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RESEARCH ARTICLE

Yeti, an essential *Drosophila melanogaster* gene, encodes a protein required for chromatin organization

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ABSTRACT

The evolutionarily conserved family of Bucentaur (BCNT) proteins exhibits a widespread distribution in animal and plants, yet its biological role remains largely unknown. Using *Drosophila melanogaster* as a model organism, we investigated the *in vivo* role of the *Drosophila* BCNT member called YETI. We report that loss of YETI causes lethality before pupation and defects in higher-order chromatin organization, as evidenced by severe impairment in the association of histone H2A.V, nucleosomal histones and epigenetic marks with polytene chromosomes. We also find that YETI binds to polytene chromosomes through its conserved BCNT domain and interacts with the histone variant H2A.V, HP1a and Domino-A (DOM-A), the ATPase subunit of the DOM/Tip60 chromatin remodeling complex. Furthermore, we identify YETI as a downstream target of the *Drosophila* DOM-A. On the basis of these results, we propose that YETI interacts with H2A.V-exchanging machinery, as a chaperone or as a new subunit of the DOM/Tip60 remodeling complex, and acts to regulate the accumulation of H2A.V at chromatin sites. Overall, our findings suggest an unanticipated role of YETI protein in chromatin organization and provide, for the first time, mechanistic clues on how BCNT proteins control development in multicellular organisms.

KEY WORDS: Chromatin remodeling, Chromosome, *Drosophila*, Yeti

INTRODUCTION

The Bucentaur (BCNT) protein family, conserved from yeast to humans, is characterized by the so-called BCNT domain, a stretch of 80 amino acids located at the C-terminal end (Fig. 1). Despite its widespread distribution in animals and plants, however, the functions of this protein family remain largely unknown (Iwashita et al., 2003). Sparse information in the literature indicate that BCNT proteins might perform important cellular functions; the yeast BCNT protein, Swc5, is a component of the ATP-dependent chromatin remodeling complex SWR1, involved in the exchange of the histone variant H2A.Z with the canonical H2A within euchromatin (Kobor et al., 2004; Mizuguchi et al., 2004). The *Drosophila melanogaster* BCNT protein, YETI, was originally defined as a kinesin-binding protein able to bind both subunits of the microtubule-based motor kinesin-I (Wisniewski et al., 2003). Related BCNT proteins have been found in both cytoplasmic and nuclear fractions (Iwashita and Naoki, 2011), and they have been shown to associate with kinetochores in chicken (Ohta et al., 2010). Additional studies have linked the BCNT proteins to craniofacial development and osteogenesis, though limited mechanistic understanding is available. First, the mouse BCNT protein, called CP27 or CFDP1, plays essential cellular functions and is required for teeth and bone development (Diekwisch et al., 1999; Diekwisch et al., 2002). Second, in mouse, downregulation of the *Cfdp1* gene correlates with the induction of cleft palate due to persistent expression of the PAX3-encoding gene in neural crest cells (Wu et al., 2008); upregulation of *Cfdp1* was also observed after experimentally induced osteodifferentiation (Bustos-Valenzuela et al., 2011). Third, the Zebrafish BCNT gene, called *Rltpr*, is developmentally expressed in the branchial arches that will give rise to the craniofacial structures (Thisse, 2004). Finally, the human *Cfdp1* gene has been shown to be a target of TFII-I transcription factors, which in turn are encoded by genes suggested to be primary candidates for Williams–Beuren disease, which causes – among others symptoms – craniofacial abnormalities (Makeyev and Bayarsaihan, 2011).

In this work, we have carried out in-depth functional studies of the *Yeti* gene and its encoded product, YETI, in *Drosophila melanogaster*. We show that the YETI protein is required for higher-order chromosome and/or chromatin organization during development. In addition, our results provide evidence suggesting that YETI interacts with HP1a and with the H2A.V-exchanging machinery and participates in the step of H2A.V deposition.

RESULTS

Associating the vital gene *l(2)41Aa* with the *Yeti* gene of *Drosophila*

We previously found that *l(2)41Aa*, a vital heterochromatin gene of *Drosophila melanogaster* (Hilliker, 1976; Dimitri et al., 2003; Rossi et al., 2007), is required for proper chromosome organization in both mitosis and meiosis (Cenci et al., 2003a). To identify the actual gene at the molecular level, we screened for P-element-induced mutations that failed to complement *l(2)41Aa* alleles and recovered two non-complementing lethal insertions, denoted *LP1* and *LP2* (see Materials and Methods). Molecular analysis showed

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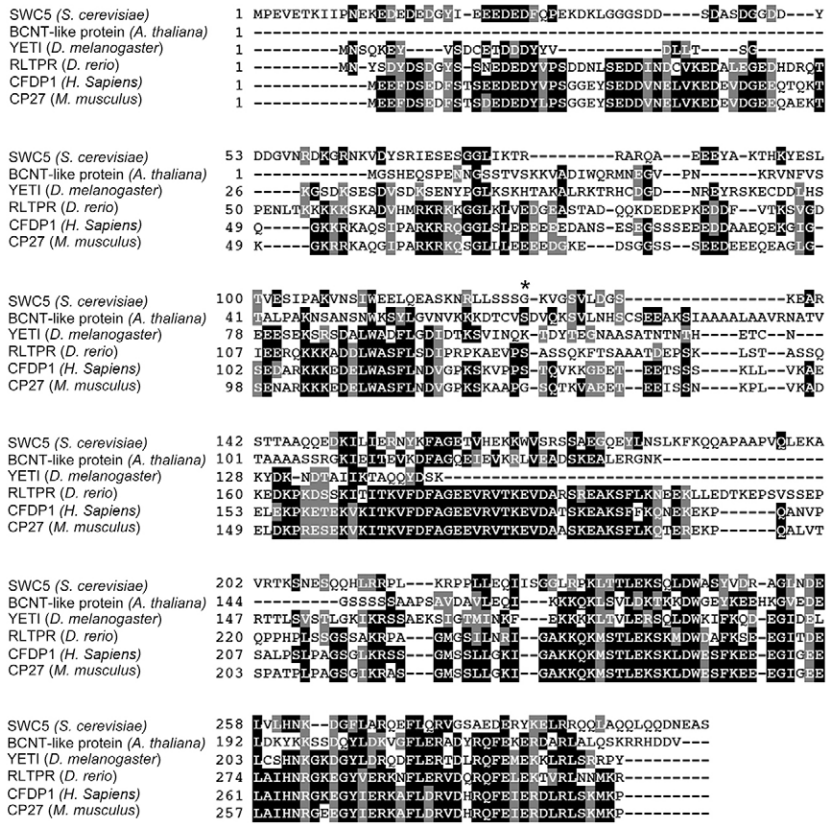

Fig. 1. The BCNT proteins. Blast alignments of YETI and other BCNT proteins sequences from humans, *Mus musculus*, *A. thaliana* and *S. cerevisiae*. Note the high conservation of protein sequence (about 45% similarity) in the last 80 residues of the C-terminal end. In the *EMS-31* lethal allele of *Yeti*, a single base pair substitution (from A to T) was found at position 316, where codon AAA for lysine is changed to a TAA stop codon. The lysine residue changed in *EMS31* allele is located at position 106 (see the asterisk).

that *LP1* is a P-insertional allele that, when homozygous, results in prolonged larval development followed by lethality before pupation. Mutant larvae exhibited severely reduced imaginal discs and brains, with melanotic masses in their hemocoel (Fig. 2A). Cytological examination of DAPI-stained salivary gland cells from *LP1/LP1* homozygous and *LP1/Df(2R)B* hemizygous larvae revealed decondensed and disorganized chromosomes (Fig. 2B); all examined polytene cells displayed such aberrant chromosome morphology, albeit at different degrees. Moreover, polytene chromosomes and nuclei of *LP1/LP1* homozygous salivary glands are about 40% smaller than those present in wild-type salivary glands.

The flanking sequences of the *LP1* insertional allele were recovered by inverse PCR, sequenced and used to search the *Drosophila melanogaster* genome database. BLAST analysis using the 3′ end flanking sequences of *LP1* revealed 100% identity with the 5′ end region of *CG40218*, also called *Yeti*, a 967-bp-long gene located in scaffold AABU01002199. The P-element was found to be inserted within the predicted start codon ATG of *Yeti* at position +2. To further verify the connection between the genetic locus *l(2)41Aa* and the *Yeti* gene sequence, we performed PCR amplification and sequencing of the *Yeti* region using genomic DNA from larvae homozygous for *EMS-31*, a lethal allele of *l(2)41Aa*. Sequence inspection revealed that *EMS-31* carried a single base pair mutation (from A to T) at position 106, where codon AAA for Lys is changed into a TAA stop codon, most likely producing a truncated protein lacking ~60% of the protein including the evolutionary conserved C-terminal region (Fig. 1). Taken together, these results indicate that the *l(2)41Aa* vital gene corresponds to the *Yeti* gene.

Northern analysis using the 0.9-kb *Yeti* cDNA as a probe revealed a transcript of the expected size (~0.9 kb) in larvae of the Oregon-R wild-type strain, which was absent in *LP1/LP1* homozygotes (Fig. 2C) indicating that *LP1* is a null allele of *Yeti*. The transcript was detected throughout development in embryos, larvae, pupae, adults and ovarian RNA (Fig. 2D). To further confirm whether the *Yeti* product is required for proper chromosome organization, we depleted the *Yeti* gene in S2 cells using double-stranded RNA (dsRNA)-mediated interference (RNAi) (Fig. 2E). The RNAi-treated cells displayed abnormally condensed chromosomes with fuzzy and loose appearance in ~70% of examined metaphases (n=457), versus the 3% baseline level recorded in mock-treated cells. Remarkably, these defects of chromosome organization strongly resemble those originally characterized in *l(41A)* mutants (Cenci et al., 2003a).

Exogenously expressed YETI protein rescues the chromosome morphology in *Yeti* mutants

If *Yeti* actually corresponds to the vital heterochromatin gene *l(2)41Aa*, then reconstituting *LP1* homozygous mutant strains with exogenous *Yeti* gene product should rescue the defective phenotypes observed in mutants. To assess whether that was the case, we generated transgenic flies carrying the full-length *Yeti* cDNA. Overexpression of the UAS-Yeti-M17 transgene transgene using *tub-GAL4* to induce the UAS promoter rescued indeed both the larval lethality and the chromosome morphology phenotypes associated with *LP1* null alleles (supplementary material Fig. S1A; details in Materials and Methods). To assess the expression of the YETI protein in *LP1/LP1*; *UAS-Yeti-M17*/*tub-GAL4* larvae, we produced polyclonal antibodies directed against the conserved C-terminal portion of the YETI protein (see Materials and Methods) and analyzed protein extracts by western blotting. A single 32-kDa band was detected in extracts from *LP1/LP1*; *UAS-Yeti-M17*/*tub-GAL4* larvae (Fig. 2F), but not in

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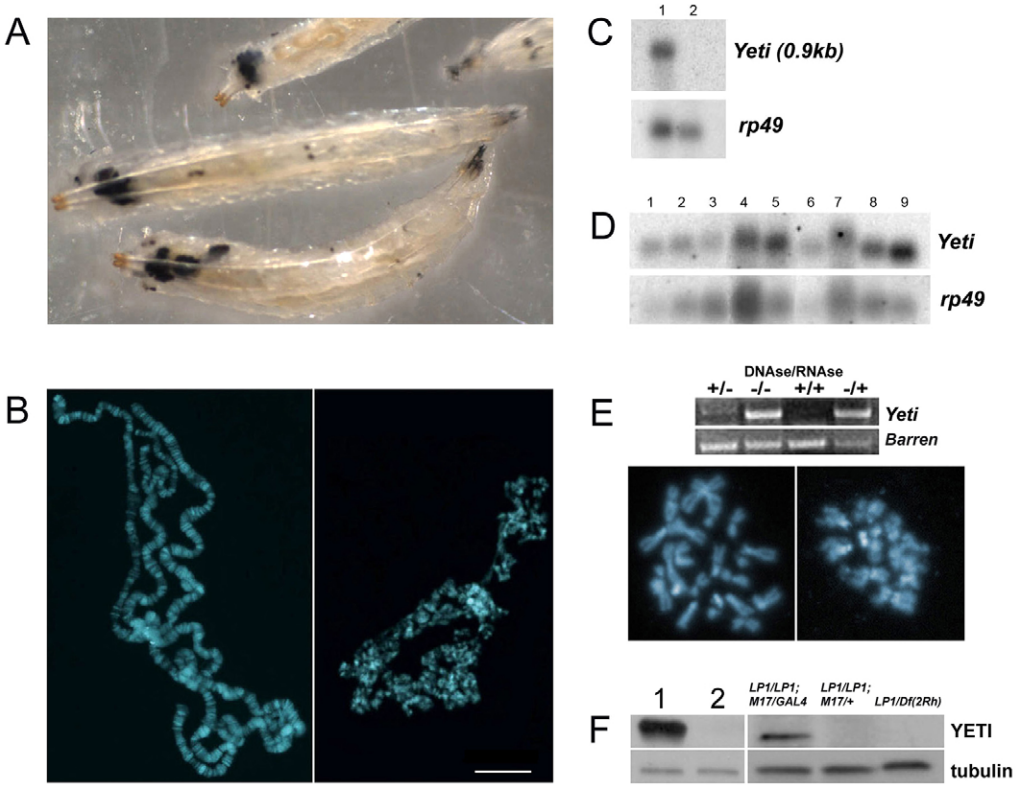

Fig. 2. Cytological and molecular analyses of the *Yeti* gene. (A) *Yeti* mutant larvae showing melanotic masses in their hemocoel. (B) DAPI-stained polytene chromosomes of Oregon-R wild-type (left panel) and *Df(2R)B/LP1* hemizygous mutant larvae (right panel). *Df(2R)B* fails to complement the *LP1* allele (see Materials and Methods). Chromosome organization in *Yeti* mutant genotypes is clearly perturbed. A similarly abnormal polytene chromosome organization was detected in *LP1* homozygotes. Scale bar: 10 μm. (C) Northern blot analysis of *Yeti* polyadenylated RNA from wild-type (lane 1) and *LP1/LP1* larvae (lane 2). (D) Northern blots of *Yeti* polyadenylated RNA from different developmental stages. The *rp49* ribosomal protein gene was used as a loading control. Lane 1, 0–3-hour-old embryos: lane 2, 9–12-hour-old embryos; lane 3, 18–21-hour-old embryos; lane 4, third-instar larvae; lane 5, larval/early pupal stage; lane 6, late pupae; lane 7, adult males; lane 8, adult females; lane 9, ovaries. (E) RNAi-mediated knockdown of *Yeti* in *Drosophila melanogaster* S2 cells. *Yeti* double-stranded RNA (15 μg) addition to S2 cell cultures strongly reduced the levels of endogenous mRNA after 72 hours as shown by RT-PCR. DNase treatment combined with RNAi shows that a residual *Yeti*-specific amplified band is due to genomic DNA (top panel). RT-PCR amplification of *barren* mRNA was used as control. Cytological analysis (bottom panel) revealed a highly defective chromosome organization in RNAi-treated cells (right panel) compared to non-RNAi controls (left panel); note the fuzzy and loose appearance of condensing chromatin and the highly disorganized morphology of sister chromatids. (F) Western blotting with anti-YETI antibodies. The YETI protein is recognized by anti-YETI antibody in total extracts of BL21 bacterial cells after 4 hours of activation with 1 mM IPTG (lane 1). YETI was not detected in extracts from non-activated BL21 cells (lane 2). A band of ~32 kDa is only detectable in protein extracts from salivary glands and brains of *LP1/LP1*; *UAS-Yeti-M17*/tub-GAL4 larvae, and is absent in *Yeti*-null mutant larvae. *Df(2Rh), Df(2R)MS41A1*, a deletion lacking the *Yeti* gene region (see Materials and Methods). The migration of YETI protein (apparent molecular mass of 32 kDa) is somewhat slower than predicted (about 27 kDa), as also reported for bovine BCNT proteins, possibly owing to the physical properties of these intrinsically disordered proteins lacking a stable three-dimensional structure (Iwashita and Naoki, 2011).

extracts from *LP1/LP1*; *UAS-Yeti-M17/+* larvae, which do not express the *M17* transgene in the absence of the *tub-GAL4* driver, or from *LP1/Df* null hemizygotes lacking the functional *Yeti* gene. These results therefore demonstrate that the *Yeti* gene (1) fully complements the vital heterochromatin gene *l(2)41Aa*, and (2) encodes a protein required for proper chromosome organization, which is highly defective in mutant strains.

A YETI–GFP fusion protein localizes to the nucleus and binds polytene chromosomes

Our previous work with *l(2)41Aa* (Cenci et al., 2003a) and the results reported thus far implicate the YETI protein in chromatin organization. To gain more understanding of how YETI might perform this function, we sought to localize the protein in salivary gland polytene chromosomes using anti-YETI in immunostaining assays, but cytological analyses failed to reveal any clear staining, suggesting that the antibody, although performing well in western blot assays of denatured protein extracts (Fig. 2F), could not react with the protein in its native conformation in whole cells. To circumvent this problem, we generated transgenic *Drosophila melanogaster* lines carrying a *UAS-Yeti-GFP* transgene (see Materials and Methods) and induced its expression in salivary glands and brains using the *elav-GAL4* driver (Fig. 3A). Anti-GFP antibody specifically detected the YETI–GFP fusion protein in total protein extracts from salivary glands of larvae carrying the *UAS-Yeti-GFP* transgene activated by *elav-GAL4* driver (Fig. 3B). GFP immunofluorescence assays in these larvae show that the YETI–GFP fusion protein is present in all nuclei and is recruited to hundreds of sites along polytene chromosome arms (Fig. 3A), yielding a staining pattern that overlaps with that produced by DAPI-staining. On mitotic cells of neural ganglia isolated from third-instar larvae, anti-GFP staining was present in nuclei but non detectable on chromosomes (supplementary material Fig. S2). Moreover, in *LP1/LP1; tub-GAL4*/*Yeti-GFP* larvae, expressing the *Yeti-GFP* transgene activated by *tub-GAL4* driver, a rescue of aberrant polytene chromosome

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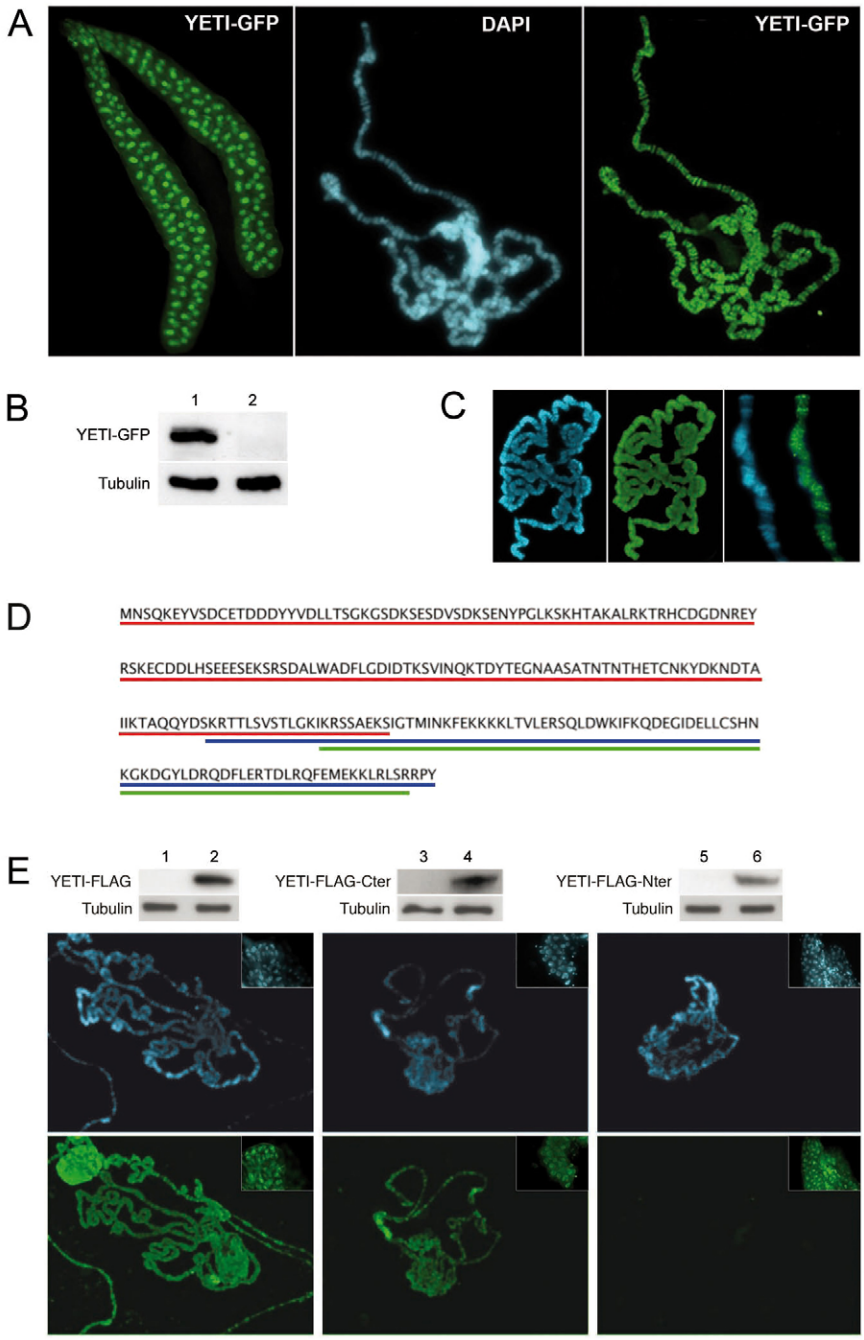

Fig. 3. Expression of YETI–GFP proteins in salivary gland cells. (A) Two lines carrying a different *Yeti-GFP* transgene and the *w elav-GAL4* driver were examined. Polytene chromosomes from *w elav-GAL4; Yeti-GFP/ Yeti-GFP* larvae were stained with DAPI (blue) and immunostained with anti-GFP (green): YETI–GFP accumulates in salivary gland nuclei (left panel) and binds polytene chromosomes arms (right panel). (B) Western blotting assay showing the specificity of GFP antibody for YETI–GFP protein in salivary gland cells in the presence (lane 1) or absence (lane 2) of *elav-GAL4* driver expression. (C) Chromosome morphology rescue by YETI–GFP protein. Polytene chromosomes from *LP1/LP1; tub-GAL4/Yeti-GFP* larvae were stained with DAPI (blue) and immunostained with anti-GFP (green). YETI-GFP (green) binds to polytene chromosomes and its expression is associated with rescue of chromosome morphology (left and middle panels). The antibody decorates the DAPI-intense or -intermediate fluorescent chromosome bands (right panel). (D) The full-length YETI amino acid sequence (241 amino acids); portions present in the truncated YETI-3×FLAG proteins are underscored: YETI-Nter3×FLAG (1–166) in red; YETI-Cter3×FLAG (145–241) in blue. The BCNT domain (80 amino acids, 159–238) is shown in green. (E) Expression of full-length or deleted YETI-FLAG tagged proteins in salivary glands specifically occurs under *elav-GAL4* driver (lanes 2, 4 and 6) but not in the absence of driver expression (lanes 1, 3 and 5). The lower panels show polytene chromosomes sequentially stained with DAPI (blue) and with anti-FLAG (green). The inserts show the detail of fixed salivary glands sequentially stained with DAPI (blue) and with anti-FLAG (green). Full-length YETI-3×FLAG and YETI-Cter3×FLAG and YETI-Nter3×FLAG truncated proteins enter salivary gland nuclei as indicated by the anti-FLAG staining of salivary gland nuclei (inserts). Full-length YETI-3×FLAG (left panel) and YETI-Cter3×FLAG (middle panel) binds to polytene chromosomes, whereas YETI-Nter3×FLAG (right panel) does not.

morphology was seen (Fig. 3C) and the staining pattern was similar to that found in *elav-GAL4; Yeti-GFP* flies (Fig. 3C). This result shows that the YETI–GFP fusion protein binds to chromatin in the absence of the YETI wild-type protein and is able to rescue the aberrant chromosomal phenotype found in *Yeti* mutants.

The BCNT domain is essential for YETI binding to chromatin

The BCNT domain is a highly conserved stretch of about 80 amino acids located in the C-terminal portion of the Bucentaur protein family (Fig. 1), the function of which is unknown. To test whether the BCNT domain of YETI is required for chromatin binding, we constructed three different transgenic *Drosophila melanogaster* lines carrying diverse *Yeti*-3×FLAG transgenes: (1) the *Yeti*-3×FLAG transgene bearing a complete open reading frame of the *Yeti* gene, (2) the *Yeti-Cter*3×FLAG transgene (containing residues 145–241), and deleted for the 5′ end, and (3) the *Yeti-Nter*3×FLAG transgene (containing residues 1–166) and lacking the 3′ end of *Yeti*, which includes the BCNT domain (at 159–238) (Fig. 3D,E; see Materials and Methods). We next induced the expression of each transgene in salivary glands by using the *elav-GAL4* driver and performed both immunostaining and western blot assays using anti-FLAG antibodies (Fig. 3E). A clear staining of salivary gland nuclei was observed using anti-FLAG in larvae expressing each of the three transgenes, showing that both truncated YETI proteins, like YETI–GFP, are able to enter the nucleus (see the inserts in Fig. 3E). However, when we immunostained polytene chromosomes, we found GFP fluorescent signals in larvae

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expressing both full-length *Yeti*-3×FLAG transgene and the *Yeti-Cter*3×FLAG-transgene, but not in larvae expressing the *Yeti-Nter*3×FLAG transgene (Fig. 3E). The results of these experiments, therefore, show that the BCNT domain of YETI is essential for chromatin binding.

Loss of *Yeti* function impairs polytene chromosome accumulation of histone H2A.V variant, nucleosomal histones and histone modification marks

The SWC5 protein, the yeast ortholog of YETI, is a component of the SWR1 ATP-dependent remodeling complex and is required for the recruitment of variant histone Htz1 into chromatin (Kobor et al., 2004; Mizuguchi et al., 2004; Wu et al., 2005). Such a role would be consistent with the observed defects in chromosome/chromatin organization in the absence of YETI function (Cenci et al., 2003a; this paper). We therefore examined the pattern of H2A.V histone variant in polytene chromosomes of wild-type and *Yeti* mutant strains by immunofluorescence methods. As shown in Fig. 4A,C, *Drosophila melanogaster* wild-type salivary glands show multiple H2A.V sites of varying fluorescence intensity along the chromosome arms and in the chromocenter, consistent with previous reports (Leach et al., 2000). *Yeti* mutants [both *LP1/LP1* homozygotes and *LP1/Df(2R)B* hemizygotes] showed instead only residual background fluorescence, with rare faint signs of banding (Fig. 4A,C). This finding prompted us to assess whether other histone types are also affected in the mutant. To address that question we also studied the distribution of H2A, H2B, H3 and H4 chromosomal histones. We found that, indeed, the abundance of Histone H2A was dramatically reduced in *Yeti* mutant polytene chromosomes, similar to the H2A.V variant (Fig. 4A,C). The levels of the H2B, H3 and H4 species are also affected on polytene chromosomes of *Yeti* mutants, but to a lesser extent compared to those of H2A.V and H2A (Fig. 4A,C). These results indicate that loss of *Yeti* function preferentially impairs the chromosomal accumulation of H2A.V and H2A.

As loss of *Yeti* affects the abundance of nucleosomal histones in polytene chromosomes, we assessed the overall level of chromosomal epigenetic marks required for the establishment and maintenance of chromatin organization and function, e.g. histone acetylation and methylation, in wild-type and mutant strains. Immunofluorescence experiments showed that levels of H3K9ac, H3K9me3, H3K4me3 histone modification marks were indeed strongly reduced in *Yeti* mutants (Fig. 4A). However, the levels of H3K9me3, H3K4me3 in the chromocenter were comparable to that of the controls (Fig. 4A,C).

Distribution of chromatin factors in *Yeti* mutants

Post-translationally modified histones are recognized by and interact with different multiprotein complexes that ensure the organization of chromatin in open or closed states, leading, in turn, to either activation or repression of neighboring gene expression. These complexes contain one or more subunits responsible for histone modification, a chromatin remodeling ATPase and other chromatin proteins (Hopfner et al., 2012; Simon and Kingston, 2013). We therefore tested a panel of known major chromatin factors, such as ISWI and DOM-A remodelers, HOAP telomeric protein (also known as Caravaggio), HP1a and Histone Deacetylase RPD3 (James et al., 1989; De Rubertis et al., 1996; Corona et al., 1999; Ruhf et al., 2001; Shareef et al., 2003; Cenci et al. 2003b; Kusch et al., 2004), for their incorporation in polytene chromosomes. The chromosomal levels of ISWI and HOAP in *Yeti* mutants were comparable to those of wild-type strains; however, those of DOM-A (Fig. 4B,C) were reduced by ~70%. In addition, we found reduced chromosomal abundance of HP1a and Histone Deacetylase RPD3. However, the HP1a levels at chromocenter were comparable to those of the controls (Fig. 4B,C).

The results thus far indicate a substantial decrease in the accumulation of both H2A.V and H2A, and of some histone modifications and chromatin factors, along polytene chromosomes of *Yeti* mutants. Western blotting assays of whole protein extracts (Fig. 5A) depicted a reduced overall protein amount of both H2A.V and H2A and specifically modified histones in *Yeti* mutants; HP1a and RPD3 also showed reduced protein levels in *Yeti* mutants.

Loss of YETI yields reduced abundance of products and transcripts from chromatin protein-encoding genes

In *Drosophila melanogaster*, the loss of chromatin remodelers, such as ISWI, leads to defects in higher-order chromosome structure similar to those found in *Yeti* mutants and affects transcriptional regulation (Corona et al., 2007). We next performed qRT-PCR assays for genes encoding H2A.V, nuclesomal histones, HP1a and RPD3, to test whether their transcript abundance is perturbed in *LP1/LP1* mutants. The results of this analysis show that the level of transcripts of genes encoding H2A.V, nucleosomal histones, HP1a and RPD3 is indeed affected in *LP1* mutants (Fig. 5B): decreased levels of transcripts for histones and chromatin marks were apparent and correlated with the reduced protein levels found by both immunofluorescence and western analyses (Fig. 4; Fig. 5A).

YETI–GFP interacts with H2A.V, HP1a and DOM-A

The *Drosophila melanogaster* histone variant H2A.V is known to play roles in HP1a recruitment (Swaminathan et al., 2005). Consistent with this, our present findings show, among other chromosomal defects, a poor content of both H2A.V and HP1a in polytene chromosomes of *Yeti* mutants (Fig. 4).

Interestingly, loss-of-function mutants for YETI, H2A.V, HP1a and DOM-A, all have a similar lethal phenotype characterized by prolonged larval development followed by lethality before pupation and by the presence of large melanotic masses in the larval hemocoel (Minakhina and Steward, 2006). Notably, DOM-A is the ATPase subunit of the Tip60 remodeling complex of *Drosophila* (Ruhf et al., 2001; Kusch et al., 2004), which catalyzes the exchange of phosphorylated H2A.V with H2A during the repair of DNA damage (Kusch et al., 2004) and is also proposed to exchange back H2A with H2A.V, thus facilitating incorporation of the latter in nucleosomes (Baldi and Becker, 2013).

Based on these observations, we decided to investigate whether YETI might interact with H2A.V, HP1a and DOM-A proteins. Using the GFP-TRAP system, we immunoprecipitated H2A.V–GFP fusion protein from extracts of salivary glands of larvae expressing H2A.V–GFP and then analyzed the immunoprecipitates by western blotting using antibodies against YETI or GFP (to visualize H2A.V). As shown in Fig. 6A, YETI was selectively found in the immunoprecipitate from H2A.V–GFP extracts, but was absent from control Oregon-R immunoprecipitate. Reciprocally, H2A.V was found in the YETI–GFP immunoprecipitate (Fig. 6B). However, HP1a was not detected in the same YETI–GFP immunoprecipitate (data not shown). Thus, we also performed GST pulldown experiments to test the interaction between YETI and HP1a. As shown in Fig. 6C,D, GST–HP1a, but not GST alone, precipitates YETI from YETI–GFP-expressing larval salivary gland extracts.

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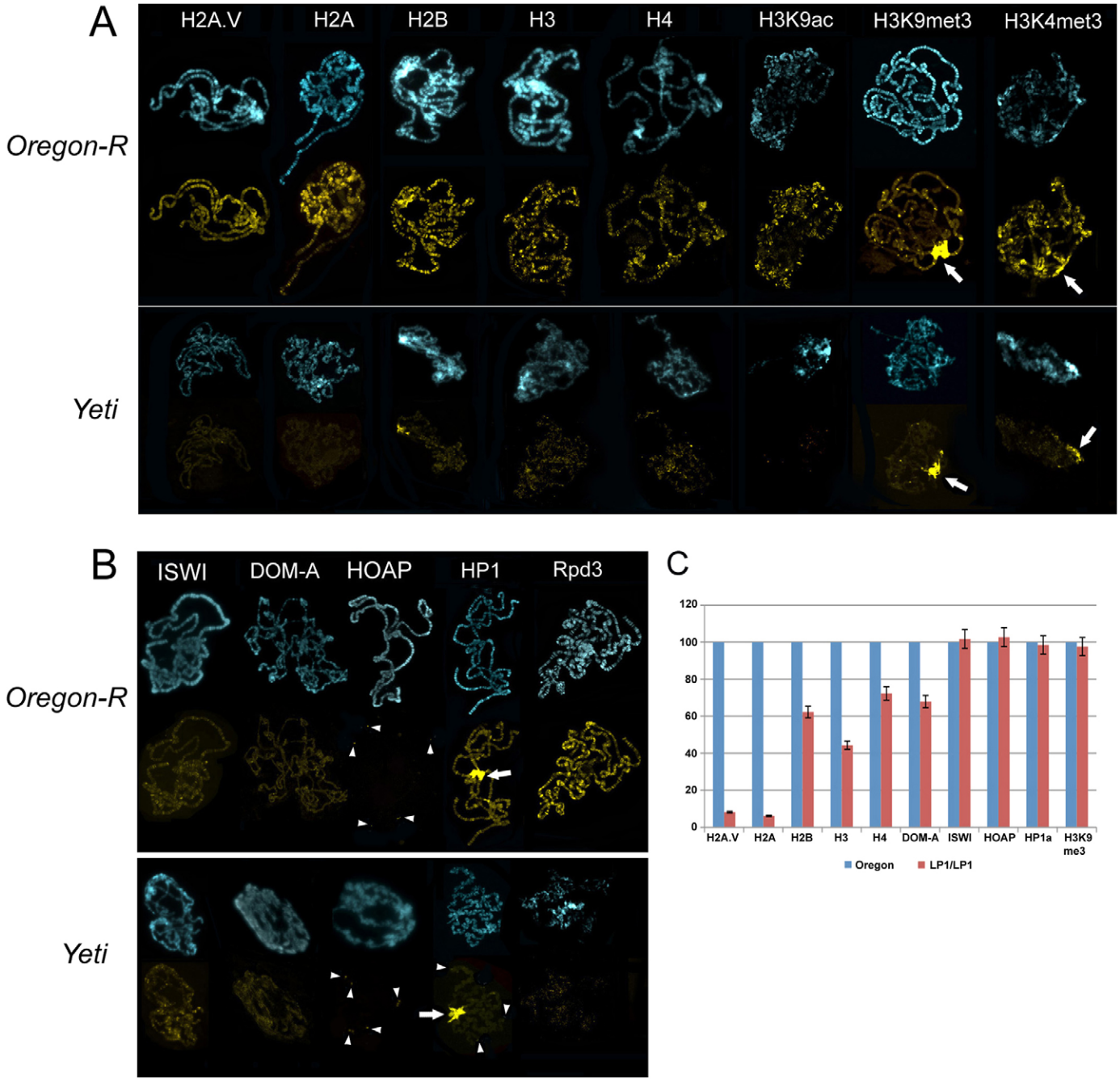

Fig. 4. Levels of H2A.V, nucleosomal histones, histone modifications and chromatin proteins in polytene chromosomes of wild-type Oregon-R and *Yeti* mutants. (A) Distribution of H2A.V, nucleosomal histones and histone marks. Not all histones and chromatin proteins tested are equally affected in *Yeti* mutants (*LP1*/*LP1*) compared to the wild-type Oregon-R. Immunofluorescence analysis reveals that levels of H2A.V and H2A are drastically compromised, whereas those of H2B, H3 and H4 are reduced to a lesser extent (62%, 45% and 72%, respectively). (B) Distribution of chromatin modifiers. Note that in *Yeti* mutants ISWI and HOAP accumulates at comparable levels to those of wild-type; telomeres stained by anti-HOAP are indicated by arrowheads. Notably, HP1, H3K9met3 and H3K4met3 still yield a significant staining of chromocenters (marked by arrows) in *Yeti* mutants, suggesting that their reduced accumulation mainly affects euchromatic chromosome arms. HP1 is also present on telomeres of *Yeti* mutants (marked by arrowheads). Chromosomes were stained with DAPI and pseudocolored in blue; antibody staining was pseudocolored in yellow. (C) Histograms showing the quantification of chromosomal levels of H2A.V, nucleosomal histones, histone modifications and chromatin proteins.

Next, we immunoprecipitated DOM-A from salivary glands extracts of *elav-Gal4; UAS-Yeti-GFP* larvae (expressing the YETI–GFP fusion protein). As shown in Fig. 7A, YETI–GFP was recovered in the immunoprecipitates with anti-DOM-A antibody. Taken together, these results suggest that DOM-A interacts with YETI. The present findings that YETI interacts both with DOM-A and with H2A.V, together with the known role of DOM-A in recruitment of H2A.V, would place DOM-A upstream of YETI and H2A.V in events involved in the recruitment of chromatin proteins.

We therefore directly asked whether DOM-A is actually responsible for the recruitment of YETI to chromatin. To this

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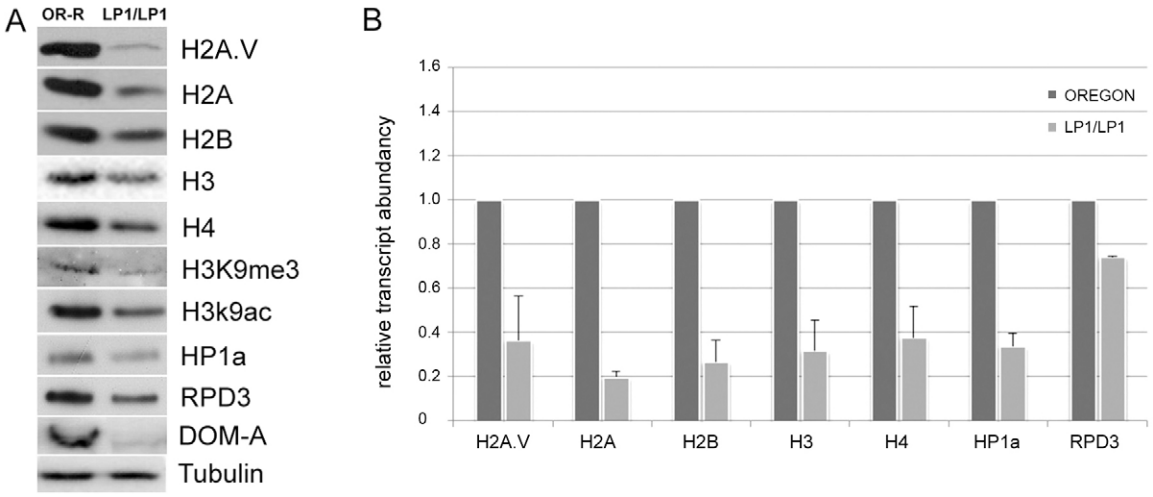

Fig. 5. Western blotting and qRT-PCR analyses in wild-type Oregon-R and *Yeti* mutants. (A) Western blotting analysis showing a decrease in protein abundance for H2A.V, chromosomal histones and chromatin marks in *Yeti* mutants compared to wild-type Oregon-R controls; each assay was repeated at least twice. (B) Quantification of the mRNA levels for histone and chromatin proteins as assessed by qRT-PCR in *Yeti* mutants and wild-type Oregon-R controls. The values were calculated from four independent experiments, with three replicates each. The results show the mean±s.d. from four different experiments with three replicates each.

aim, we examined the distribution of YETI–GFP in polytene chromosomes of homozygous larvae for *domino3* (*dom3*), a null allele of *domino*. The *domino* mutant larvae undergo prolonged larval development followed by lethality before pupation and show large melanotic masses in their hemocoel. By genetic crosses we obtained *dom3/dom3; Tub-Gal4/UAS-Yeti-GFP* larvae, such that YETI could be visualized by its GFP fluorescence, both in salivary glands *in vivo* and in fixed polytene chromosomes. Comparable amounts of YETI–GFP were present in both *dom3/dom3; Tub-Gal4/UAS-Yeti-GFP* (lane 1) and *dom3/CyS; Tub-Gal4/UAS-Yeti-GFP* control (lane 2) salivary glands (Fig. 7C). As shown in Fig. 7B, *domino* mutant salivary gland nuclei exhibit intensely fluorescent YETI signals, whereas, in contrast, only a residual background fluorescence is visible on polytene chromosomes (Fig. 7C). Thus, in *domino* mutants the YETI–GFP protein enters the nucleus but its deposition onto chromatin is abolished. Taken together, these results suggest that YETI is recruited to chromatin by DOM-A and is in turn implicated in regulating H2A.V, which is another downstream target of DOM-A.

DISCUSSION

In present study, we have carried out a functional analysis of the *Drosophila melanogaster Yeti* gene and its encoded product, YETI. The results show the *Yeti* is an essential gene and indicate a previously unrecognized role of YETI protein as a key chromatin factor, providing mechanistic clues on possible roles of the BCNT protein family in eukaryotes. In particular, based on the chromatin defects found here in *Yeti* mutants, as well as the newly emerging interactions of YETI–GFP with H2A.V, DOM-A and HP1a, we propose that YETI interacts with H2A.V-exchanging machinery, as a chaperone or as a new subunit of the *Drosophila* DOM/Tip60 complex, and participates in the step of H2A.V deposition.

The loss of YETI perturbs chromatin organization and function

The functions of the YETI protein were poorly understood thus far. We found that chromosome structure is drastically perturbed in *Yeti* mutants (Cenci et al., 2003a; Fig. 2B,E), suggesting that YETI is involved in chromosome and/or chromatin organization. YETI protein has also been proposed to bind both subunits of the microtubule-based motor kinesin-I (Wisniewski et al., 2003), and previous immunolocalization experiments have shown both a nuclear and cytoplasmic localization of epitope-tagged YETI in *Drosophila* S2 cultured cells (Wisniewski et al., 2003). In those experiments, however, it remained unclear whether YETI was located within the nuclei or associated with the nuclear membrane.

The present results clearly show that the YETI–GFP fusion protein is abundant in nuclei and associates with polytene chromosomes (Fig. 3A,C). Notably, our experiments demonstrate that the evolutionary conserved BCNT domain is indispensable for YETI binding to polytene chromosomes (Fig. 3D,E), providing the first direct evidence for an essential role of this domain in chromatin binding.

Furthermore, we find that YETI–GFP interacts with H2A.V, HP1a and DOM-A (Fig 6; Fig. 7A). DOM-A is the ATPase subunit of the Tip60 remodeling complex that, similar to the related SWR1 complex in yeast, is likely to incorporate H2A.V into nucleosomes by exchange of H2A with the variant (Baldi and Becker, 2013). Like the related yeast SWIR1 protein, DOM-A might be the platform required for the assembly of the other subunits of the Tip60 complex. In support of this view, we now show that YETI–GFP requires DOM-A to be recruited to chromatin (Fig. 7C).

The defects in higher-order chromosome structure characterized here in *Yeti* mutants are accompanied by reduced chromosomal levels of H2A.V, of nucleosomal histones and of epigenetic marks (Fig. 4). However, not all tested histones and epigenetic marks are equally affected in *Yeti* mutants compared to controls; the chromosomal levels of H2A.V and H2A are dramatically decreased, whereas those of H2B, H3 and H4 are reduced to a lesser extent (Fig. 4A,C). In addition, among tested chromatin proteins, the chromosomal levels of ISWI and HOAP remained unaffected, those of DOM-A were decreased by 30% and those of HP1a only decreased in euchromatin (Fig. 4B,C). Taken together,

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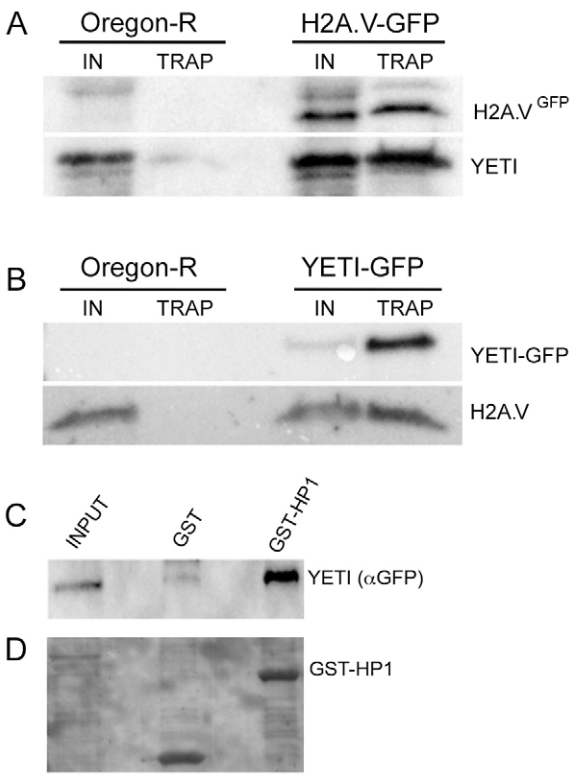

Fig. 6. YETI interacts with H2A.V and HP1a. (A) Salivary gland protein extracts from H2A.V–GFP-expressing strains and the Oregon-R wild-type strain were subjected to immunoprecipitation using the GFP-TRAP system. Input protein extracts (IN) and GFP-TRAP immunoprecipitates (TRAP) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane, then probed with anti-YETI and anti-GFP antibodies. YETI is present in the H2A.V–GFP immunoprecipitate, but is absent in Oregon-R control immunoprecipitates. (B) Inverse co-immunoprecipitation using the GFP-TRAP system in brain and salivary gland protein extracts from YETI–GFP-expressing and Oregon-R strains. Western blots were probed with anti-H2A.V and anti-GFP antibodies. H2A.V is present in the YETI–GFP immunoprecipitate but is absent from the Oregon-R control immunoprecipitate. (C) Western blotting showing that GST–HP1a (but not GST alone) interacts with YETI (as detected by anti-GFP antibody) from YETI–GFP-expressing larval brain extracts. Input is 10% of the total extract. (D) GST–HP1 and GST bands are revealed by Ponceau staining.

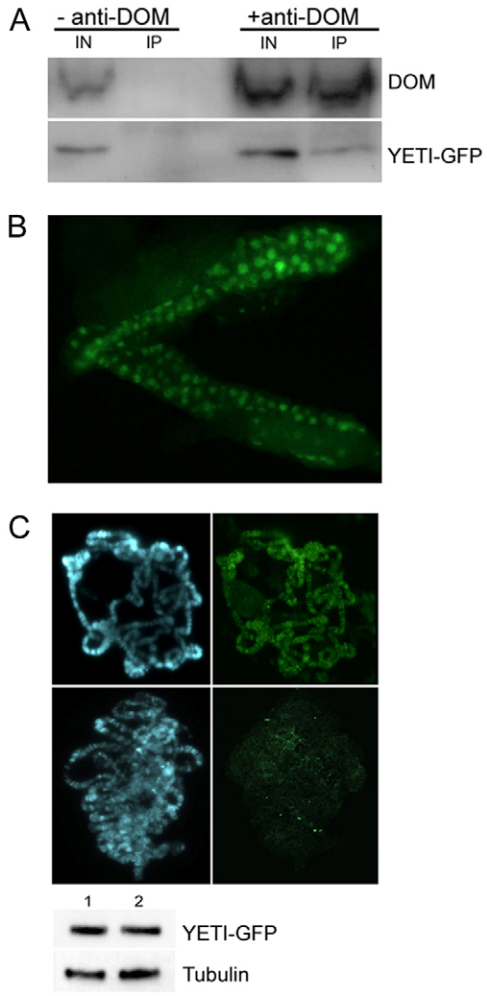

Fig. 7. DOM-A interacts with YETI–GFP and is responsible for its recruitment. (A) Immunoprecipitation (IP) of DOM-A from salivary gland extracts of *elav-Gal4; UAS-Yeti-GFP/UAS-Yeti-GFP* larvae. YETI–GFP fusion protein was co-immunoprecipitated from *elav-Gal4; UAS-Yeti-GFP* extracts only when anti-DOM-A antibodies were added. IN, input. (B) *In vivo* salivary glands of *dom3/dom3; Tub-Gal4/UAS-Yeti-GFP* larvae showing GFP fluorescence in all nuclei. Nuclei in the lower layer of the glands are out of focus and show a lower fluorescence intensity. (C) Immunostaining of polytene chromosomes of *dom3/dom3; Tub-Gal4/UAS-Yeti-GFP* and *dom3/+; Tub-Gal4/UAS-Yeti-GFP* larvae. GFP staining is present on polytene chromosomes of *dom3/+; Tub-Gal4/UAS-Yeti-GFP* larvae (above), but *dom3/dom3; Tub-Gal4/UAS-Yeti-GFP* polytene chromosomes fail to reveal any GFP signals (below). The amount of YETI–GFP is unchanged in *dom3/dom3; Tub-Gal4/UAS-Yeti-GFP* (lane 1) and *dom3/+; Tub-Gal4/UAS-Yeti-GFP* (lane 2) larvae (right panel).

these results indicate that the loss of YETI protein does not generally affect chromatin and/or chromosome organization. We can also exclude a primary defect on DNA replication, as that would result in a significant percentage of chromosome breaks (Branzei and Foiani, 2008), which are not detected in the *Yeti* mutants (Cenci et al., 2003a; Fig. 4E). Finally, qRT-PCR analysis of *Yeti* mutants showed downregulation of the genes encoding the proteins tested by immunostaining and western assays.

It is worth mentioning that the lethal phenotype of *Yeti* mutants (prolonged larval development followed by lethality before pupation and presence of melanotic masses) is highly reminiscent of that produced by mutations in genes encoding H2A.V and chromatin proteins, such as DOM-A, HP1a and *Drosophila* Rsf1 (Minakhina and Steward, 2006; Hanai et al., 2008). Interestingly, the Rsf1 remodeler interacts with H2A.V and members of the Tip60 complex (Hanai et al., 2008), suggesting a functional link between those chromatin factors. The lack of essential chromatin regulators that control chromatin architecture and gene expression might well affect blood cell differentiation, the alterations of which result in melanotic tumors. Interestingly, it has been recently found that a network of epigenetic regulators controls the establishment of hematopoietic cells in zebrafish (Huang et al., 2013).

In *S. cerevisiae* the H2A histone variant, called Htz1, is incorporated into chromatin by the SWR1 ATP-dependent chromatin-remodeling complex (Wu et al., 2005). The Swc5 subunit of the SWR1 complex, the ortholog of YETI, has been suggested to be required for eviction of H2A from nucleosomes prior to Htz1 loading carried out by Swc2 and Swr1 (Lavigne

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et al., 2009; Wu et al., 2009; Morillo-Huesca et al., 2010). Our findings that H2A.V deposition is perturbed in *Yeti* mutants and that YETI interacts with both H2A.V and DOM-A (Fig. 6A,B; Fig. 7A), strongly suggest that YETI, like his yeast homolog Swc5, might contribute to loading the variant H2A.V onto nucleosomes. The dramatic defect in H2A deposition also found in *Yeti* mutants (Fig. 4) suggests that, in YETI defective cells, H2A is removed by the DOM/tip60 complex but not replaced with H2A.V, resulting in depletion of both histones and disrupting chromatin integrity and function. A similar scenario has been proposed in yeast (Morillo-Huesca et al., 2010).

Moreover, the observation that YETI–GFP interacts with HP1a in GST pulldown assays (Fig. 6C), suggests that YETI might be an important mediator in targeting HP1a to chromatin remodeling regions. Association between HP1a and other chromatin remodeling factors has been reported (Fischer et al., 2009; Lavigne et al., 2009). Human HP1α interacts with the BRG1 protein of the SWI/SNF ATP-dependent remodeling complex, suggesting a direct role for BRG1 in the modulation of HP1α-containing higher-order chromatin structures (Nielsen et al., 2002).

How to account for cytological and molecular defects found in YETI mutant cells?

The loss of *Drosophila melanogaster* chromatin remodelers such as ISWI leads to defects in higher-order chromosome structure similar to those found in *Yeti* mutants and affects transcriptional regulation (Corona et al., 2007). H2A variants and HP1 play key roles in both transcriptional activation and repression control in different species (Stargell et al., 1993; Dhillon and Kamakaka, 2000; Piacentini et al., 2003; Jin et al., 2005; Swaminathan et al., 2005; Keogh et al., 2006; Mavrich et al., 2008; Petesch and Lis, 2008; Henikoff et al., 2009; Cryderman et al., 2011; Kotova et al., 2011; Baldi and Becker, 2013). The drastic depletion of both H2A.V and H2A found in *Yeti* mutants might either directly or indirectly impair chromatin organization and function. H2A.V knockdown in *Drosophila melanogaster* indeed results in inefficient processing of the histone mRNA (Wagner et al., 2007), giving rise to a reduction of histone gene transcription and histone protein synthesis; this can affect in turn the global transcriptional output (Celona et al., 2011).

In the light of our results, we suggest that the specific impairment of H2A.V exchange and HP1a recruitment in YETI-defective cells yields a global chromatin unbalance, which in turn reverberates on the epigenetic control of gene expression, thus producing a 'boomerang' effect with a generalized impact on chromatin organization and function.

Concluding remarks

In the past decade, growing lines of evidence indicate that mutations in genes encoding proteins directly or potentially implicated in chromatin organization and remodeling are crucial players in human developmental disorders, including craniofacial development (Bickmore and van der Maarel, 2003; Fog et al., 2012). Thus, the identification of new factors required for chromatin dynamics and organization can expand our understanding of mechanisms underpinning developmental diseases. Several studies implicate CFDP1, the human ortholog of YETI, in osteogenesis and craniofacial development (Diekwisch et al., 1999; Diekwisch et al., 2002; Thisse, 2004; Wu et al., 2008; Bustos-Valenzuela et al., 2011; Makeyev and Bayarsaihan, 2011). Future research can provide a better understanding of BCNT protein functions in chromatin organization and yield more insight into their role in developmental disorders.

MATERIALS AND METHODS

Drosophila strains and genetic crosses

Fly cultures and crosses were carried out at 25°C in standard cornmeal yeast medium. The fly line #5941 of the Bloomington Drosophila Stock Center expresses H2A.V–GFP. To induce expression of YETI–GFP we used the fly lines #5138 and #5112 containing the *Tub-Gal4* and *elav-Gal4* drivers, respectively. The T(2;3) translocation carrying the dominant *Cy* and *Tb* markers used as balancer of *Yeti* alleles is designated 'ST' in the text. *Df(2R)B* is a chromosome carrying a partial deletion of the heterochromatin of the right arm chromosome 2 (2R) that fails to complement *l(2)41A*a (Hilliker, 1976; Rossi et al., 2007) and *LP1*. *Df(2R)M41A10* is a large deletion lacking almost all the heterochromatin of 2R together with all known essential genes and gene models (Corradini et al., 2003; Rossi et al., 2007). The EMS-31 allele is an EMS-induced allele of *l(2)41A*a (Hilliker, 1976; Cenci et al., 2003a; Dimitri et al., 2003). To recover insertional alleles of the *l(2)41A*a gene, we induced mobilization of a homozygous viable and fertile P element [*P(w+)19.74.3*] that colocalizes cytologically with *l(2)41A*a in region h41 of 2R mitotic heterochromatin (Corradini et al., 2003; Fig. 2). By inverse PCR and sequencing, we found that 19.74.3 is located in scaffold AABU01002199, about 2 kb from the 5′ end of CG40218. *P(w+) 19.74.3/Cy S; delta 2-3/TM3, Sb Sr* males were generated carrying simultaneously the *P(w+)19.74.3*, heterochromatic element and the *delta 2-3* element as a constitutive source of transposase. In the germline of *P(w+)19.74.3/Cy S; delta 2-3/TM3* males, the single P element jumps all around the genome, with some preference for insertion in nearby heterochromatin (Zhang and Spradling, 1993). *P(w+)19.74.3/Cy S; delta 2-3/TM3* males were crossed to *Cy S/Sco* females to recover *P(w+)19.74.3*/Cy S* males carrying putative new insertions [*P(w+)19.74.3**] in the vital heterochromatin genes of chromosome 2. P-element-induced recessive lethal alleles of vital heterochromatic genes were selected over *Df(2R)M41A10*. Complementation tests were performed by crossing single *P(w+)19.74.3*/CyS* males to *Df(2R)M41A10/CyS* females. The absence of *P(w+)19.74.3*/Df(2R)M41A10 Cy*$^{+}$ flies among the offspring indicated that the tested *P(w+)19.74.3** chromosome carries a lethal mutation uncovered by *Df(2R)M41A10*. For each lethal mutation, balanced stocks were established. Lethal mutations were tested for their ability to complement a collection of EMS-induced lethal alleles of known genes of 2R heterochromatin. After mobilization of *P(w+)19.74.3*, out of 1077 tested chromosomes we recovered two chromosomes denoted *LP1* and *LP2* that resulted in lethality when heterozygous with *Df(2R)M41A10* and alleles of *l(2)41Aa* vital gene.

Constructs and transgenic lines

Yeti-GFP transgenic lines of *Drosophila melanogaster* were made as follows: (1) the *Yeti* cDNA was cloned in-frame with the reporter GFP sequence in the transformation vector pUASt; (2) the *pUAS-Yeti-GFP* construct was injected in pre-blastoderm *Drosophila* embryos of *w*^{1118} genotype; (3) transgenic flies were selected using the *white*$^{+}$ phenotypic marker. Transgenic lines carrying the *Yeti*-cDNA (M17 and M7) under control of UAS sequences were also made using the same experimental strategy.

Yeti-3×FLAG transgenic lines of *Drosophila melanogaster* were made as follows: full-length, N-terminal and C-terminal sequences of *Yeti* cDNA were cloned in frame with the FLAG epitope into the p3xFLAG-CMV-10 expression vector (Sigma); the different *Yeti*-3×FLAG fragments were then subcloned into the pUASt destination vector. The same procedures for injections and selection of transgenic flies were used for YETI–GFP and YETI–cDNA constructs.

Diffuse expression of *Yeti-GFP*, *Yeti*-cDNA (line M17) and *Yeti*-3×FLAG transgenes was induced by crosses with enhancer trap lines carrying the ubiquitously expressed *tub-GAL4* driver or the *elav-GAL4* driver that activates expression in larval neuroblasts and salivary glands.

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To rescue the lethal phenotype of the *LP1* null allele, *w/w*; *LP1*; *tub-GAL4/T(2;3)ST, Cy Tb* females were crossed to *w/Y*; *LP1*; *UAS-Yeti-M17/T(2;3)ST, Cy Tb* males. In the progeny of this cross, a 2:1 ratio was found between *Cy Tb* and Cy^{+} Tb^{+} progenies, the latter corresponding to the *LP1/LP1*; *UAS-Yeti-M17/tub-GAL4* flies.

Antibodies

Rabbit polyclonal antibodies against *Drosophila* YETI were raised against a synthetic peptide (CFEMEKKLRLSRRPY) located from position 227 to position 241 of the C-terminal region of the protein. Polyclonal antibodies against H2A.V, HP1a, DOM-A, ISWI, HOAP, RPD3 and GFP (for immunostaining) were kindly provided by Robert Glazer (Wadsworth Center, Div. Genetic Disorders New York State Department of Health & University at Albany, USA); Sarah Elgin (Department of Biology Washington University in St. Louis, USA); Marie-Laure Rufh; Davide Corona (Dipartimento di Biologia Cellulare e dello Sviluppo, Università degli Studi di Palermo, Italy); Laura Ciapponi (Dipartimento di Biologia e Biotecnologie, Sapienza Università di Roma, Italy); Thomas Rudolph (Institute of Genetics, Halle University, Germany); and Gianluca Cestra (Dipartimento di Biologia e Biotecnologie, Sapienza Università di Roma, Italy), respectively. The following commercial antibodies against *Drosophila* proteins were used: anti-H2A, anti-H2B, anti-H3 and anti-H4 (Millipore), anti-HP1a, anti-H3K9me3 and anti-H3K9ac (Abcam), anti-Tubulin (Sigma), anti-GFP-HRP (vector).

Cytology and immunostaining

Polytene chromosome squashes and immunolocalization experiments to *Drosophila* polytene chromosomes performed as described previously (Deuring et al., 2000). Rabbit antibodies against GFP, H3K9ac, ISWI, H2A.V or H2A, and mouse antibodies against H3met K9 were used at 1:100 dilution. Rabbit antibodies against HOAP were used at 1:50 dilution; as a secondary antibody a rabbit monoclonal Alexa-Fluor-conjugated antibody (Life technologies) was used at 1:300 dilution. Rat anti-DOM-A antibodies were used at 1:500 dilution; secondary rat monoclonal Alexa-Fluor-conjugated antibody antibodies (Life technologies) was used at 1:200 dilution. Chromosomes were analyzed by a computer-controlled Nikon Eclipse 50i epifluorescence microscope equipped with a CCD camera. Quantification of chromosomal levels of H2A.V, nucleosomal histones, histone modifications and chromatin proteins were performed using the NIS-Elements software provided by Nikon.

Cell cultures and RNAi treatments

Drosophila S2 cells were cultured at 25℃ in Shields and Sang M3 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich). RNAi treatments were carried out according to Clemens et al. (Clemens et al., 2000). Production of dsRNA and cytological analysis of RNAi-treated cells were performed according to Somma et al. (Somma et al., 2002).

RNA preparations, northern blotting and western blotting

Total RNA and proteins were extracted from larval brains and salivary glands. Total RNA preparations, and northern and western blotting were according to standard protocols (Crowley et al., 1984; Sambrook et al., 2001). Antibodies against histones and α-Tubulin were used at the following dilution: 1:2500. Rabbit, mouse and rat secondary antibodies were used at 1:5000 dilution. The bands were immunodetected using the ECL kit from BIORAD.

Quantitative RT-PCR and inverse PCR

Total RNA was extracted from wandering third-instar larvae with the Tri Reagent (Sigma) according to the manufacturer's instruction. RNA (2 γ) were retrotranscribed with the RETROscript kit (AM1710) according to the manufacturer's instruction. RT-qPCR was performed on an ABI 7300 Real-Time PCR System, according to the supplier protocol and using the following PCR conditions: 10 min at 95℃, 40×(15 s at 95℃, 1 min at the annealing/extension temp of 60℃) 10 min at 72℃. Primers and annealing temperatures were as follows: H2A-F, 5′-CGGCCAGATATTCCATTACG-3′; H2A-R, 5′-GGCTGCTCCTACTGATCCTG-3′; H2B-F, 5′-TGACACCGGAATTTCGTCGA-3′; H2B-R, 5′-TTGGTTCCCTCACTGACAGC-3′; H2A.V-F, 5′-CGAAACCGAATTCCGTAGAA-3′; H2A.V-R, 5′-GCCAGCCATTTCTGTTCAAT-3′; H3-F, 5′-GCTCGGTGCTCTTTTGGTAG-3′; H3-R, 5′-ATGGCTCGTACCAAGCAAAC-3′; H4-F, 5′-GTGTGAAGCGCATATCTGGA-3′; H4-R, 5′-TAACCGCCAAATCCGTAGAG-3′; HP1-F, 5′-CAAGCGAAAGTCCGAAGAAC-3′; HP1-R, 5′-ACCATTTCTGCTTGGTCCAC-3′; RPD3-F, 5′-CAAGGACACCAACAGCAAC-3′; RPD3-R, 5′-TCGCTGCGGCATCATTATC-3′; Rpl32-F, 5′-GCGCACCAAGCACTTCATC-3′; Rpl32-R, 5′-TTGGGCTTGCGCCATT-3′.

Data were analyzed according to Livak and Schmittgen (Livak and Schmittgen, 2001). Relative expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$); mRNA expression was normalized against the average values of RpL32. The values in Fig. 5 were calculated from four independent experiments, with three replicates each.

Genomic DNA from two to three flies was prepared as described previously (Eggert et al., 1998) and digested using either *Hha*I or *Hpa*II. DNA equivalent to approximately one-fifth of a fly was ligated using the Rapid Ligation kit (Roche). Inverse PCR was performed according to standard procedures (Dobie et al., 2001) and sequencing was performed by the MWG company. BLASTN against nonredundant database was performed on flanking sequences >25 bp in order to identify homologous gene models or repeated sequences.

Co-immunoprecipitation and GST pulldown

Wandering third-instar larvae were dissected in physiological solution; brains and salivary glands were resuspended in lysis buffer [20 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM DTT, 420 mM NaCl, 30 mM NaF, 25 mM β-glycerophosphatase, 0.2 mM Na_3VO_4, 0.1% NP-40, 0.5 mM PMSF, 1× Protease Inhibitor Cocktail (Roche)] and incubated on ice for 1 hour. GFP-TRAP assays in *Drosophila* was performed according to manufacturer's instructions (Chromotek). The proteins of interest was detected using the specific antibodies against the following proteins at the given dilutions: GFP (1:2500); Yeti (1:250); H2A.V (1:2500) and α-Tubulin (1:2500). Horseradish peroxidase secondary antibodies were all used at 1:5000. Immunodetection was performed using the ECL kit from BIORAD. For GST pulldown, 2 mg of either the GST–HP1 fusion protein or GST alone was incubated with protein extracts from 50 brains in incubation buffer (20 mM HEPES KOH, 20 mM NaF and 0.8% NP40), 1 hour at 4℃ with agitation. After incubation, GST proteins were harvested and washed several times with incubation buffer and resuspended in 1× Laemmli Buffer. Co-immunoprecipitation assays were repeated at least twice.

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Competing interests

The authors declare no competing interests.

Author contributions

All authors conceived and designed the experiments, performed the experiments and analyzed the data. P.D. wrote the paper.

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A

P *w/w; LP1 UAS-M17/St, Cy, Tb* **X** *w/Y; LP1 Tub-GAL4/St, Cy, Tb*

F1	***Cy+ Tb+***	***Cy Tb***
	164	*107*

B

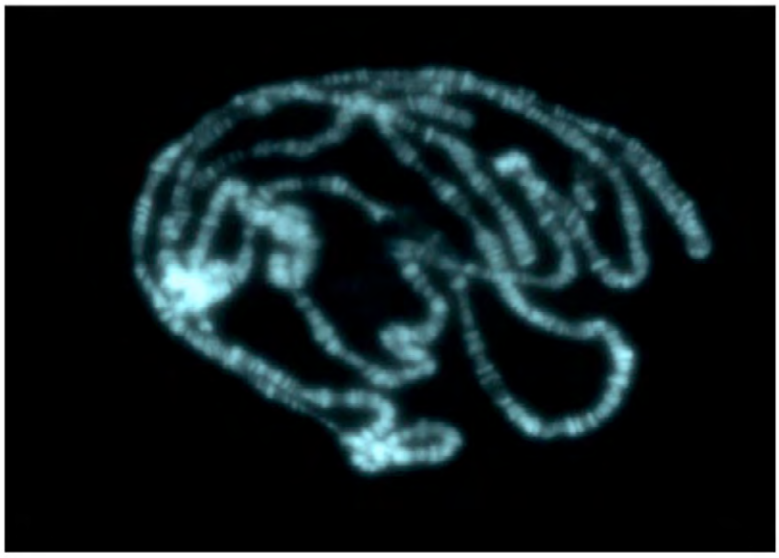

Fig. S1. Rescue of *Yeti* lethality in *LP1/LP1*; *UAS-Yeti-M17/tub-GAL4* flies. A) Results of genetic crosses where the expression of *M17* transgene is ubiquitously driven by the *tub-GAL4* driver. B) Fully rescued polytene chromosomes of *LP1/LP1*; *UAS-Yeti-M17/tub-GAL4* larvae stained with DAPI.

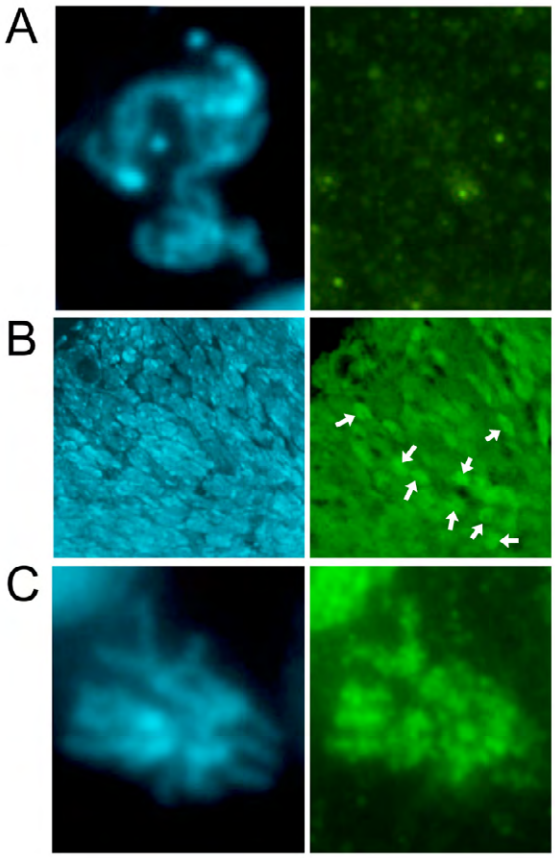

Fig. S2. Immunostaining of mitotic chromosomes and tissue from larval brains with confocal microscope. A) Mitotic chromosomes from neuroblasts of larvae expressing *Yeti-GFP* trangene under the *elev-GAL4* driver. B) Mitotic brain squashes showing nuclei stained by anti-GFP C) Mitotic chromosome from neuroblasts of larvae expressing *H2A.V-GFP* under the endogenous *H2A.V* promoter. Chromosomes are sequentially stained with DAPI and with anti-GFP. No significant GFP staning is seen on chromosomes from *elav-GAL4*; *UAS-Yeti-GFP*/*UAS-Yeti-GFP* (A, right panel); in the same larvae, GFP staining is present in mitotic nuclei and pointed by arrows (B, right panel). In the control line expressing *H2A.V-GFP, c*hromosomes are clearly stained with anti-GFP (C, right panel).