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The Polyhomeotic protein induces hyperplastic tissue overgrowth through the activation of the JAK/STAT pathway

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Epigenetic mechanisms controlling cellular proliferation are essential to animal development. Moreover, altered levels of expression of the epigenetic regulator proteins are associated with the development and progression of human diseases like cancer. We have studied the effects of high levels of Polyhomeotic (PH) protein, a member of the Polycomb Group (PcG), during the proliferation of the imaginal discs in Drosophila. Overexpression of PH protein causes induction of proliferation, accompanied with induction of JNK-dependent apoptosis. As a result, massive hyperplastic overgrowth is produced and the corresponding differentiated tissues show phenotypes related with mis-regulation of homeotic gene expression. We have found that high levels of PH upregulate the JAK/STAT pathway through the de-repression of Unpaired (UPD), the extracellular ligand of the Drosophila JAK/STAT signalling cascade. Moreover, inactivation of the JAK/STAT pathway in the presence of a large amount of PH protein greatly reduces the tissue overgrowth, demonstrating a functional role of JAK/STAT in PH-induced hyperplasia. Finally, we have observed that *decapentaplegic* and *d-myc*, two growth genes and putative targets of the JAK/STAT pathway, are also overexpressed in the PH-induced tumors. We propose that during normal development, the PcG proteins act to maintain inactive the JAK/STAT pathway. Upon cellular stress, changes in the levels of PcG proteins expression are induced and JAK/STAT is activated leading to tumor development. Our results show a functional relationship between the PcG gene expression and the JAK/STAT pathway, both of which are found to be perturbed in tumorigenesis.

Introduction

Epigenetic regulation of gene expression has been shown to be crucial for the development of organisms by maintaining cellular fates during proliferation.[1,2] Moreover, variations in both epigenetic proteins expression and patterns of histone modifications are related with the development and progression of cancer.[3-5] The *Polycomb* (*Pc*G) and *trithorax* (*trx*G) group of genes were initially discovered in Drosophila as regulators of homeotic gene expression.[6] However, studies in vertebrates as well as recent studies in Drosophila support the idea that *Pc*G and *trx*G genes also have important roles in other biological processes, such as hematopoiesis, stem cell renewal, cellular proliferation and neoplastic development.[2,7-11]

PcG and trxG are phylogenetically conserved proteins and encode for chromatin binding factors that function as multimeric complexes with unique biochemical activities.[12] Three complexes containing PcG proteins have been isolated in Drosophila: PRC1,[13] PRC2,[14,15] and PhoRC.[16] Polyhomeotic (PH), a zinc finger protein, is a subunit of PRC1 (Polycomb Repressive complex 1). PRC1 also contains Polycomb (PC), a chromodomain protein that can bind to trimethylated lysine 27 of histone H3 (H3k27me3), Sex comb extra/Ring1 (SCE), with E3 ligase activity for ubiquitylation of Histone H2A at Lysine 119, and Posterior sex comb (PSC), a Ring domain protein.[12] PRC1, PRC2 and PhoRC complexes bind to the Polycomb Response Elements, (PREs), cis-regulatory DNA elements found in PcG/trxG gene targets.[17]

High levels of the HPH1 protein, the human homolog of Drosophila PH[18] and altered levels of PcG proteins have been found in numerous types of human cancers.[19-22] However, little is known regarding how a modulation in the expression levels or in the activity of PcG proteins could influence cellular homeostasis and either be beneficial for the survival of organisms or be detrimental and lead to pathogenesis. The study of the pathways affected by altering the levels of PcG proteins should help the understanding of PcG function in these processes. As PcG proteins and developmental growth pathways are highly conserved from flies to humans,[23] such studies serve as a useful model for human cancer research.[24]

The conserved JAK/STAT cascade plays an important role in a wide spectrum of biological processes, including the response of

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the immune system and stem cell maintenance.[25] Furthermore, this pathway is deregulated in many cancers.[26-29] The canonical JAK/STAT pathway in Drosophila[30] is activated upon binding to the transmembrane receptor Domeless (encoded by *dome*), of the Unpaired (UPD) proteins, Drosophila cytokines encoded by the *outstretched* (*os*), *unpaired 2* (*upd2*) and *unpaired 3* (*upd3*) genes. DOME, in turn, associates with the JAK tyrosine kinase (encoded by *hopscotch*) and the transcription factor STAT92E (encoded by *stat92E*), resulting in the phosphorylation of STAT92E, which then enters the nucleus to regulate the transcription of its targets.[30]

Recent work has linked the epigenetic regulation of gene expression and the JAK/STAT pathway. RNAi screens in S2 cells for novel regulators of the JAK/STAT pathway have identified chromatin modifier loci, among them the PcG/trxG genes.[31,32] We have begun a systematic investigation of the phenotypes generated by both the overexpression and the inactivation of the PcG proteins during the imaginal discs growth. Here we present the results obtained of the overexpression of the epigenetic PH regulator protein and show that this produces massive hyperplastic overgrowth and that this is mediated via the JAK/STAT pathway.

Results and Discussion

High levels of Polyhomeotic induces hyperplastic overgrowth and tissue differentiation. The Drosophila *polyhomeotic* locus consists of two homologous transcription units: *polyhomeotic* proximal (*ph*-p) and *polyhomeotic* distal (*ph*-d)[33] (**Fig. 1A**), coding for two related proteins which are ubiquitously expressed.[34] We overexpressed the PH-P protein (see Materials and Methods) using the GAL4/UAS system[35] and we confirmed increased levels of the PH transcripts by quantitative RT-PCR (a 64-fold increase, data not shown) and by antibody staining (**Fig. 1G–G”**). In general, PH-overexpressing larvae remain in third instar stage longer time than wild type larvae, are bigger than wild type larvae, rarely reach the pupal stage and, if they do, die soon after. Their imaginal discs show very large overgrowths that produce multiples duplications of sections of the wing disc detected by the expression of wingless (WG) protein (**Fig. 1I–I”**, for a wild type expression of WG see **Suppl. Fig. 1B**). In addition to the high rates of proliferation (**Fig. 1H–H”**), we also observed increased apoptosis (**Fig. 1J–J”**), activation of the JNK pathway (**Fig. 1K–K”**) and activation of *reaper-lacZ* (*rpr-lacZ*) expression (**Fig. 1L-L”**). This proliferation can be considered hyperplastic because the apico/basal cellular localization[36] of either lethal giant disc or crumbs protein expression is not disrupted (data not shown).

We next studied whether over proliferated discs were able to differentiate by inducing PH-overexpressing clones and using the Gal80^{ts}/Gal4 System (see Materials and Methods).[37] The resulting differentiated appendages are bigger than wild type and show homeotic transformations (**Fig. 2D–H**). The homeotic UBX (Ultrabithorax) protein expression was examined both in PH-overexpressing clones and in wing and haltere imaginal discs of *sdGAL4;UAS-ph-GFP*/+ larvae. In wild type larvae, UBX protein is expressed in the haltere discs and in the peripodial membrane of the wing disc but not in the wing disc proper (**Fig. 2B**).[38] In contrast, UBX expression is repressed in PH-overexpressing clones in the haltere discs (**Fig. 2A–A”**) and very weakly expressed in *sdGAL4;UAS-ph-GFP*/+ wing discs (**Fig. 2C**). This very weak expression is not due to an effect of high levels of PH on *Abdominal-B* (*Abd-B*)[39] expression as Abd-B protein is not expressed in the *sdGAL4;UAS-ph-GFP*/+ wing discs (not shown).

These results indicate that the alteration of the levels of the epigenetic regulator protein PH leads to an overgrowth of differentiated tissue accompanied by apoptosis and changes in cell identity. A requirement of PH for the maintenance of homeotic gene expression has been described.[33,40] Moreover, it has been shown that inactivation of PH induces overgrowth exhibiting strong de-repression of UBX protein expression in the wing discs.[40] In contrast, we find extremely weak UBX expression in the wing discs (**Fig. 2C**). Because inactivation of PH leads to tissue overgrowth, it has been proposed that this protein might function as a tumor suppressor gene.[8] Our results show that PH overexpression also produces tissue overgrowth, thereby qualifying PH as an oncogene. That PH can behave both as a tumor suppressor gene and as an oncogene can be explained by the nature and the broad range of targets of the PcG regulation of expression. Interestingly, this dual behavior may provide a mechanism by which expression levels or activity of PcG proteins can control cellular plasticity in normal as well as in pathological development.

The JAK/STAT pathway is required for PH-induced hyperplasia. We have investigated the developmental growth pathways[23] that could be involved in the generation of the PH-induced overgrowth. We hypothesized that the PcG proteins silence those pathways that are activated in response to cellular stress. We first focused on the JAK/STAT pathway as a plausible target of the PcG proteins. We searched for putative PREs residing in the JAK/STAT pathway genes looking at the results of the ChIP-on-chip experiments performed with PcG proteins. We found that the *outstreched* gene, encoding the Unpaired (UPD) protein, and the *unpaired 2* gene, encoding Unpaired 2 (UPD2), both of which are cytokines that activate the JAK/STAT pathway, contain PREs which are bound by PcG/trxG proteins.[8,41-43] We therefore investigated the expression of *Upd* (*os*) in the PH-induced overgrowths. *Upd* (*os*) mRNA expression is seen in few cells of wild type imaginal discs[44] (**Fig. 3A**). In contrast, overgrown *sdGAL4;UAS-ph-GFP*/+ wing imaginal discs show expression of *Upd* (*os*) in many more cells (**Fig. 3E**). Quantification of *Upd* (*os*) and *Upd2* mRNA levels in *sdGAL4;UAS-ph-GFP*/+ wing imaginal discs compared to control discs by quantitative RT-PCR showed a 17-fold and a 32-fold increased in expression level, respectively (**Fig. 3M**). Using the stat-*lacZ* transgenic flies we examined whether the expression of the *stat92E* gene is also upregulated in *sdGAL4;UAS-ph-GFP*/+ wing discs as a consequence of Unpaired upregulation. **Figure 3F** shows that β-GAL expression in *sdGAL4;UAS-ph-GFP*/+; *stat-lacZ*/+ imaginal discs is increased compared with its expression in *stat-lacZ* imaginal discs (**Fig. 3B**). These results indicate that induction of high levels of PH expression and the

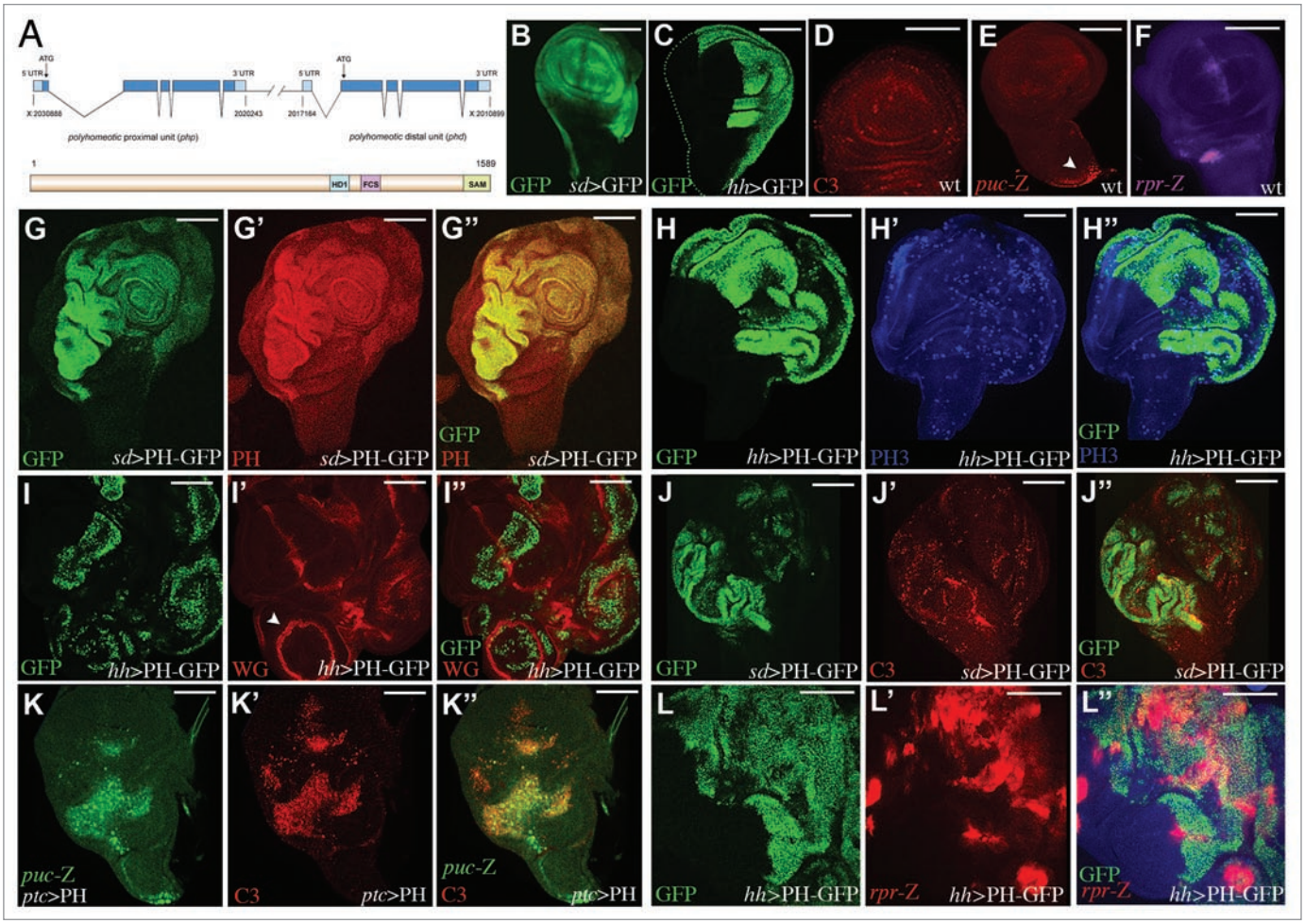

Figure 1. High levels of Polyhomeotic induces hyperplastic overgrowth and induction of apoptosis. (A) Map of the *ph* locus containing the *php* and *phd* transcription units and the structure of the PH proteins. The conserved HD1, FCS-Zn Finger and SAM domains are indicated. (B–L) show wing imaginal discs from the indicated larvae (B) GFP expression in *sd*-Gal4;UAS-GFP/+ showing the domain of *sd-Gal4* line in the wing disc pouch (C) GFP expression in *hh*-Gal4/+;UAS-GFP/+ showing the domain of expression of the *hh*-Gal4 line in the posterior compartment of the wing disc (D) Caspase 3 (C3) staining in wild type wing disc (E) β-GAL expression in *puc-lacZ*/+ (F) β-GAL expression in rpr-*lacZ*/+ (G) GFP expression in *sd*-Gal4; UAS-PH-GFP/+ (note the overgrowths) (G') PH protein expression in the same disc (G'') merge of (G and G''). (H) GFP expression in *tub*-Gal80^{ts}/ UAS-*ph*-GFP; *hh*-Gal4/+ (H') PH3 (phosphoHistone-3 expression) expression in the same disc. Note the increased proliferation in the posterior compartment due to overexpression of PH under the control of *hh*-Gal4 line (H'') merge of (H and H') (I) GFP expression in *tub*-Gal80^{ts}/UAS-*ph*-GFP; *hh*-Gal4/+ (I') WG expression in the same disc. Note the generation of multiple winglets (I'') merge of (I and I''). (J) GFP expression in *sd*-Gal4; UAS-*ph*-GFP/+ (J') caspase 3 (C3) staining in the same disc (J'') merge of (J and J'). (K) β-GAL expression in *tub*-Gal80^{ts}/UAS-*ph*-GFP; *ptc*-Gal4/*puc-lacZ* (K') caspase (C3) staining in the same disc (K'') merge of (K and K'). (L) GFP expression *tub*-Gal80^{ts}/*rpr-lacZ;* UAS-*ph*-GFP/*hh*-Gal4 (L') β-GAL expression in the same disc (L'') merge of (L and L'). Abbreviations: ph (polyhomeotic), sd (scalloped), hh (hedgehog), ptc (patched), wg (wingless), rpr (reaper), puc (puckered), C3 (Activated Caspase 3), PH3 (phosphoHistone-3).

resulting de-regulation of epigenetic control causes the activation of the JAK/STAT pathway, which in turn, induces uncontrolled proliferation.

To demonstrate the functional requirement of the JAK/STAT pathway in the PH-induced hyperplasia, the activation of the pathway was inhibited by Dome$^{\Delta CYT}$, a truncated version of DOME that lacks the intracellular protein domain involved in signal transduction.[45] *sdGAL4;UAS-dome*$^{\Delta CYT}$/+ ; *UAS-ph-GFP*/+ wing imaginal discs show a greatly reduced overgrowth phenotype (**Fig. 3L**, see Materials and Methods) indicating the functional requirement of the JAK/STAT pathway for PH-mediated induction of hyperplastic growth.

We next studied the putative JAK/STAT targets responsible for PH-induced overgrowth. It is well established that *decapentaplegic* (DPP) signalling is linked to growth (reviewed in ref. 46). Loss of DPP expression causes wing growth arrest while gain of DPP signaling causes wing overgrowth and subsequent formation of extra winglets, very similar to the winglets generated by the PH overexpression (**Fig. 1I' and I''**). We first sought to show the involvement of DPP in the PH-induced overgrowth phenotype examining *dpp* expression by in situ hybridization. We found that *dpp* expression is upregulated in the PH-induced overgrown discs (**Fig. 3G**). Additionally, we found that *dpp* mRNA expression is increased 2.6-fold in *sdGAL4;UAS-ph-GFP*/+ discs relative to

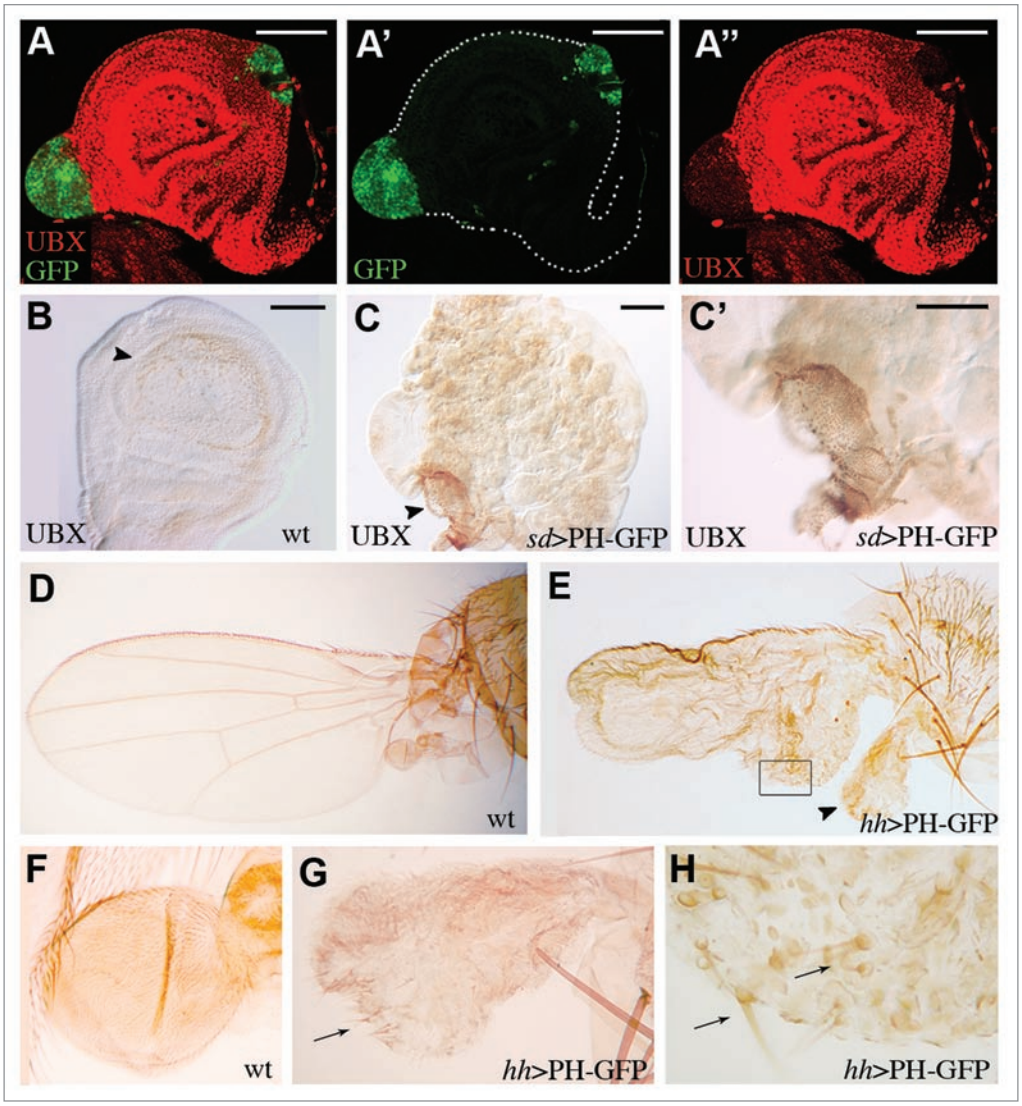

Figure 2. High levels of Polyhomeotic induces tissue differentiation and homeotic transformations. (A) Merged image of a *hs-FLP; actin* <y+> Gal4,UAS-GFP/UAS-*ph* haltere disc showing PH-overexpressing clones (green) and UBX expression (red) (A') GFP expression (green) (A'') UBX expression (red). Note the repression of UBX in the outgrowth clones (B) wild type wing imaginal discs showing the expression of UBX in the peripodial membrane (arrowhead) (C) UBX expression in a *sd-Gal4*; UAS-*ph*-GFP/+ wing disc. Note the overgrowth disc showing very weak expression in the proper disc but expression in the peripodial membrane (arrowhead) (C') Magnification of (C). (D) Adult wild type wing and haltere (E) Adult *tub*-Gal80^{ts}/UAS-*ph*-GFP; *hh*-Gal4/+ showing overgrowth wing and haltere (F) Adult wild type haltere (G) Magnification of the haltere shown in (E): note the transformation towards wing indicated by the size, shape and wing bristles (H) Magnification of the wing shown in (H): note that bristles in the posterior compartment have anterior identity. Abbreviations: ph (polyhomeotic), sd (scalloped), hh (hedgehog), UBX (Ultrabithorax).

the control wing discs (**Fig. 3M**). These results indicate that the growth gene *dpp* is activated in response to the high levels of PH. The upregulation of *dpp* expression may be due to an effect of PH on *dpp* transcription mediated through the PRE found in the *dpp* promoter[42] and not to as a consequence of JAK/STAT activation. To test this we used dpp-*lacZ* transgenic flies as a reporter for *dpp* expression. Importantly, the dpp-lacZ$^{BS3.0}$ construct[46] does not include the *dpp* PRE but does contain STAT consensus binding sites (**Suppl. Fig. 2**). As shown (**Fig. 3H–H"**), β-GAL expression is de-repressed in *tub*-Gal80^{ts}/UAS-*ph-GFP*/+;*hh*-Gal4/dpp-*lacZ* wing discs. These results argue against a direct effect of PH overexpression on *dpp* activation and suggest that *dpp* upregulation found in the PH-induced overgrowths could be a partial consequence of activation of the JAK/STAT. This is consistent with recent results showing that ectopic activation of the JAK/STAT pathway in the support cells in the Drosophila ovarian niche causes a similar increase of *dpp* mRNA expression.[47,48]

The *d-myc* oncogene is another factor involved in controlling Drosophila developmental growth. Its human homolog, c-myc is upregulated in many cancers.[49] We examined the expression of *d-myc* in PH-induced tumors in *sdGAL4;UAS-ph-GFP*/+ wing discs and found that its expression is induced in the overgrowth (**Fig. 3I–I"**). Additionally, we found that the levels of *d-myc* mRNA levels are upregulated 11.6-fold relative to control

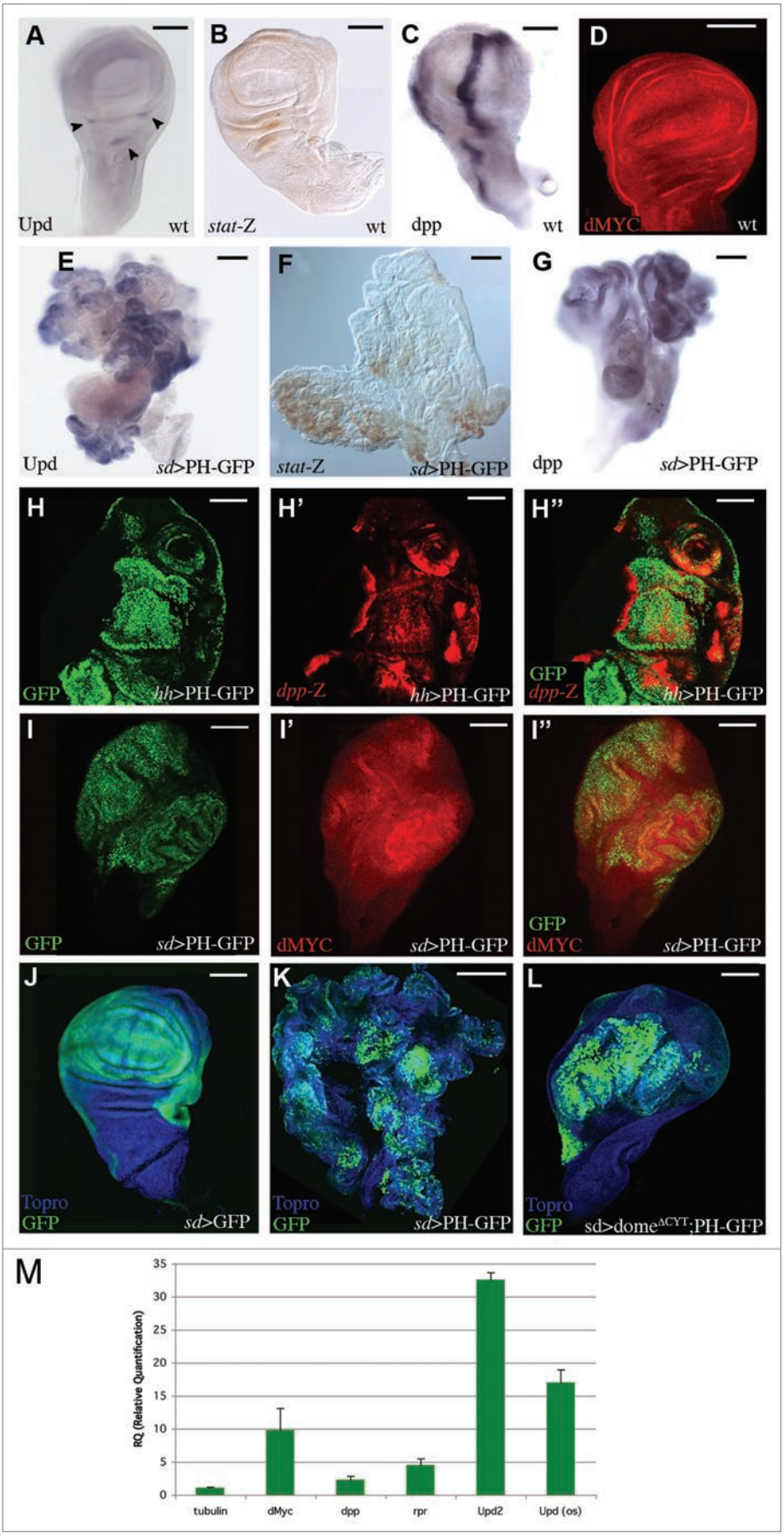

Figure 3. High levels of PH activate the JAK/STAT pathway as well as *dpp* and *d-myc* expression. Wing imaginal discs are shown in all the following panels (A) wild type expression *Upd-2* mRNA (arrows): note that only few cells of the disc show *Upd-2* mRNA (B) β-GAL expression in *stat-lacZ*/+ (C) Wild type *dpp* mRNA expression (D) d-Myc wild type expression (E) *Upd-2* mRNA expression in *sd*-Gal4;UAS-*ph*-GFP/+: note the massive proliferation and the upregulation of *Upd-2* expression (F) β-GAL expression in *sd*-Gal4;UAS-*ph*-GFP/*stat-lacZ*: note the upregulation of *stat* expression (G) *dpp* mRNA expression in *sd*-Gal4;UAS-*ph*-GFP/+ (H) GFP expression in *tub*-Gal80^{ts}/*dpp-lacZ;* UAS-*ph*-GFP/*hh*-Gal4 (H') β-GAL expression in the same disc (H") Merged of (H and H'). (I) GFP expression in *sd*-Gal4;UAS-*ph*-GFP/+ (I') d-MYC expression in the same disc (I") Merged of (I and I'). (J) GFP expression (green) in control *sd*-Gal4;UAS-GFP/+ wing disc stained with Topro (blue) (K) GFP expression in *sd*-Gal4; UAS-*ph*-GFP/+ wing disc stained with Topro (blue) (L) GFP expression in *sd*-Gal4; UAS-*dome*$^{\Delta CYT}$/+; UAS-GFP/+ wing disc stained with Topro (blue). Note that proliferation is greatly reduced in (L) compared to (K) and that (J–L) wing discs were dissected and stained from third instar stage larvae grown at the same temperature (M) Table showing the quantitative RT-PCR results. RQ: Relative Quantification of mRNA. Abbreviations: ph (polyhomeotic), Upd (Unpaired), dpp (decapentaplegic), sd (scalloped), hh (hedgehog).

wing discs (**Fig. 3M**). This result is in agreement with the results showing that stat-activated expression of c-myc can account, at least in part, for the proliferative effect associated with JAK/STAT pathway activation.[50] Taken together, these results show that the over proliferation induced by high levels of PH is mediated by the activation of the JAK/STAT pathway and that this pathway functions via activation of growth genes like *dpp* and *d-myc*.

The upregulation of *d-myc* expression could be related to the high rates of apoptosis found in the PH-induced overgrowths (**Fig. 1J–L**) that, interestingly, still allow the observed massive proliferation. Activation of apoptosis could be due to de-repression of the apoptotic genes, like *reaper* where functional PREs have been found.[51,52] We have observed a 4-fold increase of mRNA *reaper* expression (**Fig. 3M**) and activation of the expression of *reaper-lacZ* (**Fig. 1L'**). Alternatively,

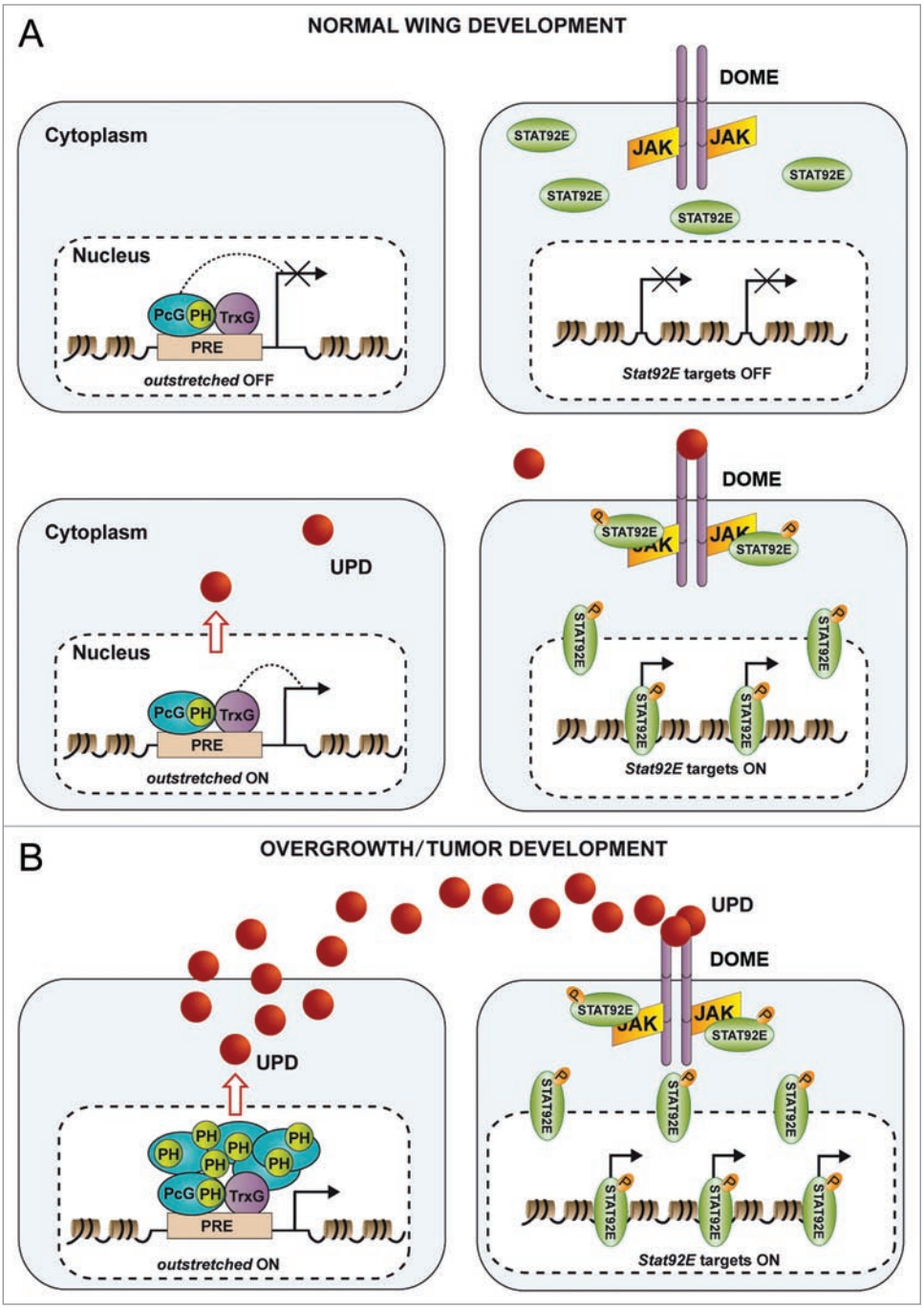

Figure 4. A model explaining PcG/trxG protein control the state of JAK/STAT pathway activation. PcG/trxG bind to the PREs located in the Unpaired coding genes, like the *outstrechted* gene, (for simplification only the PRE of the *outstrechted* gene is indicated) and epigenetically control its transcriptional state. Activation or repression of *outstrechted* is modulated in response to the expression requirements of Unpaired for normal wing development. Under pathological conditions the PcG/trxG biochemical activities or RNA expression levels of PcG/trxG are modified resulting in upregulation of Unpaired expression with subsequent aberrant activation of the JAK/STAT pathway and tumor development. Signals modulating the activity and or levels of PcG/trxG expression in normal and pathological development remain to be elucidated.

apoptosis may be additionally due to discontinuity generated in the reception of *dpp* or *wg* signals[53] that we also observe in PH-overexpressing wing discs (data not shown). However, it has been demonstrated that Drosophila imaginal cells expressing higher levels of dMyc protein are capable of inducing cell death in neighboring cells.[54,55] Therefore, the activation of *d-myc* may also contribute to the apoptosis observed in PH-induced overgrowths.

We have shown that tissue overgrowth promoted by high levels of the epigenetic protein PH is mediated, at least in part, by the activation of the JAK/STAT pathway. We propose that the PcG/trxG proteins regulate the JAK/STAT pathway

via interaction with the PREs located in the Unpaired coding genes like the *outstreched* gene[42] (**Fig. 4**). During normal wing development cytokine expression is maintained ON/OFF depending on the JAK/STAT pathway requirement for proliferation (**Fig. 4A**). However, in response to stress signals (**Fig. 4B**), epigenetic regulation mediated by PcG proteins is altered thus allowing activation of cytokine expression and the subsequent aberrant activation of the JAK/STAT pathway leading to tissue overgrowth and tumorigenesis. Signals that regulate the state of PcG activity/expression and consequent JAK/STAT modulation remain to be elucidated. We observed upregulation of the JNK pathways in the PH-induced proliferating tissues. Perhaps, as found in the processes of tissue trans-determination and tissue regeneration,[56] activation of JNK could also influence the epigenetic state of the PcG proteins and trigger over proliferation.

The experiments presented here provide the tools to pursue the basis of PcG regulation of the JAK/STAT pathway as well as highlight the interest in the study of epigenetic regulation of proliferation and its relevance to human cancer research.[49]

Materials and Methods

Drosophila strains, handling and genetics. The GAL4/UAS system[35] was used at different temperatures ranging from 17°C to 29°C with the GAL4 drivers *scalloped*-Gal4 (*sd*-Gal4) (Morata G and Calleja M, unpublished), *hedgehog*-Gal4 (*hh*-Gal4), *patched*-Gal4 (*ptc*-Gal4) (Flybase, hptt://flybase.bio.indiana.edu) and transgenic flies containing the constructs that express the PH-P protein *P*[*UAS: ph-EGFP*]$^{m\text{-}20}$,[57] and *P*[*UAS: ph*]$^{L\text{-}7}$ and the *P*[*UAS: dome*$^{\Delta CYT}$].[45] P-*lacZ* strains *dpp-lacZ*$^{BS3.0}$,[46] *puc-lacZ* (*puckered-lacZ*^{E69}),[58] *rpr-lacZ* (*reaper-4kb-lacZ*)[59] and *wg-lacZ*, (*wingless-lacZ*, Flybase), were used as reporters of the expression of the corresponding genes.

To generate PH overexpressing clones during larval development females *y*^{1}*w*^{1118}, *P[hsp70-FLP122]; actin FRT [y+] FRT Gal4, P[UAS-GFP]/SM5*,[60] were crossed with males *If/CyO; P*[*UAS: ph*]^{L7}. The larval progeny were heat shocked at 37°C degrees for 10 minutes and placed at 29° degrees until they reach the third instar larval stage. The Gal80^{ts}/Gal4 System[37] was used to control the time of expression of the PH protein using the stocks *tub*-Gal80^{ts}/*CyO; hh-Gal4/TM6B* and *ptc*-Gal4/*CyO;tub*-Gal80^{ts}/*TM6B*. To control that the reduction of growth observed in *sdGAL4;UAS-dome*$^{\Delta CYT}$/+; *UAS-ph-GFP*/+ compared to *sdGAL4; UAS-ph-GFP*/+ was not due to depletion of GAL4, the growth of imaginal discs from *sdGAL4;UAS-GFP*/+; *UAS-ph-GFP*/+ larvae was also studied at the same temperature conditions as *sdGAL4;UAS-dome*$^{\Delta CYT}$/+; *UAS-ph-GFP*/+ and *sdGAL4; UAS-ph-GFP*/+.

Immunostaining. Imaginal discs were dissected and stained as previously described.[51] The primary antibodies used were rabbit anti-GFP (1:300, Invitrogen), rabbit anti-PH[61] (1:200), mouse anti-β-gal (1:200, Promega), rabbit anti-phospho-Histone-3 (1:100, Upstate), rabbit anti-C3 (1:200, Cell Signalling Technologies), mouse anti-wingless (1:25, Iowa Hybridoma Bank), mouse anti-UBX[38] (1:20), guinea-pig anti-dMyc[62] (1:100) and mouse anti-Abd-B[39] (1:20). Secondary fluorescent antibodies were coupled to Rhodamin RedX, FYCT and Cys-5 (Jackson Immuno Research). The ABC Elite kit (Vector Laboratories) was used for stains with HRP-peroxidase following standard protocols. Images were generated using a Zeiss CDD microscope, a MicroRadiance (Bio-Rad) confocal and a LSM510 META (Zeiss) confocal microscope and subsequently processed using Adobe Photoshop CS3.

In situ hybridization. Imaginal discs of *sd*-Gal4*;* P[UAS*: ph*-EGFP]$^{m\text{-}20}$/+ larvae and of *sd*-Gal4*;* P[UAS:GFP]/+ (used as control) were hybridized with a *dpp* cDNA probe or a *upd(os)* cDNA probe following standard protocols.[63]

Quantitative reverse transcription PCR (qRT-PCR). Total RNA from 100 wing imaginal discs of experimental *sd*-Gal4*;* P[UAS*: ph*-EGFP]$^{m\text{-}20}$/+ and control *sd*-Gal4*;*P[UAS:GFP]/+ larvae was isolated as previously described.[51] One microgram of RNA was used as a template for first-strand cDNA synthesis using the High-Capacity cDNA Reverse transcription Kit (Applied Biosystems) following manufacturer directions. Real Time-PCR reactions were performed at the Lausanne DNA Array Facility using LightCycler FastStart DNA MasterSYBR Green I kit (Roche) following manufacturer instructions. The DNA sequences of the oligonucleotides used in the experiment are:

php 5'-ATC TGC TCA GGT CCA CCA AC-3' and 5'-CAG TGG AAA GGT GAC CCA CT-3'; *Upd (os)* 5'-CCA CGT AAG TTT GCA TGT TG-3' and 5'-CTA AAC AGT AGC CAG GAC TC-3'; *Upd2* 5'-AGC GCC AGC CAA GGA CGA GTT ATC-3' and 5'-TTG GCT GGC GTG TGA AAG TTG AGA-3'; *dpp* 5'-GCC AAC ACA GTG CGA AGT TTT A-3' and 5'-TGG TGC GGA AAT CGA TCG T-3'; *d-myc* 5'-CAG TTC CAG TTC GCA GTC AA-3' and 5'-AGA TAA ACG CTG CTG GAG GA-3'; *rpr* 5'-AGG CGA CTC TGT TGC GGG AG-3' and 5'-TGC GAT GGC TTG CGA TAT TTG-3'; the tubulin 5'-TCC AAT CGC AAC AAA AAA TTC A-3' and 5'-TCG TTT TCG TAT GCT TTT CAG TGT-3' was used as endogenous control. Quantified mRNA levels were expressed as relative fold change normalized to *tubulin*.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/GonzalezCC8-24-Sup.pdf

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