RESEARCH COMMUNICATION

PR-Set7-dependent methylation of histone H4 Lys 20 functions in repression of gene expression and is essential for mitosis

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The histone methyl transferase PR-Set7 mediates histone H4 Lys 20 methylation, a mark of constitutive and facultative heterochromatin. We isolated a null mutation in *Drosophila* PR-Set7 that suppresses position effect variegation, indicating that PR-Set7 indeed functions in silencing general gene expression. In PR-Set7 larval leg and eye discs, the number of cells is lower than normal, and the DNA content in these cells is significantly increased. These data show that PR-Set7-dependent methylation is essential for the process of mitosis. The methylation mark is highly stable and is maintained even in the absence of PR-Set7 protein.

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The development of a single cell into an embryo consisting of specific tissues is dependent on cascades of cell-to-cell signaling events that activate transcription factors controlling the expression of specific genes. Control of expression of these genes can be regulated at two levels: the interplay of transcription factors that bind DNA directly, and the conformation of chromatin that controls the access of the transcription factors to the DNA. Recently it has become clear that chromatin organization is regulated in part by post-translational modification of the four histone proteins that control the packaging of DNA into the nucleosome, the basic unit of chromatin.

The nucleosome consists of an octamer of two copies of the four core histones H2A, H2B, H3, and H4, around which 146 base pairs of DNA are wound (Luger et al. 1997). The N-terminal tails of the four core histones are exposed on the nucleosome surface and are targets for a

variety of enzyme-catalyzed, post-translational modifications of selected amino acids. These modifications include lysine acetylation, serine phosphorylation, ubiquitination, poly ADP-ribosylation, and lysine and arginine methylation (Stahl and Allis 2000; Zhang and Reinberg 2001). Particular histone modifications can control different states of chromatin. A particularly exciting possibility is that these modifications may transmit epigenetic information from one cell generation to the next. Histone lysine methylation is a relatively stable modification that correlates with transcriptional activation (H3-K4, H3-K36, H3-K79) or with transcriptionally repressed chromatin (H3-K9, H3-K27, H4-K20) depending on the residues that are modified (van Holde 1988; Strahl et al. 1999; Rice and Allis 2001; Zhang and Reinberg 2001).

Monomethylation of Lys 20 of histone H4 is regulated by the methyl transferase PR-Set7 (Nishioka et al. 2002; B. Xiao, C. Jing, G. Kelly, P.A. Walker, F.W. Muskett, T.A. Frenkiel, K. Sarma, D. Reinberg, S.J. Gamblin, and J.R. Wilson, in prep.), or Set8 (Fang et al. 2002), while trimethylation of the same amino acid occurs through the action of Suv4-20 (Schotta et al. 2004). Both enzymes contain an evolutionarily conserved SET domain (Jenuwein et al. 1998). The PR-Set7-mediated methylation of H4-K20 correlates with repression, as it is found in association with facultative and constitutive heterochromatin. Moreover, methylation of H4-K20 interferes with H4-K16 acetylation, a mark associated with active chromatin (Nishioka et al. 2002). In HeLa cells, the expression of PR-Set7 was found to be cell cycle-regulated, with a steep increase in late G2 and early mitosis. Further, in HeLa cells, PR-Set7 was observed specifically on mitotic chromosomes (Rice et al. 2002).

The *Drosophila* PR-Set7 ortholog is predicted to be a 76-kDa protein (CG3307) with a SET domain that is ~40% identical to the human domain. In the *Drosophila* gene disruption project, a single P-insertion into the 5'UTR of the gene was isolated. This partial loss-of-function allele, *l(3)neo41*, is hemizygous lethal at the late pupal stage. We have isolated a complete loss-of-function PR-Set7 allele in order to determine the functional importance of the gene. In the homozygous null mutants, the maternally deposited PR-Set7 protein does not perdure into the first instar larval stage, but mono-, di-, and trimethylation of H4-K20 are still present at late larval stages, when all three methyl marks disappear.

PR-Set7 mutants suppress variegation, confirming that PR-Set7 functions in silencing gene expression. PR-Set7 mutants die at the larval-to-pupal transition and show strong phenotypes in their imaginal discs; the number of cells in the discs is reduced and the content of DNA increased, suggesting a failure to complete cell division. The reduced eye phenotype seen in rare mutant escapers confirms the importance of PR-Set7 function in mitosis.

Results and Discussion

A PR-Set7 complete loss of function allele

The existing *l(3)neo41* mutation is caused by a P-element insertion into the 5'UTR of the PR-Set7 gene and is lethal at the late pupal stage over *Df(3R)red31* (Fang et

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Figure 1. *PR-Set7*²⁰ mutants lack the PR-Set7 methylase. (A) Western blot of protein extracts derived from wild-type and homozygous *PR-Set7*²⁰ tissue, probed with anti-*Drosophila* PR-Set7 antibodies. (B,C) Immunofluorescent staining of wild-type (B) and *PR-Set7*²⁰ (C) salivary gland chromosomes with anti-*Drosophila* PR-Set7 antibodies (red) and Hoechst DNA dye (blue).

al. 2002; Nishioka et al. 2002; see also Materials and Methods). Such insertions frequently result in partial loss of function of the gene (Spradling 1986). In order to obtain a complete loss of function allele, we took advantage of a new P-element insertion obtained from the *Drosophila* stock center, *PR-Set7*^{EY04668}. This allele is lethal in homozygous pupae. By mobilizing this P-element we isolated a deletion in which the entire PR-Set7 protein coding region is missing (*PR-Set7*²⁰) (Supplementary Fig. S1). *PR-Set7*²⁰ homozygotes or hemizygotes over *Df(3R)red31* show a somewhat stronger phenotype: Most animals die at the larval-to-pupal transition, with rare escapers surviving into early pupal stage. We determined the presence and perdurance of PR-Set7 protein in wild-type and mutant animals using anti-*Drosophila* PR-Set7 antibody raised in Rats against *Drosophila* recombinant PR-Set7 (Materials and Methods).

In Western blots (Fig. 1A), a band of ~100 kDa is present in extracts of wild-type ovaries, early embryos, and throughout development, suggesting that PR-Set7 is deposited in the egg during oogenesis. In extracts from homozygous *PR-Set7* animals, the ~100-kDa band is missing, while α-tubulin is clearly present. These results show that the antibody recognizes the PR-Set7 protein specifically. They further show that the maternally deposited PR-Set7 protein does not perdure into first instar larvae, since the band is missing in homozygous mutants.

Using the antibody to stain salivary gland chromosomes, we found that the PR-Set7 protein is associated with the chromocenter and with the chromosome arms, mostly with densely packed DNA (Fig. 1B), indicating that PR-Set7 is associated with facultative and constitutive heterochromatin as well as with euchromatin. The chromosomal staining appears specific for PR-Set7, as no staining is observed on *PR-Set7*²⁰ salivary gland chromosomes (Fig. 1C).

These results show that even though the protein is missing in the mutants from first instar larval stage onward, the homozygous mutant *PR-Set7*²⁰ animals can survive until late larval to early pupal stages.

PR-Set7 functions as a silencer

Based on the localization of methylated H4-K20 on salivary gland chromosomes at transcriptionally inactive regions, we proposed that this methylation functions to nucleate higher-order structures that would maintain chromatin in an inactive state (see Nishioka et al. 2002). To determine whether indeed *PR-Set7* functions as a silencer, we examined its capability to suppress variegation. When euchromatic genes such as the wild-type *white* (*w*⁺) gene in *Drosophila* are inserted into heterochromatic regions, the condensed chromatin structure of heterochromatin often spreads into the euchromatic region, resulting in full or partial inactivation of the genes (Cryderman et al. 1998, 1999). This results in variegated expression of the genes, easily seen in *Drosophila* eyes (position effect variegation, PEV).

To measure the level of expression of the *w*⁺ gene we measured the amount of red pigment in extracts of fly heads and found that both *Df(3R)red31* and the *PR-Set7*²⁰ allele function as dominant suppressors of PEV (Fig. 2A). The expression of *w*⁺ transgenes inserted into centromeric and telomeric heterochromatin of the fourth chromosome is significantly activated by the loss of function of one copy of *PR-Set7* (Fig. 2A,B). PEV of *w*⁺ insertions on chromosomes two and three were not

Figure 2. Reduction of PR-Set7 suppresses position effect variegation (PEV). (A, right) *TM3Sb*/+ fly showing variegated expression of a *w*⁺ transgene. (Left) *PR-Set7*²⁰/+ fly has red eyes because the variegated expression of the *w*⁺ transgene has been suppressed. (B) Measurement of eye-color (OD 480 nm) of extracts from different transgenic *w*⁺ lines expression, in heterozygous *PR-Set7* flies (*PR-Set7*²⁰/+, or *Df(3R)red31*/+), or wild-type flies (*Bal*/+). Deletion of one copy of the *PR-Set7* gene increases the eye color significantly, confirming the suppression of variegation. The balancers used were *TM3* and *TM6*. The transgenic lines used were 36C-72 and 118E-15 containing *w*⁺ in the telomeric heterochromatin of the fourth chromosome. 118E-10 contains a *w*⁺ transgene in the centromeric heterochromatin. All second and third *w*⁺ transgenic lines contain the transgene in the telomeric heterochromatin.

changed (Fig. 2B). This result shows that indeed *PR-Set7* functions as a repressor of gene activation and supports our previous supposition that was based only on distribution of the protein on chromosomes (Nishioka et al. 2002).

Mono-, di-, and trimethylation of histone H4-K20 is affected in PR-Set7 mutants

We investigated the mono-, di-, and trimethylated state of histone H4-K20 in wild-type and mutant larvae by antibody staining of salivary gland chromosomes and by Western blot. The staining of salivary gland chromosomes with the antibody specific for mono-, di-, or trimethylated histone H4-K20 shows that all three methyl marks have an indistinguishable distribution, with the exception of trimethyl H4-K20 appearing more abundant in centromeric heterochromatin (Fig. 3A,C,E; see also Schotta et al. 2004). This result agrees with studies demonstrating that trimethylation of H4-K20 is preferentially a pericentromeric mark (Schotta et al. 2004). On salivary gland chromosomes derived from homozygous *PR-Set7²⁰* mutants, the level of all three forms of methylated H4-K20 was reduced (Fig. 3B,D,F). This reduction was only observed in late-stage larvae and was usually stronger on the chromosome arms than in the centromeric regions. The salivary chromosome preparations were costained with antibody recognizing the transcriptionally active form of RNA polymerase II. Both wild-type and mutant glands showed strong polymerase staining, indicating that the chromosomes are intact and that the reduced levels of the methylated forms of H4-K20 are not due to degradation of the chromosomes (Fig. 3A–F).

Methylation of H4-K20 is essential for mitosis

Homozygous *PR-Set7* larvae generally die at the larval-to-pupal transition. Although the salivary glands and the chromosomes of such mutants look relatively normal, a distinct phenotype can be observed in tissues with higher rates of cell divisions such as larval imaginal discs (Fig. 4A). Imaginal discs are epithelial sacs made up of a folded, columnar epithelium on one side and a squamous epithelium on the other side (Bate and Arias 1993). The columnar discs cells are very small, only a few microns in size. The pictures shown in Figure 4A show the folded columnar epithelium of leg and eye discs from wild-type

and *PR-Set7* late third instar larvae. The mutant discs are 10%–20% smaller than the wild-type discs and contain a significantly smaller number of cells. We counted the number of cells in optical sections prepared on a confocal microscope, and compared wild-type and mutant eye discs (Supplementary Fig. S2). The amount of DNA in each cell was determined by measuring the intensity of Hoechst fluorescence (Materials and Methods). We found that in wild-type discs there are approximately four times as many cells as in the mutant discs, and the fluorescence of nuclei indicates that there is ~3.5 times more DNA in mutant nuclei compared with wild-type (Supplementary Fig. S2). Further, hetero-allelic *PR-Set7* mutants *l(3)neo41/PR-Set7²⁰* rarely survive to adulthood and die soon after eclosion. In these escapers the eye is reduced in size and irregular, consistent with the phenotype observed in the eye discs of homozygous *PR-Set7²⁰* larvae (Fig. 4B).

These results show that PR-Set7-controlled methylation of H4-K20 is essential for normal cell cycle progression. The mutant cells seem to undergo DNA replication but fail to complete mitosis.

The monomethyl H4-K20 mark is highly stable

Our analysis of PR-Set7 protein in wild-type animals reveals a protein on Western blots that appears to be significantly larger than the predicted size of 76 kDa. This difference in size could, at least in part, be due to as yet uncharacterized modifications of the enzyme. Several experiments show that our antibody specifically recognizes the Pr-Set7 protein. The protein is absent in extracts from mutant animals, and it is not detectable on mutant salivary gland chromosomes (Fig. 1A–C). Further, Flag-tagged PR-Set7 expressed in flies is recognized as a larger protein in Western blots of extracts from transgenic lines (data not shown).

The Western blots also show that mutant first instar larvae are devoid of the enzyme. Nevertheless, the homozygous mutant animals live until the late third instar larval stage. *PR-Set7* is expressed in oogenesis and is deposited into the egg and is still detectable in early embryos, resulting in the synthesis of monomethyl H4-K20, present in oocytes and early embryos. Zygotic transcription of *PR-Set7* presumably starts during embryogenesis, and PR-Set7 is present throughout development, as expected from a gene functioning in the cell cycle. The presence of monomethylated H4-K20 in the third instar

Figure 3. The mono-, di-, and trimethylation mark of H4-K20 is reduced in *PR-Set7* mutants. Immunofluorescent staining of wild-type (A,C,E) and *PR-Set7²⁰* (B,D,F) salivary gland chromosomes with anti-monomethylated (A,B), anti-dimethylated (C,D), and anti-trimethylated (E,F) H4-K20 antibodies (red) and antibody recognizing the catalytic subunit of RNA polymerase II (green). DNA dye Hoechst (blue).

PR-Set7 mutant larvae indicates that this methylation mark of histone H4-K20 is stable over several days and several cell generations, or that an additional methylase exists that controls the methylation of H4-K20 up to late larval stages.

These results agree well with observations previously obtained in HeLa cells (Rice et al. 2002). In these cells *PR-Set7* expression is controlled transcriptionally and fluctuates with different stages of the cell cycle. Both mRNA and protein levels are lowest in G1 and highest during G2/M. In parallel, methylation levels of H4-K20 also cycle but the cycling is not pronounced, suggesting that the methylation is stable over more than one cell generation.

Our results suggest that monomethylation is a prerequisite for di- and trimethylation to occur or be stabilized. At least two scenarios can explain this result: (1) Suv4-20 and a possible dimethylase may modify primarily a monomethylated H4-K20 substrate. (2) The arrest in the cell cycle in the *PR-Set7* mutant could result in reduced levels of the di- and trimethylated forms of H4-K20.

Only a partial loss-of-function allele of the newly identified *Suv4-20* gene exists (Schotta et al. 2004). Further work will be necessary to fully understand the connection between mono-, di-, and trimethylation.

Methylated histone H4-K20 functions as a repressor

Our results indicate that *PR-Set7* functions as a suppressor of gene expression and hence as a SUVAR. This ob-

servation is consistent with our previous finding that monomethylated H4-K20 is strongly associated with silent chromatin of polytene chromosomes. It is not clear why the suppression is only observed with *w⁺* transgenes inserted on the fourth chromosome. Since in this genetic interaction experiment the amount of *Pr-Set7* function is reduced by only 50%, it is possible that a stronger reduction of the enzyme would also affect the expression of *w⁺* transgenes inserted into the heterochromatin of other chromosomes. Our results suggest that the heterochromatin organization on the fourth chromosome differs from that of the other autosomes (Schotta et al. 2002).

It is likely that monomethylated H4-K20 regulates the packaging of silent chromatin domains in constitutive and facultative heterochromatin. Because *PR-Set7* mutants show a cell-cycle defect phenotype, we propose that the monomethylated H4-K20 is associated with regions containing cell-cycle genes that must remain repressed in early mitosis. Alternatively, the chromosomal regions associated with monomethylated H4-K20 may represent a flag for a cell-cycle checkpoint control or be otherwise involved in separation of chromosomes.

PR-Set7 is essential for mitosis

The cell cycle-regulated expression of *PR-Set7* (Rice et al. 2002) is consistent with the observed phenotype of *PR-Set7* mutants that go through S phase normally but enter into an endoreplication cycle. This phenotype is most easily seen in diploid tissues such as imaginal discs.

Imaginal disc cells undergo ~12 divisions from the end of embryogenesis to the late third instar larval stage (Bate and Arias 1993). Because *PR-Set7* discs have about four times fewer cells and 3.5 times more DNA, mitosis probably is affected about two cell divisions before the final development of the discs, in mid-third instar larvae. This is about the same time when the H4-K20 methyl mark begins to disappear.

Investigating the function of *PR-Set7* using a short interfering RNA (siRNA) approach in HeLa cells, Julien and Herr (2004) found no change in mitosis. In our experiments the mitosis phenotype is apparent in mutant larvae only ~10 cell divisions after the *PR-Set7* enzyme is not detectable. The discrepancy in these results may lie in the fact that the time-frame of tissue culture experiments does not allow the detection of the mitosis phenotype. However, Julien and Herr (2004) also found that cells treated with *HCF-1* and *PR-Set7* siRNA have a weaker multinucleate phenotype than when *HCF-1* siRNA is applied alone. They proposed that reduction in *PR-Set7* and resulting changes in H4-K20 methylation have a function in mitosis. Based on our results we suggest that the effect of lowering *PR-Set7* levels may arrest mitosis, resulting in fewer multinucleated cells than in cells treated with *HCF-1* siRNA alone. The *PR-Set7* phenotype may be more easily discernible in the sensitized background, when *HCF-1* is reduced.

Figure 4. In *PR-Set7²⁰* animals, the mitotic process is interrupted. (A) Leg imaginal discs (left) and eye imaginal discs (right) of wild-type and *PR-Set7* larvae stained with the DNA dye Hoechst (white). (B) Eyes of wild-type and *PR-Set7* partial loss-of-function flies. Note the smaller eye size in irregular organization of the mutant eye.

In fission yeast, *Schizosaccharomyces pombe*, the *Suv9* gene has been shown to be responsible for methylation of histone H4-K20. No role for the gene in control of gene expression could be demonstrated; rather, lack of H4-K20 methylation results in a defect in double-strand break repair and does not seem to affect mitosis (Sanders et al. 2004). It remains to be seen whether mono-, di- or trimethylation of histone H4-K20 functions in double-strand break repair in *Drosophila*.

Materials and methods

Isolation of PR-Set7 complete loss of function allele

The existing *l(3)neo41* stock is homozygous lethal at the second instar, but only lethal at the late pupal stage over *Df(3R)red31*, presumably because of the existence of an unknown lethal mutation on the *l(3)neo4* chromosome. Fly stock #15761-PR-Set7^{EY04668} was obtained from Bloomington Stock Center (Supplementary Fig. S1; Spradling 1986). To study PEV, fly stocks bearing *w⁺* transgenes inserted into centromeric or telomeric heterochromatin on the second, third, or fourth chromosome (Cryderman et al. 1998, 1999) were crossed with PR-Set7²⁰/TM3Sb or *Df(3R)red31/TM6Tb*. The eye color of F1 animals, bearing a transgene and also heterozygous for the PR-Set7 mutation, the deficiency, or a balancer chromosome was determined reading the OD 480 of an extract of 10 fly heads (Ashburner 1989).

Antibodies and Western blots

Recombinant his-tagged *Drosophila* PR-Set7 was expressed in *Escherichia coli*, purified under native conditions according to the manufacturer's protocol (QIAGEN), and used for raising polyclonal antibodies in rats. The specificity of the antibodies was checked by Western blots and the staining of wild-type and PR-Set7²⁰ tissues. Western blot analysis was performed as described (Whalen and Steward 1993). Antibodies were used in the following dilutions: α -Dm-PR-Set7 rat polyclonal 1:500; α -H4-K20 mono-, di-, and trimethyl, all rabbit polyclonal obtained from Upstate, 1:1000.

Salivary gland chromosome preparations and antibody staining

Salivary glands of third instar larvae were dissected, and polytene chromosomes were isolated and mounted on slides as described (Lis et al. 2000). Polytene chromosomes were stained with 1:100 dilution of mouse monoclonal α -Pol II antibody (Covance H5), α -Dm-PR-Set7 rat polyclonal 1:100, α -H4-K20 mono- and dimethyl rabbit polyclonal 1:100, α -H4-K20 trimethyl rabbit polyclonal (Abcam) 1:100.

Antibody staining of tissues

Antibody staining of *Drosophila* ovaries and embryos was performed as described (Whalen and Steward 1993). The CNS of third instar larvae were dissected, and mitotic chromosomes prepared as described (Ashburner 1989). Staining was visualized using a Zeiss Axioplan 2 microscope and an AxioCam HRm digital camera.

Imaginal discs of third instar larvae were stained with Hoechst. Measurements of fluorescence were done on a Zeiss Axioplan 2 microscope and AxioCam HRm digital camera. Pictures were analyzed with the Image Pro Plus imaging software. The relative units represent the intensity of fluorescence present in a defined field under identical exposures (Supplementary Fig. S2).

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