Diverse tumor pathology due to distinctive patterns of JAK/STAT pathway activation caused by different Drosophila polyhomeotic alleles

Polycomb Group (PcG) genes encode conserved epigenetic regulators that form complexes to repress target gene transcription (Schuettengruber et al. 2007). Drosophila polyhomeotic (ph) encodes a core component of Polycomb Repressive Complex 1 (PRC1) (Shao et al. 1999). Ph plays vital roles in diverse biological functions such as neurodevelopment and cell cycle regulation (Feng et al. 2011; Narbonne et al. 2004; Wang et al. 2006). Deregulation of PcG genes have also been correlated with various types of human cancer (Bracken and Helin 2009). Therefore, elucidating how ph controls cell proliferation has important implications in both basic research and human health.

We have previously reported that different ph alleles cause tissue overgrowth in different ways. While a ph null allele, phdel, causes only non-autonomous cell over-proliferation, a ph hypomorphic allele, ph505, causes both autonomous and non-autonomous cell over-proliferation (Feng et al. 2011, Figure 1, A-C). In mosaic tissues, we define over-proliferation of mutant cells as autonomous, whereas over-proliferation of genotypically wild type cells induced by mutant cells as non-autonomous. We have also elucidated the signaling pathway involved in phdel induced non-autonomous cell over-proliferation. In summary, elevated Notch activity in ph cells up-regulates the expression of JAK/STAT pathway ligands Upd homologs, which in turn activate the JAK/STAT pathway in neighboring wild type cells and cause their over-proliferation (Feng et al. 2011). In this study, we addressed why a ph null allele and a ph hypomorphic allele both cause tumors but in such different ways.

We first tested whether the same signaling pathway underlay non-autonomous over-proliferation induced by both phdel and ph505. The functions of Notch and Upd homologs in the ph505 mosaic eyes were examined with the same strategy used for phdel (Feng et al. 2011). A ph505-Notch double mutant line was generated, and eyes mosaic for this line were essentially of the same size as wild type eyes (Figure 1, D vs. F). The mosaic eye discs had normal size and normal cell proliferation level, as shown by PH3 staining, which marks mitotic cells (Figure 1, H vs. J). Moreover, the size of ph505-Notch clones was significantly reduced when compared to that of ph505 clones (Figure 1, I vs. J). These results indicated that Notch was required for both autonomous and non-autonomous over-proliferation induced by ph505.

We next recombined ph505 with upd1-3, a deficiency line that lacks all three upd homologs in the Drosophila genome (Feng et al. 2011). Mosaic analyses were then performed using this double mutant line. ph505-upd1-3 mosaic eyes were significantly smaller than ph505 mosaic eyes and were comparable to wild type eyes (Figure 1, D, E and G), indicating that tissue overgrowth was largely suppressed. PH3 staining of the double mutant mosaic eye discs showed that these discs had relatively normal size and cell proliferation level (Figure 1, H vs. K). Importantly, ph505-upd1-3 clones were also drastically reduced in size compared to ph505 clones (Figure 1, I vs. K). These results
indicated that Upd homologs are required for not only non-autonomous but also autonomous cell over-proliferations induced by ph \(^{505}\).

It is not surprising that the same signaling pathway is responsible for non-autonomous over-proliferation induced by both ph\(^{del}\) and ph\(^{505}\), and it is not completely unexpected that Notch is also required for ph\(^{505}\) induced autonomous over-proliferation, as Notch is a transcription factor that has been shown to autonomously regulate cell proliferation (ARTAVANIS-TSAKONAS and MUSKAVITCH 2010). However, the three Upd proteins are secreted ligands (ARBOUZOVA and ZEIDLER 2006; HOMBRIÆ et al. 2005) and are not expected to have any direct effect on autonomous cell proliferation. To interpret our observations, we hypothesized that ph\(^{505}\) cells still respond to Upd ligands secreted by themselves in an autocrine or paracrine manner, and therefore over-proliferate. On the other hand, ph\(^{del}\) cells were no longer responsive to Upd ligands.

To functionally test this hypothesis, we again applied the double mutant strategy, taking advantage of the fact that the genes domeless (dome, encoding the only transmembrane receptor of the Drosophila JAK/STAT pathway (BROWN et al. 2001)) and hopscotch (hop, encoding the only Drosophila JAK kinase (BINARI and PERRIMON 1994)) are also on X chromosome as is ph. We first recombined ph\(^{505}\) with two dome alleles to generate ph\(^{505}\)-dome double mutant lines. Eye discs mosaic for these lines were still significantly larger than wild type, but the size of double mutant clones was dramatically reduced, so that only a tiny portion of the disc was composed of mutant cells (Figure 2, A, B). PH3 staining indicated that non-autonomous proliferation level was still high, but autonomous proliferation largely disappeared (Figure 2, A, B). We further examined the adult eyes mosaic for such double mutant lines and found that these eyes were still much larger than wild type and similar to ph\(^{505}\) mosaic eyes in size, but they generally were not folded as seen in ph\(^{505}\) mosaic eyes (Figure 2, H, J and K).

Next we generated a ph\(^{505}\)-hop double mutant line. We found that autonomous proliferation in mosaic eye discs of this double mutant was also significantly suppressed, with mutant cells only accounted for a small portion of the whole disc. On the other hand, non-autonomous cell over-proliferation was not affected and the overall size of these discs was still significantly larger than wild type (Figure 2, C). Adult eyes mosaic for this double mutant showed similar phenotypes as those of ph\(^{505}\)-dome mosaic eyes. These eyes were still significantly larger than wild type but they were generally not folded (Figure 2, L). Therefore, the removal of either dome or hop from ph\(^{505}\) cells only suppressed autonomous over-proliferation but did not affect non-autonomous over-proliferation, making such double mutant mosaic discs phenotypically similar to ph\(^{del}\) mosaic discs.

As controls, ph\(^{del}\)-dome and ph\(^{del}\)-hop double mutant lines were also generated using the same dome and hop alleles. Mosaic analyses on eye discs showed that the removal of dome or hop from ph\(^{del}\) cells did not affect non-autonomous cell over-proliferation. It did, however, mildly reduce the mutant clone size (Figure 2, D-F), suggesting that ph\(^{del}\) cells might still have a weak response to Upd ligands. Adult eyes mosaic for these double
mutant lines were phenotypically indistinguishable from \( ph^{del} \) mosaic eyes (Figure 2, I and M-O), consistent with the above observations in mosaic eye discs.

Finally we asked why \( ph^{del} \) and \( ph^{505} \) cells responds differently to the Upd ligands secreted by themselves. We hypothesized that some of the JAK/STAT pathway modulators might be differentially expressed in \( ph^{del} \) and \( ph^{505} \) cells. To test this hypothesis, we chose TU-Tagging, a technique that enables the purification of RNA from mutant cells without having to physically isolate such cells (Miller et al. 2009). Briefly, Drosophila is unable to synthesize uridine from uracil due to the lack of phosphoribosyltransferase (UPRT). When exogenous UPRT is expressed in mutant cells by MARCM, such cells would acquire the ability to utilize uracil. If these larvae are fed with 4-thiouracil (4-TU), a uracil derivative that contains a thio group, only mutant cells would be able to use 4-TU and eventually incorporate thio-containing uridine into newly synthesized RNA. This treatment has little toxicity, and the thio-labeled RNA can be purified from total RNA using conventional biochemical methods.

We performed TU-tagging to isolate RNA from \( ph^{del} \) cells and \( ph^{505} \) cells, and used qRT-PCR to examine candidate gene expression (Figure 3A). The expression of the JAK/STAT pathway receptor dome was significantly higher in \( ph^{505} \) cells than in \( ph^{del} \) cells. A higher receptor expression might sensitize \( ph^{505} \) cells to the Upd ligands. The levels of enok and socs42a, both negative regulators of the JAK/STAT pathway (Arbouzova and Zeidler 2006; Müller et al. 2008), were also significantly higher in \( ph^{505} \) cells compared to \( ph^{del} \) cells. This might represent feedback loops that negatively regulate the pathway activity. In fact, several such negative feedback loops, in which elevated pathway activity upregulates a negative pathway regulator, have been reported in JAK/STAT pathway (Arbouzova and Zeidler 2006).

Together, we conclude that \( ph^{del} \) and \( ph^{505} \) both cause autonomous over-expression of Upd homologs in mutant cells, which represents the only driving force of cell over-proliferation in \( ph^{del} \) and \( ph^{505} \) mosaic tissues and in essence acts non-autonomously to activate JAK/STAT pathway. The different phenotypes of these two types of mosaics are due to different sensitivity of mutant cells to Upd homologs. \( ph^{505} \) mutant cells robustly respond to Upd ligands that they secreted. Therefore, Upd ligands secreted by \( ph^{505} \) cells simultaneously induce over-proliferation in both mutant and wild type cells. In contrast, \( ph^{del} \) cells are largely insensitive to Upd ligands, so that Upd ligands secreted by \( ph^{del} \) cells only induce over-proliferation in wild type but not mutant cells. Furthermore, differential expression of the JAK/STAT pathway receptor dome might underlie the different sensitivity of \( ph^{del} \) and \( ph^{505} \) cells to Upd ligands. Models of cell proliferation patterns and the underlying signaling pathways in \( ph^{del} \) and \( ph^{505} \) mosaic tissues are given in Figure 3B and 3C.

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Figure 1. - Notch and upd homologs are required for both autonomous and non-autonomous over-proliferation induced by ph^{505}. (A-C) ph^{del}, a ph null allele, only induces non-autonomous over-proliferation, while ph^{505}, a ph hypomorphic allele, induces both autonomous and non-autonomous over-proliferation. Mosaic eye discs of wild type allele (A), ph^{del} (B) and ph^{505} (C) were analyzed. ey-flp was used to induce mosaics, and mutant cells were positively labeled by GFP (green) using MARCM (Lee and Luo 1999). DNA was stained by DAPI (blue). (D-G) The removal of Notch or all three upd homologs from ph^{505} cells suppresses the enlarged eye phenotype induced by ph^{505}. Adult eyes mosaic for wild type allele (D), ph^{505} (E), ph^{505}-Notch (F) and ph^{505}-upd^{del-3} (G) were analyzed. ey-flp was used to induce mosaics. upd^{del-3} is a deletion that lacks all three upd homologs (Feng et al. 2011). To remove Notch or all three upd homologs specifically from ph^{505} cells in mosaic eyes, ph^{505}-Notch and ph^{505}-upd^{del-3} double mutant lines were generated and were used to perform mosaic analyses (F and G). (H to K) Notch and upd homologs are required for not only non-autonomous but also autonomous over-proliferation induced by ph^{505}. Eye discs mosaic for wild type allele (H), ph^{505} (I), ph^{505}-Notch (J) and ph^{505}-upd^{del-3} (K) were stained with PH3 (red), a mitotic marker. ey-flp was used to induce mosaics, and mutant cells were positively labeled by GFP (green). DNA was stained with DAPI (blue). Note that when Notch or all three upd homologs were removed from ph^{505} cells, both non-autonomous and autonomous over-proliferation was suppressed.

Figure 2. – JAK/STAT pathway is involved in autonomous over-proliferation induced by ph^{505}. (A to C) The removal of dome or hop from ph^{505} cells suppresses autonomous but not non-autonomous over-proliferation. Mosaic eye discs of wild type allele (A), ph^{505}-dome (B) and ph^{505}-hop (C) were stained with PH3 (red), which marks mitotic cells. ey-flp was used to induce mosaics. Mutant cells were labeled by GFP (green), and DNA was stained with DAPI (blue). To remove dome or hop from ph^{505} cells, ph^{505}-dome and ph^{505}-hop double mutant lines were generated and were used for mosaic analyses. (D to F) Eye discs mosaic for wild type allele (D), ph^{del}-dome (E) and ph^{del}-hop (F) were stained with PH3 (red) as controls. ey-flp was used to induce mosaics. Mutant cells were labeled by GFP (green), and DNA was stained with DAPI (blue). (G to O) When JAK/STAT pathway components dome or hop was removed from ph^{505} or ph^{del} cells in mosaic eyes, the eyes were still much larger than wild type. Adult eyes mosaic for wild type allele (G), ph^{505} (H), ph^{del} (I), two ph^{505}-dome double mutant lines with different dome alleles (J and K), ph^{505}-hop (L), two ph^{del}-dome double mutant lines with different dome alleles (M and N) and ph^{del}-hop (O) were analyzed. ey-flp was used to induce mosaics.

Figure 3. – Molecular mechanism underlying different responses of ph^{del} and ph^{505} cells to Upd ligands. (A) TU-tagging followed by Real-Time PCR revealed differential expression of JAK/STAT pathway components and major regulators. UPRT and GFP
were expressed in mutant cells by MARCM with ey-flp. Mid-3rd instar Larvae were fed with 4-TU for 10 to 12 hours. Total RNA was extracted from eye discs, labeled and purified according to the published protocols (MILLER et al. 2009). Real-Time PCR was then performed using purified RNA (and total RNA for tubulin and GFP only). GFP to tubulin ratio was 3 to 6 times higher in purified RNA than in total RNA, indicating that TU-tagging successfully enriched RNA from mutant cells. (B and C) Models of over-proliferation patterns and underlying signaling pathways in ph<sup>del</sup> (B) and ph<sup>505</sup> (C) mosaic eye discs.

**References:**


