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Epigenetic silencers and Notch collaborate to promote malignant tumours by *Rb* silencing

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Cancer is both a genetic and an epigenetic disease. Inactivation of tumour-suppressor genes by epigenetic changes is frequently observed in human cancers, particularly as a result of the modifications of histones and DNA methylation. It is therefore important to understand how these damaging changes might come about. By studying tumorigenesis in the *Drosophila* eye, here we identify two Polycomb group epigenetic silencers, Pipsqueak and Lola, that participate in this process. When coupled with overexpression of Delta, deregulation of the expression of Pipsqueak and Lola induces the formation of metastatic tumours. This phenotype depends on the histone-modifying enzymes Rpd3 (a histone deacetylase), Su(var)3-9 and E(z), as well as on the chromodomain protein Polycomb. Expression of the gene *Retinoblastoma-family protein* (*Rbf*) is downregulated in these tumours and, indeed, this downregulation is associated with DNA hypermethylation. Together, these results establish a mechanism that links the Notch–Delta pathway, epigenetic silencing pathways and cell-cycle control in the process of tumorigenesis.

Correct organ formation depends on the balanced activation of conserved developmental signalling pathways (such as the Wnt, Hedgehog and Notch pathways). If insufficient signals are received, organ growth may be deficient. By contrast, excess signalling leads to an overproduction of progenitor cells and a propensity to develop tumours[1–3]. When such hyperproliferation is associated with the capacity of cells to invade surrounding tissue and metastasis to distant organs, cancer develops[4]. Indeed, activation of the Wnt, Hedgehog and Notch pathways is a common clinical occurrence in cancers[5,6]. Curiously, activation of any of these pathways in animal models seems to be insufficient for cancer to develop, indicating that synergism with other genes is required for these pathways to produce cancer.

Cellular memory or the epigenetic inheritance of transcription patterns has also been implicated in the control of cell proliferation during development, as well as in stem-cell renewal and cancer[7,8]. Proteins of the Polycomb group (PcG) are part of the memory machinery and maintain transcriptional repression patterns[9]. The upregulation of several PcG proteins has been associated with invasive cancers[8]. Thus, increased amounts of EZH2, the human homologue of the *Drosophila* histone methyltransferase E(z)[10], is associated with poorer prognoses of breast and prostate cancers[8].

Another histone methyltransferase implicated both in gene silencing and in cancer is SUV39H1, a homologue of *Drosophila* Su(var)3-9 (ref. 11). SUV39H1 and Su(var)3-9 methylate histone H3 on lysine 9 (H3K9me), and this epigenetic tag is characteristic of heterochromatin and DNA sequences that are constitutively methylated in normal cells[12]. DNA methylation is another mechanism involved in cellular memory that actively contributes to cancer[7,13]. Indeed, numerous tumour-suppressor genes, including the retinoblastoma gene *RB*, are silenced in cancer cells by DNA hypermethylation[13]. Inactivation of the *RB* tumour-suppressor pathway is considered an important step towards malignancy[14]; thus, it is important to understand how these damaging epigenetic changes are initiated in cells that become precursors of cancer. Moreover, it is equally important to determine the connection between these processes and the developmental pathways controlling proliferation.

Forward genetic screening in suitable animals is a powerful tool with which to identify tumour-inducing genes and to reveal changes that precede neoplastic events *in vivo*. The developing eye of *Drosophila melanogaster* is a good model for such studies[15] because it is a simple and genetically well-defined organ. The growth of the eye depends on Notch activation in the dorsal–ventral organizer by its ligands Delta (human counterparts, DLL-1, -3, -4) and Serrate (human counterparts, JAGGED-1, -2)[3,16]. Here we used the 'large eye' phenotype, produced by overexpression of *Delta*, as a tool to screen for mutations that interact with the Notch pathway and convert tissue overgrowths into tumours (Fig. 1a). We isolate one mutation, *eyeful*, that combined with Delta induces metastatic tumours. *eyeful* forces the transcription of two hitherto unsuspected growth and epigenetic genes, *lola* and *pipsqueak* (*psq*). The identification of *eyeful* has been a starting point from which to unravel crosstalk between the Notch and epigenetic pathways in growth control and tumorigenesis. The fact that many epigenetic factors are involved in cancer suggests that these processes may be more generally involved in tumorigenesis than at first it might seem.

Genetic screen and isolation of *eyeful*

To identify genes that interact with the Notch pathway and that influence growth and tumorigenesis, we used the Gene Search (GS) system[17] to screen for genes that provoked tumours when co-expressed with Delta in the proliferating *Drosophila* eye (Fig. 1a).

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The *ey–Gal4* line was used for both eye-specific and ubiquitous induction, resulting in the transactivation of *UAS*-linked genes throughout the proliferating eye discs (Fig. 1b). It was through such a screen that we isolated the *GS88A8* line. Generalized overexpression of *Delta* by *ey–Gal4* (hereafter termed *ey–Gal4 > Dl*) produces mild eye overgrowth[18] (Fig. 1c, d). In most of the flies in which the *GS88A8* line was coexpressed with *Delta*, tumours developed in the eyes (Fig. 1e, f). Moreover, in ~30% of the mutant flies, secondary eye growths were observed throughout the body (Fig. 1e–i), and in some flies the whole body filled up with eye tissue. These secondary eye growths had ragged borders, indicating invasion of the mutant tissue into the surrounding normal tissue (Fig. 1g–i). As a result, we named the *GS88A8* line '*eyeful*'.

We next undertook a developmental analysis of the tumours (Fig. 2). To facilitate analyses, we generated a triple mutant strain carrying the *eyeful*, *UAS–Dl* and *ey–Gal4* transgenes all on the same chromosome (*ey–Gal4 > eyeful > Dl*; see Supplementary Information). In this strain, mutant eye discs showed massive uncontrolled overgrowth (some discs were more than five times their normal size; Fig. 2b–d). In most discs, the epithelial cells had lost their apical–basal polarity (Fig. 2c), and some had a disrupted basement membrane (Fig. 2d) and grew without differentiating.

We extended these results to the wing disc. First, we used *dpp–Gal4* to direct coexpression of *eyeful* and *Delta* along the anterior–posterior boundary of the wing (perpendicular to the endogenous Delta domain along the dorsal–ventral boundary: Fig. 2e–h). In a normal wing disc, the *dpp–Gal4* driver typically establishes a stripe of green fluorescent protein (GFP) expression with a sharp border at the boundary (Fig. 2e). Whereas wild-type (or single *eyeful*) cells expressing GFP conformed with this pattern (data not shown), some of the *eyeful* and *Delta* cells were found outside this stripe (Fig. 2h), indicating that the mutant cells can disseminate and invade adjacent regions of the disc. Second, the *MS1096–Gal4* line was used to direct expression in the dorsal wing disc compartment. Under these conditions, the wing tissue grew massively and aggressively, and the mutant tissue failed to differentiate (Fig. 2i). Together with the results in Fig. 1e–i, these observations suggest that, when coupled with *Delta* overexpression, an excess of the gene products flanking the *eyeful* insertion site induces the formation of tumours capable of metastasising.

eyeful enforces the transcription of *lola* and *psq*

We isolated and sequenced the genomic DNA flanking the *eyeful* P-element (Fig. 3a and Supplementary Table S1 and Fig. S1a). *eyeful* is inserted in an intron of the gene *longitudinals lacking* (*lola*), which is known to be a chief regulator of axon guidance[19–21]. *lola* encodes 25 messenger RNAs that are produced by alternative splicing and that generate 19 different transcription factors. All of the different isoforms share four exons that encode a common amino terminus, which contains a BTB or POZ domain. In addition, all but one of these transcription factors are spliced to unique exons encoding one or a pair of zinc-finger motifs[19,22].

The GS P-elements allow Gal4-dependent inducible expression of sequences flanking the insertion site in both directions[17] (Fig. 1a). The nearest gene in the opposite direction to transcription of *lola* is the *psq* gene (Fig. 3a). This gene encodes nine variants produced by alternative splicing and alternative promoter use, generating four different proteins. Three of the *psq* isoforms contain a BTB or POZ domain in the N terminus, and a histidine- and glutamine-rich region downstream of this domain. Two of the BTB-containing isoforms and the isoform that lacks this domain contain four tandem copies of an evolutionarily conserved DNA-binding motif, the Psq helix–turn–helix (HTH) motif[23–25].

psq was initially identified for its 'grandchildless' and posterior group defects[23,26] and was subsequently shown to have a role in retinal cell fate determination[24]. Psq is essential for sequence-specific targeting of a PcG complex that contains histone deacetylase (HDAC) activity[9]. Psq binds to the GAGA sequence[27,28], which is present in many Hox genes and in hundreds of other chromosomal sites[9].

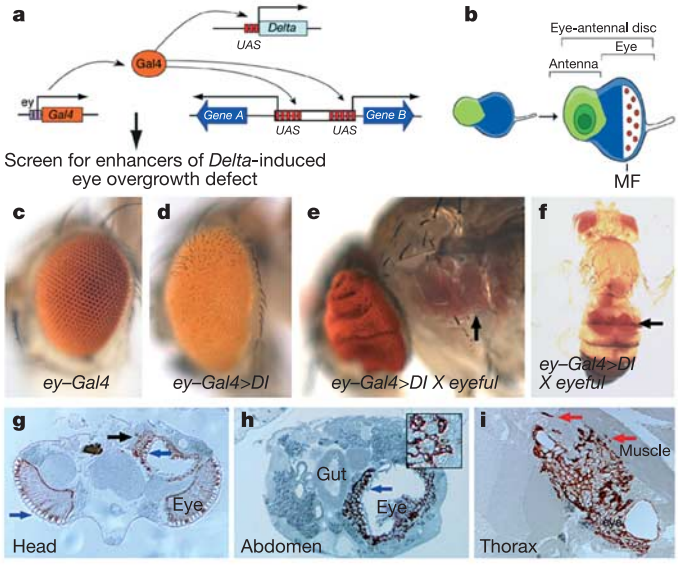

Figure 1 | Isolation of tumour-initiating genes in *Drosophila melanogaster*. **a**, Design of the GS screen for enhancers of the *Delta*-induced eye overgrowth defect. Flies carrying the *ey–Gal4* and *UAS–Delta* (*ey–Gal4 > Dl*) constructs were crossed to randomly inserted GS lines (D.F.-M. and M.D., unpublished data). In these flies, Gal4 binds to the *UAS* sites and causes coexpression of *Delta* and the gene or genes adjacent to the GS P-element insertion in the developing eye disc. **b**, Schematic of *ey–Gal4* expression (blue) in the cycling cells anterior (to the left) to the morphogenetic furrow (MF) in the larval eye-antennal disc. Postmitotic retinal differentiating cells (red circles) are indicated. **c**, Control *ey–Gal4* adult eye. **d**, *ey–Gal4 > Dl* adult 'large eye'. **e**, **f**, Adults from the cross of *eyeful* to *ey–Gal4 > Dl* show massive eyes and secondary eye growths (arrows). **g–i**, Sections through a head (**g**), abdomen (**h**) and thorax (**i**) carrying secondary eye growths. Note the ragged borders of these eye growths (black arrow). Lenses (blue arrows) and dispersed groups of retinal cells (red arrows) are indicated. Inset shows a detail of ommatidal arrangement in these eye growths.

Both polymerase chain reaction with reverse transcription (RT–PCR) and *in situ* hybridization experiments (Supplementary Fig. S1b–g) confirmed that transcription of *lola* and *psq* was influenced by *eyeful* in response to Gal4 activation.

Both genes seem to contribute to the tumour phenotype

To determine whether *lola* and/or *psq* was responsible for the tumour phenotype, we tested 11 enhancer promoter (EP) P-elements inserted into the *lola* and *psq* region (Supplementary Fig. S1a). In contrast to the GS lines, the EP lines allows Gal4-dependent inducible expression of sequences flanking only one end of the P-element[29]. We found that none of the EP lines induced tumours (Supplementary Table S1); thus, we reasoned that the deregulation of both genes might be required to produce the tumours.

The complexity of *lola* and *psq* loci, which together produce 23 proteins, hampers identification of the transcripts responsible for the *eyeful* phenotype by gain-of-expression mutants (that is, by expressing individual or combinations of isoforms). Therefore, we resolved this issue by isolating point mutations that reverted the phenotype caused by deregulated expression of *lola* and *psq*. In this analysis, the chemical mutagen ethyl-methane sulphonate (EMS) was used to induce preferentially single nucleotide changes.

The parental *eyeful* GS line was viable in *trans* with deficiencies that removed both *lola* and *psq* (data not shown). In contrast, a set of 14 EMS-induced mutations on the *eyeful* chromosome failed to complement these deficiencies and were found to be alleles of *psq* or *lola* (Fig. 3b–f and Supplementary Figs S2 and S3). We sequenced the EMS-induced mutations that best recovered a normal eye size. Each individual mutation had a single base change or a small deletion that considerably altered the predicted Psq or Lola proteins.

All psq^{-} mutations induced on the *eyeful* chromosome prevented *eyeful* from producing eye tumours and metastases (Fig. 3b, c, e). Three alleles affected the BTB domain (psq^{rev2}, psq^{rev7} and psq^{rev9}), and three other alleles contained either a premature stop codon that would produce truncated proteins lacking the Psq HTH repeats (psq^{rev4} and psq^{rev14}) or a missense mutation that would change a conserved amino acid in the third Psq HTH repeat (psq^{rev12}). All $lola^{-}$ mutations induced on the *eyeful* chromosome, including the presumptive null allele ($lola^{rev6}$; Fig. 3d, f), reduced eye tumour size but still permitted sporadic secondary growth (Supplementary Information).

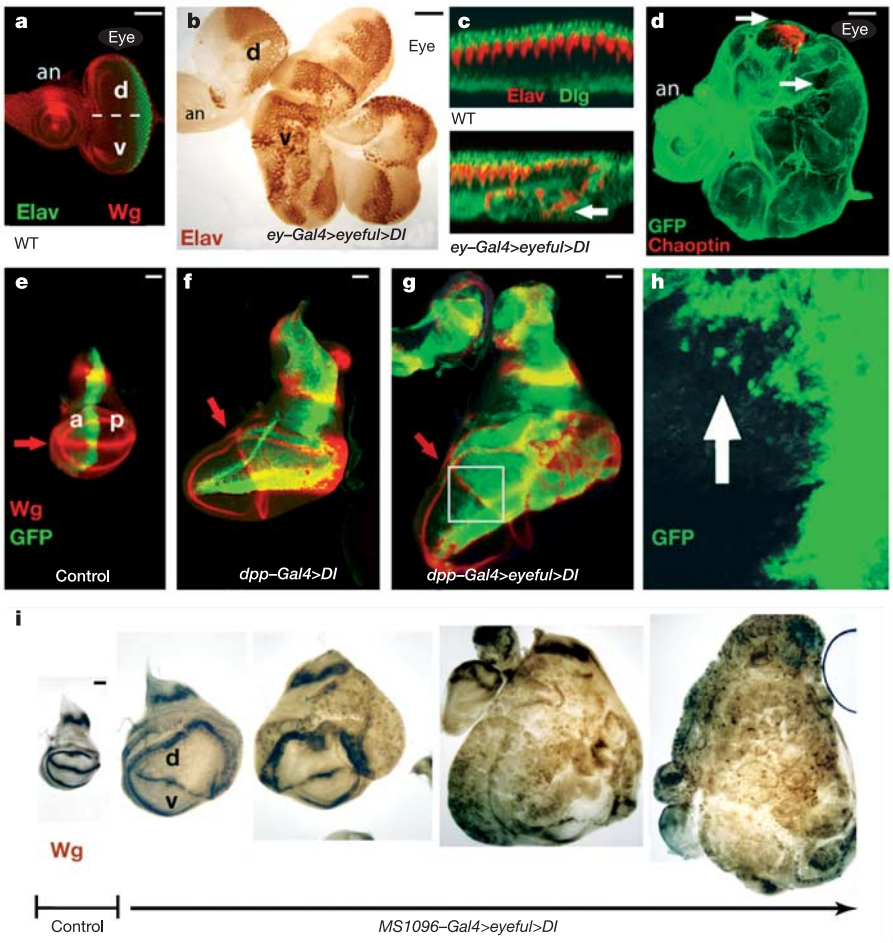

Figure 2 | *eyeful* collaborates with *Delta* to cause malignant tumours. **a**–**c**, Wild-type (**a**) and 7-day-old mutant (**b**) eye discs. Dorsal eye cells are refractory to Delta[33–35] and overgrowth occurs predominantly in the ventral region. The dorsal–ventral boundary is indicated. Elav marks the nuclei of photoreceptor neurons. **c**, Vertical confocal section of a wild-type (top) and a mutant (bottom) eye disc dissected from a 9-day-old larva. Cell membranes are highlighted by Dlg (green) staining. The disc is a bilayer epithelium comprising the squamous epithelium (or 'peripodial membrane') and the main disc epithelium where the nuclei of photoreceptor neurons (red) are apically located. Note the disorganization of the nuclei of neurons (arrow) in the mutants. **d**, Disruption (arrows) of the basement membrane visualized by GFP-tagged Viking/Collagen IV (green). Retinal differentiation (Chaoptin, red) is apparent only in a dorsal spot. **e**, Control *dpp–Gal4 > GFP* wing disc size. The anterior–posterior boundary is indicated; Wg staining marks the dorsal–ventral boundary (red arrows). **f**, *dpp–Gal4 > Dl* wing disc overgrowth. **g**, **h**, *dpp–Gal4 > eyeful > Dl* wing disc massive overgrowth (**g**) and invasive migratory cellular behaviour (arrow, **h**). **i**, Evolution of wing disc tumours caused by coexpression of *eyeful* and *Delta* in the MS1096–Gal4 line. Ventral wing cells are refractory to Delta[33]; thus, overgrowth in these discs initiates in the dorsal region but progressively extends to occupy the whole disc (all discs are at the same magnification). Scale bars, 50 μm.

These data show an unequal contribution of Psq and Lola in this process, whereby Psq is the most important factor in the tumorigenic phenotype. The BTB subfamily of transcriptional repressors includes the human oncogenes *BCL6* and *PLZF*. In these oncogenes, the BTB domain is crucial for oncogenesis through the recruitment of PcG and HDAC complexes[30,31]. We therefore speculated that deregulated Psq and Lola could lead to tumorigenesis by epigenetic processes and that *Drosophila* counterparts of HDACs and PcG proteins might be involved in the progression of these tumours. Indeed, we found genetic evidence that both Lola and Psq function as epigenetic silencers *in vivo* (Supplementary Fig. S4).

Loss of trimethylation of histone H3 on K4 in tumours

We therefore attempted to determine the specific epigenetic mechanisms through which deregulation of Psq and Lola might induce tumorigenesis in conjunction with *Delta* overexpression. Methylation of histone on lysine is a central modification in both epigenetic gene control and in large-scale chromatin structural organization[12]. For example, trimethylation of histone H3 on K4 (H3K4me3) is associated with the active transcription of genes and open chromatin structure. By contrast, histone hypoacetylation and H3K9 and H3K27 methylation are characteristic of heterochromatin state and gene silencing[12,32] (Fig. 4a). To determine whether any changes in these epigenetic markers might coincide with the induction of tumorigenesis, we immunolabelled eye discs with antibodies against specific histone H3 modifications. Because dorsal eye disc cells are refractory to Delta[33–35], the dorsal region of the discs provided an internal control for these studies.

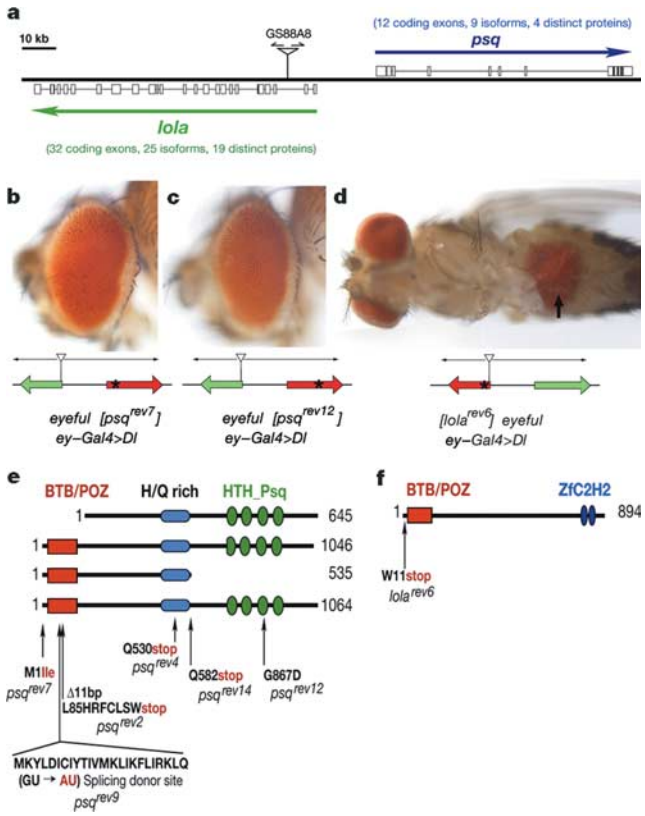

Figure 3 | *eyeful* is a complex mutation affecting two BTB family genes, *lola* and *psq*. **a**, Genomic organization of the *lola* and *psq* genes in the cytological region 47A11-B1 and the *eyeful* GS insertion site. **b**–**d**, EMS-induced revertants of *eyeful* involve mutations in *psq* (**b**, **c**) or *lola* (**d**). Arrow indicates a large secondary eye growth in the presumably null $lola^{rev6}$ revertant. **e**, **f**, Schematic of the four Psq variant proteins (**e**) and a zinc-finger-containing Lola variant protein (**f**) showing the position of the mutations. Numbers refer to the longest, Psq-PB and Psq-PC, variants (**e**).

With the exception of some scattered cells, a prominent loss or strong reduction of H3K4me3 was observed in the ventral region of the mutant discs (Fig. 4b). Notably, although the loss of *Notch* in clones does not affect this epigenetic tag[36], overexpression of *Delta* caused a significant reduction in staining for H3K4me3 (Fig. 4b). The H3K4me3 depletion was already apparent in discs showing moderate hyperplasia and thus preceded neoplasm formation. We could not reproducibly resolve changes in other epigenetic tags (such H3K9me3 and H3K27me2); perhaps more sensitive methods or antibodies might facilitate detection of such changes.

Histone deacetylation and methylation in tumours *in vivo*

H3K4 methylation is thought to be permissive for maintaining and propagating activated chromatin and is thought to neutralize repressor tags by precluding binding of the HDAC complex and impairing SUV39H1-mediated H3K9 methylation[12]. Thus, H3K4me3 depletion may contribute to tumour formation by permitting aberrant chromatin silencing (Fig. 4a). We found that a 50% reduction in dosage of the HDAC gene *Rpd3* (Fig. 4c) or of *Su(var)3-9* (Fig. 4d) decreased the tumour phenotype dominantly. In contrast, reducing the activity of the H3K4 histone methyltransferase genes[9] *Trx* (known as *ALL1* or *MLL* in humans) or *Ash1* (Fig. 4a), which would be expected to deplete the H3K4me3 tag further, did not visibly enhance the tumours (data not shown).

E(z) when complexed with the Extra sex combs (Esc) protein becomes a histone methyltransferase[10]. The E(z)–Esc complex and its mammalian counterpart Ezh2–Eed show specificity for H3K27 but may also target H3K9 (refs 10, 12, 37, and Fig. 4a). The complex also contains the HDAC Rpd3, and this association with Rpd3 is conserved in mammals[38]. H3K27 methylation facilitates binding of the chromodomain protein Pc (HPC in humans)[37,39], which

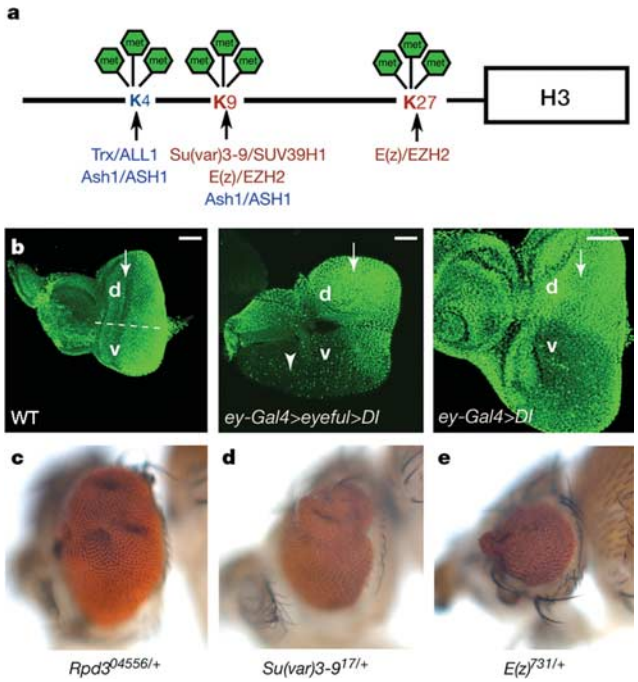

Figure 4 | Aberrant histone modifications associated with the tumours. **a**, Overview of site-selective specificity of histone methyltransferases for distinct lysine (K) positions in the N terminus of histone H3. Histone methyltransferases associated with active chromatin are blue and those associated with silenced chromatin are red. **b**, Wild-type and altered patterns of H3K4me3 in the tumour (*ey–Gal4* > *eyeful* > *Dl*) and overgrowth (*ey–Gal4* > *Dl*) genotypes. The furrow (arrows) and the dorsal and ventral regions of the eye discs are indicated. **c**–**e**, Representative adult eyes of *ey–Gal4* > *eyeful* > *Dl* flies carrying a mutant copy of the HDAC gene *Rpd3* (**c**), *Su(var)3-9* (**d**) and *E(z)* (**e**). Scale bars, 50 μm.

then creates a repressive chromatin state that is a stable silencer of genes.

Although loss of *E(z)* does not cause proliferation defects within discs[37,40], halving the *E(z)* gene dosage dominantly suppressed tumorigenesis (Fig. 4e), indicating that histone methylation by the E(z)–Esc complex is also a prerequisite for the excessive proliferation of these tumours. Accordingly, Esc^{-} or Pc^{-} mutations also notably reduced the tumours (Supplementary Information).

Together, these findings suggest that the development of these tumours involves, at least in part, changes in the structure of chromatin brought about by covalent modifications of histones. These changes probably switch the target genes from the active H3K4me3 state to a deacetylated H3K9 and H3K27 methylation silent state.

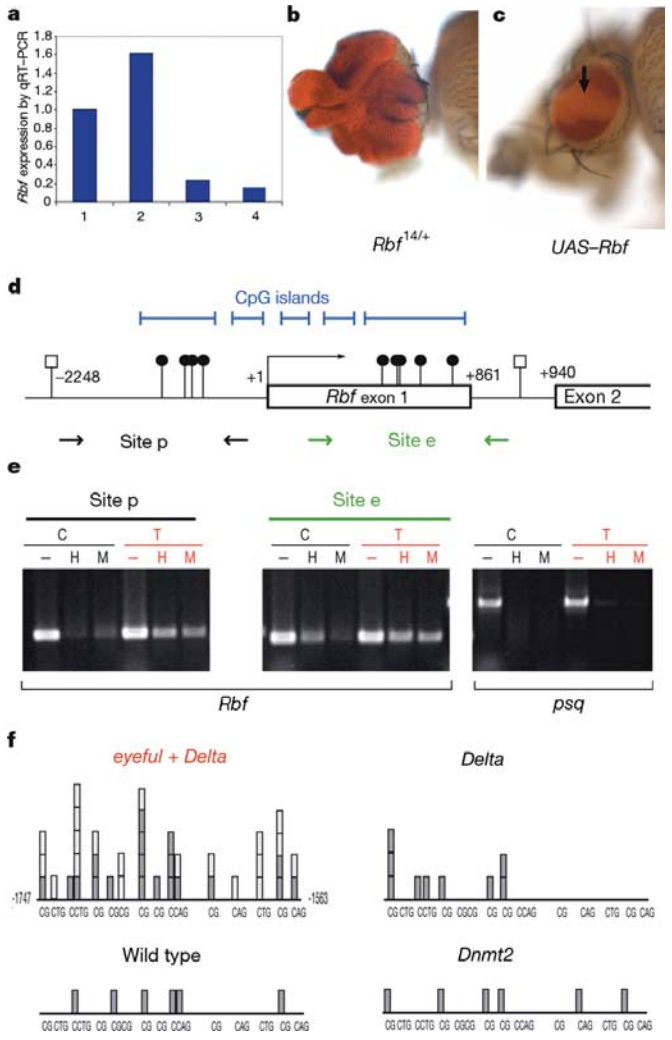

Figure 5 | Silencing of *Rbf* is associated with promoter DNA hypermethylation. **a**, *Rbf* gene expression was analysed by quantitative real-time PCR. The relative levels of *Rbf* normalized to *Rp49* (as a loading control) signals are shown for 5-day-old eye discs from wild type (lane 1), *ey–Gal4 > eyeful* (lane 2), *ey–Gal4 > eyeful > Dl* (lane 3) and *ey–Gal4 > Dl* (lane 4). **b**, A mutated allele of *Rbf* (Rbf^{14}) enhances the tumour defect of *ey–Gal4 > eyeful > Dl* flies. **c**, In contrast, eye-specific overexpression of *UAS–Rbf* rescues the tumours. Arrow indicates transposon silencing (see Supplementary Fig. S4). **d**, CpG-rich islands in the *Rbf* promoter and exon 1. The upstream region of *Rbf* overlaps with a predicted gene named *CG13359*, which is not conserved in *Drosophila pseudoobscura*. *Hind*III (squares) and *Hpa*II/*Msp*I (circles) sites are indicated. **e**, Methylation-sensitive restriction digests followed by PCR amplification. PCR yields products only when the region is methylated at each *Hpa*II/*Msp*I site. Shown are representative gels for *Rbf* and *psq* (as a control for a gene expressed in the tumours) of w^{1118} (C) and *ey–Gal > eyeful > Dl* (T). Uncut (–) DNA was used as a loading control and as a control for the PCR reaction. **f**, DNA methylation in the *Rbf* region in mutant and control eye disc cells as determined by bisulphite genomic sequencing (Supplementary Information). The number and position of the methylated cytosines (out of a possible 13) are indicated by filled rectangles (light grey squares denote hemi-methylation).

Silencing of *Rbf* in overgrowth and tumours

From the above data, we considered that the tumours might form as a result of aberrant gene silencing. If so, then the expression of genes involved in cell-cycle control is likely to be altered in the mutant cells. We compared the transcription of 12 tumour-related genes in the mutant and wild-type discs. Transcription of the gene *Rbf*, a fly homologue of the *RB*/*Rb* family of genes[41], was strongly down-regulated in this assay (and even in *ey–Gal4 > Dl* flies; Fig. 5a). A second *Rb* gene, *Rbf2*, remained unchanged in the different genetic backgrounds, highlighting the specificity of *Rbf* silencing (data not shown).

We found that *Rbf* depletion seems to be intricately associated with tumorigenesis: first, reducing *Rbf* gene dosage by 50% enhanced tumour growth (Fig. 5b); second, re-establishing *Rbf* expression in the eye (using an *UAS–Rbf* transgene) consistently prevented eye tumours and occurrence of secondary growths (in 100 flies examined; Fig. 5c).

Inactivation of *RB1* in retinoblastoma, a form of eye cancer in children, can occur through DNA hypermethylation of the promoter[13]. Unlike in mammals, however, there is little cytosine methylation of the genome in *Drosophila* during developmental stages[42,43], and its potential role during tumorigenesis is unknown. DNA methylation seems to depend on one DNA methyltransferase, Dnmt2, that preferentially methylates cytosine at CpT or CpA sites[44–47]. The fly genome also encodes one methyl-CpG DNA-binding MBD2/3 protein[48]. Because there are no known *Dnmt2* loss-of-function mutations, we could not test the role of this gene in tumorigenesis.

Nevertheless, we tested whether the CpG islands that we observed in the *Rbf* gene were potential targets for repression by DNA methylation by two methods (Fig. 5d). First, we used methylation-sensitive restriction enzymes analysis (Supplementary Information), which showed that the regions around the promoter and transcription start site of the *Rbf* gene were susceptible to methylation (Fig. 5d, e). This approach showed aberrant DNA hypermethylation of *Rbf* in the *eyeful* and *Delta* eye discs (Fig. 5e) and mild hypermethylation in *Delta* discs (data not shown); however, at best only very mild methylation was detected in discs from wild-type flies or from flies with the control *psq* gene (Fig. 5e).

Second, we carried out direct bisulphite sequencing of genomic DNA from mutant discs (Fig. 5f). This approach confirmed the notable increase in methylated DNA in *eyeful* and *Delta* discs when compared with wild-type discs (and a moderate increase in methylated DNA in the *Delta* discs). Hypermethylation of the *Rbf* promoter was not simply the result of *de novo* transcription of *Dnmt2* (*ey-Gal4 > Dnmt2*; Fig. 5f), indicating that activation of the Notch pathway is a crucial step in this *de novo* hypermethylation of *Rbf*.

Discussion

Here we have used *Drosophila* genetics to search for genes that collaborate with the Notch pathway during tumorigenesis *in vivo*. We have identified Psq and Lola as decisive factors to foment tumour growth and invasion when coactivated with the Notch pathway. These proteins are presumptive transcription repressors that contain a BTB domain and sequence-specific DNA-binding motifs and behave as epigenetic silencers *in vivo*.

In addition, we have identified crosstalk between the Notch

pathway and different epigenetic regulators. It is likely that alterations in this crosstalk provoke the aberrant epigenetic repression (and perhaps also derepression) of genes that contributes to cellular transformation. We have identified the *Rbf* gene as one target for this epigenetic regulation and shown that *Rbf* depletion directly contributes to the tumours.

We propose that the sequence of events that leads to these tumours commences with hyperactivation of the Notch pathway, which initiates gene repression. Subsequently, or at the same time as Notch, Psq–Lola could bind to the silenced genes and enforce silencing by recruitment of HDAC or PcG repressors. Given the conservation of the Psq-like HTH domains in Psq and of BTB domains, it seems likely that other transcriptional repressors containing such domains strongly influence the tumour-inducing capacities of HDACs and PcG repressors in human cancers.

Finally, the collaboration between PcG-mediated cellular memory and the Notch pathway may have implications in other processes controlled by Notch, including the second mitotic wave in the *Drosophila* eye[49,50], and the organization of eye and wing growth[3,16]. In these processes, the memory mechanism could ensure that cells kept a record of the Notch signals received at an earlier stage or when the progenitor cells were closer to the Delta source. In this way, they might remain proliferative without having to receive continuous instructions from Notch. Likewise, such a situation could be conceived for tumorigenesis. The oncogenic signals could opportunistically take advantage of the memory mechanism to fix and to maintain their instructions of continuous proliferation in progenitor or stem cells, thereby fostering tumour growth and metastasis.

METHODS

***Drosophila* husbandry and the genetic screen.** The *GS88A8* (*eyeful*) and *GS71A5* lines and other mutant stocks and transgenes are described in the Supplementary Information.

GS element mapping. Genomic DNA flanking the P-element insertions in the *GS88A8* and *GS71A5* lines were recovered by inverse PCR (http://www.fruitfly.org/about/methods) and sequenced.

***In situ* hybridization and antibody staining.** We carried out *in situ* hybridization and antibody staining as described in Supplementary Information.

EMS reversion mutagenesis and phenotypic analyses. Details of mutagenesis and phenotypic analyses are described in the Supplementary Information.

Real-time PCR. Real-time PCR was done with a BioRad iCycler in the presence of SYBR Green I according to the manufacturer's instructions. The primers of the *Rbf* gene and the control *Rp49* gene were designed with Primer Express software (Applied Biosystems). To assess the expression of *Rbf* and *Rp49* (as a loading control), we extracted total RNA from antennal-eye imaginal discs with Trizol Reagent (Invitrogen). We prepared complementary DNA with an Enhanced Avian RT-PCR kit (Sigma) using oligo $(dT)_{23}$ primers and 2 μg of total RNA. Expression levels were calculated as a ratio between the signals from *Rbf* and those from *Rp49*. The nature of the PCR products was confirmed by agarose gel electrophoresis and melting curve analysis.

Bisulphite and methylation analysis. Genomic DNA was isolated from third instar larvae or dissected eye discs and the DNA was analysed by methylation-sensitive enzyme digestion and direct bisulphite sequencing as described in the Supplementary Information.

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