Loss of the Polycomb group gene *polyhomeotic* induces non-autonomous cell overproliferation

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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, 2006). 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 (Tokimasa *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 discs 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—*ph^{del}*—from two viable *P*-element insertion lines by using a flippase recombination target (FRT)-based genetic technique (Parks *et al*, 2004). In *ph^{del}*, the *ph* locus was deleted without disrupting the surrounding genes (Figs 1A, 5A). As expected, *ph^{del}* failed to complement with several known *ph* alleles, but complemented with genes adjacent to the *ph* locus on both sides. The phenotypes of *ph^{del}* mosaic eyes were indistinguishable from those of *l(X)MB342* (Fig 1B,C). Moreover, a transgene, *p{ph-d⁺}*,

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Fig 1 | *ph^{del}* clones in mosaic eyes induce non-autonomous overgrowth. (A) Diagram of the genomic region surrounding *ph* genes, showing DNA fragments that are deleted in the deficiency lines *l(X)MB342* and *ph^{del}*. (B) Scanning electron microscopy images showing mosaic eyes with different genotypes. Mosaic eyes of *l(X)MB342* and *ph^{del}* induced by *ey-flp* are enlarged and misshapen. The overgrowth phenotype of *ph^{del}* mosaic eyes can be suppressed by one copy of the *p{ph-d⁺}* transgene, but the rescued eye has a rough surface. Two copies of *p{ph-d⁺}* transgene are needed for a full rescue (supplementary Fig S4 online). (C) Mosaic eye imaginal discs were dissected at the wandering larval stage and stained with DAPI (blue). Clones homozygous for WT or *ph^{del}* are marked by GFP (green). Compared with WT, the *ph^{del}* mosaic eye disc is larger, but *ph^{del}* clones occupy a smaller portion of the disc. One copy of the *p{ph-d⁺}* transgene suppresses the overgrowth phenotype of the *ph^{del}* mosaic eye disc and restores normal morphology of *ph^{del}* clones. (D) Adult eyes of *ey-flp*-induced mosaics of WT and *ph^{del}* show that *ph^{del}* mosaic eyes are overgrown and lack mutant clones. When *ph^{del}* is associated with a *w⁻* chromosome, the mosaic eye is mainly red. Conversely, when *ph^{del}* is associated with a *w⁺* chromosome, the mosaic eye is mainly white. DAPI, 4,6-diamidino-2-phenylindole; FRT, flippase recombination target; GFP, green fluorescent protein; *ph*, *polyhomeotic*; WT, wild type.

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 repressible 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

Fig 2 | ph^{del} clones stimulate overproliferation of surrounding wild-type cells, but also cause autonomous apoptosis and defective differentiation. Confocal images show eye imaginal discs of wandering larvae containing clones of WT and ph^{del} (green) labelled for BrdU, PH3, TUNEL and Elav (all shown in red). DAPI was used to mark nuclei (blue). Mosaic discs of ph^{del} have increased BrdU and PH3 labelling throughout the discs and elevated TUNEL labelling in ph^{del} clones. Elav staining is absent from ph^{del} clones behind the morphogenetic furrow. BrdU, 5-bromo-2-deoxyuridine; DAPI, 4,6-diamidino-2-phenylindole; Elav, embryonic lethal abnormal vision; GFP, green fluorescent protein; PH3, phospho-histone H3; TUNEL, TdT-mediated dUTP nick end labelling; WT, wild type.

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 ph^{del} 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 ph^{del} 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 ph^{del} mosaic eye discs (Fig 2; supplementary Fig S3A online). Conversely, TdT-mediated dUTP nick-end labelling (TUNEL) staining showed that apoptosis

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^{del}-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^{del}-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^{del}-upd^{d1-3}* double mutant) has the same effect as *ph^{del}-N* double mutant. (C) Elevated levels of Notch protein are detected in the inner surface of *ph^{del}* clones and massive amounts of Upd protein are detected within the lumen of *ph^{del}* 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.

was increased in *ph* mutant clones (Fig 2). Consistent with the fact that *ph* cells are missing from the adult ommatidia (Fig 1D), *ph^{del}* 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^{del}* 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^{del}* 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.

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–Eyg–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 (Eyg). 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^{d1-3}*—which deletes the genomic region from X:18133021 to X:18206733 that includes genes of all three JAK/STAT ligands. *ph^{del}-upd^{d1-3}* mosaic eyes were significantly smaller than *ph* mosaic eyes (Fig 3B), and the PH3 signal in *ph-upd^{d1-3}* 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^{del}* 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).

ph⁵⁰⁵ is phenotypically different from *ph^{del}*

On the basis of their studies of *ph⁵⁰⁵*—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^{del}* and *ph⁵⁰⁵* mosaic eyes. We found that *ph^{del}* is phenotypically different from *ph⁵⁰⁵*; *ph⁵⁰⁵* clones in mosaic eye discs overproliferated autonomously, but *ph^{del}* clones did not (Fig 4A compared with Fig 1C). Moreover, *ph^{del}* clones formed single-cell-layer cavities (supplementary Fig S4 online), but *ph⁵⁰⁵* clones did not. Conversely, we also found that *ph^{del}* and *ph⁵⁰⁵* mosaic eyes are similar in other aspects. The overgrowth phenotypes of both *ph^{del}* and *ph⁵⁰⁵* were fully suppressed by the *p{ph-d⁺}* transgene (Fig 4A; supplementary Fig S2 online), suggesting that they are both caused by loss of Ph. In addition, similarly to *ph^{del}* mosaic eyes, the enlarged *ph⁵⁰⁵* mosaic adult eyes were mainly composed of wild-type cells. As shown in Fig 4B, when *ph⁵⁰⁵* was associated with a w^- chromosome, the mosaic eye was mainly red. Conversely, when *ph⁵⁰⁵* was associated with a w^+ chromosome, the mosaic eye was mainly white. Elva antibody staining indicated that *ph⁵⁰⁵* cells did not differentiate into photoreceptor neurons (Fig 4C). Therefore, we infer that to form the enlarged adult eyes, wild-type cells within *ph⁵⁰⁵* mosaic eyes must also overproliferate. In short, *ph^{del}* and *ph⁵⁰⁵* mosaic eyes both overgrow, but *ph^{del}* induces non-autonomous cell overproliferation, whereas *ph⁵⁰⁵* induces both non-autonomous and autonomous cell overproliferation. Nevertheless, for both alleles, the adult ommatidia are composed of wild-type cells only.

ph⁵⁰⁵ 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^{del}* and *ph⁵⁰⁵* at the molecular level. *ph⁵⁰⁵* is a mutation created by two rounds of EMS treatment (Dura et al, 1987). We sequenced the genomic region of *ph⁵⁰⁵* 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^{del}* 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^{del}* is obviously a null allele, the truncated Ph proteins encoded by *ph⁵⁰⁵* might retain partial Ph function. To test this possibility, we examined the lethal phase of *ph⁵⁰⁵* and *ph^{del}*. Both *ph⁵⁰⁵* and *ph^{del}* mutants died in the embryonic stage, but *ph⁵⁰⁵* mutants died later than *ph^{del}* mutants (Fig 5B). Moreover, we also noticed that two copies of the *p{ph-d⁺}* transgene were required to rescue fully the adult viability and mosaic eye phenotypes of *ph^{del}*, but one copy of the same transgene was sufficient to rescue these phenotypes of *ph⁵⁰⁵* (supplementary Fig S2 online). Taken together, we conclude that *ph^{del}* is a *ph*-null allele, but *ph⁵⁰⁵* 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

Fig 4 | ph^{505} clones in mosaic eyes induce non-autonomous cell overproliferation. (A) WT, ph^{505} and ph^{del} mosaic eye discs dissected from wandering larvae were stained with DAPI (blue). Homozygous mosaic clones are labelled with GFP (green). Both ph^{505} and ph^{del} mosaic eye discs are larger than those of WT, but ph^{505} cells have overproliferated and occupy a large portion of the eye disc, whereas ph^{del} cells occupy a small portion of the disc. However, the overgrowth phenotype of both ph^{505} and ph^{del} mosaic eye discs can be suppressed by the $p\{ph-d^+\}$ transgene. (B) Similarly to ph^{del} mosaic eyes (Fig 1D), the adult ph^{505} mosaic eyes are mainly composed of WT cells. When ph^{505} is associated with a w^- chromosome, the mosaic eye is mainly red. Conversely, when ph^{505} is associated with a w^+ chromosome, the mosaic eye is mainly white. (C) ph^{505} mosaic eye discs of wandering larval stage were stained with Elav antibody (red). ph^{505} cells (GFP positive) do not differentiate into photoreceptor neurons. DAPI, 4,6-diamidino-2-phenylindole; FRT, flippase recombination target; GFP, green fluorescent protein; WT, wild type.

Fig 5 | ph^{505} is not a completely null allele. (A) Nature of mutation of ph^{505} and ph^{del} . DNA sequencing data showed that ph^{505} carried nonsense mutations in Gln 398 of $ph-d$ and Gln 749 of $ph-p$. The breakpoints of the ph^{del} deficiency line, X:2006972 and X:2029518, were also verified by DNA sequencing. All exons of $ph-d$ and $ph-p$, except for the first exon of $ph-p$, which only encodes 12 amino acids, were deleted in ph^{del} . (B) Phenotypic analyses of ph^{del} and ph^{505} embryos. Both ph^{del}/Y and ph^{505}/Y hemizygous males are embryonic lethal. All ph^{del}/Y embryos die at a similar stage soon after initiation of germ band retraction. These embryos also show defects in dorsal closure. ph^{505}/Y embryos die after germ band retraction has finished; many of them are able to develop grooves between segments.

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). ph^{505} and ph^{600} were a gift from N.B. Randsholt. Deficiency lines ph^{del} and upd^{d1-3} 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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