Epigenetic regulation of facultative heterochromatinisation in *Planococcus citri* via the Me(3)K9H3-HP1-Me(3)K20H4 pathway

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Summary

Using RNA interference (RNAi) we have conducted a functional analysis of the HP1-like chromobox gene *pchet2* during embryogenesis of the mealybug *Planococcus citri*. Knocking down *pchet2* expression results in decondensation of the male-specific chromocenter that normally arises from the developmentally-regulated facultative heterochromatinisation of the paternal chromosome complement. Together with the disappearance of the chromocenter the staining levels of two associated histone modifications, *tri*-methylated lysine 9 of histone H3 [Me(3)K9H3] and *tri*-methylated lysine 20 of histone H4 [Me(3)K20H4], are reduced to undetectable levels. Embryos treated with double-stranded RNA (dsRNA) targeting *pchet2* also exhibit chromosome abnormalities, such as aberrant chromosome condensation, and also the presence of metaphases that contain 'lagging' chromosomes. We conclude that PCHET2 regulates chromosome behavior during metaphase and is a crucial component of a Me(3)K9H3-HP1-Me(3)K20H4 pathway involved in the facultative heterochromatinisation of the (imprinted) paternal chromosome set.

Supplementary material available online at
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Introduction

Early work on *Coccidae*, especially the mealybug *Planococcus citri* and on the fungus gnat *Sciara coprophila*, showed that reciprocal crosses are not always equivalent (Brown, 1966; Crow, 2000). In male mealybug embryos an entire haploid set of paternal chromosomes becomes facultatively heterochromatinised and genetically inert at the early cleavage (blastoderm) stage of development; in females both parental sets remain euchromatic and active (Hughes-Schrader, 1948; Brown and Nur, 1964; Chandra and Brown, 1975). In *Sciaridae*, a similar phenomenon has been observed during late blastoderm, just before cellularization, where the paternal X chromosomes are selectively eliminated in male embryos (reviewed by Metz, 1938). Cytological study of X:autosome translocations has mapped the controlling element regulating this selective elimination to heterochromomere II adjacent to the X-centromere, indicating that X-centromere function might be affected by the adjacent heterochromatin by a spreading effect (Crouse, 1960a; Crouse, 1960b) (reviewed in John, 1988).

Although seen as curiosities in their time, this early work by Crouse (Crouse, 1960a; Crouse, 1960b) quite accurately anticipated work in mammals where the relationship between heterochromatin and parent-of-origin effects (chromosome imprinting) was found to exist (Lyon and Rastan, 1984). For example, imprinted facultative heterochromatinisation of the paternal X chromosome occurs in all tissues of marsupials (Cooper, 1971). In the mouse, paternal X-chromosome inactivation occurs during early pre-implantation embryogenesis (Krietsch et al., 1982) and is maintained in the trophectoderm (Takagi and Sasaki, 1975) but is reversed in the inner cell mass before random X-inactivation in the embryo proper (Mak et al., 2004; Okamoto et al., 2004). More recent work on epigenetic silencing of imprinted genes reveals similarities to imprinted X-chromosome inactivation. For example, inactivation of the paternal *H19* gene results in the formation of an inactive heterochromatin-like domain that is inimical to gene expression (Banerjee et al., 2000). Despite these similarities between imprinting in insects and mammals, especially at the level of cytologically visible heterochromatin, no unifying mechanism has yet been identified. In mammals, CpG DNA methylation has been found to be important for silencing of imprinted genes, X-chromosome inactivation and genome stability (Li et al., 1993; Panning and Jaenisch, 1996; Gaudet et al., 2003). However, in mealybugs, CpG DNA methylation does not appear to play a role in the silencing of paternal chromosome set because paternally derived chromosomes are hypomethylated at CpG dinucleotides with respect to maternal chromosomes in both male and female embryos (Bongiorni et al., 1999).

Previously, we have explored the possibility that another, more atavistic, mechanism may underpin imprinting phenomena, namely epigenetic gene regulation involving the HP1 class of non-histone proteins (James and Elgin, 1986;

Singh et al., 1991). HP1 proteins are highly conserved and play a role in gene silencing in organisms as diverse as fission yeast (with no DNA methylation) and mice (Singh and Georgatos, 2002). In fission yeast, targeting to and assembly of heterochromatin that contains HP1 at specific genomic sites involves components of the RNA interference pathway and this involvement is likely to be conserved in higher organisms (reviewed in Grewal and Elgin, 2002). Indeed, inactivation of the X-chromosome in mammals requires the expression of X (inactive)-specific transcript gene (*Xist*) (Penny et al., 1996; Lee and Jaenisch, 1997), although no link between *Xist* expression and HP1 recruitment has so far been demonstrated. HP1s are also an activator of gene expression, with some genes requiring an HP1-containing heterochromatic environment for correct expression (Hearn et al., 1991; Lu et al., 2000); other euchromatic genes require HP1s for stabilisation of their elongating transcripts (Piacentini et al., 2003; Vakoc et al., 2005).

Studies of the molecular mechanisms by which HP1s mediate changes in chromatin structure (and therefore changes in gene expression) have focussed on the interaction of the HP1 chromodomain with *tri*-methylated lysine 9 of histone H3 (Me(3)K9H3). Me(3)K9H3 results from the activity of Suv39 histone methyl transferases (HMTases) (Rea et al., 2000) and is thought to form a binding site for HP1s. Structural analysis has shown that the Me(3)K9H3 histone tail inserts itself into the binding groove of the HP1 chromodomain (Nielsen et al., 2002). This interaction is relatively weak (association constant in the μM range) and highly dynamic (Cheutin et al., 2003; Festenstein et al., 2003). Binding of HP1s to Me(3)K9H3 also appears to be part of an epigenetic cascade in mammals: HP1s bound to Me(3)K9H3 recruits a K20H4 HMTase that *tri*-methylates Lys20 on histone H4 (Me(3)K20H4) (Kourmouli et al., 2004; Kourmouli et al., 2005; Schotta et al., 2004). The pathway from Me(3)K9H3 to Me(3)K20H4 via HP1s is thought to be important for the assembly of HP1-containing heterochromatin and gene silencing (Kourmouli et al., 2004; Schotta et al., 2004), but its role in HP1-regulated gene activation is unclear.

We have recently described the distribution of an HP1-like protein and the two associated histone modifications, Me(3)K9H3 and Me(3)K20H4, in the mealybug *P. citri*. Using an antibody against *Drosophila* HP1 (C1A9 antibody) (James and Elgin, 1986), we showed that a protein of similar mass (29 kD) and that shares the *Drosophila* HP1 epitope was preferentially associated with the paternal, male-specific, heterochromatic chromocenter in *P. citri* embryos, and that this distribution colocalised with Me(3)K9H3 and Me(3)K20H4 staining (Bongiorni et al., 2001; Bongiorni and Prantera, 2003; Cowell et al., 2002; Kourmouli et al., 2004). By contrast, acetylation of histone H4 (AcH4; a marker of gene activity) was found to be absent on the male-specific heterochromatic chromocenter (Ferraro et al., 2001). Interestingly, a recent study on the inactivation of the human X-chromosome has shown a similar colocalisation of HP1 with Me(3)K9H3 and Me(3)K20H4 histone modifications (Chadwick and Willard, 2004); AcH4 of the mammalian X chromosome is known to be depleted on the inactive X chromosome (Jeppesen and Turner, 1993). These data have led to the suggestion that the Me(3)K9H3-HP1-Me(3)K20H4 pathway is an evolutionarily conserved mechanism for epigenetically silencing large chromosomal domains by facultative heterochromatinisation (Chadwick and Willard, 2004).

In this paper, we explore the relationship of the Me(3)K9H3-HP1-Me(3)K20H4 pathway to facultative heterochromatinisation in *P. citri* embryos. To that end, we have investigated the effect that interference of expression of the *P. citri* HP1-like protein PCHET2 has on chromosome behaviour, and Me(3)K9H3 and Me(3)K20H4 histone modifications. The *pchet2* cDNA was isolated by low-stringency hybridisation using the *Drosophila* HP1 chromobox as a probe (Epstein et al., 1992). Using double-stranded RNA interference (dsRNAi) (Fire et al., 1998) we show that knocking down *pchet2* expression in embryos leads to loss of C1A9 staining concomitant with a loss of staining for both Me(3)K9H3 and Me(3)K20H4, and the generation of abnormal cytological morphologies of heterochromatin and chromosomes. These data confirmed that *pchet2* encodes the HP1-like protein responsible for the epitope recognised by C1A9 antibody. Consistent with previous data, that Me(3)K9H3 is the primary epigenetic histone modification associated with heterochromatin (Kourmouli et al., 2004; Kourmouli et al., 2005), we found that Me(3)K9H3 remains associated with decondensed chromocenters in nuclei of the gut and the Malphigian tubules, which undergo developmental reversal of heterochromatinisation (Brown and Nur, 1964; Nur, 1967); HP1 and Me(3)K20H4 in the same nuclei are either dispersed or absent, respectively.

Our data provide evidence that the Me(3)K9H3-HP1-Me(3)K20H4 pathway is likely to be involved in parent-of-origin-specific facultative heterochromatinisation in mealybugs. We suspect that this finding will have wider use and be applicable to other organisms where such imprinting phenomena have been described, including yeast, insects and mammals.

Results

HP1 and histone tail post-translational modifications during facultative (de-) heterochromatinisation

Facultative heterochromatinisation in the nuclei of male mealybugs does not occur simultaneously in all cells of the embryo but takes place as a wave beginning at one end of the mid-cleavage embryo spreading to the other (Bongiorni et al., 2001). To investigate the expression and colocalisation of HP1 with the Me(3)K9H3 and Me(3)K20H4 histone modifications during this wave of facultative heterochromatinisation we stained mid-cleavage male embryos with the C1A9 monoclonal antibody and specific antibodies against Me(3)K9H3 (Cowell et al., 2002; Tamaru et al., 2003; Ringrose et al., 2004) and Me(3)K20H4 (Kourmouli et al., 2004). Fig. 1A shows a region of a male mid-cleavage embryo where a wave of facultative heterochromatinisation is spreading from the bottom left hand corner to the top right hand corner. Within this region we have focused on two sectors (labeled B and C) that are shown magnified in Fig. 1B,C respectively. In Fig. 1B facultative heterochromatinisation of the paternal chromosomal set has taken place in most nuclei and the chromosomes have gathered together to form bright, DAPI-positive chromocenters. These nuclei also show bright staining with the C1A9 antibody (Fig. 1B') and an antibody against Me(3)K9H3 (Fig. 1B''); the merged image reveals colocalisation of the DAPI-positive chromocenters with C1A9

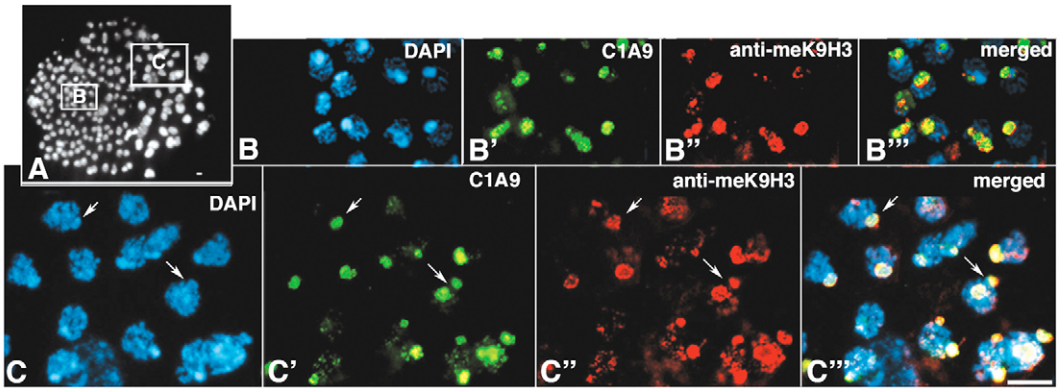

Fig. 1. Localisation of C1A9 and Me(3)K9H3 antibodies to nuclei in mid-cleavage embryos (128-256-nuclei embryos) undergoing facultative heterochromatinisation. (A) Embryo region where the wave of facultative heterochromatinisation is spreading from the bottom left corner to the top right corner. Boxed area B shows nuclei that have completed heterochromatinisaton and contain DAPI-positive chromocenters (see magnified image in B), whereas boxed area C shows nuclei still undergoing heterochromatinisation, many of which have no overt DAPI-positive chromocenters (see arrows pointing to nuclei in C). The nuclei in B are labeled with C1A9 antibody (B') and with the anti-Me(3)K9H3 antiserum (B'') and the merged image in B''' shows colocalisation of DAPI-positive chromocenter with C1A9 and Me(3)K9H3 staining. The DAPI-stained nuclei in C were stained with C1A9 antibody (C') and with the anti-Me(3)K9H3 antiserum (C''). Although the pattern of Me(3)K9H3 staining is more spread out in these nuclei compared with those that have completed heterochromatinisation (see C'' and B'') the merged image in C''' shows that the Me(3)H9H3 pattern largely colocalises with C1A9 staining. Bars, 10 μm.

staining and staining against Me(3)K9H3 (Fig. 1B'''). The nuclei in Fig. 1C show a section of the embryo that has yet to complete facultative heterochromatinisation. In many nuclei, the male-specific chromocenter (arrows in Fig. 1C) is not obvious. However, staining of the nuclei shows that the patterns of C1A9 (Fig. 1C') and Me(3)K9H3 (Fig. 1C'') are still localised together in all nuclei, even in those that have yet to form recognisable DAPI-positive chromocenters (see merged image in Fig. 1C''' and arrows in 1C). This indicates that Me(3)K9H3 localisation to the presumptive chromocenter is an important determinant for chromocenter formation. Consistent with this, we found that staining of male nuclei that have undergone the reversal of heterochromatinisation (Fig. 2A) still show strong focal staining of Me(3)K9H3 over the decondensed chromocenter (Fig. 2B,D). By contrast, the C1A9 staining is grainy and dispersed, indicating that the C1A9 antigen is delocalised during de-heterochromatinisation (Fig. 2C and merged image in Fig. 2E).

Staining of mid-blastoderm embryos with the anti-Me(3)K20H4 antibodies revealed a picture similar to that found for Me(3)K9H3 (Fig. 1B-B'''). In sectors of the embryo where DAPI-positive chromocenters (Fig. 3A) had formed, Me(3)K20H4 (Fig. 3A'') was found to colocalise with both C1A9 (Fig. 3A') and DAPI staining (see merged image in Fig. 3A'''). In regions of the embryo where chromocenter formation was ongoing (Fig. 3B), Me(3)K20H4 (Fig. 3B'') colocalised with C1A9 staining (Fig. 3B'; see merged image in Fig. 3B'''). However, in adult gut, Me(3)K20H4 staining of nuclei that underwent developmental reversal of heterochomatinisation (Fig. 3C), showed a picture very different to that found with Me(3)K9H3 (Fig. 2B-D). Whereas we again found that C1A9 antibody staining was grainy and loosely associated with the decondensed chromocenter (Fig. 3C'), there was an almost complete loss of Me(3)K20H4 nuclear staining (Fig. 3C'' and merged image in Fig. 3C'''); instead, we found its distribution throughout the cytoplasm. To test that this cytoplasmic staining was not the result of non-specific staining of a cross-reactive gut protein while trying to stain against Me(3)K20H4, we set up two types of control experiments. In the first, we immunostained male gut preparations with an anti-histone H4 antibody, and found that in reverted cells the antibody signal is diffuse and distributed through the nuclei and cytoplasm (see supplementary material Fig. S1). In the second, we immunostained female gut tissues with the anti-Me(3)K20H4

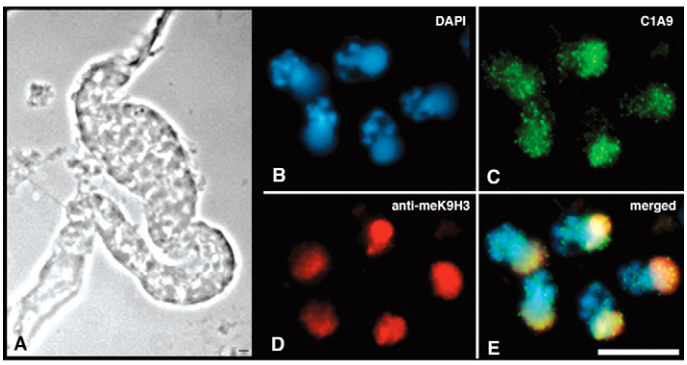

Fig. 2. C1A9 and Me(3)K9H3 staining in nuclei of cells from a tract of a male gut undergoing developmental reversal of heterochromatinisation. (A) Dissected gut of a male mealybug. (B) DAPI staining of nuclei, showing that the chromocenters of nuclei undergoing reversal of heterochromatinisation are decondensed and have lost their focal morphological appearance. (C,D) C1A9 staining of these nuclei is grainy and dispersed over the nucleus (C), whereas Me(3)K9H3 is still concentrated over the decondensed chromocenter (D). (E) Merged image. Bars, 10 μm.

antibody and found no evidence of a cytoplasmic signal (see supplementary material Fig. S2). These findings strongly indicate that the cytoplasmic immunostaining we observed in cytological preparations of male gut stained with anti-Me(3)K20H4 antibody is not an arte-fact but the result of H4 displacement and/or degradation.

pchet2 regulates male-specific facultative heterochromatinisation

We decided to investigate the role played by the mealybug HP1-like protein in the male-specific developmental facultative heterochromatinisation of the paternal chromosome set. To identify the HP1-like gene whose product is recognised by the C1A9 monoclonal antibody (mAb) we decided to amplify HP1-like sequences from *P. citri* genomic DNA by using redundant PCR primers deduced from known HP1 cDNA sequences (see Materials and Methods). Using this strategy we consistently amplified one fragment from *P. citri* genomic DNA which, when sequenced, corresponded to the *pchet2* cDNA that had been isolated in a previous screen for *P. citri* HP1-like cDNAs (Epstein et al., 1992). To determine whether this was the antigen recognised by the C1A9 mAb we used the *pchet2* cds in RNA interference (RNAi) experiments. Accordingly, embryos at various stages of development were released from dissected gravid females and soaked in either a solution containing dsRNA targeting *pchet2* or two control solutions containing no interfering RNA or dsRNA targeting *pchet1*.

We took partial or complete loss of C1A9 immunostaining of male-specific chromocenters as indicative of successful interference of gene expression. The effect of the *pchet2* RNAi was also confirmed by western blotting; reduction in PCHET2 protein was observed in whole-cell extracts from embryos treated with dsRNA targeting *pchet2* (see Fig. S3 in supplementary material). On average, 35% of *pchet2*-dsRNA-treated blastoderm male embryos showed the loss of C1A9 staining in all or most of nuclei ($P<0.0001$ compared with mock-treated embryos). As shown in Fig. 4, in embryos treated with dsRNA targeting *pchet2*, where no immunostaining of C1A9 was seen (Fig. 4A''), dramatic changes in chromatin organisation were observed. DAPI staining of male embryos after 4 hours of RNAi showed an almost complete absence of the male-specific chromocenter (compare Fig. 4A and Fig. 4B). Male embryos treated this way could only be distinguished from female embryos by the presence of faintly differentiated chromatin regions – remnants of former chromocenters (arrow in Fig. 4A'). By sharp contrast, mock RNAi-treated embryos showed typical DAPI-positive and C1A9-positive chromocenters (Fig. 4B,B',B''). In addition, embryos treated with *pchet1*-dsRNA (same size RNA as *pchet2* and a related HP1 family member) showed neither morphological changes in the chromocenter nor changes in C1A9 staining (Fig. S4 in supplementary material) indicating that the effects are particular to *pchet2* dsRNA.

A more detailed time course experiment revealed that, even 2 hours after soaking in *pchet2* dsRNA, male cleavage-stage nuclei are affected; nuclei showed weak, grainy staining with the C1A9 antibody (Fig. 5A') and a relatively normal chromocenter (Fig. 5A). Mitotic cells were particularly vulnerable to the effects of *pchet2* dsRNA and appeared to have lost almost all C1A9 staining by 2 hours (Fig. 5B'). After 4 hours of exposure to *pchet2* dsRNA, no C1A9 staining was observed (Fig. 5C',D') and only remnants of the chromocenter were seen (arrows in Fig. 5C,D). Importantly, after 4 hours of exposure male nuclei were also negative for Me(3)K9H3 (Fig. 5C'') and Me(3)K20H4 staining (Fig. 5D''). Thus, interference with *pchet2* expression in cleavage-stage embryos results in the loss of heterochromatic characteristics of the male-specific chromocenter: loss of PCHET2 protein, loss of heterochromatin-associated histone modifications and loss of

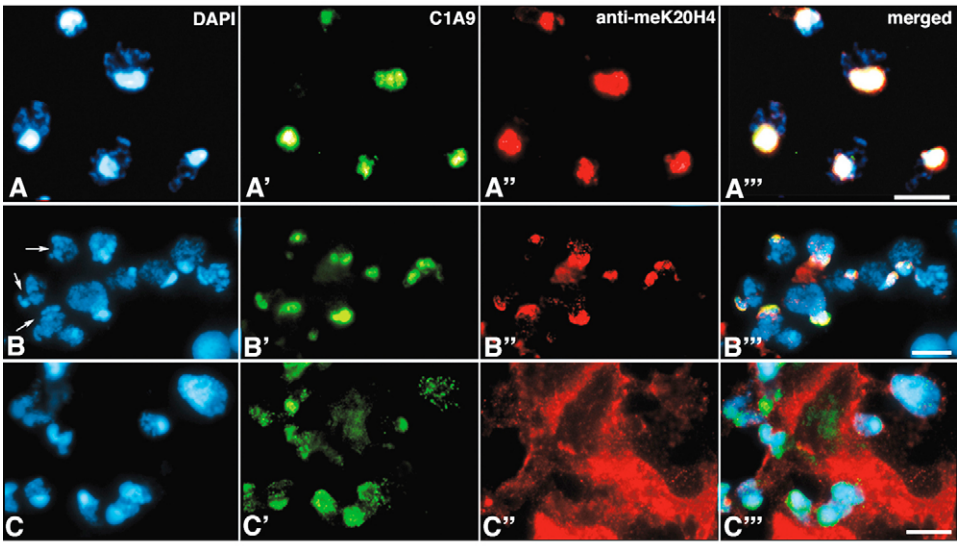

Fig. 3. Localisation of Me(3)K20H4 to nuclei either undergoing facultative heterochromatization or developmental de-heterochromatinisation. (A) DAPI-stained nuclei from a mid-cleavage stage embryo (128-256-nuclei embryo) that has undergone facultative heterchromatinisation. A clear, strongly-staining, chromocenter can be seen in each nucleus. The nuclei shown in A were labeled with C1A9 antibody (A') and with the anti-Me(3)K20H4 antiserum (A''); the merged image in A''' shows co-incidence of DAPI-positive chromocenters with C1A9 and Me(3)K20H4 staining. The nuclei in B are from another region of the same embryo that has yet to complete heterochromatinisation and several of them have no overt DAPI positive chromocenters. The DAPI-stained nuclei in B were stained with C1A9 antibody (B') and with the anti-Me(3)K20H4 antiserum (B''). Whereas the Me(3)K20H4 staining is more spread out in these nuclei compared with those that have completed heterochromatinisation (see B'' and A'') the merged image (B''') shows that the Me(3)H20H4 pattern largely colocalised with C1A9 staining. (C) DAPI-stained nuclei taken from cells of adult tissues that undergo developmental reversal of heterochromtinisation. C1A9 staining is dispersed and has a grainy appearance over the nucleus (C'). There is no staining of Me(3)K20H4 in the nucleus; rather, Me(3)K20H4 is found in the cytoplasm (C''). (C''') Merged image. Bars, 10 μm.

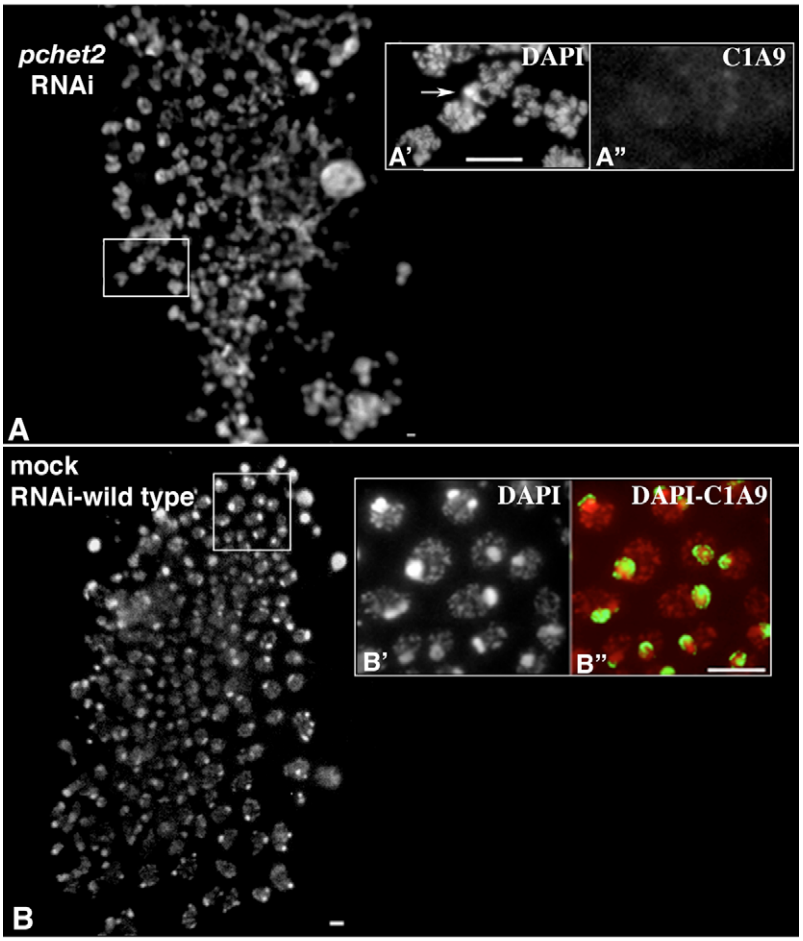

Fig. 4. Mid-cleavage male embryos treated for 4 hours with *pchet2* dsRNA exhibit a dramatic loss in facultative heterochromatinisation of the paternal chromosome set. (A) DAPI staining of a 128-256-nuclei male embryo treated for 4 hours with *pchet2* dsRNA showing a loss of strongly staining DAPI-positive chromocenters. These male embryos can only be distinguished from female embryos because of male-specific chromocenter remnants (arrow in A'). (A") C1A9 staining is not detectable in these embryos. (B-B") In control mock-treated embryos typical, bright, DAPI-positive chromocenters can be seen (B and B') that are also positive for C1A9 staining (merged image in B"); DAPI staining (red), C1A9 staining (green). Bars, 10 μm.

chromocenter morphology. Importantly, the lack of Me(3)K9H3 (Fig. 5C") was not due to a reduction in the protein levels, because western blot analysis revealed no difference in Me(3)K9H3 levels between mock- and dsRNA-treated embryos (see supplementary material Fig. S5). This indicates that the loss of PCHET2 protein at cleavage stage is crucial for nuclear and chromosomal organization (see chromosomes from *pchet2* dsRNA-treated embryos in Fig. 6), which is reflected in epitope-masking of Me(3)K9H3 (Fig. 5C") in *pchet2*-dsRNA-treated embryos. The loss of Me(3)K20H4 staining (Fig. 5D") correlates with a loss of protein levels, as determined by western blotting (see supplementary material Fig. S5).

Cleavage-stage embryos are particularly sensitive to *pchet2*-RNAi treatment; embryos at later stages appeared refractory to *pchet2* dsRNA treatment. When S-shaped (gastrula) male embryos were exposed to *pchet2* dsRNA for 4 hours, the effect was minimal: there was no effect on chromocenter morphology and staining with either C1A9 antibody or anti-Me(3)K9H3 and anti-Me(3)K20H4 antibodies was unchanged (not shown).

The analysis of mitotic chromosomes from dsRNA-treated cleavage-stage embryos also revealed sex-independent structural defects (Fig. 6A-C) with respect to mock-treated normal-shaped chromosomes (Fig. 6F). We observed failure of proper chromosome condensation, with chromosomes exhibiting a string-like appearance (Fig. 6A) and the presence of chromosome fragments in many metaphases (arrows in Fig. 6B,C). We also observed a significant number of abnormal metaphases (approximately 36% per embryo, $P<0.01$, with respect to mock-treated embryos), which contained one or two chromosomes displaced from the metaphase plate (arrows in Fig. 6D,E).

Discussion

Facultative heterochromatinisation is phyogenetically conserved and represents the paradigm of how chromosomes and chromosome regions are epigenetically changed in their expressibility during development (Brown, 1966; Lyon and Rastan, 1984). Insights into conserved mechanisms that are likely to underpin facultative heterochromatinisation have been aided by the current interest in post translational modifications of histones, the histone code (Jenuwein and Allis, 2001; Turner, 2005). Recent work has shown that two histone modifications, Me(3)K9H3 and Me(3)K20H4, are part of an epigenetic pathway – the Me(3)K9H3-HP1-Me(3)K20H4 pathway – for heterochromatin assembly (Chadwick and Willard, 2004; Kourmouli et al., 2004; Kourmouli et al., 2005; Schotta et al., 2004) (see Introduction). Here, we have undertaken functional in vivo experiments to investigate the role of this epigenetic pathway in facultative heterochromatinisation.

Me(3)K9H3 appears to be the primary determinant of facultative heterochromatinisation in the mealybug. Me(3)K9H3 localises to the presumptive chromocenter during the wave of facultative heterochromatinisation in mid-cleavage embryos, as does Me(3)K20H4 (Figs 1 and 3). However, unlike Me(3)K20H4, Me(3)K9H3 is robust and remains associated with the decondensing chromocenters in male nuclei that undergo developmental reversal of heterochromatinisation (Fig. 2D). By contrast, the Me(3)K20H4 staining in these nuclei is strikingly different: Me(3)K20H4 is excluded from the nucleus and localises to the cytoplasm (Fig. 3C" and merged image in 3C"'). The absence of Me(3)K20H4 staining in the nucleus indicates that either the Me(3)K20H4 modification is removed by potential demethylases and/or the histone itself is removed by a histone replacement mechanism (Ahmad and Henikoff, 2002). The loss of the Me(3)K20H4 from the nucleus is, we suggest, one of the first steps in

disassembling the chromocenter before its complete decondensation. The cytoplasmic staining might reflect the accumulation of the Me(3)K20H4 histone that has been replaced and/or that the Me(3)K20H4 HMTase becomes excluded to the cytoplasm where it tri-methylates K20 on newly synthesised H4 histone.

Identification of *pchet2* as the HP1-like gene in mealybug enabled us to investigate the role played by HP1 in the Me(3)K9H3-HP1-Me(3)K20H4 pathway. As with Me(3)K9H3 and Me(3)K20H4, PCHET2 localises to the paternal chromosomes in male embryos before the overt morphological appearance of the chromocenter and gave us our first indication that PCHET2 is likely to be required for chromocenter formation (Fig. 1). During developmental de-heterochromatinisation PCHET2 becomes delocalised and has a grainy appearance over the decondensing Me(3)K9H3-positive chromocenter (Fig. 2C). Clearly, Me(3)K9H3 is not the sole factor that maintains the tight localization of PCHET2 to the heterochromatic chromocenter. The delocalization of PCHET2 during developmental de-heterochromatinisation coincides with the loss of Me(3)K20H4 staining and we strongly suspect that these events are related because it is known that the HP1 proteins recruit the K20H4 HMTrimethylases to heterochromatin (Kourmouli et al., 2004; Kourmouli et al., 2005; Schotta et al., 2004). Indeed, the de-heterochromatinisation of the male-specific heterochromatin in mealybugs appears to be the reverse of the epigenetic pathway described in mammals (see Introduction) – loss of Me(3)K20H4 is concomitant with HP1 delocalisation, with the robust Me(3)K9H3 methylation remaining on the decondensing chromocenter. This reversal of the Me(3)K9H3-HP1-Me(3)K20H4 epigenetic pathway might be conserved and operate in mammals when the inactive X chromosome is de-heterochromatinised (reprogrammed) in developing oocytes (Gartler and Goldman, 1994). Additionally, in mealybugs, it will be of interest to see whether the robust Me(3)K9H3 is the imprint carried into the fertilised egg by the sperm and whether it then nucleates the facultative heterochromatinisation that takes place at the mid-blastoderm stage.

After 4 hours of exposure to *pchet2* dsRNA we observed dramatic effects in mid-blastoderm embryos that are undergoing facultative heterochromatinisation (Figs 4, 5). The chromocenter was almost completely decondensed making it difficult to distinguish between male and female embryos (compare Fig. 4A and 4B), and we could detect neither Me(3)K9H3 nor Me(3)K20H4. The loss of Me(3)K9H3 staining in the *pchet2* RNAi-treated embryos is consistent with the model where K9H3 trimethylation spreads and is maintained at the chromocenter through the recruitment of a H3-specific HMTase by HP1 (Schotta et al., 2002). The loss of the Me(3)K20H4 staining is likewise explained by the lack of recruitment of a K20H4 HMTase through the absence of PCHET2 (Kourmouli et al., 2004; Kourmouli et al., 2005; Schotta et al., 2004). However, the failure to detect changes in Me(3)K9H3 protein levels by western blotting in embryos treated with *pchet2* dsRNA (supplementary material Fig. S5) indicates that the antibody epitope of Me(3)K9H3 in *pchet2*-interfered embryos becomes masked and inaccessible. By contrast, Me(3)K20H4 protein levels are reduced in *pchet2*-dsRNAi-treated embryos compared with wild-type (supplementary material Fig. S5). This epitope-masking effect of the *pchet2* dsRNA on Me(3)K9H3 indicates that

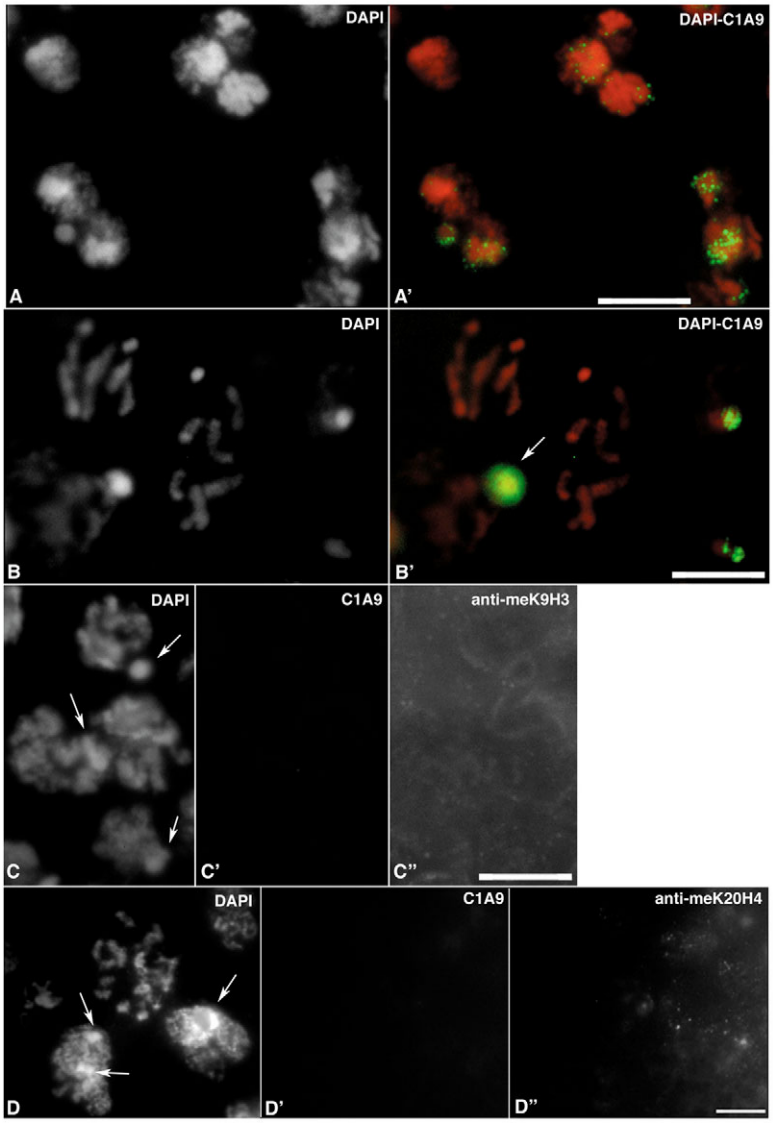

Fig. 5. *pchet2* dsRNA has an effect after 2 hours and mitotic chromosomes are particularly sensitive to the treatment. (A) DAPI staining of a 128-256-nuclei male embryo treated for 2 hours showing a decondensing chromocenter and detectable C1A9-staining that is becoming dispersed and more grainy in appearance (A'). Mitotic chromosomes show a marked reduction in C1A9 staining. (B,B') Both mitotic chromosomes and an interphase nucleus are stained with DAPI and C1A9, respectively. In B' the mitotic chromosomes are not stained with C1A9, whereas the interphase nuclei still possess a C1A9-positive chromocenter (arrow in B'). After 4 hours the chromocenters have been almost completely disassembled, with only remnants remaining (arrows in C and D). The remnants did not stain with C1A9 (C',D') or Me(3)K9H3 (C''), or with Me(3)K20H4 (D''). Bars, 10 μm.

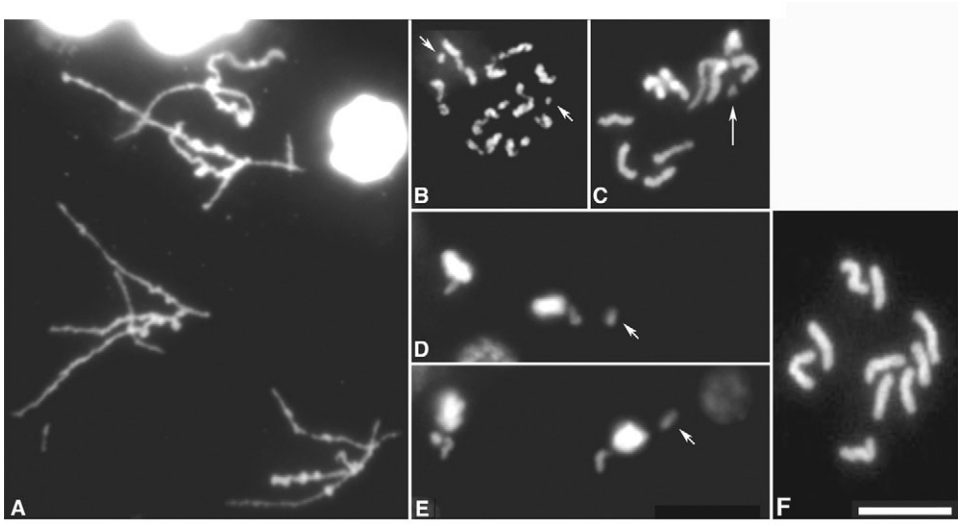

Fig. 6. *pchet2*-dsRNA-treated embryos of both sexes exhibit defects in chromosome condensation and integrity as well as segregation defects. (A-E) DAPI-stained nuclei from *pchet2*-dsRNA-treated 128-256-stage embryos often possess highly elongated chromosomes that result from incomplete condensation. Mitotic figures also contain 'dot' chromosomes (arrows in B and C). *pchet2*-RNA-treated embryos exhibit segregation defects, showing chromosomes that are displaced from the metaphase plate (arrows in D and E). For comparison, a normal metaphase from mock-treated embryos is shown in F. Bar, 10 μm.

PCHET2 might be the primary determinant in organising heterochromatin in mid-cleavage male embryos and loss of PCHET2 in *pchet2*-interfered embryos results in a catastrophic loss of genome integrity. This result changes the emphasis of the role of HP1 in the Me(3)K9H3-HP1-Me(3)K20H4 pathway to one that is primary rather than seconday and intermediary.

Mid-cleavage male embryos are particularly sensitive to exposure to *pchet2* dsRNA (Figs 4, 5) because later gastrula-stage embryos are resistant to treatment with *pchet2* dsRNA (data not shown). We suspect that this is because the blastoderm stage of development represents a particularly sensitive time for heterochromatin formation. The nuclei are rapidly dividing in the egg syncytium before cellularisation of the embryo, and large amounts of heterchromatin proteins, which may be limiting in quantity, are required as the wave of facultative heterochromatinisation proceeds across the embryo (Bongiorni et al., 2001; Bongiorni and Prantera, 2003). Changes in the level of PCHET2 at this time could dramatically inhibit the proper assembly of the paternal chromosomes into heterochromatin and thereafter into the chromocenter. A similarly sensitive stage in development was identified in *Drosophila* many decades ago (reviewed in Spofford, 1976). Heat shocking *Drosophila* embryos at the mid- to late-blastoderm stage (when cell division rate is high and heterochromatin is first observed in nuclei) results in suppression of position-effect variegation (PEV). Heat shock during gastrulation, when cell division has slowed down and the requirement for heterochromatin proteins has decreased, has little effect on PEV (Singh, 1994).

pchet2-dsRNA-treated embryos also exhibit features of genomic instability that are independent of the sex of the embryo (Fig. 6). Chromosomes from *pchet2*-dsRNA-treated embryos showed aberrant condensation (Fig. 6A-C). Some were highly elongated and string-like (Fig. 6A), whereas other appeared like dots that may have resulted from drastic undercondensation of interstitial chromosomal material or from fragmentation of chromosomes (arrows in Fig. 6B,C). One family of proteins that might be affected by the treatment is the structural maintenance proteins (SMC), which regulate various aspects of chromosome condensation and chromosome segregation (Losada and Hirano, 2005). Indeed, our observation that in a significant number of metaphase plate some chromosomes show a delay in and/or a precocious escape from congregation (arrows in Fig. 6D,E), is indicative of an effect on a sub-class of SMC proteins, the cohesins. Cohesins are required to maintain sister chromatid cohesion for proper disjunction at metaphase (Bernard and Allshire, 2002). Detailed studies of the HP1-like protein in fission yeast, SWI6, show that mutant yeast exhibit chromosome segregation defects (Ekwall et al., 1995) that result from the loss of cohesin subunit Psc3 from heterochromatin (Nonaka et al., 2002). In *Drosophila*, HP1-null mutant embryos exhibit a lagging chromosome phenotype at anaphase (Kellum and Alberts, 1995). Whereas this phenotype was originally ascribed to chromosome undercondensation, it now seems more likely to be a defect in telomere capping resulting in ectopic telomere-telomere associations (Fanti et al., 1998; Cenci et al., 2003). However, we have not observed any loss of telomere-capping function in *pchet2*-dsRNA-treated mealybug embryos. Therefore, it remains an open question whether the release of cohesin subunits from the holocentric mealybug chromosomes or their undercondensation results in the metaphase-plate anomalies seen in *pchet2* RNAi-treated embryos.

In conclusion, we have shown that, in mealybugs, the HP1-like protein PCHET2 is required for facultative heterochromatinisation of the paternal chromosome set in male mealy bugs. It is a crucial component of the Me(3)K9H3-HP1-Me(3)K20H4 pathway because loss of PCHET2 results in the loss of staining of both Me(3)K9H3 and Me(3)K20H4; the former is likely to be due to epitope masking whereas the latter is due to reduced Me(3)K20H4 histone. It will be of great interest to investigate X-chromosome inactivation in mice carrying mutations of *Hp1* genes and the effect of these mutations on the Me(3)K9H3-HP1-Me(3)K20H4 pathway.

Materials and Methods

Mealybug cultures

The mealybug *Planococcus citri* (Homoptera, Coccoidea) was raised in our laboratory on sprouting potatoes at 26°C inside glass food containers covered with gauze. The potatoes were kept in the dark to sprout for 1 month before use.

Immunofluorescence microscopy

Chromosome spreads from embryos were obtained as previously described

(Bongiorni et al., 1999; Bongiorni et al., 2001). Chromosome spreads from adult male tissues were obtained by fixing male third-instar larvae in Bradley-Carnoy solution. Fixed males were then dissected in a drop of 45% glacial acetic acid on siliconised coverslips and squashed on microscope slides. Slides were frozen in liquid nitrogen and the coverslips popped off with a razor blade (Bongiorni et al., 2004). Immediately after preparation, slides were rehydrated in 1× PBS. The primary antibodies used were anti-HP1 mouse monoclonal C1A9 antibody (James and Elgin, 1986) kindly provided by Barbara Wakimoto (University of Washington, Seattle, WA) at 1:10 dilution, the anti-Me(3)K9H3 rabbit R2B3 antibody (Cowell et al., 2002) at 1:300 dilution and the anti-Me(3)K20H4 rabbit antibody (Kourmouli et al., 2004) at 1:300 dilution. Immunostaining was performed according to Cowell et al. (Cowell et al., 2002). The secondary antibodies used were Alexa Fluor-488-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR; 1:100 dilution) and Alexa Fluor-594-conjugated goat anti-rabbit antibody (Molecular Probes; 1:600 dilution). Slides were counterstained with 0.2 μg/ml DAPI (Boehringer, Mannheim) in 2×SSC for 5 minutes and mounted in antifade medium (DABCO, Sigma). Negative controls were obtained by incubating slides with the secondary antibodies only. Immunofluorescent preparations were observed and documented as previously described (Bongiorni et al., 1999; Bongiorni et al., 2001), using filter combinations suitable for the different fluorochromes (Chroma Technology Corp., Rockingham, VT).

Cloning of *pchet2*

Genomic DNA from *P. citri* was obtained according to Savakis and Ashburner (Savakis and Ashburner, 1985). PCR amplification of HP1-like genes from *P. citri* genome was performed using primers deduced from alignments of DNA sequences of *Hp1* from *Drosophila melanogaster* and *Drosophila virilis*, and from *pchet1* and *pchet2* sequnces from *P. citri*. The following pair of primers from the chromodomain amplified an *HP1*-like fragment: forward, 5′-AATGGAAGGGCTATSSCGA-3′; reverse, 5′-GTSRATKACCAKTYGTGGA-3′ (S, G or C; R, A or G; K, G or T; Y, C or T).

The PCR amplification of *P. citri* genome was performed using those primers and under the following conditions: 94°C for 1 minute (denaturation), 46°C for 2 minutes (annealing), 72°C for 2 minutes (polymerization). DNA fragments were extracted and purified from agarose gel using the QUIAquick extraction kit (Qiagen). The amplified genomic fragments were inserted in the cloning vector pGEM-T (pGEM-T easy kit, Promega) in the molar ratio 3:1 and cloned in *E. coli* DH5α competent cells, following the manufacturer's instructions. The sequences of amplified fragments were determined by an automatic DNA sequencer (ABI Prism). Sequence homology data were obtained by BLAST analysis (Altschul et al., 1997).

Generation of dsRNA

The *pchet2* cDNA cloned into pGEM-T vector (Promega) between the T7 and SP6 promoter sequences, was used as a template for in vitro transcription by using the RiboMAX large-scale RNA production system T7 (Promega). In this system, generation of dsRNA requires a T7 RNA polymerase promoter sequence at both 5′-ends of the antiparallel *pchet2* cDNA strands. Moreover, the transcription efficiency increases if the two T7 promoter sequences are 5′ flanked by a short DNA sequence. Consequently, we stepwise replaced the 3′ SP6 promoter with a T7 promoter by two PCR reactions, at the same time adding two short sequences at both ends of the amplicon. For the first PCR reaction we used the following primers (underlined, T7 sequence; italic, vector sequence): forward: 5′-*GACGGCCAGTGAATTG*-TAATACGA-3′; reverse 1: 5′-CACTATAGGG*TACTCAAGC*-3′. The product of this first PCR was amplified again using the following primers (underlined: T7 sequence; italic: vector sequence): forward: 5′-*GACGGCCAGTGAATTG*-TAATACGA-3′; reverse 2: 5′-CCAAGTAATACGACTCACTATAGGG-3′. The *pchet2* cDNA, together with the 5′ and 3′ T7 flanking promoters, was then amplified using the forward primer and the reverse 2 primer. The PCR product was purified, and directly used for in vitro RNA transcription (Ribomax system; Promega). The DNA template was removed after RNA synthesis adding RQ1 RNase-free DNase (1 U/μg). The RNA mixture that contains both strands of RNA were then denaturated at 65°C and allowed to anneal slowly by cooling to room temperature (Somma et al., 2002). For quality control an aliquot of each dsRNA was analysed on standard non-denaturing agarose gel to confirm the size and integrity of the dsRNA.

dsRNA treatment of mealybug embryos – RNAi experiments

The *pchet2* dsRNA was precipitated with ammonium acetate and isopropanol and then dissolved in soaking buffer (Maeda et al., 2001). Embryos were soaked in 500 μl of a 20-40 μg/ml dsRNA solution, and incubated at 26°C for 2-4 hours. Embryos were dissected and immunostained and the phenotypes were observed under a fluorescence microscope (see Bongiorni et al., 2001; Bongiorni and Prantera, 2003). The negative controls involved soaking the embryos in the buffer alone, without dsRNA. Moreover, as additional control we soaked embryos for 2-4 hours (in the same buffer at the same concentrations as above) in a solution containing a dsRNA of the same size as *pchet2* but corresponding to the cDNA of *pchet1*, a chromodomain-containing gene not involved in facultative heterochromatinisation (Epstein et al., 1992). Exposure time to the soaking solution was limited to a maximum of 4 hours because embryos do not survive for longer times after release from the mother, even in a mock-interfering solution. χ^2 analysis was applied for statistic evaluation of significance of dsRNA effects.

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