physical interaction with DNA polymerase α. (A) dPrSet7 co-immunoprecipitates with DNA polymerase α in soluble extracts of *Drosophila* embryos. Proteins were precipitated using anti-dPrSet7 antibodies (dPrSet7) or pre-immune serum (PI), and proteins eluted from the precipitates with 2% SDS were subjected to western blotting with anti-polymerase and anti-dPrSet7 antibodies. The expected positions of dPrSet7 and polymerase are shown by comparison to whole-cell crude extracts (wce). A small region has been removed from this gel to allow correct alignment of the lanes. (B) V5-tagged dPrSet7 co-immunoprecipitates with DNA polymerase α in chromatin extracts from S2 cells. Proteins were precipitated using anti-V5 antibodies and the blots probed with anti-polymerase or anti-V5 antibody. Shown are results from three independent experiments using cells expressing V5-dPrSet7 (o/e1, 2,3) and wild-type (wt) cells (wt1,2,3). Lanes 1–6 show crude chromatin extracts (ip substrate) and 7–12 are elutions from the immunoprecipitates with 2% SDS (ip elutes).

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polymerase was detected in V5 immunoprecipitates from these cell lines (Fig. 1B). The interaction with the polymerase can be seen in both chromatin fractions (Fig. 1B) and soluble extracts for both endogenous (Fig. 1A) and V5-tagged (data not shown) protein.

To determine whether a synthetic interaction between the two proteins could be detected in whole flies, we generated *Drosophila* strains where double-stranded RNA (dsRNA) against dPrSet7 was under the control of the GAL4-UAS promoter. Crossing these strains with flies expressing GAL4 under the control of the Actin5C (Act5C) promoter, to express dsRNA in all cells, resulted in significant reduction of the levels of dPrSet7 mRNA in third-instar larvae (supplementary material Fig. S1A) and pupal lethality (data not shown), precluding further analysis. We therefore chose to look specifically at the effects of dPrSet7 reduction in wing discs, by crossing flies expressing dPrSet7 dsRNA with flies carrying the *MS1096-GAL4* driver, to drive GAL4 expression in the wing. Fig. 2A shows that there was a substantial decrease in the level of dPrSet7 protein in wing discs on expression of dPrSet7 dsRNA. Despite this, wing discs from dPrSet7 dsRNA flies showed no discernable phenotype or change in the density of cells present (supplementary material Fig. S1B,C). This suggests that the levels of dPrSet7 can be substantially reduced in the wing without affecting cell viability.

We also obtained a fly line in which the levels of the polymerase could be reduced by the expression of dsRNA. This dsRNA, when controlled by an Act5C promoter, significantly reduced the amount of polymerase mRNA in larvae (supplementary material Fig. S1D). When expressed specifically in the wing disc we observed a strong reduction in the amount of polymerase protein compared to wild-type flies (Fig. 2B). This line was then crossed with flies that had reduced dPrSet7, to generate flies that under-expressed both proteins in the wing discs. Fig. 2C shows that reduction of polymerase alone in the wing disc had quite a strong effect on wing development. Although dPrSet7 alone had no apparent effect on wing development, reduced levels of both proteins significantly enhanced the atrophied wing phenotype caused by the polymerase knockdown, suggestive of a genetic interaction (100 wings were examined in each case, and the penetrance of the phenotype within each group was 100%). These data suggest that dPrSet7 interacts with the DNA polymerase α in both S2 cells and intact flies.

Depletion of dPrSet7 affects S phase progression in S2 cells and flies

Given that DNA polymerase α is a central replication protein, the interaction between the polymerase and dPrSet7 could play a direct role in DNA replication. To determine whether this was the case, we looked to see if there was a synergistic effect on replication when the levels of dPrSet7 and polymerase were simultaneously reduced.

We first looked at how reduction of dPrSet7 levels alone affected DNA replication in S2 cells using dsRNA interference. Two different dsRNAs from different parts of the protein were used to confirm that the effects were specific to dPrSet7 depletion. For both dsRNAs, by day 3, no dPrSet7 protein was visible in total extracts from treated S2 cells (Fig. 3A). The proliferation of cells with reduced dPrSet7 was decreased by ~40% compared to mock-depleted cells (Fig. 3B). Between day 3, when PrSet7 was no longer visible, and day 7, depleted cells went through 2.6 doublings compared to 4.2 doublings for control cells. Flow cytometric analysis suggested that 10% more cells accumulated at the S and G2 phases of the cell cycle (Fig. 3C,D).

If a second round of dsRNA was carried out and the cells were allowed to grow for a second week in the absence of dPrSet7, no cell proliferation could be detected (Fig. 3B). Again flow cytometry suggested that the blockage occurred at late S/G2.

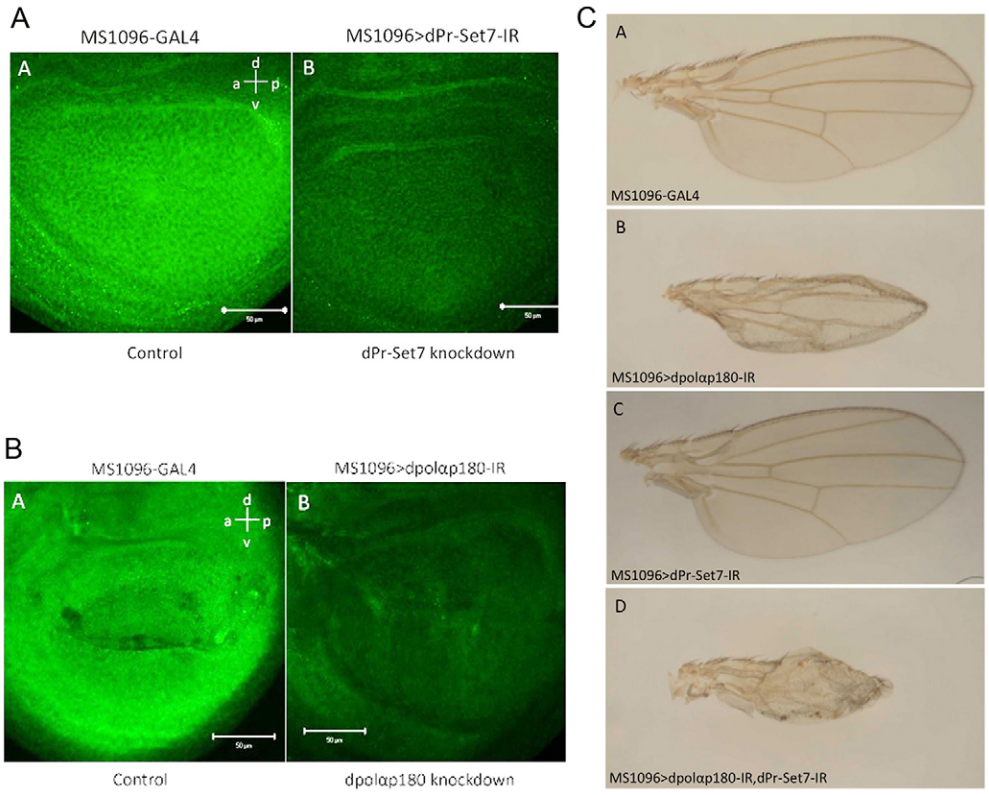

Fig. 2. dPrSet7 shows a genetic interaction with DNA polymerase α. (A) Expression of dsRNA for dPrSet7 reduces the dPrSet7 protein level in wing imaginal discs. The dPrSet7 protein levels were measured by confocal microscopy for dPrSet7 knockdown flies and control flies. Disc orientation is: a, anterior; p, posterior; d, dorsal; v, ventral. (B) Expression of DNA polymerase α dsRNA reduces the polymerase protein level in wing imaginal discs. Levels of polymerase protein were measured by confocal microscopy in polymerase knockdown and control fly wings. Disc orientation as in A. (C) *dPrSet7* genetically interacts with *dpolαp180*. Light microscopy images of representative adult wings: (C, panel A) *control*, *MS1096-GAL4*; (C, panel B) polymerase knockdown, *MS1096-GAL4>dpolap180-IR*; (C, panel C) dPrSet knockdown, *MS1096-GAL4>dPr-Set7-IR*; (C, panel D) double knockdown. Flies were developed at 28°C. Scale bar: 50 μm.

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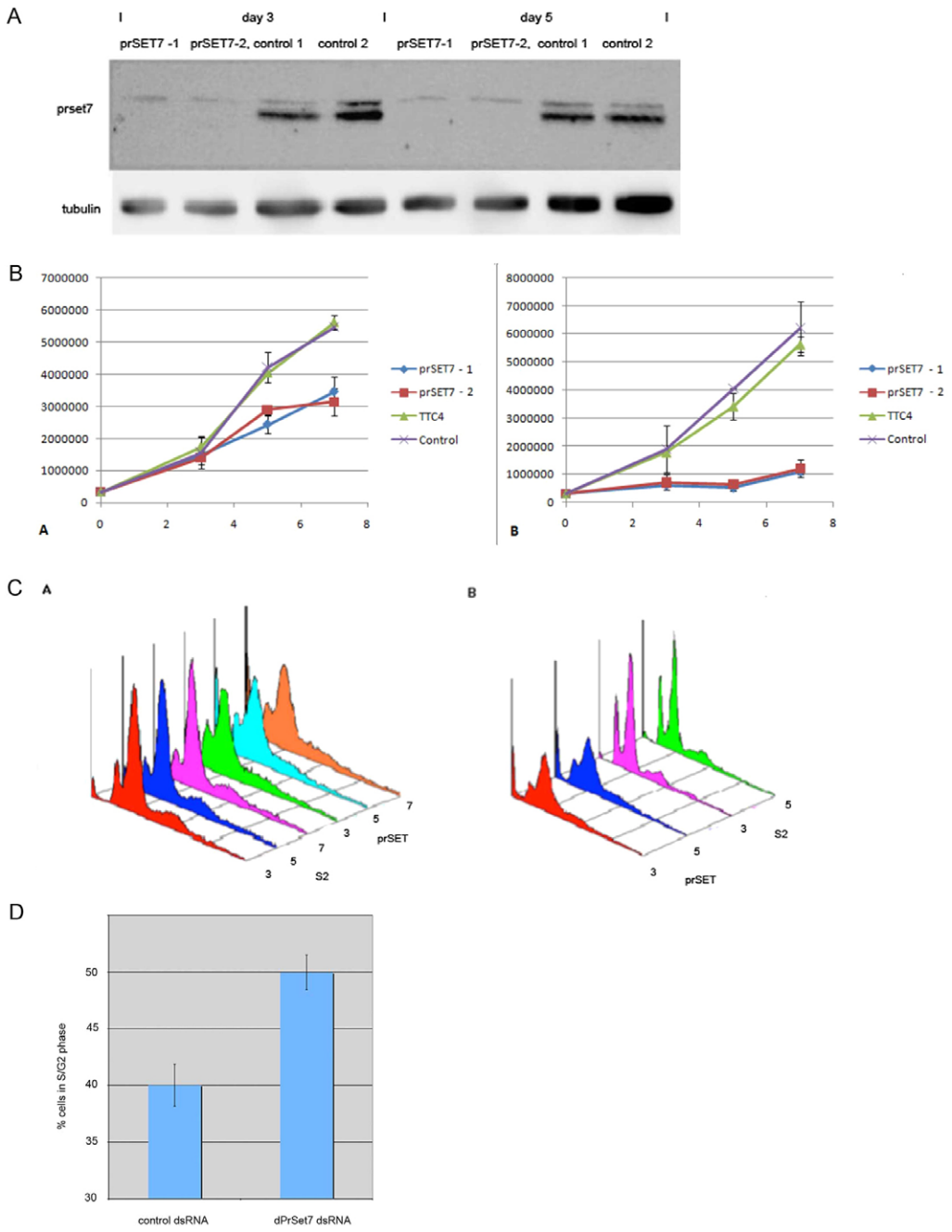

Fig. 3. Depletion of dPrSet7 from S2 cells by dsRNA interference causes decreased proliferation and slow progression of the cells through G2. (A) Depletion of dPrSet7 protein by two non-overlapping dsRNAs. Samples from day 3 and 5 are shown. For each, the order of loading is dPrSet7-1 dPrSet7-2, control 1, control 2. Tubulin is shown as a loading control. (B) Cell proliferation is decreased by treatment with dPrSet7 dsRNA. Proliferation profiles of cells treated with dPrSet7 dsRNA (dPrSet7-1 or dPrSet7-2) and control cells (TTC4 and control) were determined by cell counting at 3, 5 and 7 days after dsRNA addition (left panel). They were also counted 3, 5 and 7 days after a second dsRNA treatment on day 7 (right panel). (C) Treatment with dPrSet7 dsRNA alters the cell cycle of S2 cells: C, panel A shows slow cytometric analysis of control cells (S2) and cells treated with dPrSet7-1 dsRNA on days 3, 5 and 7 of week 1. C, panel B shows slow cytometric analysis of control cells (S2) and cells treated with dPrSet7-1 dsRNA on days 3 and 5 of week 2. For each profile, the first peak represents G1 cells and the second G2. (D) Treatment with dPrSet7 dsRNA increases the percentage of S2 cells in late S/G2. The percentage of cells in G1 or S/G2 (where G1+S/G2=100) were calculated for three independent flow cytometric analyses as described in C. Shown are the combined data for the S/G2 cells (the G1 data are not included but is the exact reverse of this).

This suggests that, even though loss of dPrSet7 is not immediately lethal, the protein is required for continued long-term proliferation of cells.

The cells appear to be stalled in late S/G2, as levels of phosphorylated H3S10 (a marker for mitotic cells) were not increased over those seen in mock-depleted cells (data not shown). Stalling of cells at late S/G2 is consistent with dPrSet7-depleted S2 cells having a defect in the completion of DNA replication. Depleted cells also showed an increase in staining for H2AvD – the *Drosophila* homologue of H2Ax (supplementary material Fig. S2). This suggests that there is an increase in DNA damage, which is also consistent with a replication role for

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dPrSet7, and could contribute to the late S/G2 block observed in these cells.

We then looked at the effects of knockdown of DNA polymerase α in S2 cells. Cells with reduced levels of polymerase ceased proliferation and showed drastic changes in morphology very rapidly under all conditions tested (data not shown). This precluded the possibility of using S2 cells to test the role of the interaction in DNA replication.

We therefore looked at the effect of single and co-depletion of dPrSet7 and DNA polymerase α on BrdU incorporation in wing discs. Individual depletion of DNA polymerase α and dPrSet7 both caused a decrease in BrdU incorporation, and the double depletion showed a much larger reduction (Fig. 4A,B), although this was additive rather than synergistic, precluding definitive conclusions about a specific role for the dPrSet7 and polymerase interaction in DNA replication.

Reduction of DNA polymerase-α levels causes reduced H4K20 monomethylation in flies

A decrease in dPrSet7 could influence S phase kinetics and DNA replication by causing a decrease in the amount of the polymerase protein. However, no change in polymerase levels could be detected in a dPrSet7 knockdown fly (supplementary material Fig. S3A).

Studies in human cells have suggested that PrSet7, and methylation of H4K20, is itself important for DNA replication. We therefore looked to see whether loss of the polymerase had any effect on dPrSet7 activity. It was not practical to study this in S2 cells, as depletion of the polymerase alone had such a deleterious effect. In addition, although treatment of S2 cells with dsRNA targeting dPrSet7 caused reduction of dPrSet7 protein to below the level of detection by day 3, levels of monomethylated H4K20 did not show a comparable reduction (Fig. 5A). Between

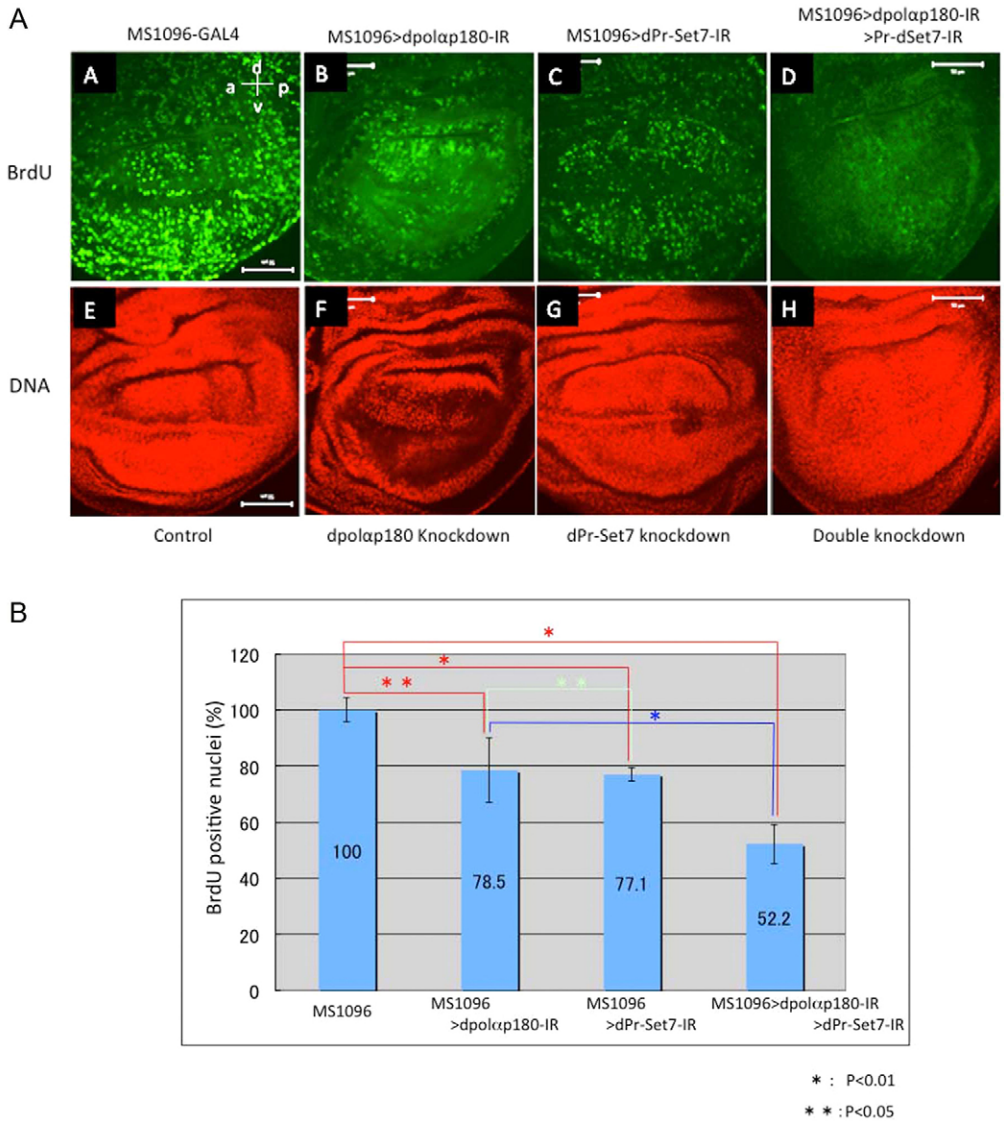

Fig. 4. Depletion of dPrSet7 reduces DNA replication in larval wing discs. (A) Confocal microscopy images of representative third-instar larval wing discs showing BrdU incorporation assays for control (A, panels A,E), polymerase knockdown (A, panels B,F), PrSet7 knockdown (A, panels C,G) and double knockdown (A, panels D,H) flies. Green, anti-BrdU; Red, DNA stained with propidium iodide. Scale bars: 50 μm. (B) Quantification of BrdU-positive nuclei in wing imaginal discs in control (*MS1096-GAL4*), polymerase knockdown (*MS1096-GAL4 >dpolαp180-IR*), dPr-Set7 knockdown (*MS1096 >dPr-Set7-IR*) and double knockdown (*MS1096-GAL4 >dpolαp180-IR >dPr-Set7-IR*) flies. All values are relative to that for control flies. P values for comparisons are as shown (Students *t*-test).

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days 3 and 5, dPrSet7-depleted cells increased in number 2.6 fold in the absence of significant amounts of dPrSet7 protein. Dilution of existing methylated H4K20 residues, even in the absence of turnover, should result in the loss of 60% of the methylation. However, the observed decrease relative to wild type was only ~6%.

By contrast reduction of dPrSet7 in wing imaginal discs caused significant reduction in the levels of monomethylated H4K20 (Fig. 5B; supplementary material Fig. S3B). Levels of monomethylated H4K20 in the wing discs of DNA polymerase α knockdown flies, also showed a marked reduction (Fig. 5C; supplementary material Fig. S3C), comparable to knockdown of dPrSet7 itself. This suggests that the interaction between the polymerase and dPrSet7 is necessary for the dPrSet7 activity to have an effect on H4K20.

DNA polymerase α depletion cannot be rescued by overexpression of dPrSet7

Depletion of DNA polymerase alone in wing discs causes a strong phenotype in wing morphology. Given that we also saw a concomitant decrease in dPrSet7 methylation activity, we looked to see whether overexpression of dPrSet7 in polymerase-depleted wing discs could rescue the wing phenotype. We generated *Drosophila* lines in which the levels of DNA polymerase α were decreased by dsRNA, and dPrSet7 was overexpressed. As shown in Fig. 5D this does not rescue the wing phenotype caused by a decrease in DNA polymerase α. In fact, the resultant wings appeared slightly more defective, suggesting that in the absence of the correct levels of polymerase, excess dPrSet7 negatively affects cellular activity and development.

Drosophila PrSet7 interacts with PCNA in S2 cells and flies

In human cells, PrSet7 has been seen to interact with PCNA, leading to the suggestion that its involvement in DNA replication is mediated through PCNA. Given that DNA polymerase α is present at replication forks, it was possible that dPrSet7 localisation was mediated by an interaction with DNA polymerase α rather than PCNA in *Drosophila*.

To determine whether the interaction between PrSet7 and PCNA was conserved in *Drosophila*, we examined V5 immunoprecipitates from S2 cells expressing V5-tagged dPrSet7 for the presence of PCNA. PCNA was detected in immunoprecipitates from chromatin extracts (Fig. 6A), although not in soluble extracts (data not shown).

We also looked for a genetic interaction between the two proteins in *Drosophila* wing discs. PCNA knockdowns alone gave a severe wing phenotype and no further enhancements were observed for the double knockdown. The experiment was

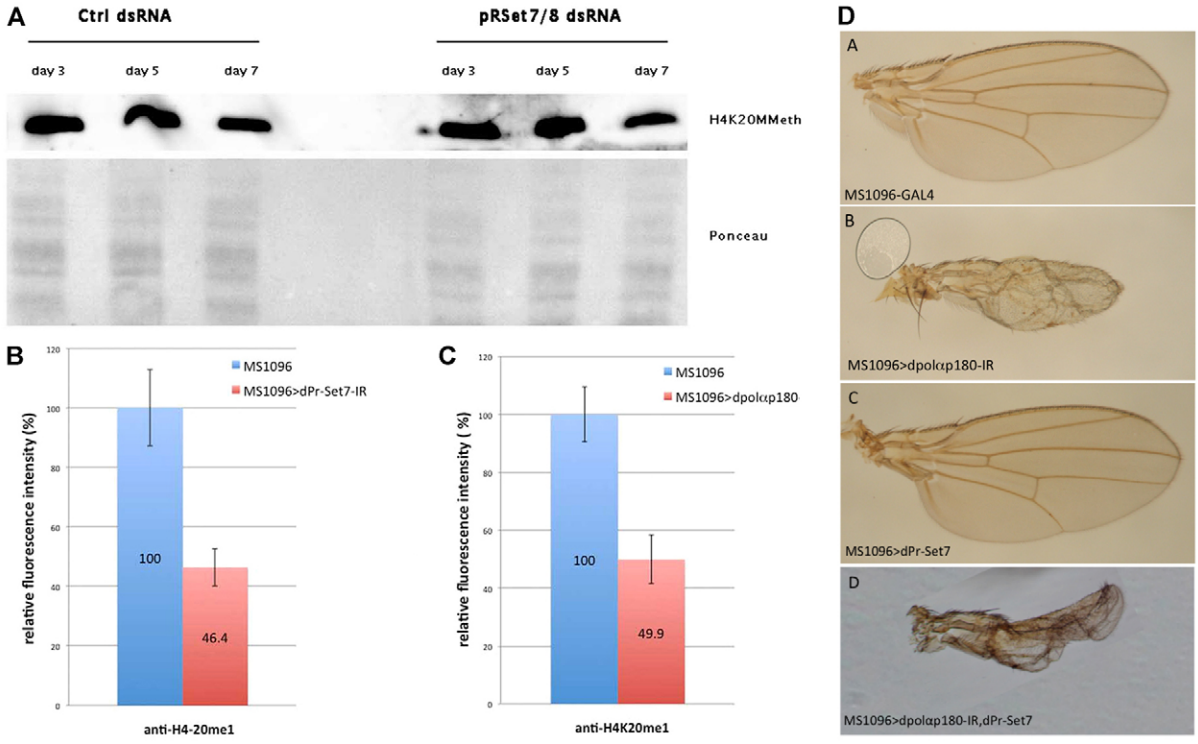

Fig. 5. Depletion of DNA polymerase α causes a significant decrease in H4K20 monomethylation in wing discs which cannot be rescued by overexpression of dPrSet7. (A) S2 cells expressing dPrSet7 dsRNA still show significant H4K20 monomethylation. Samples treated with dPrSet7 dsRNA (dPrset7/8) and control samples (Ctrl) were analysed for H4K20 monomethylation at 3, 5 and 7 days after dsRNA addition. The monomethylated H4K20 band is marked H4K20MMeth. Total protein, visualised by Ponceau staining prior to antibody development, is shown as a loading control. (B) Expression of dPrSet7 dsRNA reduces H4K20 monomethylation in wing imaginal discs. Quantification of the monomethylated H4-K20 signal in wing imaginal discs. The H4-K20 monomethyl signal intensities in dPrSet-7 knockdown flies (*MS1096-GAL4 >dPr-Set7-IR*) is shown relative to control flies (*MS1096-GAL4*). $P<0.01$ (Welch's *t*-test). (C) Expression of polymerase α dsRNA reduces H4K20 monomethylation in wing imaginal discs. Quantification of the monomethylated histone H4K20 fluorescent signal in wing imaginal discs. The H4K20 monomethyl fluorescent signal intensities in polymerase knockdown flies (MS1096 >dpolαp180-IR flies) are shown relative to control flies (MS-GAL4 flies). $P<0.01$ (Student's *t*-test). (D) Overexpression of *dPr-Set7* enhances the atrophied wing phenotype induced by *dpolαp180*-knockdown. Light microscope images of representative adult wings. (D, panel A) control (*MS1096-GAL4*); (D, panel B) polymerase knockdown (*MS1096 >dpolαp180-IR*), (D, panel C) dPrSet7 overexpression (*MS1096-GAL4 dPr-Set7*), (D, panel D) B plus C (*MS1096-GAL4 >dpolαp180-IR >dPr-Set7*). Flies were developed at 28°C.

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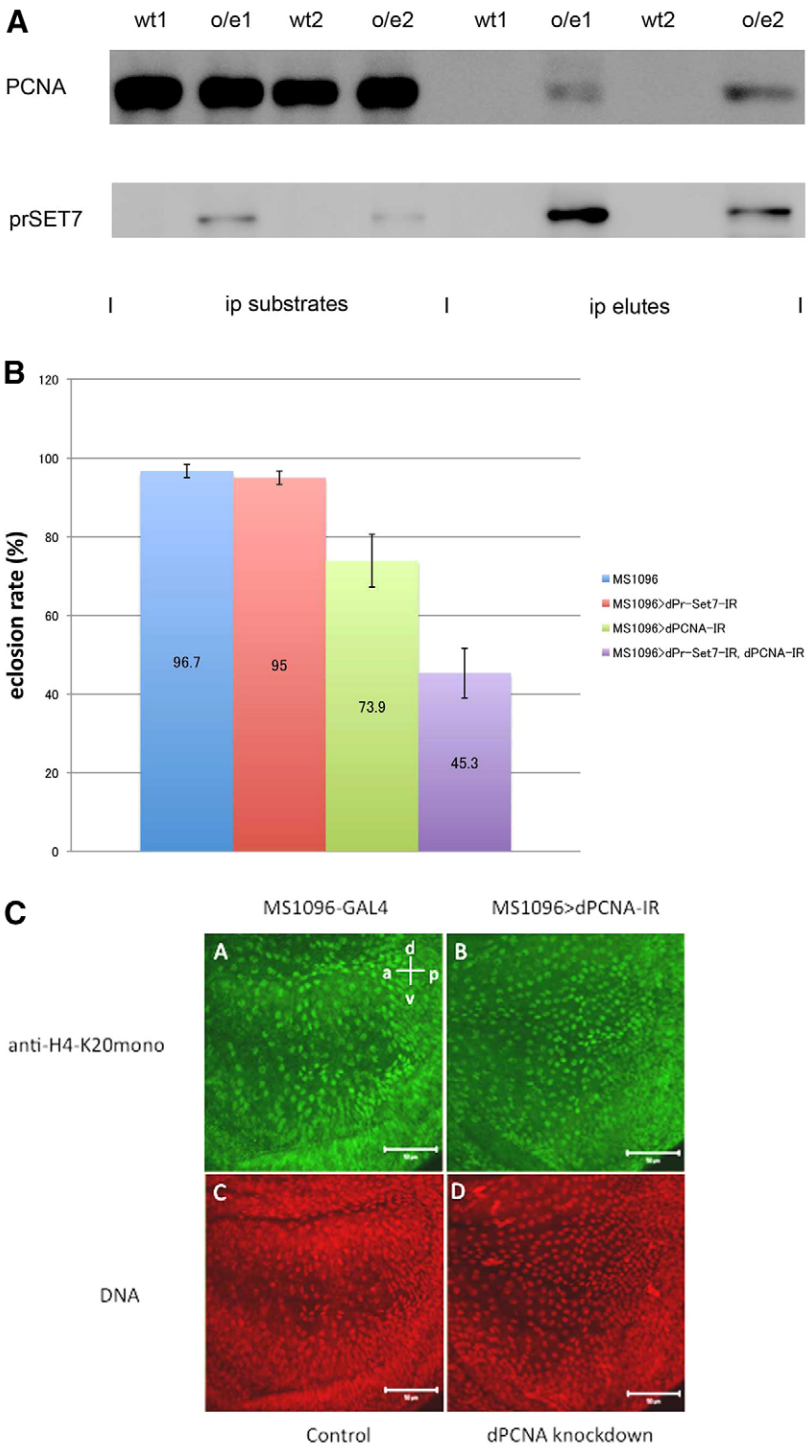

Fig. 6. dPrSet7 interacts with PCNA in S2 cells and flies. (A) PCNA co-immunoprecipitates with dPrSet7 in chromatin extracts from S2 cells. Proteins were precipitated with antiV5 antibodies and the blots probed with anti-PCNA or anti-V5 antibody. Shown are two independent experiments using cells expressing V5-dPrset7 (o/e 1/2) and S2 cells (wt 1/2). Lanes 1–4 show crude chromatin extracts and 5–8 elutions from immuno-precipitates with 2% SDS. (B) A genetic interaction between *dPr-Set7* and *PCNA* is suggested from eclosion rate analysis. Third-instar larvae ($n=60$) from a control line (*MS1096*), and lines with reduced dPrSet7 (*MS1096>dPr-Set7-IR*), reduced PCNA (*MS1096 >dPCNA-IR*), and the double knockdown (*MS1096 >dPr-Set7-IR, dPCNA-IR*) were collected and developed at 28°C. Mean results from three independent experiments are shown. (C) H4K20 monomethylation is not reduced by PCNA-knockdown in wing discs. Shown are representative examples of confocal images of wing discs stained with anti-H4-K20 monomethyl antibody for control (C, panels A,C) and PCNA knockdown (C, panels B,D) flies. Disc orientation is as Fig. 2A. Green, anti-H4-K20 monomethyl; red, DNA stained with propidium iodide. Scale bars: 50 μm.

performed at two different temperatures (28°C and 25°C) to determine whether the effect was more visible at lower temperatures, but no differences were observed. The MS1096-GAL4 driver used in these experiments is designed to give wing-specific expression. However, we observed that in addition to wing defects, the viability of PCNA knockdown flies was substantially decreased, suggesting leaky expression. The decreased severity of the viability phenotype for PCNA knockdown with this driver gave us the opportunity to compare the effects of single and double knockdowns of PCNA and dPrSet7 in flies. For this experiment, the flies were cultured on rich medium to enhance the possibility of detecting an interaction. Although reduction of dPrSet7 had no effect on viability, a double knockdown of PCNA and dPrSet7 had a significantly lower eclosion rate than a PCNA knockdown alone (Fig. 6B), consistent with a genetic interaction between the two proteins.

We also looked to see whether reduced methylation activity was observed in PCNA knockdowns. However decreased levels of PCNA did not significantly alter H4K20 monomethylation in the wing discs (Fig. 6C; supplementary material Fig. S4).

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Chromatin association of *Drosophila* PrSet7 decreases as S phase progresses

In human cells, interaction with PCNA is necessary for correct proteolysis of PrSet7 through the $Cul4^{Cdt2}$ pathway. This leads to a significant decrease in the total amount of PrSet7 as S phase progresses. To determine whether dPrSet7 showed the same pattern of expression, *Drosophila* cells were synchronised at the start of S phase (Crevel and Cotterill, 2012) (Fig. 7A), and the total level of dPrSet7 noted at various times after release. Fig. 7B shows that there was no significant decrease in the level of dPrSet7 as the cells moved through S phase. Given that the interaction with PCNA was only observed on chromatin, it was possible that chromatin-associated dPrset7 showed variation, but that this was masked by the behaviour of other pools of dPrset7. We therefore carried out a similar analysis, but looked only at dPrSet7 in chromatin fractions at each time point. As can be seen in Fig. 7B, chromatin-bound dPrset7 showed a marked decrease during the first 30 minutes of S phase, and then remained at a low and constant level for the rest of the cycle. In addition the levels of H4K20 monomethylation increased as the cells traversed S phase, consistent with observations in mammalian cells.

Drosophila PrSet7 interacts with PCNA and the DNA polymerase α through distinct regions

To determine whether PCNA and DNA polymerase bound to dPrSet7 at the same site, yeast two-hybrid analysis was used to define the region of interaction for each protein.

A small region of the DNA polymerase in the N-terminus (amino acids 1–100) was found to be sufficient to allow an interaction with dPrSet7 (Fig. 8A,B). This region does not have any specific assigned function but is well conserved between the DNA polymerases from different species.

Deletions of PCNA suggested that the interaction region was contained in amino acids 201–260 (Fig. 8C), a region that is involved in binding of PCNA to RFC, FEN1, and CIP1, and also contains the DNA-binding site.

The region of dPrSet7 required to interact with both proteins was contained in amino acids 344–544. This excludes the SET domain, but as has been noted by others (Centore et al., 2010), also contains a putative PIP degron (NREMTDFFPVRR). Constructs of dPrSet7 without the degron region (aa 344–530) no longer interacted with PCNA, suggesting that the putative PIP box did indeed provide the interaction site with PCNA (Fig. 8C). However the PIP box deletion constructs were still able to interact with the polymerase (Fig. 8B). This suggests that DNA polymerase interacts with dPrSet7 at a distinct site from PCNA.

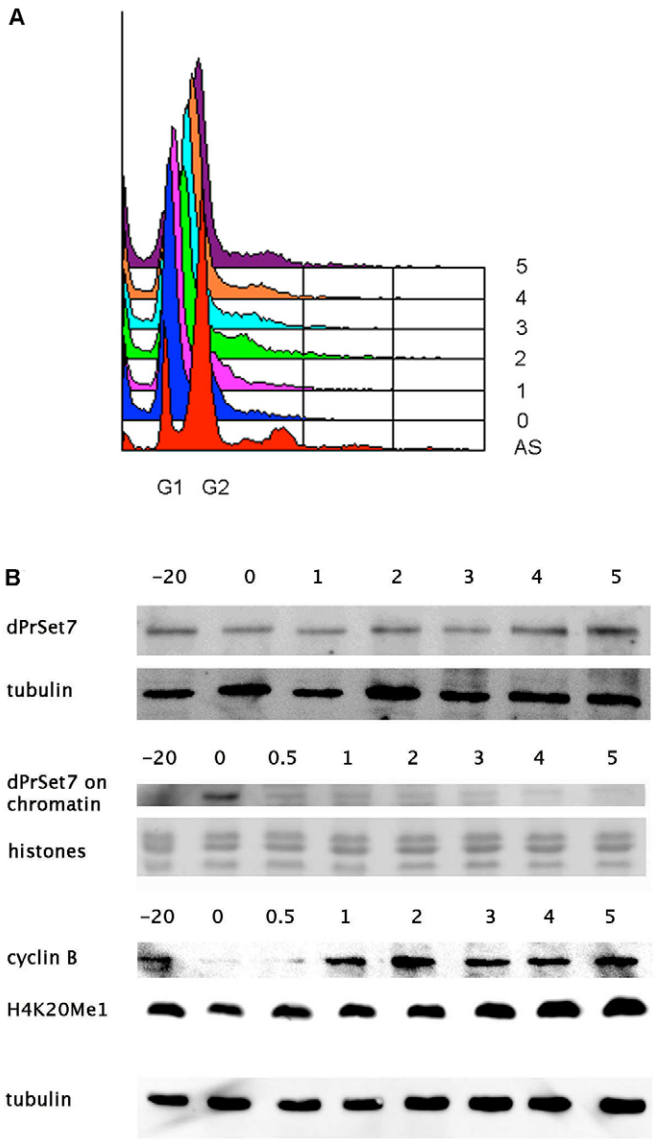

Fig. 7. Levels of dPrSet7 bound to chromatin decrease as S phase progresses. (A) Flow cytometry of S2 cells showing synchronisation. AS, asynchronous cells; 0, cells after 20 hours in HU (mostly G1); and 1, 2, 3, 4 and 5, 1–5 hours after HU removal, respectively. (B) Chromatin-bound, but not total dPrSet7 protein levels, decrease as S phase progresses in S2 cells. Cells were synchronised, and samples were taken before the block (−20, mostly G2 cells), and then at 0, 1, 2, 3, 4 and 5 hours after release from the HU block. These were either monitored for dPrSet7 levels directly using antibodies against the endogenous protein (dPrSet7) or used to prepare chromatin samples which were also monitored for dPrSet7 (dPrSet7 on chromatin). Total cell extracts were also analysed for the presence of monomethylated H4K20 (H4K20Me1), and cyclin B (cycB) as a marker of cell cycle progression. Tubulin is shown as a loading control for whole cells extracts, and histones, visualised by Ponceau staining prior to antibody development, are shown as a chromatin-loading control.

DISCUSSION

The role of dPrSet7 and its interaction with DNA polymerase α in DNA replication

Several previous publications have documented a role for the PrSet7 protein in DNA replication, in mammalian cells (Tardat et al., 2007; Jørgensen et al., 2007), S2 cells (Sakaguchi et al., 2012) and whole flies (Karachentsev et al., 2005; Sakaguchi and Steward, 2007). Our results are also consistent with a replication role for dPrSet7, however the observation that S2 cells divide several times in the absence of visible dPrSet7 protein might indicate that the requirement for PrSet7 is facilitative rather than essential. This does not rule out an important role for H4K20 monomethylation, as reduction of dPrSet7 protein levels in S2 cells does not cause immediate loss of this modification (Fig. 7; Sakaguchi et al., 2012). Perhaps in the absence of new modification, feedback stabilisation of H4K20 monomethylation occurs. This could happen through the l(3)mbt protein, which has been shown to play a role in the stabilisation of H4K20 monomethylation (Sakaguchi et al., 2012). It could also be mediated by prevention of the removal of H4K20 monomethylation [e.g. by the demethylase PHF8 (Liu et al., 2010) or by conversion to di and tri forms]. Alternatively a sub

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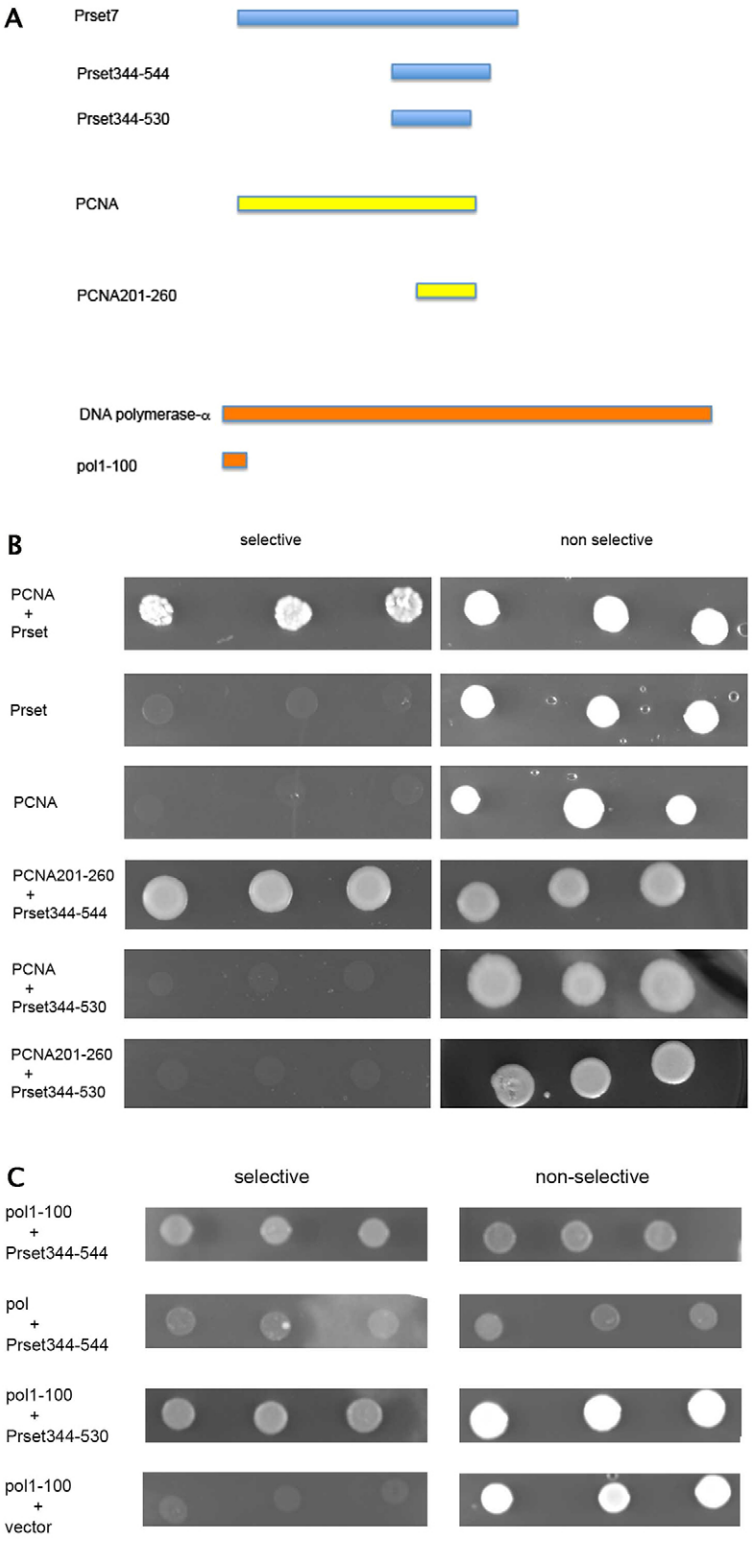

Fig. 8. PCNA and DNA polymerase α interact with dPrSet7 at proximal, but distinct, sites. (A) Deletions of dPrSet7 used in two-hybrid screening. (B) The PCNA–dPrSet7 interaction requires the dPrSet7 PIP box. Data are shown for: full-length dPrSet7 and PCNA proteins (PCNA + Prset); dPrSet7 (prset) and PCNA (PCNA) vector controls; the smallest mapped interacting regions of dPrSet7 (344–544) and PCNA (201–260) (PCNA201–260 +prset344–544); full-length PCNA with the PIP-box dPrSet7 deletion (344–530) (PCNA+prset344–530) and PCNA201–260 with the PIP-box dPrSet7 deletion (PCNA201–260+prset344–530). Three independent colonies are shown for each interaction and the experiment was repeated at least three times. (C) The polymerase–dPrSet7 interaction is independent of the dPrSet7 PIP box. Data are shown for: the smallest interacting regions of dPrSet7 and polymerase (pol1–100 +prset344–544), full-length polymerase with dPrSet7344–544 (pol +prset344– 544), the dPrSet7 PIP box deletion with pol1–100 (pol1–100 +prset344–530) and the pol1–100 vector control (pol1–100 + vector). Three independent colonies are shown for each interaction and the experiment was repeated at least three times.

population of PrSet7 could be maintained on chromatin at the expense of other populations. Consistent with this latter possibility, we have observed differential control of chromatin-bound and cytoplasmic dPrSet7 protein. The complete block to proliferation of S2 cells, which we observed after prolonged periods without PrSet7 protein, could be due to levels of H4K20 monomethylation falling below a critical threshold. However, it could also be wholly or partially due to loss of dPrSet7 function in one of the other cellular processes in which it has been implicated (e.g. transcription).

The continued proliferation of both wing (this publication) and eye (Karachentsev et al., 2005) discs after depletion of dPrSet7 is also consistent with a facilitative role. In this case, depletion of dPrSet7 causes significant loss of H4K20 monomethylation. Although this might suggest some differences in the control of the modification, it could equally

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be explained by the cells monitored in these tissues having undergone more divisions.

Both general [chromatin compaction (Abbas et al., 2010; Centore et al., 2010; Wu et al., 2010)] and specific [directly effects at origins or replication (Tardat et al., 2010)] models have been advanced for the role of PrSet7 in replication. A facilitative role for PrSet7 is reconcilable with either of these models.

Although our results are consistent with a replication role for PrSet7, we cannot conclude that the interaction between PrSet7 and the polymerase is important for replication. In wing discs, although a reduction in the level of dPrSet7 caused decreased BrdU incorporation, co-depletion of polymerase and dPrSet7 did not show synergistic effects as might be expected. However, we cannot entirely exclude a role for the interaction in DNA replication, as depletions of both proteins were suboptimal.

DNA polymerase α is needed for expression of dPrSet7 activity

DNA polymerase transcription is controlled by E2f (Yamaguchi et al., 1997), and PrSet7 has been shown to affect expression of E2f-related genes (Abbas et al., 2010). Therefore dPrSet7 could control the level of DNA polymerase protein in the cell. However, loss of dPrset7 had no effect on polymerase levels. A second possibility is that PrSet7 could directly affect polymerase activity. There is precedent for this, as human PrSet7 has been shown to methylate PCNA on lysine 248, which enhances the interaction between PCNA and FEN1 (Takawa et al., 2012). Loss of PCNA methylation slowed the maturation of Okazaki fragments, slowed DNA replication and induced DNA damage. Although at present we cannot exclude a role for polymerase modification by dPrSet7, our studies suggest at least one other role for the interaction. Reducing the levels of polymerase in wing discs causes decreased H4K20 monomethylation. No loss of modification is seen on reduction of PCNA levels, which also causes S phase defects, suggesting that the change is not simply due to a non-specific S phase defect. In addition, we have seen that overexpression of dPrSet7 makes the wings more, rather than less, defective. Perhaps in the absence of controlled loading of the methylase, the excess activity is free to methylate incorrectly [inappropriate sites or other proteins such as p53 (Shi et al., 2007; Driskell et al., 2012)] adding to the phenotype observed. Deleterious effects have also been observed on inappropriate dPrSet7 expression in human cells.

The interaction between dPrSet7 and PCNA

We also show that, in agreement with previous observations in human cells, *Drosophila* PrSet7 interacts with PCNA, and that this occurs at a site distinct from the site of polymerase interaction. In human cells, the PCNA interaction is responsible for the breakdown of a substantial proportion of total PrSet7 through the $CRL4^{Cdt2}$ pathway (Abbas et al., 2010; Centore et al., 2010; Jørgensen et al., 2011; Oda et al., 2010; Tardat et al., 2010) as S phase progresses. However, although dPrSet7 contains a good match to the PIP degron sequence (Centore et al., 2010), significant loss of total dPrSet7 during S phase progression was not observed in S2 cells. Removing 30 kDa from the N-terminus of dPrSet7, which shows no homology to human PrSet7, did not restore S phase degradation. This precludes the possibility that N-terminus overrides the effects of the PCNA interaction to maintain dPrSet7 levels for a different cellular function at other phases of the cell cycle. However analysis of the fraction of dPrSet7 bound to chromatin showed a marked decrease in protein levels as S phase progressed. In addition, H4K20 monomethylation showed variation consistent with that seen in mammalian cells. This suggests that S2 cells use the same mechanism as human cells to control chromatin-bound PrSet7, but, in addition, these cells contain a separate pool of PrSet7, not subject to the same controls, which could be responsible for additional cellular functions of PrSet7.

DNA polymerase α as a mediator of PrSet7 methylase activity

Previous studies on the control of PrSet7 activity have addressed the question of inactivation and removal of the protein to prevent inappropriate activity, however little is understood about the events surrounding PrSet7 chromatin loading. Our observations could be explained by a role for the polymerase either in loading of dPrSet7 onto chromatin, or activation of the methylase activity. The observation that polymerase interacts with dPrSet7 in soluble extracts as well as on chromatin, leads us to favour a loading function. This is also consistent with previous observations that PrSet7 activity could be observed *in vitro* in the absence of other proteins (Nishioka et al., 2002; Fang et al., 2002; Rice et al., 2002).

We therefore propose a model where DNA polymerase α modulates the loading of a sub-population of PrSet7 onto chromatin early in S phase. Once loaded, PrSet7 is appropriately located to methylate histone H4 and other chromatin-associated substrates as required. Given that DNA polymerase α does not arrive at origins of replication until the later stages of initiation, this would prevent the over-replication induced by premature binding of PrSet7 to origins (Tardat et al., 2010). It would also stop inhibition of the HBO1-catalysed acetylations, which are associated with the initiation of DNA replication, and which others have shown to be inhibited by the presence of the histone-binding domain of PrSet7 (Yin et al., 2008). During the synthesis phase of DNA replication, there is close proximity between DNA polymerase α and PCNA. This would provide an opportunity for interaction between PCNA and PrSet7, which could lead to PrSet7 being targeted for destruction. Whether PrSet7 is directly transferred between the polymerase and PCNA, or dissociates from the polymerase and rebinds independently is not clear. The transfer between polymerase and PCNA binding could provide a natural window of opportunity for the modulation of PrSet7 activity in specific regions of chromatin, or on fork stalling due to DNA damage. In the case of DNA damage there could still be a requirement for PCNA, via the PIP box, to stabilise the PrSet7–chromatin interaction, and allow an opportunity for the loading of repair factors, as has been observed by others (Abbas et al., 2010).

MATERIALS AND METHODS

Antibodies and reagents

Primary antibodies were purchased from Abcam (V5 and PCNA mouse monoclonal antibodies); Sigma (mouse anti-tubulin monoclonal antibodies): Rockland (rabbit anti-histone H2AvD); and Upstate (anti-monomethyl-H4K20) (Karachentsev et al., 2007). Rabbit polyclonal antibodies against DNA polymerase α and the mouse monoclonal antibody against the DNA polymerase α 180-kDa subunit were as reported previously (Melov et al., 1992; Yamaguchi et al., 1992). Rabbit anti-dPrSet7 polyclonal antibodies were raised against His-tagged full-length protein, and affinity purified against overexpressed protein before use. Horseradish peroxidase (HRP)-labelled secondary antibodies for western blotting were from Thermo Scientific (anti-rabbit and anti-mouse). Secondary antibodies for immunostaining (Alexa-Fluor-594-conjugated anti-rabbit, and Alexa-Fluor-488-conjugated anti-mouse and anti-rabbit) were from Molecular Probes, Oregon, USA.

Drosophila cell culture

S2 cells were from the *Drosophila* Genomics Resource Center and were propagated in Schneiders *Drosophila* medium from Lonza, with 10% foetal calf serum from Gibco and penicillin/streptomycin from Sigma.

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Fly maintenance

Fly stocks were cultured at 25℃ on standard food (3% dry yeast, 3% cornmeal, 5% glucose, 1.5% agar, 0.5% propionic acid and 0.25% ethyl-parahydroxybenzoate). Rich food for the eclosion assay was 4% dry yeast, 9% cornmeal, 10% glucose, 0.8% agar, 0.5% propionic acid and 0.25% ethyl parahydroxybenzoate.

Stable S2 cells lines

The full-length *Pr-Set7* gene was cloned into the *Kpn1* and *Not1* sites of pMT/V5hisA (Invitrogen) such that it was His- and SV5-tagged at the C terminus, and under the control of the inducible metallothionein promoter. The construct was introduced into S2 cells along with pCoBlast using the calcium phosphate procedure, and stably transfected cells selected using blasticidin according to the manufacturer's instructions. The N-terminal was removed by internal PCR using the internal primer 5′-(ATG)GCGGGGCAACCCCGA-CGCA-3′ and a vector primer, followed by intra-molecular ligation.

dsRNA interference in S2 cells

RNAi experiments were performed as described previously (Crevel et al., 2007) using two non-overlapping regions of dPrSet7. All primers contained 5′ T7 RNA polymerase binding sites plus the site-specific sequences: nt151–851 (5′-CCCAAGCGGAAAGACTGC-3′ and 3′-AGTTCGAAGCTCCGATTGATC-5′); and nt 1301–2001 (5′-AGCA-GCAGCAGGATGATATC-3′ and 3′-CGGCTCGATGTCGTCCTT-5′). Where a second week of RNAi was performed the cells were re-exposed to the dsRNA on day 7 and the dsRNA procedure repeated as for the first week.

Knockdown and overexpression fly lines

The yellow white fly was used as a control strain. The *MS1096*-GAL4 and the UAS-GFP-IR lines were from the Bloomington *Drosophila* stock center (Indiana), the RNAi line *UAS-dpolα p180-IR* (stock number 6349R-3) from the National Institute of Genetics (Mishima) and *UAS-dPCNA-IR* (stock number 108384) from Vienna *Drosophila* RNAi Center (VDRC). To express GAL4, flies were cultivated at 25℃ or 28℃. The PrSet knockdown flies were constructed by the cloning of a 500-bp DNA fragment for *dPrSet7* into pWIZ (Lee and Carthew, 2003) in a head to head orientation. To generate dPrSet overexpression flies, full-length dPrSet7 was cloned into pUAST and expression lines generated by P-element-mediated germ line transformation (Spradling, 1986). F1 transformants were selected on the basis of white-eye colour rescue (Robertson et al., 1988). A line carrying the transgene on chromosome III was used in this study.

Fly genotypes

Controls were: *Act5C*-GAL4 (*yw;+;Act5C-GAL4/+*); MS1096-GAL4 (*MS1096-GAL4/yw*;+;+); GFP control (*MS1096GAL4/w;+;+/UAS-GFP-IR*). dPrSet7 dsRNA lines were: Act5C >*dPr-Set7-IR* (*yw;+; Act5C-GAL4/UAS-dPr-Set7-IR*); MS1096>dPr-Set7-IR (*MS1096-GAL4/w*;+;+/*UAS-dPr-Set7-IR*). DNA polymerase dsRNA lines were: Act5C> *dpolαp180-IR* flies (*yw*;+; *Act5C-GAL4/UAS-dpolαp180-IR*); MS1096 >*dpolαp180-IR* (*MS1096-GAL4/w*;+;+/*UAS-polαp180-IR*). Double mutants were: MS1096 >*dpolαp180-IR* dPr-Set7 (*MS1096-GAL4/w*;+/*UAS-dPr-Set7;UAS-dpolαp180-IR*/+). dPrSet7 overexpression lines were: MS1096> dPr-Set7 (*MS1096-GAL4/w*;+/*UAS-dPr-Set7*); MS1096>*dpolαp180-IR* >dPr-Set7(*MS1096-GAL4/w*;+/*UAS-dPr-Set7;UAS-dpolαp180-IR*/+).

Protein blotting

SDS-PAGE-separated proteins were transferred onto Hybond-ECL (GE) and developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore). Visualisation and quantification were performed using the Fujifilm LAS-4000 imaging system.

Flow cytometry

Harvested cells were fixed using 50% ethanol in PBS. Immediately before use, cells were transferred to PBS containing 1% glucose, 10 µg/ml RNase, 1 mM EDTA, 0.5% Triton X100 and 50 µg/ml propidium iodide to stain DNA. Flow cytometry was carried out on a CYTOMICS 500 (Beckman) and analysed using CXP software.

Immunofluorescence

S2 cells were deposited on polylysine-treated coverslips and fixed using 4% paraformaldehyde in 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM Pipes, 5.5 mM glucose, pH 6.1 (Maiato et al., 2003). After permeabilisation in PBS, 1% BSA and 0.1% Triton X-100, coverslips were processed using appropriate antibodies, the DNA counterstained with DAPI, and coverslips were mounted in mounting medium Vectashield (Vector) and analysed.

Third-instar larvae were dissected in PBS. Wing discs were fixed in 4% paraformaldehyde (25℃ for 15 minutes), washed with 0.3% Triton X-100 in PBS and blocked in 0.10% Triton X-100 in PBS containing 10% goat serum (25℃ for 20 minutes). After incubation with mouse anti-dpolαp180 monoclonal antibody (1:100), rabbit polyclonal anti-PrSet7 (1:100) or rabbit polyclonal anti-monomethyl-H4K20 (1:200), (4℃ for 16 hours), they were washed with 0.3% Triton X-100 in PBS and incubated with the appropriate secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) (1:400; 25℃ for 2 hours). After extensive washing with 0.1% Triton X-100 in PBS and PBS, samples were mounted in Fluoroguard antifade reagent (Bio-Rad) and examined by confocal laser scanning microscopy (Carl Zeiss LSM510, Jena, Germany). DNA was stained with propidium iodide (PI).

For quantification, the signal intensities of 30 nuclei per wing imaginal disc, from several independent samples, were quantified using Image J software and analysed using the Student's *t*-test (dpolαp180 knockdowns) or Welch's *t*-test (dPr-Set7 knockdowns). For anti-dpolαp180 in PrSet7 knockdown fly wing imaginal discs, signal intensities of five wing imaginal discs were quantified using Image J software and statistically analysed by Student's *t*-test.

BrdU labelling of wing discs

Third-instar larvae were dissected in PBS. The imaginal discs were suspended in Grace's insect medium and incubated in 75 µg/ml 5-bromo-2′-deoxyuridine (BrdU; Roche) (25℃ for 1 hour). After fixing in Carnoy's fixative (ethanol:acetic acid:chloroform, 6:1:3; 25℃ for 20 minutes), then 80% ethanol, 50 mM glycine buffer (pH 2.0) (−20℃ for 16 hours), BrdU incorporation was visualized using the BrdU Labelling and Detection Kit I (Roche).

BrdU-positive nuclei were counted in whole-wing pouches from three discs of independent strains. Statistical analysis was by Student's *t*-test.

Preparation of wing samples

Adult wings were placed on a slide glass, covered in isopropanol, and Hoyer's medium was added before the isopropanol was dry (Ashburner, 1989). The samples were covered with a glass coverslip and incubated at 65℃ for 16 hours.

Quantitative RT-PCR

Total RNA was isolated from third-instar larvae using Trizol® Reagent (Invitrogen). 1 µg aliquots were reverse transcribed with oligo(dT) primer using a high fidelity RNA-PCR kit (TaKaRa). Real-time PCR was performed with a SYBR Green kit (TaKaRa) in the Applied Biosystems 7000 Real-time system (1 µl of reverse transcribed sample per reaction). Levels of specific mRNAs were investigated by the Ct comparative method (Morrison et al., 1998). The *RpL32* (*Rp49*) gene was used as an endogenous reference gene. Experiments were performed on three independent RNA preparations.

Immunoprecipitation

Samples were prepared using the Proteojet extraction kit (Fermentas). All steps were carried out at 4℃. Cells were collected by centrifugation at 3800 ***g***, washed twice with PBS, and resuspended in cold cell lysis buffer plus DTT and protease inhibitors. They were incubated on ice for 10 minutes and centrifuged at 16,300 ***g***. The supernatant was re-centrifuged and the resulting supernatant is the cytoplasmic fraction. The pellet was incubated in nuclear lysis buffer for 15 minutes and centrifuged to produce the nuclear extract (supernatant) and the chromatin fraction (pellet). Before use the chromatin was treated with DNase for 30 minutes and centrifuged at 16,300 ***g*** and the resulting supernatant used for immunoprecipitation.

Antibody coupling to Protein-A–Sepharose and immunoprecipitation were as described previously (Crevel et al., 2007).

Two hybrid analyses

All dPrSet7 constructs were in pGADT7, and PCNA and polymerase constructs in pGBKT7. Yeast manipulations were as described previously (Brent and Finley, 1997). For each interaction, three independent clones were grown for 24 hours to comparable optical density (OD) and 3 µl spotted onto selective or control plates. All interaction experiments were repeated two or three times.

S2 cell synchronisation

This was performed as described (Crevel and Cotterill, 2012) to produce cell populations that progressed synchronously through S phase in 4–6 hours.

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Competing interests

The authors declare no competing interests.

Author contributions

R.S., G.C., J.P., O.S., R.N. AND M.S. planned and carried out the experiments and contributed to the writing of the manuscript. M.Y. and S.C. planned the experiments and wrote the manuscript.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.144501/-/DC1

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Supplementary figure legends

Fig S1

A) Expression of dsRNA for dPrSet7 reduces dPrSet7 mRNA levels in 3rd instar larvae. Levels of Pr-Set7 mRNA in Act5C>*dPr-Set7-IR* larvae and larvae from *Act5C*-GAL4 controls were measured by quantitative RT-PCR. Results from three independent mRNA preparations are shown.

B) Depletion of dPr-Set7 by dsRNA interference does not affect the wing phenotype of adult flies**.** A representative wing is shown for dPrSet7 knockdown flies, GFP control flies and wt flies. Flies were developed at 28 ºC.

C) Quantitation of cell density in the wings of control (100%) and dPrSet7 depleted (102%) flies. Cells present in the region surrounded by L4, L5, and pcv were counted from five independent flies and expressed as a percentage of the control, $P>0.1$ (student t-test).

D) Expression of dsRNA for DNA polymerase-alpha reduces polymerase mRNA levels in 3rd instar larvae. Levels of dpol p180 mRNA level in *Act5C-GAL4>dpol α p180-IR* larvae and larvae from *Act5C-GAL4* controls were measured by quantitative RT-PCR. Results from three independent dsRNA preparations are shown.

Fig S2

Immunofluorescence analysis showing that phospho-H2AvD staining is increased in S2 cells treated with dPrSet7 dsRNA (dPrSet7-1/dPrSet7-2) compared to untreated (control1) or mock treated cells (control 2). Nuclear DNA is stained with DAPI.

Fig S3

A Confocal microscope image of representative wing disc showing immunostaining of dPrSet7-knockdown wing imaginal disc with anti-dpolα antibody. (A) control (B) dPr-Set7 knockdown. Discs are oriented as in 1 C: Scale bar indicates 50 m.

B Quantification of data from S4A. The pol signal intensity in dPr-Set7 knockdown flies is shown relative to that of control flies. $P>0.1$ (Student's t-test).

C Expression of dPrSet7 dsRNA reduces H4K20 mono-methylation in wing imaginal discs. Representative confocal microscopy results are shown for GFPdsRNA and dPrSet7 knockdown relative to control discs. Discs are oriented as described in 1C. Green- anti-

H4-K20 monomethyl : Red - DNA stained with propidium iodide. Scale bar indicates 50 μm.

D Expression of polymerase-alpha dsRNA reduces H4K20 mono-methylation in wing imaginal discs. Representative confocal microscopy results are shown for control flies (*MS1096-GAL4*) (A, C) and dpol p180 knockdown flies (*MS1096> dpol p180-IR*) (B, D). Discs are oriented as in 1C: Green - anti-H4-K20-monomethyl. Red - DNA stained with propidium iodide. Scale bar indicates 50 m.

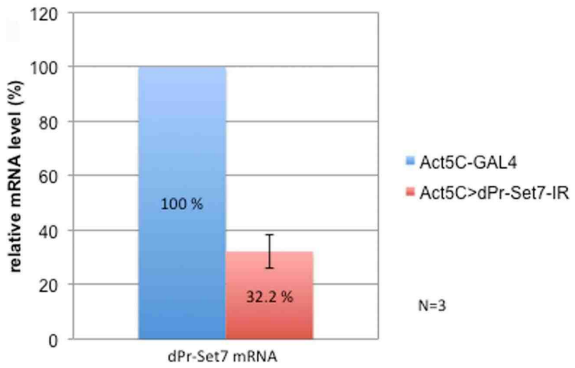
Fig S4

Expression of PCNA dsRNA does not reduce H4K20 mono-methylation in wing imaginal discs. Quantification of mono-methylated histone H4-K20 fluorescent signal in wing imaginal discs. The H4-K20 mono-methyl fluorescent signal intensities in PCNA knockdown flies (MS1096> PCNA-IR flies) are shown relative to control flies (MS-1096 flies). $P<0.01$ (Student's t-test).

Supplementary figures

S1

A



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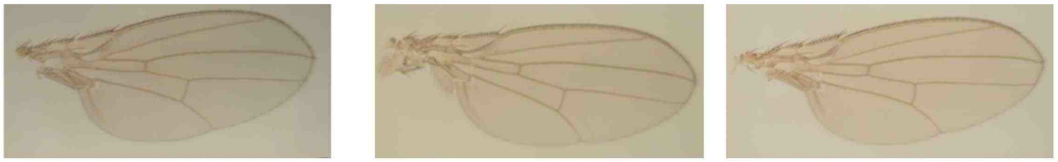

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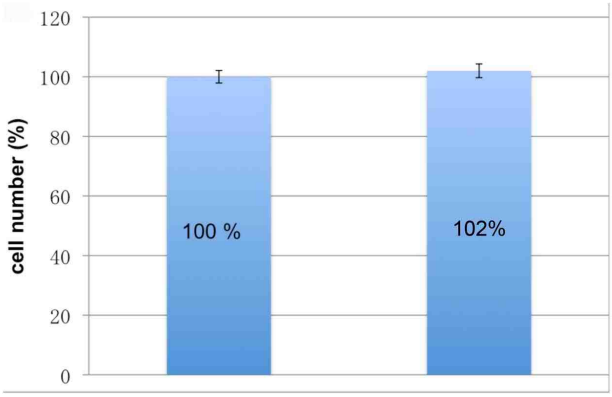

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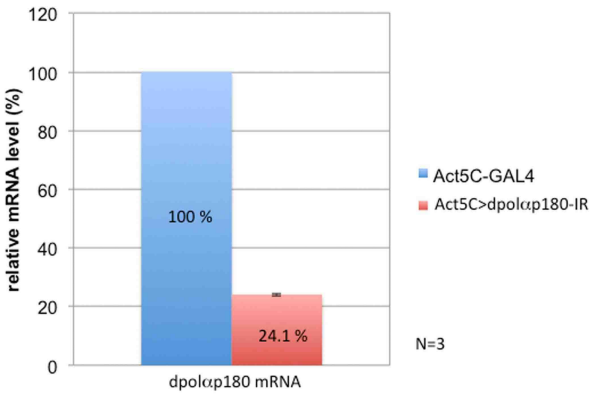
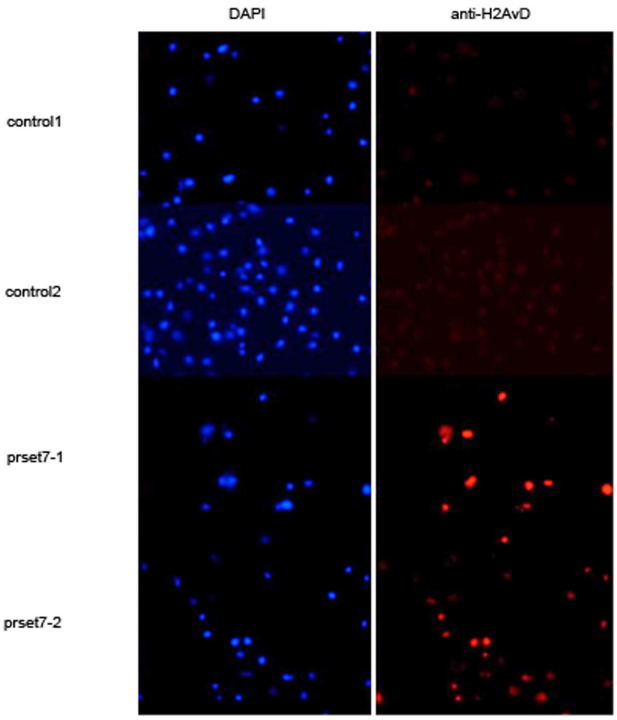
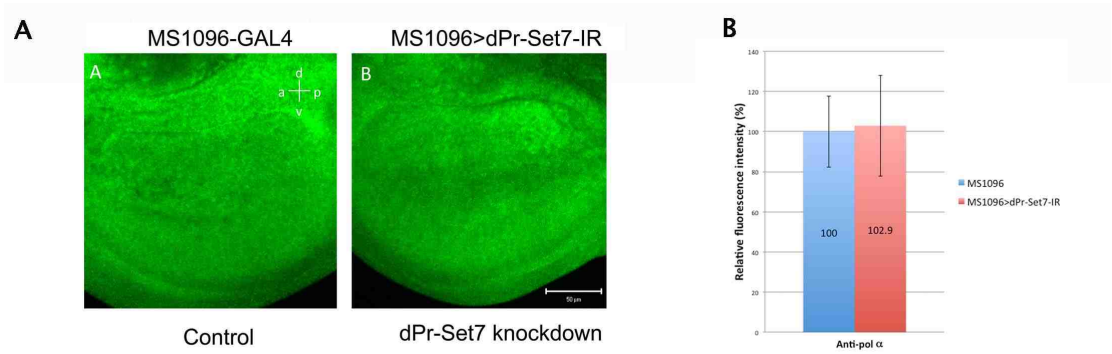

120
100
80
60
40
20
0
relative mRNA level (%)
100 %
24.1 %
dpolαp180 mRNA
Act5C-GAL4
Act5C>dpolαp180-IR
N=3

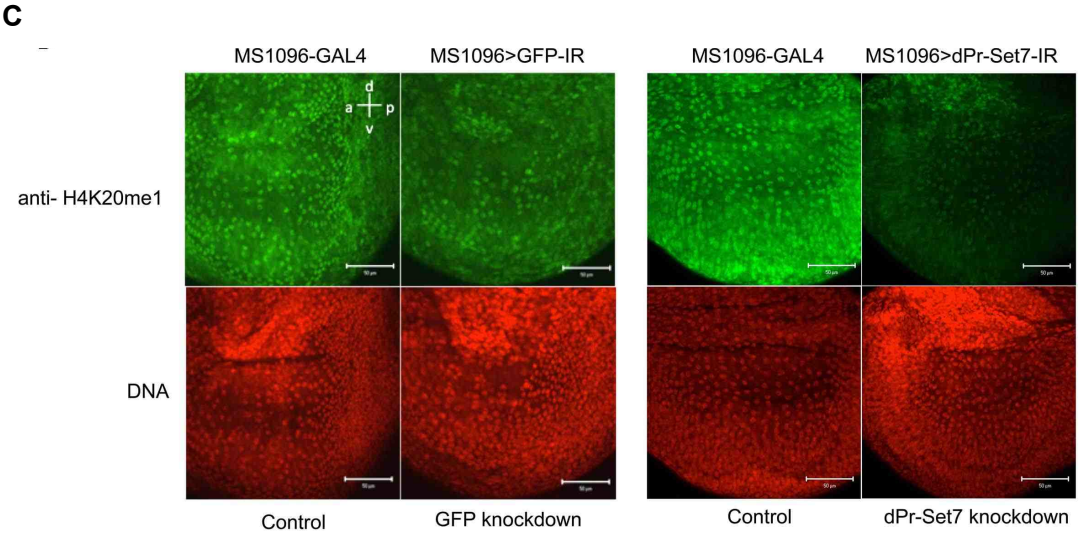
S2

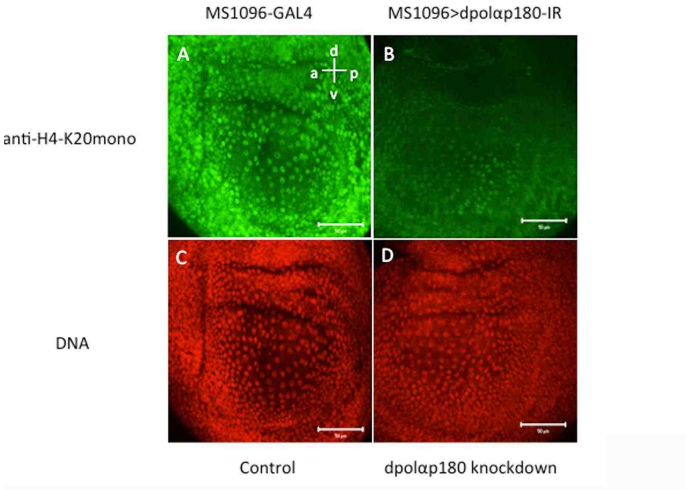
E

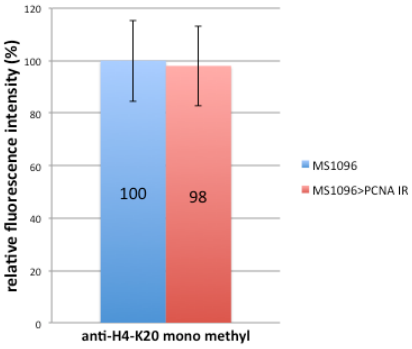


S3


A
MS1096-GAL4
MS1096>dPr-Set7-IR
A
B
d
a
p
v
Control
dPr-Set7 knockdown
B
Relative fluorescence intensity (%)
140
120
100
80
60
40
20
0
100
102.9
MS1096
MS1096>dPr-Set7-IR
Anti-pol α


C
MS1096-GAL4
MS1096>GFP-IR
MS1096-GAL4
MS1096>dPr-Set7-IR
anti- H4K20me1
DNA
d
a
p
v
Control
GFP knockdown
Control
dPr-Set7 knockdown


D
MS1096-GAL4
MS1096>dpolαp180-IR
anti-H4-K20mono
DNA
A
B
C
D
d
a
p
v
Control
dpolαp180 knockdown


120
100
80
60
40
20
0
relative fluorescence intensity (%)
100
98
anti-H4-K20 mono methyl
MS1096
MS1096>PCNA IR