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The enhancer of trithorax and polycomb gene *Caf1/p55* is essential for cell survival and patterning in *Drosophila* development

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SUMMARY

In vitro data suggest that the human *RbAp46* and *RbAp48* genes encode proteins involved in multiple chromatin remodeling complexes and are likely to play important roles in development and tumor suppression. However, to date, our understanding of the role of *RbAp46*/*RbAp48* and its homologs in metazoan development and disease has been hampered by a lack of insect and mammalian mutant models, as well as redundancy due to multiple orthologs in most organisms studied. Here, we report the first mutations in the single *Drosophila RbAp46*/*RbAp48* homolog *Caf1*, identified as strong suppressors of a *senseless* overexpression phenotype. Reduced levels of *Caf1* expression result in flies with phenotypes reminiscent of Hox gene misregulation. Additionally, analysis of *Caf1* mutant tissue suggests that *Caf1* plays important roles in cell survival and segment identity, and loss of *Caf1* is associated with a reduction in the Polycomb Repressive Complex 2 (PRC2)-specific histone methylation mark H3K27me3. Taken together, our results suggest suppression of *senseless* overexpression by mutations in *Caf1* is mediated by participation of Caf1 in PRC2-mediated silencing. More importantly, our mutant phenotypes confirm that Caf1-mediated silencing is vital to *Drosophila* development. These studies underscore the importance of *Caf1* and its mammalian homologs in development and disease.

KEY WORDS: Caf1, *Drosophila*, Epigenetics, Polycomb, RbAp48, Senseless

INTRODUCTION

The development of complex multicellular organisms requires the precise patterning of a wide range of appendages, organs, tissues and cell types. To a large extent, this task is accomplished by the reiterative use of a limited number of conserved pathways and proteins. However, we are only beginning to understand how pathways and proteins can be used repeatedly and still achieve developmental diversity. Chromatin remodeling is an important mechanism that allows cells to stably yet reversibly lock in repressed or active chromatin states, thereby restricting the fate of the cell during development (Kadonaga, 1998; Schulze and Wallrath, 2007; Vermaak et al., 2003).

Drosophila Chromatin Assembly Factor 1 (Caf1, also known as p55; *RbAp48* – FlyBase) is a 55 kDa protein containing seven WD repeats and α-helical regions in the N and C termini, and binds directly to histone H4 (Song et al., 2008; Tyler et al., 1996). The *Caf1* gene has homology to two human genes, retinoblastoma associated protein 46 (*RbAp46*; *RBBP7* – Human Gene Nomenclature Database) and retinoblastoma associated protein 48 (*RbAp48*; *RBBP4* – Human Gene Nomenclature Database) (Tyler et al., 1996; Verreault et al., 1996). Caf1 shares 87% and 84% amino acid identity with human RbAp48 and RbAp46, respectively, and is therefore likely to prove an excellent tool for studying the functional and developmental roles of the human proteins (Tyler et al., 1996). *Drosophila* Caf1, first identified as a component of the Chromatin Assembly Factor 1 complex (CAF-1) which acts in nucleosome assembly following DNA replication, is a component of many chromatin remodeling complexes (Tyler et al., 1996). Caf1 is also found as a component of the Nucleosome Remodeling Factor (NURF) and, like its human homologs RbAp46 and RbAp48, is a component of retinoblastoma (RB)-containing complexes (Korenjak et al., 2004; Martinez-Balbas et al., 1998; Qian and Lee, 1995; Qian et al., 1993; Taylor-Harding et al., 2004).

Caf1 is also a member of the Polycomb group (PcG) complex Polycomb Repressive Complex 2 (PRC2), along with Extra Sex Combs (ESC), Suppressor of Zeste 12 [SU(Z)12] and Enhancer of Zeste [E(Z)] (Muller et al., 2002; Tie et al., 2001). The role of PcG proteins has been best characterized in silencing of Hox genes, although PcG silencing also occurs at many non-*Hox* loci (Bello et al., 1998; Chen and Rasmuson-Lestander, 2009; Dura and Ingham, 1988; Netter et al., 1998; Pelegri and Lehmann, 1994; Schuettengruber et al., 2007). PRC2 silences genes by effecting histone modifications, particularly trimethylation of histone H3 at lysine 27 (Czermin et al., 2002) (for a review, see Schuettengruber et al., 2007). Beyond its important role in development, PRC2 has also been implicated in cancer pathways. For example, in mammals, aberrant epigenetic modification of PRC2 target genes is associated with colorectal cancer (Widschwendter et al., 2007).

With participation in such diverse complexes, *Drosophila* Caf1 is likely to have important roles in multiple aspects of development. Until now, mutant or reduced expression phenotypes of *Caf1* homologs have been reported in yeast, *Arabidopsis* and *C. elegans*, but no mutations in *Drosophila Caf1* have been identified and genetically characterized (Bouveret et al., 2006; Guitton and

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Berger, 2005; Hennig et al., 2003; Jullien et al., 2008; Lu and Horvitz, 1998; Ruggieri et al., 1989). Furthermore, reduced expression of the mammalian *Caf1* homologs *RbAp46* and *RbAp48* is observed in human cancers, and in vitro studies suggest both genes may act as tumor suppressors, but the lack of *Caf1* mouse models hinders our understanding of its potential role in human disease (Guan et al., 2001; Guan et al., 1998; Ishimaru et al., 2006; Kong et al., 2007; Li et al., 2003; Pacifico et al., 2007; Thakur et al., 2007).

Chromatin remodeling adds a unique level of transcriptional regulation to the signaling pathways that control development by stably repressing genes in a heritable manner, and may therefore be of particular importance to genes and pathways that are re-used in development. The *Drosophila senseless* (*sens*) gene is one example of a gene that is reiteratively employed. Specifically, Sens functions in a number of processes in both the embryo and larval imaginal tissues, including the compound eye. In the third instar eye disc, Sens is necessary for differentiation of the R8 photoreceptor, the founding cell of each ommatidium (Frankfort et al., 2001). Continued expression of Sens is necessary for maintenance of R8 fate during pupal development (Xie et al., 2007). Sens is also necessary for formation of interommatidial bristles (Domingos et al., 2004; Frankfort et al., 2004; Morey et al., 2008).

Recently, we performed a screen in *Drosophila* for dominant modifiers of a disorganized eye phenotype caused by overexpression of *senseless* (Pepple et al., 2007). Here, we report the identification of the complementation group *S(ls)3*, comprising three loss-of-function alleles, as the first mutations in *Drosophila Caf1*. In the current study, we describe functional and developmental consequences of *Caf1* gain- and loss-of-function in *Drosophila*. Our observations indicate that severe loss of Caf1 expression results in cell death, consistent with key roles for Caf1 in chromatin remodeling complexes necessary for basic cellular functions. However, at intermediate levels of wild-type Caf1 expression, we observe homeotic transformations and eye phenotypes consistent with a major role for Caf1 in Polycomb silencing. We present evidence suggesting that the genetic interaction between *Caf1* and *sens* is mediated by Polycomb silencing. These results suggest that Caf1 is essential not only for basic cellular functions and survival, but also for maintenance of cellular identity and normal body plan.

MATERIALS AND METHODS

Fly stocks and mosaic analysis

Caf1 alleles generated in our laboratory were reported previously as *S(ls)3* (Pepple et al., 2007). The *Caf1 Genomic Rescue* construct (*Caf1GR*) was generated by recombineering a fragment from *BACR32A03* into *pACMAN* (Venken et al., 2006). *pACMAN* vector was a gift from Hugo Bellen (Jan and Dan Duncan Neurological Research Institute, Houston, TX, USA). The *UAS-Caf1* construct was generated by insertion of the *Caf1* cDNA (*LD33761*, *Drosophila* Genomics Resource Center) into *pUAST-attB* (Bischof et al., 2007). Transgenic flies were obtained by injection of *Caf1GR* or *UAS-Caf1* into the second chromosome insertion site of the *VK1* line (Venken et al., 2006). Other fly stocks used include w^{1118}, *lz-GAL4*, *UAS-GFP* and *UAS-sens/FM7 (ls)*. *eyFLP; FRT82B p{w+} cl/TM6B*, Dp^{49FK-1} c^{1}/*SM5*, cn^{1}, *w;* Dp^{a1bw1} sp^{1}/*CyO*, *w;* $E2f2^{76Q1}$ cn^{1} bw^{1}/*CyO*, $E2f^{07172}$/*TM3*, $E(z)^{731}$, $Rpd3^{04556}$ ry^{506}/*TM3* ry^{RK} Sb^{1} Ser^{1}, esc^{1}, *UAS-pb* and *UAS-Antp* were obtained from the Bloomington *Drosophila* Stock Center. $Caf1^{short}$, $Caf1^{med}$ and $Caf1^{long}$ alleles were recombined onto the *FRT82B* chromosome. Mosaic heads were generated by crossing mutant alleles on *FRT* chromosomes with *eyFLP; FRT82B p{w+} cl/TM6B*, obtained from the Bloomington *Drosophila* Stock Center (Newsome et al., 2000).

Mapping and sequencing

Three rounds of P-element mapping were performed to localize *S(ls)3* mutations to a 50 kb region as described (Zhai et al., 2003). Sequencing was performed by the MD Anderson DNA Analysis Facility and Macrogen. Sequence analysis was performed using Sequencher software (Genecodes).

Light microscopy

Tangential sections of the adult retina were performed as described (Tomlinson and Ready, 1987). Images were acquired with a Zeiss Axioplan 2 microscope, Zeiss Axiocam digital camera and Axiovision software. Adobe Photoshop software was used to resize images and adjust brightness and contrast.

Scanning electron microscopy (SEM)

Samples were prepared as described previously (Pepple et al., 2007).

Antibody staining and confocal microscopy

The following antibodies were used: rabbit anti-Caf1 (1:1000, AbCam); guinea-pig anti-Sens (1:5000, a gift from Hugo Bellen); mouse anti-active-Caspase 3 (1:1000, R&D Systems); rat anti-Elav (1:500, Developmental Studies Hybridoma Bank); and rabbit anti-H3K27me3 (1:200, Lake Placid). The following secondary antibodies were all used at 1:500: Alexa-conjugated goat anti-rabbit secondary antibody (Molecular Probes), goat anti-guinea pig Cy3 (Jackson ImmunoResearch), goat anti-rat Cy3 (Jackson ImmunoResearch) and goat anti-rat Cy5 (Jackson ImmunoResearch). For histone methylation staining experiments, discs were dissected and stained as described previously (Fan and Bergmann, 2010). For all other staining experiments, eye-antennal imaginal discs were dissected and stained, and images acquired, as described previously (Pepple et al., 2007). Adobe Photoshop and Gimp software were used to process image brightness, color, contrast and noise, and to merge channels.

RESULTS

Mutations in the gene *Chromatin Assembly Factor 1* (*Caf1*) interact with *senseless*

Overexpression of *UAS-sens* under control of *lozenge-GAL4* (hereafter abbreviated *ls*) results in eyes with a disruption of the regular hexagonal array of ommatidia in the adult retina. This disruption is caused, in part, by formation of extra interommatidial bristles (Pepple et al., 2007). Previously, we have reported the isolation of a three-member lethal complementation group, *S(ls)3*, that was discovered in a screen for mutations that dominantly modify *ls* (Pepple et al., 2007). Each of the three alleles of *S(ls)3* dominantly suppresses the extra bristles and disrupted ommatidial array of *ls* to a similar degree (Fig. 1B-I), indicating a role in the Sens pathway. We performed P-element recombination mapping (Zhai et al., 2003) to narrow the lethal mutations to a 50 kb region containing 15 genes. Sequencing of all exons from this region revealed mutations in the gene *Caf1* (*CG4236*) in all three alleles (Fig. 1A). *Caf1* encodes a 430 amino acid protein with seven WD repeats (Song et al., 2008; Tyler et al., 1996). $Caf1^{long}$ contains a G-to-A transition that changes a conserved amino acid from glycine to asparagine. The mutation in $Caf1^{med}$ truncates the protein in its third WD repeat. The $Caf1^{short}$ mutation, which would truncate the protein in the first WD repeat, occurs in the first exon and probably results in a null allele due to nonsense-mediated decay (Valencia-Sanchez and Maquat, 2004).

To test whether the lethality of the three mutants comprising *S(ls)3* is due to mutations in *Caf1*, we generated flies containing a 12 kb genomic rescue construct, designated *Caf1GR*, designed to ensure all regulatory regions of *Caf1* are included (not shown). This resulted in the inclusion of several other genes: *Rpb7*, *CG31344*, *Art3*, *mRPS10* and the 3′ end of *CG12241*. The *Caf1GR* construct rescues the lethality of the most severe truncation allele,

Caf1^{short}, in combination with either *Caf1*^{med} or *Df(3R)ED5664*, a deficiency that uncovers *Caf1*. Rescued flies are viable and appear phenotypically normal, indicating that the lethality of *Caf1*^{short} is due to mutations within the 12 kb region.

Reduced *Caf1* activity disrupts segment identity

We attempted to rescue flies homozygous or trans-heterozygous for *Caf1* alleles with expression of a *UAS-Caf1* construct under the control of *Ubiquitin-GAL4* (*Ubi-GAL4*). In a wild-type background, *Ubi-GAL4/UAS-Caf1* flies are viable and phenotypically normal. *Ubi-GAL4/UAS-Caf1; Caf1*^{short}*/Caf1*^{long} flies are also viable and are phenotypically normal, suggesting that lethality of the mutant alleles is due to mutations in *Caf1* (data not shown). Both *Ubi-GAL4/UAS-Caf1; Caf1*^{short}*/Caf1*^{med} and *Ubi-GAL4/UAS-Caf1; Caf1*^{short}*/Caf1*^{short} flies raised at 25°C survive to become pharates, though few adults eclose. Pharates dissected live from pupae and the few adults that do eclose appear phenotypically normal (data not shown). Given the differential ability of *UAS-Caf1* to rescue different allelic combinations at 25°C, we reasoned that raising *Ubi-GAL4/UAS-Caf1; Caf1*^{short}*/Caf1*^{med} flies at 18°C would allow us to analyze the effects of reduced levels of Caf1. All flies of this genotype raised at 18°C have disorganized kidney-shaped eyes, as well as homeotic transformations of the aristae to leg-like structures that occasionally terminate in claws. Other defects, including duplications of scutellar bristles and wing defects, are sometimes seen.

We performed a series of temperature shift experiments and determined that the eye and antennal phenotypes are dependent on the temperature during the third larval instar (data not shown). *Ubi-GAL4/UAS-Caf1; Caf1*^{short}*/Caf1*^{med} flies raised at 25°C during third instar have normal eyes and antennae (Fig. 2A,B,E). By contrast, flies raised at 18°C have small, disorganized eyes (Fig. 2C,D,F). In Fig. 2D, the transformed arista terminates in a claw (arrow). Tangential sections of adult eyes from animals raised at 18°C display abnormalities in the arrangement of rhabdomeres, including loss of small central rhabdomeres (Fig. 2G, arrowheads). These defects are probably due to insufficient levels of *Caf1* given the temperature-sensitive nature of the *GAL4-UAS* system (for a review, see Duffy, 2002) and are reminiscent of phenotypes caused by overexpression of Hox genes (Bello et al., 1998). Hox genes are major targets of the PcG complexes, which are responsible for establishing and maintaining silencing of Hox genes in regions where their expression would interfere with proper anterior-posterior patterning (Schuettengruber et al., 2007). In addition to eye and antennal defects, wing defects are seen in many animals

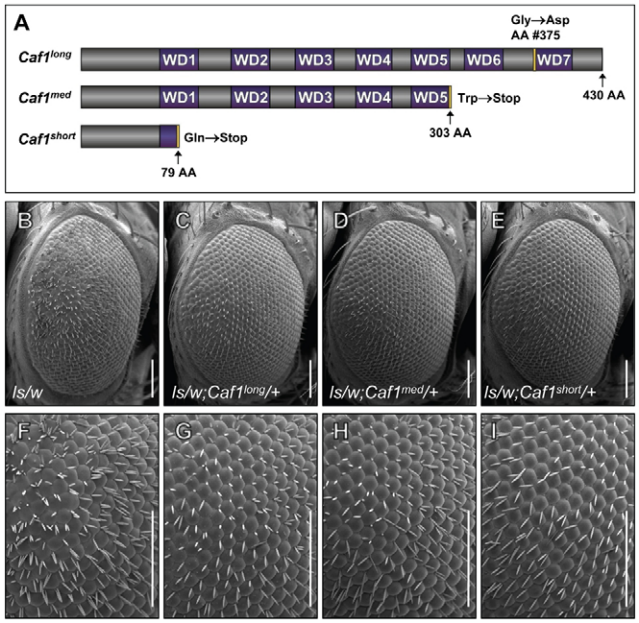

Fig. 1. Loss of *Caf1* function suppresses an ectopic *senseless* phenotype. (**A**) Three mutations in *Caf1* resulting in amino acid substitution or truncation of the Caf1 protein are shown. Purple boxes indicate WD repeats. The positions of mutations are indicated by yellow vertical bars. (**B**,**F**) *lz-GAL4, UAS-sens*/+ (*ls*) flies have disorganized eyes characterized by extra bristles. Three lethal mutations in *Caf1* can dominantly suppress the disorganized eyes and extra bristles of the *ls* phenotype. (**C**,**G**) *ls*/+; *Caf1*^{long}/+. (**D**,**H**) *ls*/+; *Caf1*^{med}/+. (**E**,**I**) *ls*/+; *Caf1*^{short}/+. F-I are magnified regions of the eyes shown in B-E, respectively. Scale bars: 100 μm.

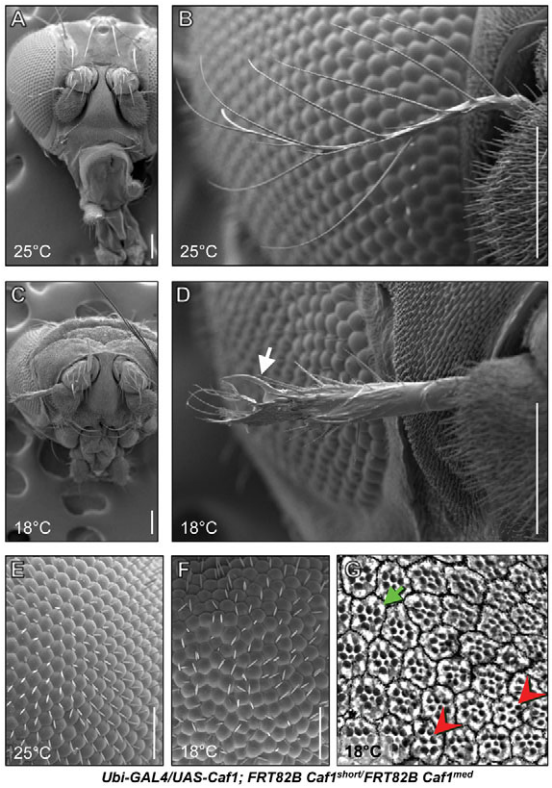

Fig. 2. Reduced levels of *Caf1* expression result in homeotic transformations and loss of neuronal structures in the eye. *Caf1* mutant flies expressing *UAS-Caf1* under the control of *Ubi-GAL4* were raised at 25°C or 18°C during larval development to modulate the level of *Caf1* expression. (**A**,**B**) Flies raised at 25°C during larval development have normal head morphology (A; eye and arista magnified in B). (**C**,**D**) A fly raised at 18°C during larval development has disorganized eye structure (C; eye and arista magnified in D) and altered arista morphology, resembling transformation to tarsus. The arrow in D indicates a claw-like structure. The eye of a fly raised at 18°C during larval development is disorganized; many interommatidial bristles are missing (**F**) in contrast to the eye of a fly raised at 25°C during larval development (**E**). (**G**) A section through the eye of a fly raised at 18°C during larval development reveals several ommatidia missing small rhabdomeres (two examples are indicated by red arrowheads). A normally constructed ommatidium is indicated by the green arrow. The genotype of all flies in this figure is *ubi-GAL4/UAS-Caf1;Caf1*^{short}*/Caf1*^{med}. Scale bars: 100 μm.

raised at 18°C during larval development; an example of a fly with reduced wings and scutellar bristle duplication is shown in Fig. S1 in the supplementary material.

Caf1 is required for cell survival

Larvae homozygous for *Caf1*^{med} or *Caf1*^{short} appear grossly normal but mature slowly and survive to first or second instar. Many *Caf1*^{long} larvae survive to third instar, but die shortly after forming a disorganized pupa. Homozygous *Caf1* germline mutant clones are not recovered. To assess the role of *Caf1* in *Drosophila* development and overcome the problem of larval lethality, we attempted to make clones using *heat shock-Flippase* (*hs-FLP*). *Caf1*^{long} clones survive well in the *Drosophila* eye (data not shown). However, *hs-FLP* clones of *Caf1*^{short} and *Caf1*^{med} do not survive in adults, suggesting cell death or proliferation defects. To recover clones of *Caf1*^{short} and *Caf1*^{med}, we used the *ey-FLP;FRT cell-lethal* system, which generates eyes composed mostly of homozygous mutant tissue and has the advantage of marking any remaining heterozygous ommatidia with w^+ (Newsome et al., 2000). We used two approaches to analyze mutant eyes: light microscopy (LM), which reveals the gross structure of the eye and can differentiate between homozygous and heterozygous tissue but does not reveal the fine details of ommatidial architecture; and scanning electron microscopy (SEM), which shows the detailed structure of the eye but does not allow identification of clones.

Eyes mutant for *Caf1*^{long} have large clones of mutant tissue with only a subtle disruption in the ommatidial array, leading to a slightly disorganized appearance (Fig. 3B,F,J). Misplaced bristles are occasionally seen in these eyes and many bristles are shorter than wild type. By contrast, *Caf1*^{short} eyes contain very little mutant tissue (white tissue in Fig. 3K). The remaining eye, composed mostly of heterozygous tissue, is small, has irregular ommatidial structure, and is almost completely devoid of bristles (Fig. 3C,G,K). This suggests impaired cell viability or proliferation in *Caf1*^{short} homozygous tissue. Consistent with these results, no adult *Caf1*^{short} clones are recovered in the thorax using *Ubx-FLP; FRT 82B Sb*^{63} *P{w*$^+$*y*$^+$*}*, whereas large wild-type or *Caf1*^{long} clones are recovered in most animals of the correct genotype with this technique (data not shown). Approximately 20% of flies with *ey-FLP*-induced *Caf1*^{short} or *Caf1*^{med} clones have extra or missing appendages on the head, including the ocelli, antennae and maxillary palps. In some cases, structures are only partially duplicated, leading to a branched appearance. An example of a fly with a duplicated antenna is shown in Fig. 3D and enlarged in Fig. 3H. Although the original publication of the *eyFLP; FRT p{w*$^+$*} cl* chromosome described the use of an eye-specific version of *ey-GAL4*, with our stock, we have observed clones outside of the eye proper, including the peripodial epithelium and the antennal disc (Newsome et al., 2000). Therefore, although adult clones outside the eye field are not marked, these duplicated structures are probably due to *Caf1* clones outside the eye field.

Overexpression of *Caf1* resembles the *Caf1* loss-of-function phenotype

To assess the effects of varying levels of *Caf1* expression, we generated an *ey-GAL4,UAS-Caf1* recombinant chromosome to overexpress *Caf1* in the eye antennal disc. These flies are viable

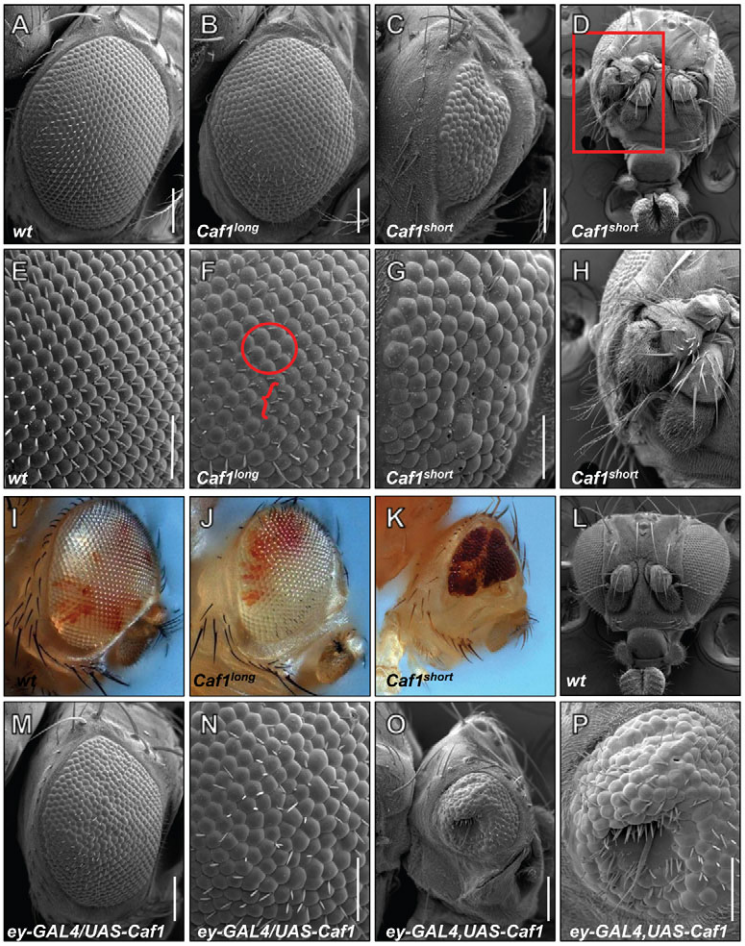

Fig. 3. Altered expression of *Caf1* disrupts eye and head development.. (B-D,F-H,J,K) The *ey-FLP; cl* technique was used to generate eyes composed mostly of *Caf1* mutant tissue. (**A**,**B**,**E**,**F**) Eyes mostly homozygous for *Caf1*^{long} (B,F) are slightly disorganized compared with wild type (A,E). Some interommatidial bristles (IOBs) are missing (circle in F) or occasionally occur at adjacent vertices (bracket in F). (**I-K**) Homozygous *Caf1*^{long} clones produced with the *ey-FLP; cl* technique are large (white tissue in J), similar to wild-type (I), while *Caf1*^{short} clones are small compared with remaining heterozygous tissue (K). (**C**,**G**,K) Eyes generated with the *ey-FLP; cl* technique and the *Caf1*^{short} allele are small, highly disorganized and almost completely devoid of bristles (C,G), and are composed mostly of heterozygous tissue (K). (**D**,**H**) An example of a fly of this genotype with a duplicated antenna is shown in D, with area in red rectangle enlarged in H. (**L**) The head of a wild-type fly. (**M**,**N**) The eyes of *ey-GAL4* +/+ *UAS-Caf1* flies raised at 25°C are large and disrupted, with reduced numbers of IOBs (M; magnified in N). Increased dose of *Caf1* in *ey-GAL4, UAS-Caf1* homozygous flies results in smaller, more highly disorganized eyes. (**O**,**P**) An example with an ectopic macrochaete is shown in O and magnified in P. Scale bars: 100 μm.

when raised at 18°C, 25°C and even 30°C. The eyes of *ey-GAL4/UAS-Caf1* flies raised at 25°C are disorganized and slightly smaller than wild type (Fig. 3M), and resemble the *Caf1^{long}* phenotype (Fig. 3J). Additionally, the eyes of these flies have reduced numbers of interommatidial bristles (Fig. 3N). When raised at 30°C, the eyes are smaller and more disorganized, probably owing to the increased activity of GAL4 at higher temperatures in *Drosophila* (Duffy, 2002). Eyes and heads of *ey-GAL4, UAS-Caf1* homozygotes raised at 25°C have an even more severe phenotype reminiscent of *Caf1* complete loss-of-function: the eyes are extremely small and ommatidial structure is highly disrupted. Occasionally, ectopic structures are seen in these eyes (Fig. 3O,P). Outside the eye, duplications and deletions of other head structures are seen frequently in *ey-GAL4, UAS-Caf1* homozygotes, similar to *Caf1^{short}* clones (data not shown).

Eyes with *Caf1^{short}* clones have excess apoptosis and disrupted early development

Even when using the *ey-FLP; FRT cl* technique, *Caf1^{short}* clones are small during third instar, and the spacing of developing R8 photoreceptors is disrupted (see Fig. S2 in the supplementary material). *Caf1^{short}* clones generated with this technique are rare at 6 hours of pupal development and almost completely absent by 24 hours (data not shown). To determine whether the small size, loss of mutant tissue and disorganization of *Caf1^{short}* eyes is due in part to apoptosis, we stained third instar eye discs with an antibody against active Caspase 3 (Fan and Bergmann, 2010). Normally, programmed cell death occurs in the *Drosophila* eye disc during pupal development to remove excess cells during differentiation of secondary and tertiary pigment cells, and formation of the hexagonal lattice (Frohlich, 2001). At late third instar, control eye discs using the *eyFLP; FRT 82B P{w+} cl* chromosome show very little Caspase 3 staining (Fig. 4A,C). Consistent with their large adult size, third instar *ey-FLP; FRT P{w$^+$} cl/FRT Caf1^{long}* eye discs also show very low levels of active Caspase 3 (Fig. 4D,F). Elav staining in these eye discs shows a nearly regular array of developing photoreceptors, similar to that of control discs (compare Fig. 4E with 4B). By contrast, eye discs with homozygous clones for *Caf1^{short}* are small and have extensive Caspase 3 staining (Fig. 4G,I). Notably, the majority of Caspase 3 staining in discs harboring *Caf1^{short}* clones is observed anterior to Elav staining, implying that most cell death occurs anterior to the furrow in cells that would normally be proliferating. The arrangement of Elav-positive photoreceptor clusters is also irregular (Fig. 4H). Similar to *ey-FLP; FRT82B Caf1^{short}/FRT82B P{w$^+$} cl* eye discs, significant apoptosis marked by Caspase 3 staining occurs in the anterior region of the eye disc in *ey-GAL4, UAS-Caf1* third instar eye discs (Fig. 4J-L). Increased apoptosis is also observed in the *Ubx-FLP; FRT82B Caf1^{short}/FRT82B ubi-GFP* wing disc (see Fig. S3 in the supplementary material).

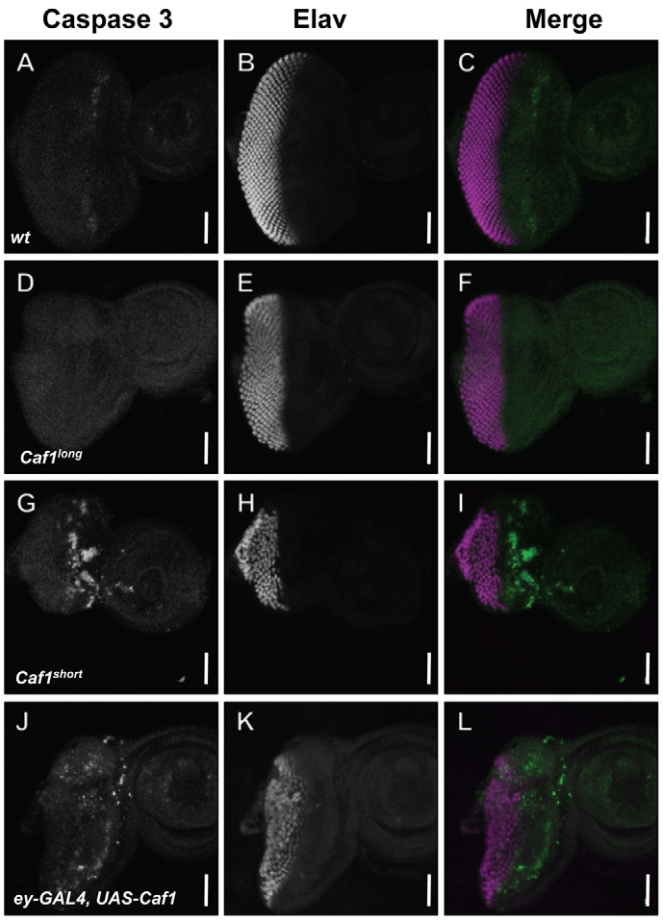

Fig. 4. Neural differentiation is disrupted and programmed cell death is increased in *Caf1^{short}* mutant eye discs. (**A-I**) Control eye discs (*ey-FLP; FRT82B/FRT82B p{w$^+$} CL*; A-C) and *Caf1^{long}* eye discs (*ey-FLP; FRT82B Caf1^{long}/FRT82B p{w$^+$} CL*; D-F) stained for active Caspase 3 (A,D, and green in C,F) show only diffuse and sporadic staining, while *Caf1^{short}* eye discs (*ey-FLP; FRT82B Caf1^{short}/FRT82B p{w$^+$} CL*; G-I) display bright patches of Caspase 3 staining. (**J-L**) Similar to *Caf1^{short}* discs, Caspase 3 staining is increased in *ey-GAL4, UAS-Caf1* discs. Elav staining (B,E,H,K; magenta in C,F,I,L) marks the position of differentiating photoreceptors, which is highly disrupted in *Caf1* mutant discs. Scale bars: 100 μm.

The *ls* eye phenotype can be rescued by overexpression of Hox genes

The homeotic transformations observed in *Caf1* mutant flies partially rescued by *Caf1* cDNA expression (Fig. 2) suggest that PRC2 function may be impaired when levels of Caf1 are low, resulting in ectopic expression of one or more Hox genes. To test whether overexpression of Hox genes could account for dominant suppression of the *ls* phenotype by mutations in *Caf1*, we crossed *ls* flies to *UAS* lines for several Hox genes, including *proboscipedia* (*pb*), *abdominal-A* (*abd-A*), *Deformed* (*Dfd*), *Ultrabithorax* (*Ubx*), *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*) and *labial* (*lab*). Overexpression of Hox genes alone in the eye also leads to a disorganized eye phenotype; each of the above *UAS* lines, when crossed to *lz-GAL4* alone, also generates disorganized eyes (data not shown). Therefore, suppression of *ls* can only occur if levels of Hox and Sens activity functionally cancel each other out. Consistent with our hypothesis, ectopic expression from two lines, *UAS-Antp* and *UAS-pb,* results in strong suppression of the *ls* disorganized eye phenotype (Fig. 5B,C,F,G) compared with *ls* crossed to *UAS-GFP* (Fig. 5A,E).

Mutations in *Polycomb Group* genes suppress the *ls* phenotype

As Caf1 is a component of PRC2, we hypothesize that suppression of *ls* by *Caf1* mutations is due to loss of PRC2 function and predict that mutations in other *PcG* genes should also dominantly suppress the *ls* phenotype. To test this prediction, we crossed *ls* flies to mutations in *PRC1* and *PRC2* genes. The *PRC2* complex mutations

Su(z)12^{2}, *Su(z)12*^{3}, *Su(z)12*^{4}, *Rpd3*^{303}, *Rpd3*^{04556}, *E(z)*^{731}, *esc*^{1} and *esc*^{2} each suppressed the *ls* phenotype, as did *PRC1* mutations *Polycomb*^{3} (*Pc*^{3}) and *Pc*^{15} (Fig. 5D,H; data not shown). We also tested mutations in genes encoding factors from other Caf1-containing complexes. Mutations in three members of the dREAM complex were suppressors of *ls* (see Fig. S4 in the supplementary material), consistent with previous observations of cooperation between PcG and dREAM complex members (Dahiya et al., 2001; Kotake et al., 2007; Tonini et al., 2004).

Caf1 interacts genetically with a *Polycomb Group* gene

We tested for a genetic interaction with the *PRC1* gene *Pc* (Fig. 5I). Males heterozygous for the *Pc*^{15} allele display ectopic sex combs on the second and sometimes third legs, consistent with homeotic transformation of second or third legs to first leg. Crossed to wild type, we observe that an average of 82% of male *Pc*^{15}/+ flies have an ectopic sex comb on at least one posterior leg. By contrast, only 12.3% of *Pc*^{15}/*Caf1*^{short} and 30% of *Pc*^{15}/*Caf1*^{long} double heterozygote male flies have one or more ectopic sex combs. Thus, *Caf1* is a strong dominant suppressor of the *Pc*^{15} phenotype. Although this result seems consistent with a Trithorax Group-like activity for Caf1, mutations in other bona fide PcG members sometimes interact genetically in a similar manner; these data suggest that like these PcG members, Caf1 can be placed in the Enhancer of Trithorax and Polycomb (ETP) group (for a review, see Fedorova et al., 2009). Furthermore, ETP-like activity of Caf1 is not surprising, given its aforementioned participation in multiple chromatin remodeling complexes. To confirm that the interaction is specific to *Pc*, we repeated this analysis with the *Pc*^{3} allele, which also results in ectopic sex combs on second and sometimes third legs. Eighty-five percent of *Pc*^{3}/+ males exhibit ectopic sex combs, compared with 64% of *Pc*^{3}/*Caf1*^{long} flies ($P<0.06$; 170 *Pc*^{3}/+ and 88 *Pc*^{3}/*Caf1*^{P9} flies counted over six replicates). There was no significant difference between *Pc*^{3}/+ and *Pc*^{3}/*Caf1*^{long}. Thus, although mutations in *Caf1* suppress *Pc*^{3} less strongly than *Pc*^{15}, the genetic interaction is consistent between the two alleles.

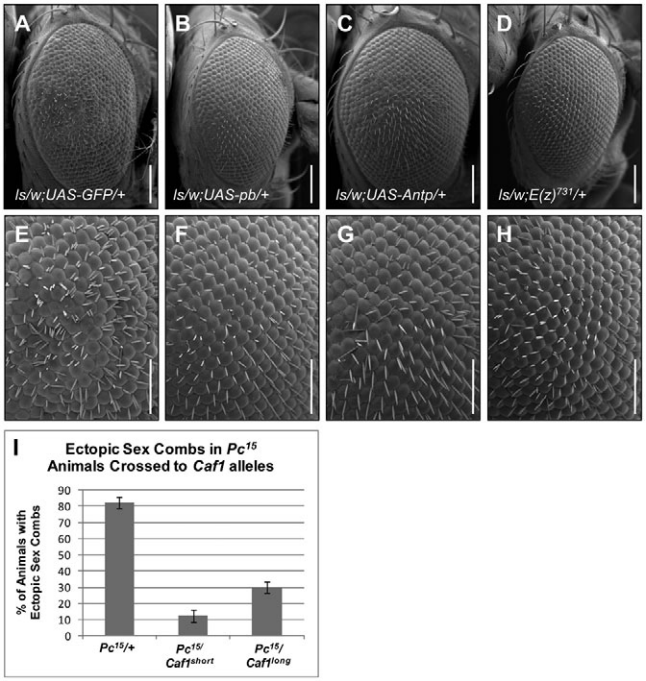

Fig. 5. The *ls* phenotype is suppressed by ectopic expression of Hox genes. Genetic interactions between *sens*, *Hox*, *Caf1* and *PcG* are shown. (**A**,**E**) Similar to *ls,* the eyes of *lz-GAL4, UAS-sens/w; UAS-GFP/+* flies are disorganized and have numerous ectopic bristles (A; magnified in E). (**B**,**C**,**F**,**G**) The eyes of *lz-GAL4, UAS-sens/w; UAS-pb/+* (B; magnified in F) and *lz-GAL4, UAS-sens/w; UAS-Antp/+* (C; magnified in G) flies have a more wild-type appearance, with reduced numbers of interommatidial bristles and a more regular pattern of hexagonal ommatidia. (**D**,**H**,**I**) *ls* is also dominantly modified by mutations in *PcG* members, including *E(z)* (D; magnified in H). In addition to its interaction with *sens*, *Caf1* interacts genetically with *Pc* (I). Eighty-two percent of *Pc*^{15}/+ animals have at least one ectopic sex comb. By contrast, only 12.3% of *Pc*^{15}/*Caf1*^{short} animals have ectopic sex combs ($P<0.0000001$) and 30.0% of *Pc*^{15}/*Caf1*^{long} animals ($P<0.000001$). For each genotype, six separate crosses were scored. A total of 297 *Pc*^{13}/+, 212 *Pc*^{13}/*Caf1*^{short} and *156 Pc*^{13}/*Caf1*^{long} animals were counted. Scale bars: 100 μm.

Caf1 mutant tissue is deficient in a PRC2-dependent histone methylation mark

The PRC2 complex is associated with trimethylation of histone 3 at lysine 27 (H3K27me3) (Czermin et al., 2002) due to the methyltransferase activity of the E(z) protein. The H3K27me3 mark is associated with repression of gene transcription. Our previous results suggest that loss of *Caf1* results in a reduction in PRC2 activity, and therefore predicts a reduction in global levels of H3K27me3. To test this, we generated *Caf1*^{short} and *Caf1*^{long} clones in the eye disc using *ey-FLP* and stained third instar discs with an antibody against H3K27me3 (Fig. 6). The H3K27me3 mark is reduced in clones of both *Caf1* alleles compared with surrounding heterozygous and wild-type tissue. A similar reduction of H3K27me3 in *Caf1* clones is seen in the wing disc (see Fig. S5 in the supplementary material). Taken together, these results

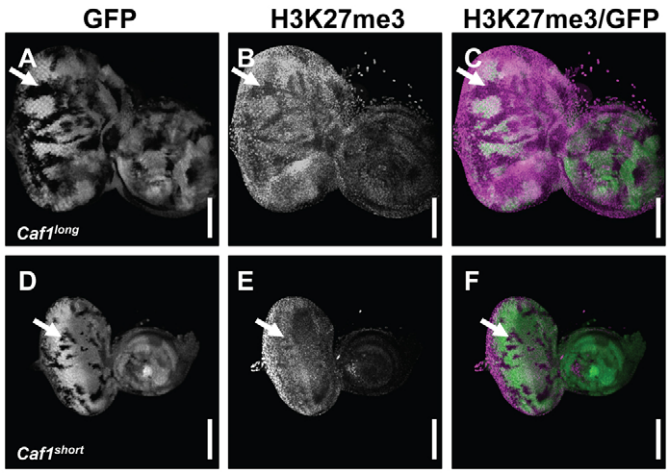

Fig. 6. Trimethylation at lysine 27 of histone 3 is reduced in *Caf1* mutant clones. (**A-C**) Genotype of eye discs shown is *ey-FLP; FRT82B Caf1*^{long}*/FRT82B ubi-GFP*. (**D-F**) Genotype of eye disc in *ey-FLP; FRT82B Caf1*^{short}*/FRT82B ubi-GFP*. Clones are marked by absence of GFP staining (A,D; green in C,F). Reduced staining of H3K27me3 (B; magenta in C) is observed in *Caf1*^{long} clones (A; green in C). H3K27me3 (E; magenta in F) is also reduced in *Caf1*^{short} clones (D; green in F). Examples of clones are marked by arrows. Scale bars: 100 μm.

suggest that at least some of the phenotypes and genetic interactions we observe with partial or complete loss of *Caf1* are due to loss of PRC2 activity and H3K27 trimethylation.

DISCUSSION

Loss or reduction of *Caf1* disrupts development via altered PRC2 activity

Several lines of evidence suggest that the participation of Caf1 in PcG complexes may account for many of the phenotypes we observe in flies with altered expression of *Caf1*. First, *Caf1* loss-of-function clones in the eye have phenotypes ranging from slight disorganization and bristle defects (Fig. 3B,F,J) to almost complete loss of homozygous tissue in adults (Fig. 3C,G,K), and incomplete rescue of *Caf1* results in adult eyes that are small and disorganized (Fig. 2C,F). Clones of many *PcG* genes have similar phenotypes in the eye. Loss of *E(z)* or *Pc* causes mild defects in differentiation in the third instar disc, but clones fail to survive in adults (Brook et al., 1996; Janody et al., 2004). An analogous situation occurs in $Caf1^{short}$ clones, where expression of Elav, which marks differentiating neurons, is present in *Caf1* clones at third instar but $Caf1^{short}$ tissue is largely missing in the adult. Derepression of Hox genes could account for these phenotypes, as ectopic expression of many Hox genes in the eye field causes small disorganized eyes in adults (Plaza et al., 2001).

Second, flies with incomplete rescue of *Caf1* display a range of homeotic phenotypes, notably transformation of arista to leg (Fig. 2C,D). Similar homeotic transformations, including antenna-to-leg transformations, are a hallmark of mutations in *PcG* genes (Denell, 1973; Lewis, 1978; Wang et al., 2006). We also observe a genetic interaction between *Caf1* and the *PRC1* gene *Pc*, as mutations in *Caf1* are able to dominantly suppress the homeotic transformation of second or third leg to first leg in Pc^{15}/+ males (Fig. 5).

Third, the disrupted patterning of $Caf1^{short}$ mutant heads may also result from PcG dysfunction (Fig. 3). It is possible that these patterning defects are non-cell autonomous and may be an indirect result of widespread apoptosis in the eye disc (Fig. 4). Normally, when an imaginal disc is injured, remaining cells proliferate and assume correct identities, leading to a perfectly patterned adult structure (McClure and Schubiger, 2007) (for a review, see Bergmann and Steller, 2010). This type of regeneration requires that some determined cells must change their fates and involves substantial chromatin remodeling. Under specific circumstances, the disc can regenerate with incorrect patterning, leading to duplication, deletion or transformation of structures, a phenomenon referred to as transdetermination (McClure and Schubiger, 2007). Levels of many *PcG* transcripts are increased in transdetermining imaginal discs, and heterozygous mutations in *PcG* genes can enhance transdetermination in regenerating imaginal discs (Klebes et al., 2005). Therefore, one interpretation of the patterning defects in $Caf1^{short}$ mutant discs is that under the stress of widespread apoptosis, the remaining heterozygous tissue is haploinsufficient for the chromatin remodeling activity required to properly regenerate and pattern the injured disc. Consistent with this interpretation, no extra or missing appendages are observed in flies with $Caf1^{long}$ clones, which show less active Caspase 3 staining at third instar.

Finally, in *Caf1* mutant tissue, we observe a reduction in levels of the H3K27me3 mark, which is associated with inactive chromatin and PRC2 activity (Fig. 6). These data are consistent with a disruption of PRC2 function as a result of loss of Caf1 and represent the first in vivo evidence that Caf1 is an essential member of this chromatin remodeling complex in an animal model.

Sens and Caf1 may act in parallel competing pathways

One obvious question arises from the current study: why were multiple *Caf1* alleles identified in a screen for modifiers of the *sens* overexpression phenotype? Moreover, it is surprising that mutations in *Caf1* were not identified in previous *Drosophila* modifier screens involving *PcG* or *Rb* pathway members (Ambrus et al., 2009; Janody et al., 2004; Steele et al., 2009). We propose that the link between *sens* and *Caf1* is due to the role of Caf1 in PcG-mediated silencing.

Recent evidence suggests that Sens and Hox proteins can compete for binding at overlapping sites at an enhancer of the *rhomboid* (*rho*) locus (Li-Kroeger et al., 2008). When the Hox protein Abdominal-A (Abd-A) binds, transcription of *rho* is activated, whereas binding by Sens leads to repression of *rho*. In the embryo, this mechanism acts as a molecular switch to allow differentiation of either chordotonal organs (under control of Sens) or hepatocyte-like cells called oenocytes (by the action of Abd-A). We propose that a similar mechanism underlies the suppression of the Sens overexpression phenotype (Fig. 7). We hypothesize that one or more targets of Sens in the eye contain similar overlapping sites that can be bound by either Sens or a Hox protein. During normal development, these loci are bound by neither Sens nor Hox in undifferentiated cells posterior to the furrow, as no Hox genes are known to be widely expressed in the eye field (Hueber and Lohmann, 2008). In the absence of both types of factors, these loci are transcriptionally active, and are necessary to ultimately attain the proper fates of the cells in which they are expressed. When Sens is overexpressed, as in *ls*, Sens binds to its recognition site in the downstream loci, repressing transcription. Repression of these genes initiates a cascade leading to a change in cell fate; for example, some of the cells that would normally become secondary or tertiary pigment cells now become bristle precursors, giving rise to the extra bristles of *ls*. However, when one copy of *Caf1* is lost, a slight derepression of the Hox genes occurs due to loss of PcG activity. Hox proteins are now able to compete with Sens for the overlapping binding sites, tipping the balance towards activation of downstream genes and attainment of normal cell fate – effectively suppressing *ls*. The ability of ectopic expression of *pb* and *Antp* in the eye to suppress *ls* is consistent with this hypothesis. Suppression of *ls* by Hox proteins is particularly significant given that ectopic expression of Antp alone in the eye field leads to a small and disorganized eye (Bello et al., 1998; Plaza et al., 2008; Plaza et al., 2001). As Sens activity is exquisitely sensitive to Hox proteins, especially in the eye, our screen for modifiers of a *sens* overexpression phenotype was therefore ideal for identifying mutations in *Caf1*.

Previous studies have explored pro-apoptotic roles of Hox proteins and anti-apoptotic roles of Sens. It is therefore possible that one effect of Hox gene derepression in *ls* eyes suppressed by *Caf1* may be restoration of an apoptotic fate in cells that would otherwise form bristle precursors due to ectopic Sens. Abd-A expression in the abdomen during normal third instar larval development leads to apoptosis of proliferating neuroblasts of the central nervous system, and ectopic expression of other Hox genes can also cause neuroblast apoptosis (Bello et al., 2003). Accordingly, survival of neuroblasts is dependent on PcG activity to repress Hox gene expression (Bello et al., 2003). Furthermore, expression of Sens is necessary in the *Drosophila* embryonic salivary gland to prevent apoptosis (Chandrasekaran and Beckendorf, 2003). Thus, one possible mechanism for suppression of *ls* by *Caf1* mutations is that in the *ls* eye, Sens may promote the

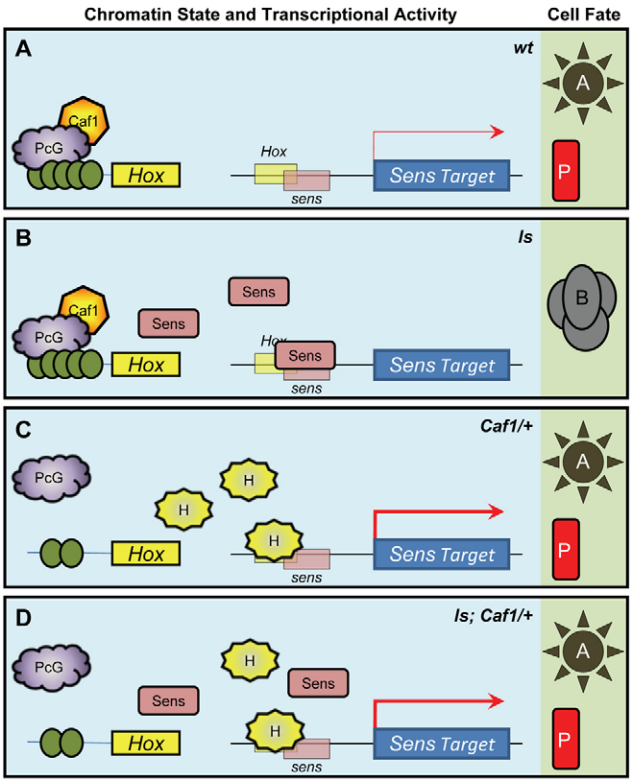

Fig. 7. A model for suppression of *ls* by mutations in *Caf1*. We propose that overlapping Sens- and Hox-binding sites occur in one or more Sens targets that are necessary to suppress bristle fate. (**A**) In the absence of Sens, as occurs in non-neuronal eye cells, the normal transcriptional state is on. Activity of this gene suppresses bristle fate and/or promotes other cell fates, such as pigment cell (oblong cell marked P) or apoptosis (starburst labeled A). Hox genes are not transcribed due to repressive chromatin state induced and maintained by PcG complexes with the participation of Caf1. (**B**) When excess Sens is introduced into the cell by *ls*, Sens occupies its binding site in downstream targets and transcription is halted. Downstream, cells that would normally achieve fates such as pigment cells or apoptosis instead become bristle precursors (cluster of grey cells labeled B). (**C**) Cells heterozygous for *Caf1* mutations have fewer functional PcG complexes and therefore some level of Hox gene derepression due to haploinsufficiency of *Caf1*. Some Hox protein (H) is able to bind its site in Sens target genes, which remain on and no fate change occurs. (**D**) In the sensitized *ls* background, haploinsufficiency of *Caf1* also leads to derepression of Hox genes. Ectopic Hox proteins compete with excess Sens produced by *lz-GAL4,UAS-sens* for binding sites, and ens is unable to repress transcription of its target genes, thus preventing fate change and production of ectopic interommatidial bristles.

ectopic bristle fate partly by repressing apoptotic genes in cells normally fated to die, whereas in the *ls* eye suppressed by mutations in *Caf1*, ectopic Hox proteins may promote apoptosis and prevent bristle formation. We were unable to detect increased Ubx expression by antibody staining; however, a very small increase in one or more Hox proteins may be all that is necessary to change the transcriptional state of downstream loci and prevent the ectopic bristles and other defects in the highly sensitized *ls* eye – especially in the eye field, where no *Hox* genes are known to be highly expressed. Furthermore, the fact that multiple Hox proteins can recognize the same DNA binding site offers the possibility that the competitive effect of each Hox protein type on genes with overlapping Sens/Hox binding sites would be additive (Hueber and Lohmann, 2008). Therefore, although loss of one copy of *Caf1* may only cause a small derepression of any one Hox gene, mild derepression of many Hox genes collectively can lead to strong repression of the *ls* phenotype.

Caf1 has multiple roles in *Drosophila* development

Biochemical evidence suggests that Caf1 is a member of multiple complexes that effect gene regulation through chromatin remodeling, suggesting that it is a vital component of the cell's arsenal of chromatin modifying factors (Henikoff, 2003). Although our results suggest that disruption of PRC2 function may be the most important consequence of Caf1 gain- or loss-of-function, many phenotypes we observed in *Caf1* mutant tissue are also reminiscent of mutations in members of other complexes previously shown to contain Caf1. It is not surprising that all three alleles of *Caf1* in the current study are homozygous lethal, and that $Caf1^{short}$ cells have poor viability, considering that Caf1 has been found in the NURF and CAF-1 complexes, which have fundamental roles in nucleosome assembly and spacing (Bulger et al., 1995; Kamakaka et al., 1996; Martinez-Balbas et al., 1998; Tyler et al., 1996; Verreault et al., 1996). The apoptosis we observe in eyes with $Caf1^{short}$ clones is also consistent with a role for Caf1 in the dREAM (*Drosophila* Rbf, E2F2, and Myb-interacting proteins) complex. Members of the E2f family of transcription factors can complex with Dp proteins and bind short recognition sites to activate transcription (Brehm and Kouzarides, 1999; Classon and Harlow, 2002; Korenjak and Brehm, 2006; Macaluso et al., 2006). When Rb binds the E2f-Dp complex, transcription is repressed. Like *Caf1* homozygotes, homozygous *rbf1* null flies die in early larval development (Du and Dyson, 1999). Fully *rbf1*-deficient embryos display increased apoptosis, a phenotype reminiscent of the increased active Caspase-3 staining seen anterior to the morphogenetic furrow in eye discs with $Caf1^{short}$ clones (Fig. 4).

The mammalian homologs of *sens*, *Growth Factor Independence 1* (*Gfi1*) and *Gfi1b* are essential to the development of multiple cell types and have been implicated as oncogenes (Duan et al., 2005; Gilks et al., 1993; Gilks et al., 1995; Grimes et al., 1996; Hochberg et al., 2008; Karsunky et al., 2002; Kazanjian et al., 2004; Liao et al., 1995; Person et al., 2003; Schmidt et al., 1998; Wallis et al., 2003; Yucel et al., 2003) (for reviews, see Duan and Horwitz, 2003; Hock and Orkin, 2006). Therefore, the possibility that Caf1 links Sens with the activity of PcG complexes through parallel, competing pathways has implications for both *Drosophila* development and the activity of *Gfi1* family members in human development and disease, and warrants additional study beyond the scope of the present work. Our results underscore the importance of Caf1 to diverse processes, including cell survival and tissue identity, and highlight the participation of Caf1 in multiple chromatin remodeling complexes. Further studies are needed to fully assess the importance of *Caf1* in *Drosophila* development, as well as its developmental role in other chromatin remodeling complexes.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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