Polycomb group (PcG) proteins are conserved epigenetic regulators that are linked to cancer in humans. However, little is known about how they control cell proliferation. Here, we report that mutant clones of the PcG gene polyhomeotic (ph) form unique single-cell-layer cavities that secrete three JAK/STAT pathway ligands, which in turn act redundantly to stimulate overproliferation of surrounding wild-type cells. Notably, different ph alleles cause different phenotypes at the cellular level. Although the ph-null allele induces non-autonomous overgrowth, an allele encoding truncated Ph induces both autonomous and non-autonomous overgrowth. We propose that PcG misregulation promotes tumorigenesis through several cellular mechanisms.

Keywords: polyhomeotic; non-autonomous cell overproliferation; JAK/STAT pathway; Notch signalling; Upd homologues

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INTRODUCTION

Polycomb group (PcG) genes are epigenetic regulators that are conserved throughout metazoa. They were first identified in Drosophila as repressors of HOX genes (reviewed in Bienz & Müller, 1995). Follow-up investigations showed that PcG genes have key roles in various biological processes, such as embryonic development, stem-cell maintenance, genomic imprinting and X-chromosome inactivation (reviewed in Sparmann & Lohuizen, 1995). Mutation or loss of PcG genes usually results in dysfunctional cells with unidentifiable cell fates and can thereby induce or facilitate tumorigenesis (Sánchez-Beato et al, 2006; Ballestar & Esteller, 2008; Bracken & Helin, 2009). The Drosophila PcG gene polyhomeotic (ph) is a key component of the polycomb repressive complex 1 (PRC1; Francis et al, 2001). Mutation or loss of expression of ph homologues has been found in human cancers (Tokiama et al, 2001; Deshpande et al, 2007), but little is known about the role of Ph in the control of cell proliferation.

A recent study has indicated that loss of Ph induces autonomous cell proliferation in mosaic eye discs (Martinez et al, 2009). Here, we report that ph cells in the mosaic eye disc fail to differentiate into photoreceptor neurons; instead, they form unique cavity-like structures that secrete JAK/STAT signalling ligands—Unpaired (Upd) and its homologues—to stimulate proliferation of surrounding wild-type cells in a non-autonomous manner.

RESULTS AND DISCUSSION

ph causes non-autonomous overgrowth in mosaic eyes

In a previous study, we isolated an ethyl methanesulphonate (EMS)-induced recessive lethal mutant, l(X)MB342, that alters cell fates in the Drosophila brain (Wang et al, 2006). Subsequently, we found that this mutant also caused cell overproliferation in mosaic eyes (Fig 1B). l(X)MB342 is a deficiency line that uncovers an approximately 40 kb genomic region and fails to complement with three complementation groups (ph, Pgd and wapl; Fig 1A). To determine which genes were responsible for the enlarged eyes, we examined the mosaic eye phenotypes of various mutant lines within this genomic region. No eye enlargement resulted from any Pgd or wapl alleles (supplementary Fig S1A online). However, we observed various, including opposite, phenotypes in eyes mosaic for different ph alleles that were all ph-null alleles (Dura et al, 1987; Boivin et al, 1999; supplementary Fig S1B online).

The ph locus consists of two tandemly duplicated genes—ph-p and ph-d—which are functionally redundant (Deatrick et al, 1991). All known ph alleles were generated by many rounds of mutagenesis to inactivate both genes, and the nature of their mutations has not been characterized at the molecular level (Dura et al, 1987; Boivin et al, 1999). These features might explain why different ph alleles have different phenotypes, and they also highlight the need for a ph-null allele with a clean genetic background. Therefore, we generated a ph deficiency line—phdel—from two viable P-element insertion lines by using a flippase recombination target (FRT)-based genetic technique (Parks et al, 2004). In phdel, the ph locus was deleted without disrupting the surrounding genes (Figs 1A, 5A). As expected, phdel failed to complement with several known ph alleles, but complemented with genes adjacent to the ph locus on both sides. The phenotypes of phdel mosaic eyes were indistinguishable from those of l(X)MB342 (Fig 1B,C). Moreover, a transgene, p(pH-d+),...
which carries a 10-kb \( ph-d \) genomic fragment, could rescue the lethality and overproliferation phenotype of \( ph^{del} \) (Fig 1B; supplementary Fig S2 online). Together, these data demonstrate that loss of Ph causes overgrowth in mosaic eyes.

We next performed MARCM (mosaic analysis with a repres- sible cell marker) analyses for \( ph^{del} \) in eye discs. Although \( ph^{del} \) mosaic discs were enlarged at the third instar larval stage, homozygous mutant cells—that were positively labelled
with green fluorescent protein (GFP)—occupied a smaller proportion of ph mosaic eye discs than did control clones in wild-type mosaic discs (Fig 1C), suggesting that ph causes overproliferation in a cell non-autonomous manner—that is, only in wild-type cells. To test this idea, we labelled the ph chromosome with w− and the wild-type chromosome with w+. In the resulting mosaic eyes, most—if not all—of the ommatidia were red. By contrast, in wild-type mosaic eyes, patches of white and red ommatidia were observed (Fig 1D). Conversely, when the ph chromosome was labelled with w+ and the wild-type chromosome was labelled with w− in phdel mosaic eyes, we observed mainly white eyes with a few red cells, which might represent heterozygous cells that did not undergo recombination (Fig 1D). These results provide further evidence that loss of Ph induces non-autonomous overgrowth and indicate that ph cells do not differentiate into ommatidia.

Cellular abnormalities in phdel mosaic discs

We then characterized cell proliferation, apoptosis and differentiation in ph mosaic discs by using different molecular markers. 5-Bromo-2-deoxyuridine (BrdU) labelling and phospho-histone H3 (PH3) staining—which mark the S and M phases of mitotic cells, respectively—showed that overproliferation occurred only in the wild-type cells (GFP-negative) of the phdel mosaic eye discs (Fig 2; supplementary Fig S3A online). Conversely, TdT-mediated dUTP nick-end labelling (TUNEL) staining showed that apoptosis...
was increased in ph mutant clones (Fig 2). Consistent with the fact that ph cells are missing from the adult ommatidia (Fig 1D), ph<sup>del</sup> clones after the morphogenetic furrow were negative for Elav (embryonic lethal abnormal vision; Fig 2; supplementary Fig S3B online), indicating that they do not differentiate normally. We tracked the fate of ph cells through the late developmental stages and found that some were retained in the adult brains as clusters of undifferentiated cells attached to the surface of optic lobes (supplementary Fig S3C online).

Remarkably, ph<sup>del</sup> clones in the mosaic eye discs formed unique single-cell-layer cavities. As indicated by the apical and subapical complex marker atypical protein kinase C (aPKC; Wodarz et al., 2000), the apical side of ph cells faced the inner surface of the cavities (supplementary Fig S4A online). ph<sup>del</sup> clones in wing and leg discs formed similar cavity-like structures (supplementary Fig S4B online). aPKC antibody staining indicated that although ph clones form unique three-dimensional structures, individual ph cells maintain their apical–basal cell polarity.

Fig 3 | Notch and Upd homologues are involved in ph-induced non-autonomous cell overproliferation. (A) In mosaic eyes, removal of Notch from ph mutant clones (ph<sup>del</sup>-N double mutant) completely suppresses the eye overgrowth phenotype. Concomitantly, increased PH3 labelling in the ph mosaic eye discs is also eliminated. However, the autonomous apoptosis phenotype and cavity-like structure of ph clones are retained. (B) Removal of upd from ph mutant clones (ph<sup>del</sup>-upd double mutant) alone does not suppress either the eye overgrowth phenotype or increased PH3 labelling in ph mosaic eye discs. However, removal of all three upd homologues from ph mutant clones (ph<sup>del</sup>-upd<sup>del1–3</sup> double mutant) has the same effect as ph<sup>del</sup>-N double mutant. (C) Elevated levels of Notch protein are detected in the inner surface of ph<sup>del</sup> clones and massive amounts of Upd protein are detected within the lumen of ph<sup>del</sup> clones. DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; PH3, phospho-histone H3; TUNEL, TdT-mediated dUTP nick end labelling; ph, polyhomeotic; Upd, unpaired; WT, wild type.

Notch is required for ph-induced overgrowth
In Drosophila, mutations in several tumour suppressor genes, such as ept, vps25 and Uba1, have been reported to cause non-autonomous overgrowth (Moberg et al., 2005; Thompson et al., 2005; Vaccari & Bilder, 2005; Herz et al., 2006; Lee et al., 2008). In all such cases, the Notch–Eyeg–Upd–JAK/STAT pathway is involved. Notch activity is increased in the mutant clones, which induces overexpression of the ligand Upd through the transcription factor eyegone (Eyeg). Upd is then secreted from the mutant cells and activates the JAK/STAT pathway in neighbouring cells, inducing overproliferation. Therefore, we first investigated whether ph cells induced non-autonomous overgrowth through the same signalling pathway.
A genetic interaction assay suggested that the Notch–Upd–JAK/STAT signalling pathway might also have a key role in ph-induced non-autonomous overgrowth (supplementary Fig S5 online). To verify the role of Notch and Upd signalling in ph-induced overgrowth in vivo, we generated ph-N and ph-upd double mutant lines. As shown in Fig 3A, the size of ph-N mosaic eyes was comparable with that of the wild type, indicating that the overgrowth phenotype of ph mosaic eyes is significantly, if not completely, suppressed by the loss of Notch from ph mutant clones. We also noticed that ph-N mosaic eyes were still mainly red. As the wild-type chromosome was w+ and the ph-N chromosome was w−, this result indicates that ph-N cells were also missing from the mosaic eyes (Fig 3A). To exclude the possibility that ph-N double mutant cells died before the induction of overproliferation, we examined ph-N clones in mosaic eye discs at the wandering larval stage. ph-N clones were viable and morphologically identical to ph mutant clones, but the overall size of ph-N mosaic discs was significantly smaller than that of ph mosaic discs (Fig 3A). Moreover, PH3 staining showed that cell proliferation of ph-N mosaic discs was reduced to a level similar to the wild-type (compare Fig 3A with Fig 2), whereas TUNEL staining showed that the ph-N cells still had higher rates of apoptosis (Fig 3A). These results demonstrate that Notch activity is required for ph-induced non-autonomous overproliferation, but not for the ph-induced autonomous increase in apoptosis and defective differentiation. Moreover, the cavity-like morphology was retained in ph-N clones.

**Upd homologues are redundant in ph-induced overgrowth**

Next, we examined the mosaic eye phenotypes of the ph-upd double mutant. Unexpectedly, ph-upd mosaic eyes were phenotypically indistinguishable from ph mosaic eyes (Fig 3B). Thus, removal of upd from ph clones is not sufficient to block ph-induced non-autonomous overgrowth. These results seem to be inconsistent with our genetic interaction data, which suggests the involvement of the JAK/STAT pathway (supplementary Fig S5 online). In the Drosophila genome, however, there are three genes that encode potential JAK/STAT pathway ligands—upd, upd2 and upd3—which form a cluster (Harrison et al., 1998; Gilbert et al., 2005; Hombría et al., 2005). We therefore postulated that these three upd homologues might contribute redundantly to ph-induced overgrowth. To test this hypothesis, we created a deficiency line—upd<sup>Del−1</sup>—which deletes the genomic region from X:18133021 to X:18206733 that includes genes of all three JAK/STAT ligands. ph<sup>del</sup>–upd<sup>Del−1</sup> mosaic eyes were significantly smaller than ph mosaic eyes (Fig 3B), and the PH3 signal in ph-upd<sup>Del−1</sup> mosaic discs was similar to that of wild-type discs (Fig 3B). These observations indicate that the three Upd homologues act redundantly in ph-induced overgrowth.

Finally, we asked whether the Notch and Upd proteins were increased in ph<sup>del</sup> mosaic eye imaginal discs. We observed that Notch protein accumulated strongly on the inner surface of ph-induced cavity-like structures (that is, the apical domain of ph cells), whereas the Upd protein was secreted into the lumen of the cavities (Fig 3C).

**ph<sup>505</sup> is not a completely null allele**

To clarify why the two ph alleles produced different phenotypes, we first compared the nature of the mutations in ph<sup>del</sup> and ph<sup>505</sup> at the molecular level. ph<sup>505</sup> is a mutation created by two rounds of EMS treatment (Dura et al., 1987). We sequenced the genomic region of ph<sup>505</sup> that contains ph-d and ph-p genes. The results showed that this ph allele carried two nonsense mutations in Gln 398 of ph-d and Gln 749 of ph-p, respectively (Fig 5A). DNA sequencing also verified that ph<sup>del</sup> was a deficiency line that uncovered all exons of ph-d and ph-p except for the first exon of ph-p, which only encodes 12 amino acids (Fig 5A).

While ph<sup>del</sup> is obviously a null allele, the truncated Ph proteins encoded by ph<sup>505</sup> might retain partial Ph function. To test this possibility, we examined the lethal phase of ph<sup>505</sup> and ph<sup>del</sup>. Both ph<sup>505</sup> and ph<sup>del</sup> mutants died in the embryonic stage, but ph<sup>505</sup> mutants died later than ph<sup>del</sup> mutants (Fig 5B). Moreover, we also noticed that two copies of the p(ph-d<sup>−/+</sup>) transgene were required to rescue fully the adult viability and mosaic eye phenotypes of ph<sup>del</sup>, but one copy of the same transgene was sufficient to rescue these phenotypes of ph<sup>505</sup> (supplementary Fig S2 online). Taken together, we conclude that ph<sup>del</sup> is a ph-null allele, but ph<sup>505</sup> is not. This difference at the molecular level might contribute to the different cellular phenotypes of these two ph alleles.

In summary, we have shown that mosaic clones homozygous for the ph-null allele induce overproliferation of surrounding wild-type cells through Notch–Upd–JAK/STAT signalling, whereas mosaic clones homozygous for a ph hypomorphic allele that encodes truncated Ph proteins induce both autonomous and non-autonomous cell overproliferation. These results highlight an important but largely overlooked phenomenon: different mutations in the same gene might induce tumours and cancers through distinct cellular mechanisms, depending on the nature of the

**ph<sup>505</sup> is phenotypically different from ph<sup>del</sup>**

On the basis of their studies of ph<sup>505</sup>—a long-accepted ph-null allele—Martinez et al (2009) recently reported that loss of Ph induces cell-autonomous overgrowth in mosaic eye discs, which conflicts with our results. Therefore, we conducted a series of comparative studies between the phenotypes of ph<sup>del</sup> and ph<sup>505</sup> mosaic eyes. We found that ph<sup>del</sup> is phenotypically different from ph<sup>505</sup>; ph<sup>505</sup> clones in mosaic eye discs overproliferated autonomously, but ph<sup>del</sup> clones did not (Fig 4A compared with Fig 1C). Moreover, ph<sup>del</sup> clones formed single-cell-layer cavities (supplementary Fig S4 online), but ph<sup>505</sup> clones did not. Conversely, we also found that ph<sup>del</sup> and ph<sup>505</sup> mosaic eyes are similar in other aspects. The overgrowth phenotypes of both ph<sup>del</sup> and ph<sup>505</sup> were fully suppressed by the p(ph-d<sup>−/+</sup>) transgene (Fig 4A; supplementary Fig S2 online), suggesting that they are both caused by loss of Ph. In addition, similarly to ph<sup>del</sup> mosaic eyes, the enlarged ph<sup>505</sup> mosaic adult eyes were mainly composed of wild-type cells. As shown in Fig 4B, when ph<sup>505</sup> was associated with a w+ chromosome, the mosaic eye was mainly red. Conversely, when ph<sup>del</sup> was associated with a w− chromosome, the mosaic eye was mainly white. Elva antibody staining indicated that ph<sup>del</sup> cells did not differentiate into photoreceptor neurons (Fig 4C). Therefore, we infer that to form the enlarged adult eyes, wild-type cell clumps within ph<sup>505</sup> mosaic eyes must also overproliferate. In short, ph<sup>del</sup> and ph<sup>505</sup> mosaic eyes both overgrow, but ph<sup>del</sup> induces non-autonomous cell overproliferation, whereas ph<sup>505</sup> induces both non-autonomous and autonomous cell overproliferation. Nevertheless, for both alleles, the adult ommatidia are composed of wild-type cells only.
mutations and/or genetic backgrounds. This fact adds another layer of complexity to cancer pathology.

METHODS

Fly stocks and genetics. l(X)MB342 and p{ph-d}+ have been described previously (Wang et al, 2006). ph505 and ph600 were a gift from N.B. Randsholt. Deficiency lines phdel and updd1–3 were generated using an FRT-dependent deletion technique (Parks et al, 2004). Other fly strains were collected from the Bloomington Drosophila Stock Center. All flies were maintained on standard cornmeal medium at 25 °C. MARCM clones in imaginal discs were induced by ey-flp or hs-flp.

Immunohistochemistry. Antibody staining and BrdU labelling were performed as described (Wang et al, 2006). TUNEL staining was performed using In situ Cell Death Detection Kit (TMR Red) from Roche, according to the manufacturer’s instructions. The following primary antibodies were used: mouse Elav (9F8A9), mouse BrdU (G3G4) and mouse Notch extracellular domain (C458.2H; Developmental Studies Hybridoma Bank at the University of Iowa); rabbit PH3 (Upstate Biotechnology); rabbit Upd (a gift from D. Harrison); and rabbit aPKC (Santa Cruz Biotechnology). All images of immunofluorescent staining were collected using a Leica SP5 X confocal microscope and processed with Adobe Photoshop.

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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