RESEARCH ARTICLE

The Polycomb group protein CRAMPED is involved with TRF2 in the activation of the histone *H1* gene

Jean-Michel Gibert · François Karch

Abstract CRAMPED (CRM), conserved from plants to animals, was previously characterized genetically as a repressive factor involved in the formation of facultative and constitutive heterochromatin (Polycomb silencing, position effect variegation). We show that *crm* is dynamically regulated during replication and identify the Histone gene cluster (*His-C*) as a major CRM target. Surprisingly, CRM is specifically required for the expression of the Histone *H1* gene, like the promoter-bound transcription factor TRF2. Consistently with this, CRM genetically interacts and co-immunoprecipitates with TRF2. However, the Polycomb phenotypes observed in *crm* mutants are not observed in *TRF2* hypomorphic mutants, suggesting that they correspond to independent roles of CRM. CRM is thus a highly pleiotropic factor involved in both activation and repression.

Introduction

Polycomb group proteins (PcG) constitute one of the best characterized groups of chromatin regulators. These silencing factors, involved in the formation and the maintenance of facultative heterochromatin, were initially identified genetically in *Drosophila* by the homeotic phenotypes that their mutations cause through the ectopic expression of *hox* genes. However, genome-wide studies have subsequently shown that *hox* genes represent only a small subset of PcG targets (Schuettengruber et al. 2009). The products of most Polycomb group genes have been shown to be components of multimeric protein complexes with particular histone-modifying activities (Klymenko et al. 2006; Nekrasov et al. 2005; Saurin et al. 2001; Schuettengruber and Cavalli 2009; Simon and Kingston 2009). These complexes are highly conserved between flies and mammals, some are even present in plants (Birve et al. 2001; Levine et al. 2002).

Genetic screens have identified many genes that positively or negatively interact with *PcG* genes. Positively interacting genes often encode members of PcG complexes. However, particular factors identified genetically as *PcG* genes have not been identified in purified PcG complexes, suggesting that they may interact only transiently with PcG complexes members. For example, *super sex combs* (*sxc*), encoding the glycosyltransferase Ogt, may be particularly limiting for Polycomb silencing because one of the Ogt targets is the PcG protein Polyhomeotic (Gambetta et al. 2009). Even components of PcG complexes can be also involved in PcG independent processes. For example, the PcG protein Pho is also a component of the chromatin remodeling complex, INO80, involved in stress-induced transcription and DNA repair (Klymenko et al. 2006). Genes negatively interacting with PcG genes are called *Trithorax* group genes. Several genes initially categorized as *PcG* genes also function as *TrxG* genes in different assays (Gildea et al. 2000). Thus, a new functional category called enhancers of Trithorax and Polycomb (ETP) has been established. Depending on circumstances, these ETP are more limiting for activation or repression (Salvaing et al. 2006).

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A few genes whose mutations genetically interact with PcG genes are still not well characterized. One of these genes is *cramped* (*crm*), a gene conserved from animals to plants (Ehsan et al. 2004; Yamamoto et al. 1997). A conserved MYB/SANT domain suggests that CRAMPED (CRM) binds DNA or histone tails (Boyer et al. 2004; Ehsan et al. 2004). Consistent with this, CRM can be detected on *Drosophila* polytene chromosomes (Gibert et al. 2007; Yamamoto et al. 1997). The *crm* mutant males die as pharate or occasionally manage to hatch, but are very weak. They have swollen aristae. In addition, they have ectopic sex combs on posterior legs and on the second tarsal segment of the first leg. The *crm* was therefore classified as a *PcG* gene (Yamamoto et al. 1997). However, CRM has not been found yet in any purified Polycomb complexes (Klymenko et al. 2006; Nekrasov et al. 2005; Saurin et al. 2001). Furthermore, *crm* mutants show several phenotypes usually not typically observed in PcG mutants (wing margin notches), suggesting that it may function independently of the classic PcG proteins. Here, we show that *crm* does indeed have PcG-independent functions and is also involved in transcription activation with the TATA binding protein (TBP)-related factor 2 (TRF2), a component of core promoter recognition complexes.

Results

In vivo cytological characterization of CRM In order to analyze the in vivo localization and dynamic of CRM, we generated CRM fluorescent fusion proteins with the fluorochromes EBFP2, mCherry or Venus (Ai et al. 2007; Nagai et al. 2002; Shaner et al. 2004). The fusion constructs were all integrated in the same genomic environment at 22A with the PhiC31 transgenesis method (Bischof et al. 2007) (with the exception of EBFP2-CRM inserted in 51D) and were expressed under the control of the Gal4 upstream activating sequence to allow conditional expression (Brand and Perrimon 1993). To verify that our CRM–fluorophore fusions are biologically active, we expressed them ubiquitously (using a Gal4-expressing line under the control of the tubulin promoter) and found that the fusion proteins rescue *crm* mutant phenotypes giving rise to flies fully viable and fertile. In vivo Venus–CRM chromosomal binding was analyzed on salivary gland polytene chromosomes, using the weak and leaky expression of *Gal4* from the *HS-Gal4* transgene in the absence of heat shock. Observation of intact salivary glands gave two major information. First, when compared to the level of RFP–H2Av, it is visible that the level of Venus–CRM varies across nuclei. (Fig. 1a, b, c). This is unlikely to be due to a non-homogenous expression of HS-Gal4. Indeed, when

expressed under the control of Hs-Gal4 in salivary glands, nuclear GFP is uniformly expressed unlike mCherry–CRM (Supplementary Fig. 1). It might reflect posttranscriptional or posttranslational regulation of Venus–CRM. Second, we distinctively observed a single bright band of Venus–CRM in some of the nuclei where the general nucleoplasmic signal is weaker (Fig. 1g–l). In many other nuclei, we observe a high level of nucleoplasmic fluorescence that obfuscates discrete sites on the chromosomal arms (Fig. 1d–f). Co-expression of a fluorescent fusion of the histone H2Av shows that the band corresponds to a chromosome section (Fig. 1g–l). This band is located in the nuclear periphery, near the chromocenter, visualized with a fluorescent fusion of HP1 (Fig. 2a–c). Due to its location and size, this bright band was reminiscent of the histone gene cluster (*His-C*) (Liu et al. 2006). We confirmed it by co-localization with a fluorescent fusion of the histone cluster-specific marker, DLsm11 (Liu et al. 2006) (Fig. 2d–f).

Our observation in whole mount salivary glands suggests that CRM is dynamically regulated. We analyzed CRM binding pattern on salivary gland polytene chromosome spreads using antibodies directed against CRM (Yamamoto et al. 1997). We observed that the pattern was variable between individual chromosome spreads. In order to assess whether it was due to technical artifacts or correlated to a dynamic process such as replication, we co-stained chromosomes with antibodies against proliferating cell nuclear antigen (PCNA) (Fig. 3). In the nuclei outside the S-phase (without PCNA staining), CRM is mostly detected on the histone locus and more weakly in many bands across all chromosomes (Fig. 3a). In the nuclei in the early S-phase (many PCNA binding sites in euchromatic regions), most CRM signals co-localize with PCNA, but CRM is present without PCNA on His-C (Fig. 3b). Finally, in the nuclei in the late S-phase (PCNA observed on the chromocenter), there is much less co-localization of CRM and PCNA despite the persistence of many PCNA bands, but both proteins are enriched on the chromocenter (Fig. 3c). Note also the persistence of CRM on His-C and more weakly on a few sites (in particular some puffs) (Fig. 3c). Altogether, with the pattern of Venus–CRM in whole mount salivary glands, our observations indicate that CRM is always present on His-C, and that in early S-phase, the CRM protein co-localizes extensively with PCNA.

CRM regulates histone H1 transcription in salivary gland The *His-C* is a complex made up of around 100 repeats ~5 Kb in length, each containing single copies of the transcription units for the linker histone (*H1*) and the four core histones (*H2A*, *H2B*, *H3*, and *H4*). *His-C* is known to be bound by the heterochromatic proteins HP1

Fig. 1 In vivo imaging of CRM fluorescent protein fusions. **a–c** Whole mount third larval instar salivary gland showing Venus–CRM (*green*; **a**, **c**) and RFP–H2Av (*red*; **b**, **c**). **d–l** Individual salivary gland nuclei with mRFP–H2Av (*red*) and different level of Venus–CRM (*green*). In some nuclei with a low level of Venus–CRM, a single bright band corresponding to a chromosome section is visible
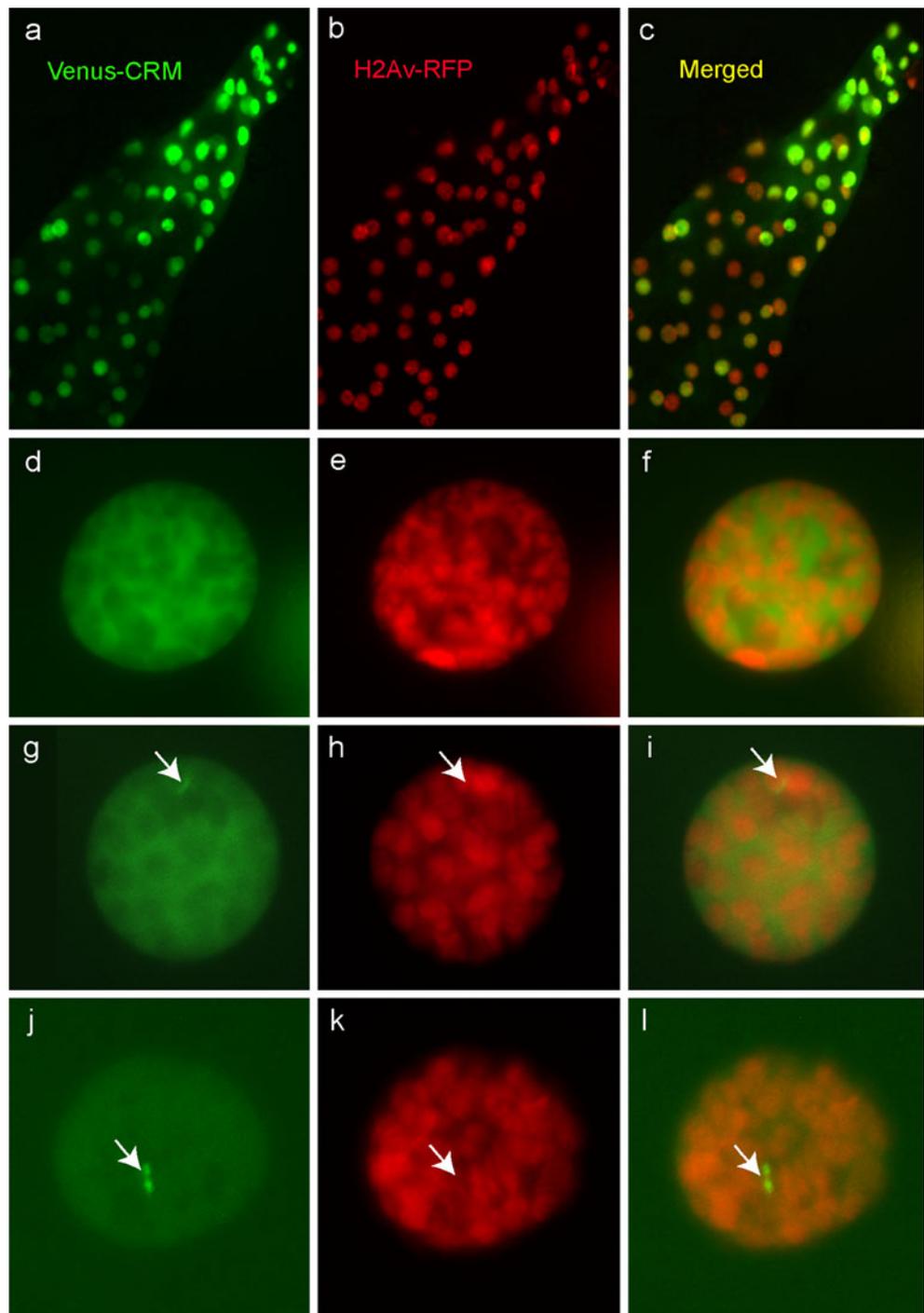
and Su(var)3-9 (Ner et al. 2002; van Steensel and Henikoff 2000). *His-C* is repressed by Su(var)3-9, which is responsible for the formation of compact heterochromatin at this locus (Ner et al. 2002). As *crm* was previously characterized as a suppressor of variegation using the chromosomal rearrangement w^{m4h} (Yamamoto et al. 1997), CRM association with the *His-C* suggested a role for CRM in the establishment or the maintenance of repressive heterochromatin at the *His-C*.

In order to test this, we analyzed the expression levels of the five histone genes (*H1*, *H2A*, *H2B*, *H3*, and *H4*) by real-time PCR on *wild-type* or *crm* mutant salivary glands. Surprisingly, we find that loss of *crm* has an effect on histone gene activation rather than repression. In *crm* mutants, the levels of *H2A*, *H2B*, *H3*, or *H4* expression remains unchanged, while the levels of H1 expression is reduced 30 to 40 times (Fig. 4a; Student's *t* test, $p<0.05$). Western blot analysis of H1 protein level in wild-type (WT)

Fig. 2 CRM is present on the histone locus. In vivo imaging of Venus–CRM (*green*; **a**, **c**) in combination with mCherry–HP1 (*red*; **b**, **c**) and mCherry–CRM (*red*; **d**, **f**) in combination with the histone locus marker DLsm11–Venus (*green*; **e**, **f**). The Venus–CRM bright band is located close to the chromocenter (**c**) and the mCherry–CRM bright band co-localizes with the histone locus (**d**–**e**)
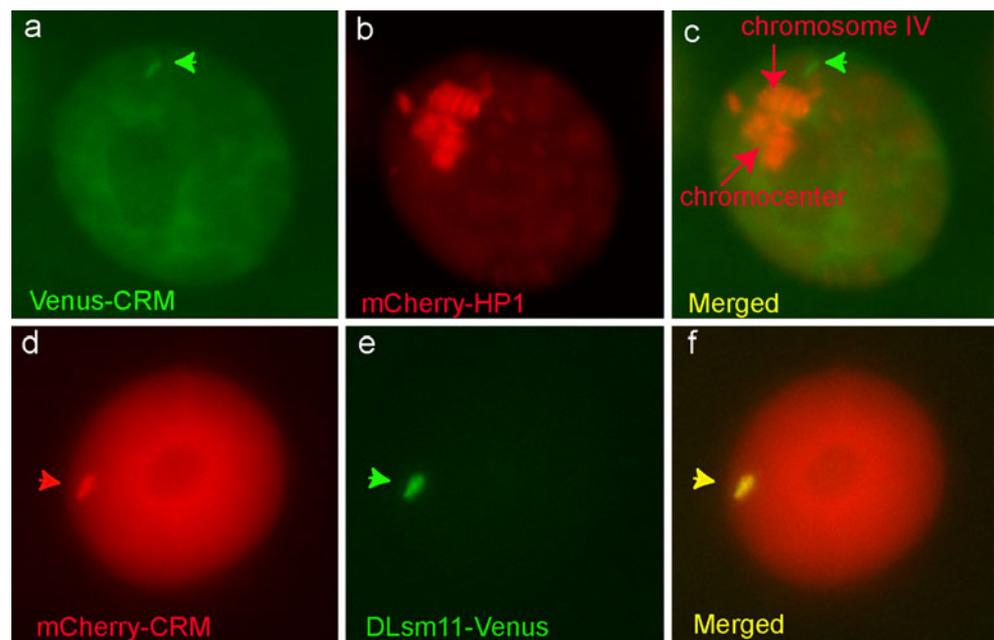
or *crm* mutant larvae shows that it is strongly reduced in *crm* mutants (Fig. 4b). In addition to a specific role of CRM on the expression of H1, the linker histone, this reveals also a previously unknown role of *crm* in the activation of gene expression.

CRM cooperates with TRF2 in the activation of H1 and in the development of the wing The specific requirement of *crm* for *H1* expression correlates well with the observation that the histone H1 gene is regulated differently than the other core histones; the genes encoding core histones require the TBP for expression, whereas *H1* requires the TRF2 (Isogai et al. 2007). Thus, TRF2 and CRM appear to be specifically required for the expression of *H1*. As *TRF2* mutants display the same wing margin phenotype seen in *crm* mutants, we decided to test if they function together, using the *Drosophila* wing as an assay (Kopytova et al. 2006). We analyzed the wing phenotypes of *crm-trf2* double mutants using the hypomorphic allele $TRF2^{G0039}$ (Bashirullah et al. 2007). $TRF2^{G0039}$ wings are smaller but have intact wing margin. The *crm* mutants show small wings with a few notches. We observe a strong increase in the wing margin notching in *crm-trf2* double mutants, indicating a strong genetic interaction (Fig. 5a–d). To confirm the genetic interaction between CRM and TRF2, we performed a reciprocal analysis. We used transgenic flies expressing dominant-negative forms of CRM in which specific conserved domains are deleted (Gibert et al. 2011). One of our mutants (deletion of amino acids 736–821) behaves as a strong dominant mutant. Its ubiquitous expression in a wild-type background using tub-gal4 causes larval lethality before metamorphosis. When we drove its

expression in the wing disc using *bi-gal4* we obtained flies with a strong wing growth defect (Fig. 5f). This phenotype is dose sensitive as two doses of the *UAS-crm-delV* leads to extremely small wings (Fig. 5f, asterisks). The co-expression of an *mCherry-Trf2* fusion (see below) with the dominant mutant cancels the effect of the dominant mutant and rescues the wing growth (Fig. 5h). This observation suggests that expression of *mCherry-Trf2* titrates the dominant mutant or compensates for titration of Trf2 by the dominant mutant. We, therefore, tested whether CRM and TRF2 could be co-immunoprecipitated. Indeed, co-immunoprecipitation experiments using extracts from embryos expressing Venus–CRM demonstrate that CRM and TRF2 exist in vivo in a complex (Fig. 6). Interestingly, whereas we observe two bands for TRF2 in the input, the upper one is much more abundant than the lower one in the co-immunoprecipitation, suggesting that a modified form of TRF2 interacts with CRM. In conclusion, our findings show that CRM and TRF2 cooperate closely in the activation of H1 and potentially other targets.

CRM and TRF2 co-localize on His-C but are not strictly required for the recruitment of one another To further substantiate the interaction between CRM and TRF2, we have generated a fluorescent fusion of TRF2. Figure 7 (a, b, c and d) reveals in vivo co-localization of EBFP2-CRM, mCherry–TRF2, and DLSM11–Venus in salivary gland nuclei, indicating that CRM and TRF2 are present simultaneously on *His-C*. On squashed salivary gland, polytene chromosomes Venus–CRM and TRF2 co-localize on *His-C*, which is their major binding site (Fig. 7e–h). We

Fig. 3 Co-immunostaining for CRM (*green*) and PCNA (*red*) on salivary gland polytene chromosomes. The different phases of the replication cycle can be identified thanks to PCNA pattern: G1 phase (*left*), early S-phase (*middle*), late S-phase (*right*)
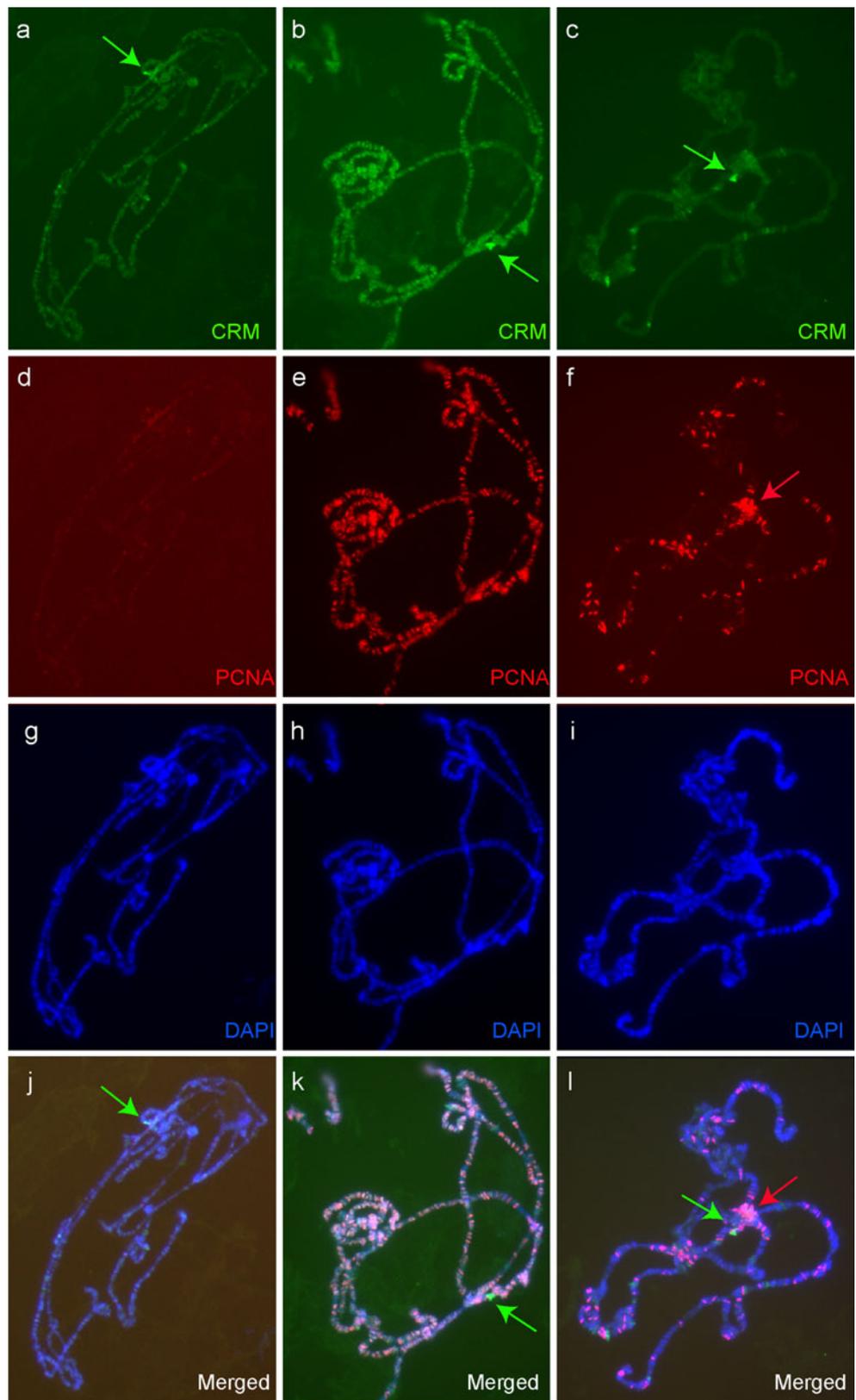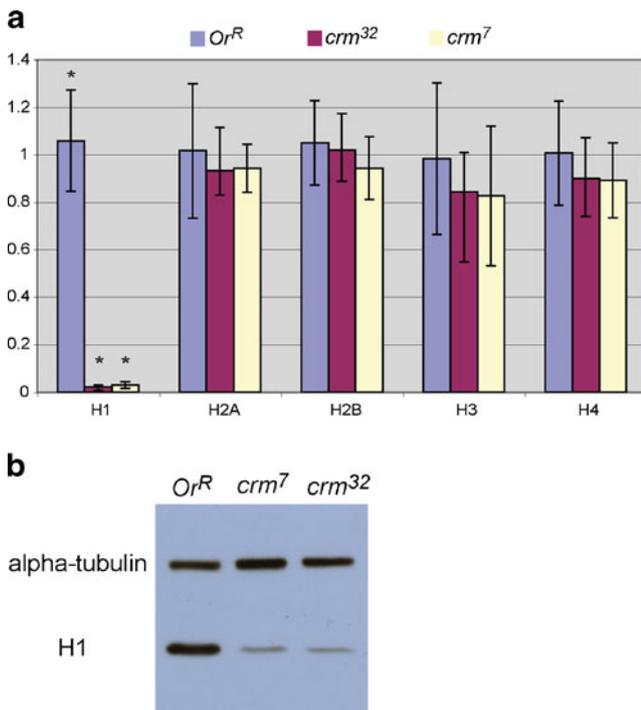
Fig. 4 Expression of *H1* is altered in *crm* mutants. **a** Real-time PCR analysis of the expression levels of the five histone genes in salivary glands of wild-type (*Or^R*) and *crm* mutant (*crm^32* or *crm^7*) third instar larvae. Expression of the histone genes was normalized with the control genes EF1g, RP49, TBP, GapdHI, and RNApolII; ±1 standard deviations are represented. The expression level of *H1* is reduced 30 to 40 times in *crm* mutants compared to WT (*p*<0.05). No significant difference is observed for the genes encoding the core histones. **b** Western blot analysis of H1 protein level in salivary glands of WT (*Or^R*) or crm mutant larvae (*crm^7* or *crm^32*)

analyzed whether CRM and TRF2 are required for the recruitment of one another on *His-C* in salivary glands. We found that CRM can be detected on *His-C* in *Trf2* hypomorphic mutants and reciprocally, TRF2 can be detected on *His-C* in crm null mutants (Fig. 8). Therefore, they are not particularly limiting for the recruitment of each other on *His-C* and seem to be able to bind *His-C* independently. However, we observed that in *Trf2* mutants, a higher level of CRM is detected on the chromocenter (Fig. 8g–i). Furthermore, we observed that chromosome condensation is altered in *crm* mutants, as previously reported in salivary glands of *H1 RNAi* larvae (Siriaco et al. 2009).

CRM role on His-C is independent of PcG factors In order to test whether CRM role on His-C could be dependent on PcG proteins we stained salivary gland polytene chromosomes with antibodies against CRM and posterior sex comb (PSC), a well-characterized component of the Polycomb complex PRC1. We observed few co-localizations on chromosome arms, but PSC clearly does not co-localize

with CRM on His-C (Fig. 9). In agreement with this, no binding of PcG proteins on His-C have been reported until now (Schwartz et al. 2006).

Discussion

Yamamoto et al. (1997) characterized *crm* as a repressive factor, dynamically regulated during the cell cycle, and involved in position effect variegation and Polycomb silencing. The belonging to the *PcG* gene family is based on the appearance of additional sex comb on the posterior legs of *crm* mutants, as well as on the genetic interactions (enhancement) with mutations in individual genes of the *PcG* family. Furthermore, *crm* mutation also behaves as suppressor of the variegated eye phenotype caused by the relocation of the *white^+* next to heterochromatin (*w^{m4h}*). Using fluorescent fusions we confirm that CRM is dynamically regulated during the cell cycle. By co-staining with PCNA, we identify early S-phase as the peak of CRM production where CRM is widely present on chromosomes at many regions, co-localizing extensively with PCNA. In the late S-phase, it is present at much fewer sites but can be detected on the chromocenter where it co-localizes with PCNA. Together with its requirements for chromatin regulation (in particular centromeric heterochromatin), co-localization of CRM and PCNA suggests a role of CRM in the transmission of epigenetic information during replication, as previously suggested by Yamamoto et al. (1997).

We report here the previously unknown localization of CRM on the Histone cluster and its involvement in the activation of the Histone 1 gene. No PcG protein has been reported to bind His-C and we clearly do not detect PSC, a member of the PcG complex PRC1, on His-C. It is therefore likely to correspond to a role of crm independent of traditional PcG proteins. Our observation that *crm* is involved in the activation of gene transcription contradicts the earlier classification of the *crm* gene as a *PcG*/ heterochromatic gene. CRM could be a general activator required for the activation of particular *PcG* and *Su(var)* genes. In agreement with this interpretation, mutations in *H1* have been shown to act as suppressors of PEV (Lu et al. 2009). The strong underexpression of *H1* in *crm* mutants could thus be responsible for the phenotypes observed in *crm* mutants. This is likely the case for chromosome condensation defects, which are observed in *H1* RNAi larvae (Siriaco et al. 2009). However, downregulation of *H1* does not explain all our observations. For example, the strong reduction of *H1* in *Trf2* mutants is not associated with any Polycomb phenotypes as *Trf2* pharates do not have ectopic sex combs. A second possibility is that *crm*

Fig. 5 a–d *crm* and *TRF2* interact genetically for the development of the wing margin. In contrast to wild-type flies (**a**) *crm^32* mutants have smaller wings with notches (*asterisks*) (**b**). *TRF2^G0039* hypomorphic mutants die during metamorphosis or as pharate adults. Wings dissected out of the pupal case are smaller but look mostly normal, with intact wing margins (**c**). In contrast, double mutants *crm^32 TRF2^G0039* have a larger region of the wing margin missing (*asterisks*) (**d**). All pictures were taken at the same magnification. The *line* represents the same region of the wing for easier comparison. **e–h** The co-expression of TRF2 suppresses *crm*-dominant mutant phenotypes. Expression of a *crm* deletion mutant in the wing disc using the *bi-Gal4* driver leads to a strong and dose sensitive growth defect (**f** one dose, small frame (*asterisk*), two doses of the dominant mutant transgene). Expression of *mCherry-Trf2* with the same driver has no effect on the wing growth (**g**), but in combination with the *crm*-dominant mutant it rescues the growth phenotype (**h**). Note that *bi-Gal4* is a hypomorphic allele of *bi*
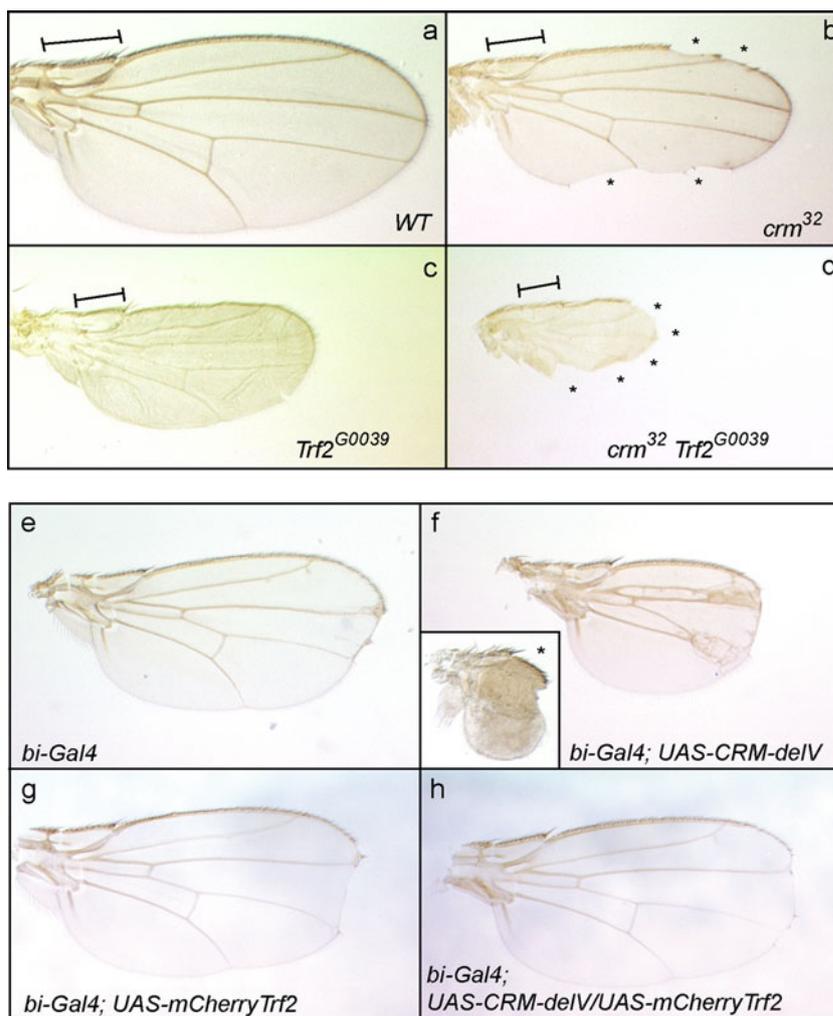
plays distinct roles in both activation and repressive processes, probably depending on the partners with which it associates. This is typical of members of the ETP group such as the GAGA factor (Gildea et al. 2000). Interactions with particular factors such as TRF2 might condition the specific requirement of *crm* for particular targets. This does not seem to be the case for *His-C*, as CRM can be detected
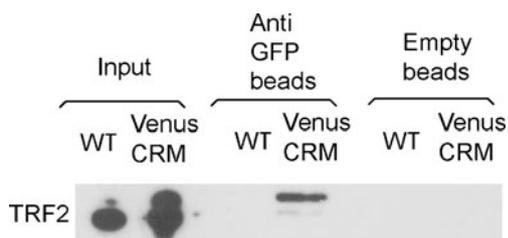
Fig. 6 Venus–CRM and TRF2 co-immunoprecipitate in embryonic extracts. CO-IP with anti-GFP beads and WT embryonic extracts (without Venus–CRM) or with empty beads and Venus–CRM embryonic extracts were used as controls

on *His-C* in TRF2 mutants. Therefore, CRM and TRF2 may not be the components of a stable complex. Their co-immunoprecipitation likely reflects their common recruitment to *His-C* (and potentially other genomic loci). However, we observed more CRM on the chromocenter in *Trf2* mutants which suggests that TRF2, at least indirectly, controls some aspects of CRM chromosomal localization. CRM is one of the few factors known to be involved with TRF2 in gene activation. Indeed, although TRF2 is an essential factor, component of core promoter recognition complexes, and involved in the regulation of more than 1,000 genes in S2 cells, very few of its cofactors are known (Isogai et al. 2007). The best characterized are TFIIA and TFIIB, components of the basal transcription machinery (Rabenstein et al. 1999). Interestingly, the mammalian protein TIP2 was shown recently to physically interact with TRF2 and Polycomb proteins (Pitulescu et al. 2009), showing, like our findings, that particular factors can have connection with both TRF2 and Polycomb silencing.
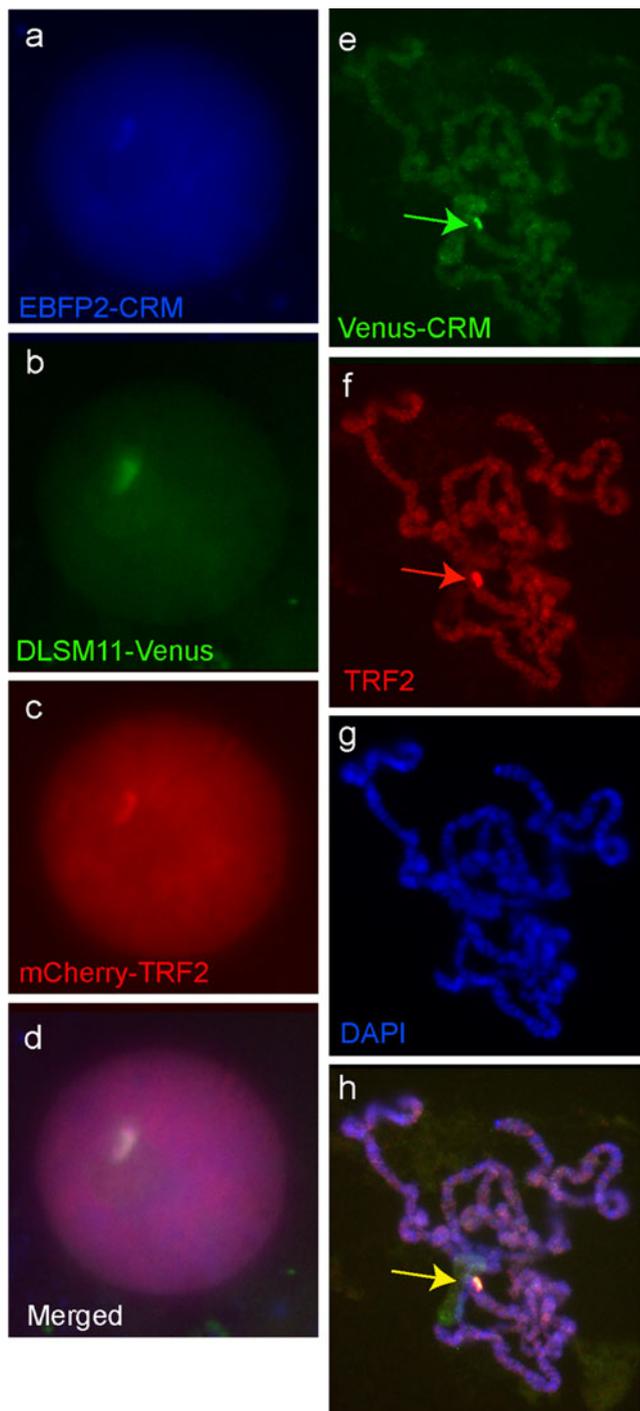
Fig. 7 CRM and TRF2 co-localize in vivo on *His-C*. In vivo imagining of the fluorescent fusion protein EBFP2-CRM (**a**), dLsm11–Venus (**b**), and mCherry–TRF2 (**c**) in a salivary gland nucleus. Venus–CRM and TRF2 co-localize on *His-C* on squashed polytene chromosomes. Staining with a mouse anti-GFP antibody (**e**, *green*) and a rabbit anti-TRF2 antibody (**f**, *red*) on squashed salivary gland polytene chromosomes from larvae expressing Venus–CRM, *blue* (**g**, **h**) *Dapi*. The *His-C* is the strongest binding site of both proteins

Methods

Construction of transgenic flies expressing fluorescent fusions

We amplified the coding sequences of Venus (Nagai et al. 2002), mCherry (Shaner et al. 2004), and EBFP2 (Ai et al. 2007) by PCR using primers with floating restrictions sites (5′, *Eco*RI; 3′, *Bgl*II). The PCR products were cloned in pGEM-T Easy (Promega) and sequenced. The coding sequence of the fluorochrome was then cut by *Eco*RI and *Bgl*II and cloned into the pUASTattB vector (Bischof et al. 2007) opened with *Eco*RI and *Bgl*II to constitute respectively pUASTVenus-attB, pUASTmCherry-attB, and pUASTEBFP2attB. We amplified the coding sequence of CRM, HP1, and TRF2 by PCR using primers with the floating restriction sites. TRF2 clones were made using the short isoform (Kopytova et al. 2006). The PCR products were cloned in pGEM-T Easy. The coding sequence of CRM, HP1, and TRF2 were cut by the appropriate enzymes and cloned into PUASTVenus(mCherry or EBFP2)attB opened with *Bgl*II and *Xba*I to construct in-frame fusions. The constructs were integrated into the landing sites 22A (Venus–CRM, mCherry–HP1), 58A (mCherry–TRF2), or 51D (EBFP2–CRM) on the second chromosome by injection of embryos with a source of PhiC31 integrase on the X chromosome (Bischof et al. 2007).

Fly cultures

Fly crosses were done at 25°C using balancer chromosomes and standard agar–corn medium. The *crm*[7] and *trf2*[G0039] were previously described (Bashirullah et al. 2007; Yamamoto et al. 1997). The *crm*[32] allele was generated by imperfect excision of the P element inserted in the stock *crm*[EY05302] (Flybase).

Fluorescence microscopy

Observation and image capture were made on an Axioplan microscope (Zeiss) with an Optronix camera and Magna-Fire software. We used the leaky driver HS::GAL4, without heat shock, to induce a moderate expression of the fluorescent constructs in the salivary glands. Salivary glands were observed in 0.7% NaCl.

Expression analysis

The expression of each of the histone genes was measured by real-time PCR in wild-type (*Oregon*[R]) and mutant (*crm*[32] and *crm*[7]) salivary glands. We used three biological samples per condition and three technical replicates per biological sample. Thirty pairs of salivary glands were

Fig. 8 CRM and TRF2 can bind independently on *His-C*. Immunostaining for CRM or TRF2 in WT or *crm* or *Trf2* mutants. CRM and TRF2 can be detected on *S* in the absence of one another. We detect more CRM on the chromocenter in Trf2 mutants (**g**, **i**)
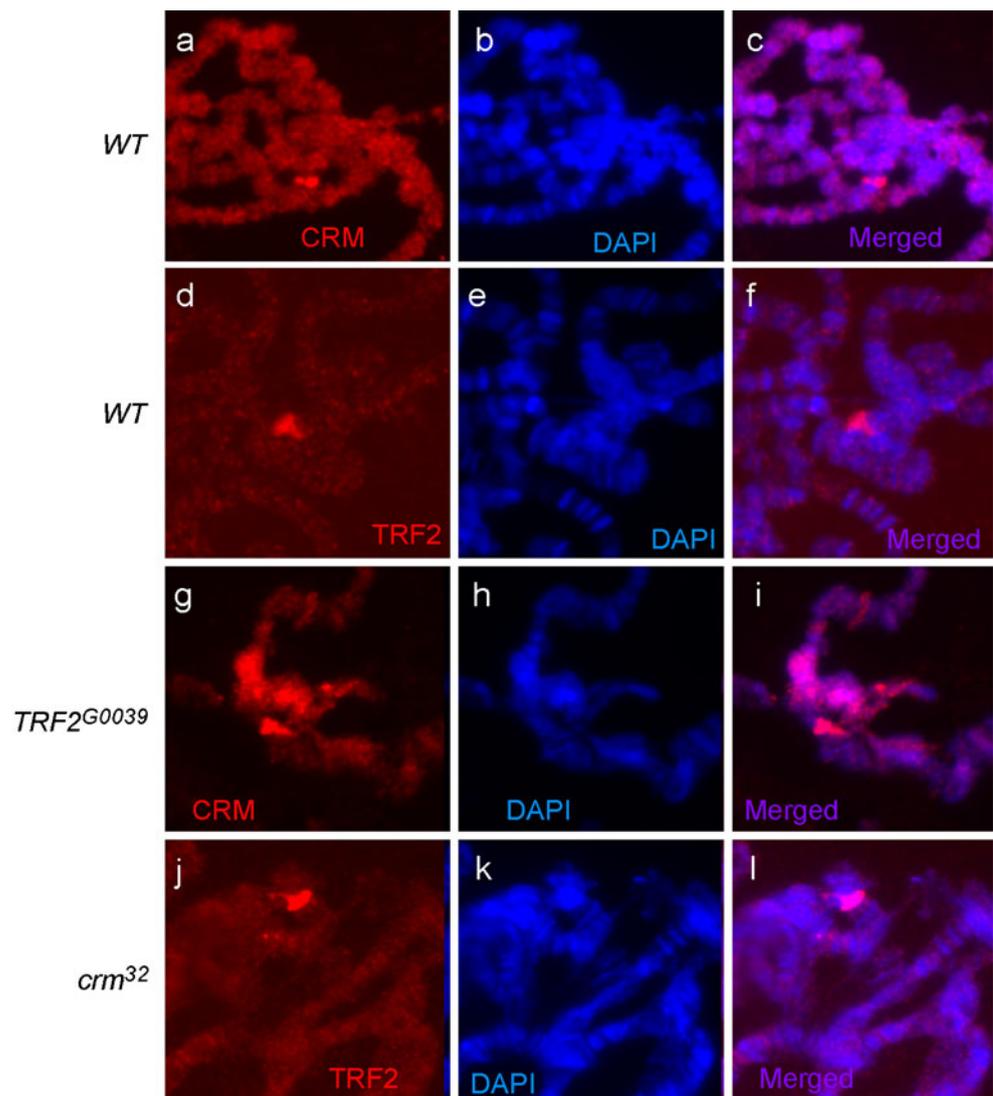
dissected for each biological replicate. cDNA were synthesized using Trizol extracted total RNAs. The genes *EF1g*, *RP49*, *TBP*, *GapdH1*, and *RNApolII* were used as controls to normalize the data.

The primers used were the following:

Histone H1 (Forward: CCCAAAAGTTAGCGC CATTC; reverse: TGACCACGGCCGATTTTAAG)

Histone 2A (Forward: GCAAAGTCCCGCT CAAACC; reverse:CCGGAGCAAACGGTGAATAC)

Histone 2B (Forward: ACAAGCGCTCGACCAT CAC; reverse:CCAACTCTCCAGGCAAAAGC)

Histone 3 (Forward: AAGCCCCACCGCTATCG; reverse: CTCTTTTGGTAGCGACGAATTTC)

Histone 4 (Forward: GAGGCAAAGGCTTGG GAAAG; reverse: TGGATGTTATCACGCAG CACTT)

TBP (Forward: CGCGCATCATCCAAAAGC; reverse: GCCGACCATGTTTTGAATCTTAA).

EF1g (Forward: GTGTTCATGTCGTGCAATCTCA; reverse: CGCCTTGCGCATCTTGT)

RP49 (Forward: GCGCACCAAGCACTTCATC; reverse: TTGGGCTTGCGCCATT)

Gapdh1 (Forward: ATTTCGCTGAACGA TAAGTTCGT; reverse: CGATGACGCGGTTG GAGTA)

pol II (Forward: CCTTCAGGAGTACGGCTAT CATCT; reverse:CCAGGAAGACCTGAGCAT TAATCT)

Immunostaining on squashed polytene chromosomes

Immunostaining on squashed polytene chromosomes from WT (y w) or UAS-VenusCRM; HS::GAL4 larvae were done as previously described (Gibert et al. 2007). The squashes of polytene chromosomes were made with a slightly different protocol (Spierer et al. 2008).
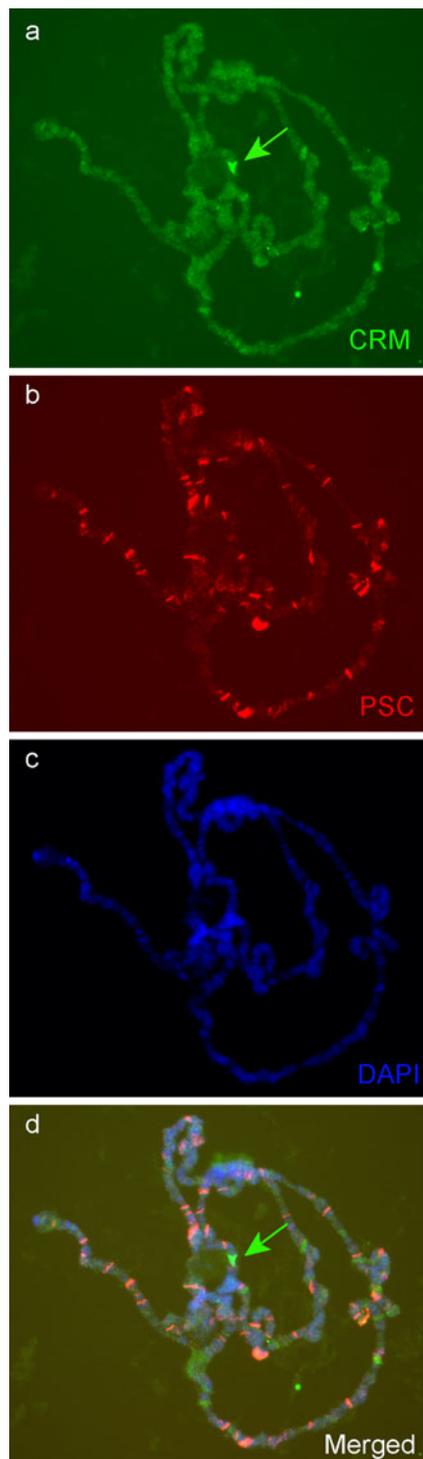
Fig. 9 The Polycomb group protein PSC does not co-localize with CRM on His-C immunostaining of CRM (**a**, **d**, *green*) and PSC (**b**, **d** *red*) on salivary gland polytene chromosomes. CRM but not PSC is present on His-C (*arrows*)

We used the following antibodies: monoclonal mouse anti-GFP (3E6, Qbiogen); polyclonal rabbit anti-GFP (ab6556, abcam); polyclonal Rabbit anti-CRM (Yamamoto et al. 1997); polyconal rabbit anti-TRF2 (Rabenstein et al. 1999); mouse

monclonal anti-PSC (Hybridoma bank); and mouse mono-clonal anti-PCNA (PC10, Santa Cruz Biotechnology).

Co-immunoprecipitation and Western blots

Immunoprecipitations of Venus–CRM were done as de-scribed previously (Rozenblatt-Rosen et al. 1998), with minor modifications using dechorionated embryos express-ing Venus–CRM driven by tub::GAL4. The composition of the immunoprecipitation (IP) buffer was 50 mM Tris, pH 8.0, 200 mM NaCl, 0.1 mM EDTA, and 0.1% NP40. One tablet of complete protease inhibitor (Roche) was added per 10 ml of IP buffer. The protein A agarose beads were purchased from PIERCE. The anti-GFP antibody used for the IP was the mouse monoclonal antibody 3E6 (QBiogen). Western blots were performed using rabbit polyclonal anti-TRF2 (Rabenstein et al. 1999). As negative controls, we used extracts from wild-type embryos (without Venus–CRM) or agarose beads without antibodies or with anti-GST antibodies.

For the H1 western blot we used rabbit antibody against *Drosophila* H1 (Active Motif, catalog number 39575) and mouse monoclonal antibody against alpha-tubulin (Sigma, T9026) as loading control. Twenty-five pairs of salivary glands for each genotype were dissected and homogenized in 40 µl of loading buffer. Ten microliters were used for the Western blot.

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