putzig Is Required for Cell Proliferation and Regulates Notch Activity in *Drosophila*[□]

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We have identified the gene *putzig* (*pzg*) as a key regulator of cell proliferation and of Notch signaling in *Drosophila*. *pzg* encodes a Zn-finger protein that was found earlier within a macromolecular complex, including TATA-binding protein-related factor 2 (TRF2)/DNA replication-related element factor (DREF). This complex is involved in core promoter selection, where DREF functions as a transcriptional activator of replication-related genes. Here, we provide the first in vivo evidence that *pzg* is required for the expression of cell cycle and replication-related genes, and hence for normal developmental growth. Independent of its role in the TRF2/DREF complex, *pzg* acts as a positive regulator of Notch signaling that may occur by chromatin activation. Down-regulation of *pzg* activity inhibits Notch target gene activation, whereas Hedgehog (Hh) signal transduction and growth regulation are unaffected. Our findings uncover different modes of operation of *pzg* during imaginal development of *Drosophila*, and they provide a novel mechanism of Notch regulation.

INTRODUCTION

In a developing organism, cell proliferation and apoptosis must be strictly coordinated with patterning processes to correctly shape the organs. Thus, it is not surprising that all major morphogenetic and developmental signaling pathways have been involved in the regulation of cell proliferation and apoptosis and that they have been linked to numerous cases of cancer formation in mammals (Maillard and Pear, 2003; Ruiz i Altaba, 1999). In *Drosophila*, a large body of work shows that several of these pathways act in concert in the coordination of cell survival and death. For example, overexpression of Notch causes vast overgrowth, whereas inhibition of Notch activity by overexpression of its antagonist Hairless results in tissue loss and apoptosis (Artavanis-Tsakonas *et al.*, 1999; Müller *et al.*, 2005). Indeed, the combined activity of Hedgehog (Hh), Decapentaplegic (Dpp), and Notch is required to promote reentry into the cell cycle after a developmentally regulated G1 arrest in the eye anlagen of *Drosophila* larvae (for review, see Thomas, 2005). Moreover, it was shown that Hh signaling promotes cellular growth by transcriptional activation of G1 cyclins *Cyclin D* and *Cyclin E* (Duman-Scheel *et al.*, 2002). However, to this end, the understanding of the molecular principles that connect these pathways to either control of cell cycle or apoptosis remains largely fragmentary (Thomas, 2005).

Cell cycle entry requires the activity of G1-S cyclins that eventually activate dE2F1, a transcription factor that induces transcription of downstream genes required, e.g., for DNA replication (Duronio *et al.*, 1995). In *Drosophila*, transcriptional activation of replication-related genes encoding, for

example, proliferating cell nuclear antigen (PCNA) or DNA-polymerase α subunit involves also DNA replication-related element factor (DREF) that recognizes DNA replication-related element (DRE) response elements (Hirose *et al.*, 1993, 2001; Hyun *et al.*, 2005). DREF can be part of a macromolecular complex including TRF2, a TATA-binding protein-related factor that binds to a subset of selected promoters, including one promoter in the PCNA gene (Hochheimer *et al.*, 2002). TRF2 has been isolated from several different organisms, where it is required for transcription of replication-related genes and key developmental genes as well (Hochheimer and Tjian, 2003). The TRF2/DREF complex consists of more than a dozen proteins, including several known chromatin-remodeling components. Three of them confer chromatin activation, whereas two others, including p160, resemble regulators of insulator function (Hochheimer *et al.*, 2002; Hochheimer and Tjian, 2003). Interestingly, p160 was recently found to enhance position effect variegation and hence chromatin silencing and to be associated with interband regions of polytene chromosomes (Eggert *et al.*, 2004). To this end, the biochemical activity and functional specificity of most of the proteins within the TRF2-complex, i.e., their role in transcriptional activation or in chromatin remodeling, however, remain elusive (Hochheimer and Tjian, 2003).

We have isolated the Zn-finger protein p160 as a genetic suppressor of Hairless activity, prompting our interest in its role during *Drosophila* development and especially during Notch signaling. In vivo RNA interference resulted in tiny larvae and developmental delay, which is why we named the corresponding gene *putzig* (*pzg*). We present the first in vivo evidence that *pzg* is essential for fly survival by regulating cell cycle entry and progression. In addition, we show that *pzg* encodes a key regulator of the Notch signaling pathway and that it is involved in histone modification and chromatin activation. Interestingly, this activity is independent of DREF, suggesting context dependence of Pzg activity.

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MATERIALS AND METHODS

Gain- and Loss-of-Function Studies on *pzg*

Flies were raised on standard fly food; information on strains can be found at <http://flybase.bio.indiana.edu/>. Tissue-specific overexpression or RNA interference (RNAi) induction was achieved with the Gal4/UAS system (Brand and Perrimon, 1993; Nagel *et al.*, 2002). To induce strong RNAi activation, crosses were performed at 25°C, with the exception of *da-Gal4*, which was analyzed at 18°C due to early larval death. All stocks used in this study are described in the Supplemental Material.

A *pzg* full-length cDNA clone (LD15904) was obtained from Open Biosystems (Heidelberg, Germany). A 699-base pair polymerase chain reaction (PCR) product including the C-terminal part of *pzg* (base pairs of cDNA 2005-2704) was used for the generation of the RNAi-construct; the cloning strategy was according to Nagel *et al.* (2002), with the exception that pUAST instead of pUASTdsGFP was used. Transgenic flies were generated according to standard methods. Phenotypic analyses were performed with transgenes located on different chromosomes. Most of the data shown were obtained with a second chromosomal insertion line, which behaved qualitatively and quantitatively comparable with all other lines tested. To visualize the expression domain in some of the experiments, *UAS-pzg-RNAi* (II.) was recombined with *enGFP-Gal4*.

Antibody Generation and Verification of *pzg*-RNAi

Guinea pig and rat anti-Pzg antibodies were raised against the C-terminal part of Pzg (base pairs of cDNA 2005-2991) fused to maltose binding protein (PINEDA, Berlin, Germany). Reduction of Pzg protein levels by *pzg*-RNAi was measured by Western analyses of protein extracts from *da-Gal4* > *UAS-pzg-RNAi* larvae versus *da-Gal4* > *UAS-lacZ* control larvae: 100 first instars were frozen in liquid nitrogen and shaken rigorously. They were transferred to a new tube, homogenized in 30 μ l of SDS-loading buffer (250 mM Tris-HCl, pH 7.6, 0.001% bromophenol blue, 5% [vol/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, and 40% [vol/vol] glycerol), and immediately boiled for 10 min. After 10-min centrifugation, 15 μ l of the supernatant was run on standard 10% polyacrylamide gel. Blots were probed with a mixture of guinea pig anti-Pzg (1:1000) and rabbit anti-actin (1:250; Sigma-Aldrich, St. Louis, MO) antibodies. Secondary antibodies, coupled to alkaline phosphatase (1:500), were obtained from Jackson Laboratories (Dianova, Hamburg, Germany). The signals were quantified by densitometry of dry membranes using the WinCAM software program (Cybertech, Berlin, Germany) and normalized with actin control.

Immunoprecipitation, Cross-linked Chromatin Immunoprecipitation (XChIP), and Semiquantitative PCR Analysis

Immunoprecipitations were essentially performed according to Nagel *et al.* (2005) by using protein extracts of ~500 wild-type embryos and 100 wild-type imaginal discs, respectively. For immunoprecipitations, we used guinea pig anti-Pzg antibodies at 1:100 dilutions; and for detection, we used rat anti-Pzg at 1:500 and rat anti-Ci 2A1 at 1:2 dilutions (Motzny and Holmgren, 1995).

Three independent immunoprecipitations of formaldehyde cross-linked chromatin (XChIP) from 0- to 14-h embryos were done according to Cavalli *et al.* (1999). Antibodies used were mouse anti-DREF (1:100; Hirose *et al.*, 1996), guinea pig anti-Pzg (1:100), and guinea pig preimmune sera (1:25) as mock control. The precipitated DNA was dissolved in 20 μ l of H₂O double distilled water, and 1 μ l was used for PCR reaction. Primer pairs from the promoter regions of *Enhancer of split m8* [*E(spl) m8*], *Notch*, *Suppressor of Hairless* [*Su(H)*], and *vestigial* were used in this study. Additionally, primer pairs amplifying the *Polymerase α* gene promoter were used as positive control, whereas the 3' untranslated region (UTR) of *E(spl) m8* was chosen as an unrelated negative control. The number of cycles used for amplification was 35, which was within the linear range of the amplification judged by different number of cycles and the amount of input DNA used. We took 7- μ l samples every two cycles from the 31st to the 35th cycle to determine the linear range of amplification. Signals were quantified using the histogram function of ImageJ software (<http://rsb.info.nih.gov/ij/>).

For chromatin immunoprecipitations of first instars (~14–15 h after hatching), we used the ChIP Assay Kit according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). For precipitation, we used rabbit ab850 anti-trimethylated H3-K4 (1:100; Abcam, Cambridge, United Kingdom). Rabbit preimmune sera (1:25) served as mock control. Of the precipitated DNA, 1.5% was used per PCR reaction.

The primer sequences and PCR conditions used to amplify XChIP DNA are described in Supplemental Material.

Antibody Stainings of Tissues and Documentation of Phenotypes

Antibody staining was performed as described in Müller *et al.* (2005). Antibodies used in this study are listed in Supplemental Material. 5-Bromo-2'-deoxyuridine (BrdU) labeling was for 45 min and standard protocols were

followed thereafter (de Nooij *et al.*, 1996). Immunostaining of polytene chromosomes was performed according to Salvaing *et al.* (2003), by using mouse anti-DREF (1:50) and guinea pig anti-Pzg (1:1000). Secondary antibodies coupled to fluorescein, Cy3, or Cy5 were purchased from Jackson Laboratories. Adult wings were dehydrated in ethanol and mounted in Euparal (Roth, Karlsruhe, Germany).

Wing size was determined using ImageJ software for pixel measurements. For phenotypic comparisons, experiments were done in parallel under identical conditions, and they were repeated at least twice. To test statistical significance, *p* values were calculated according to Student's *t* tests (<http://www.physics.csbsju.edu/stats/t-test.html>). Pictures were taken with Nomarski optics on a Zeiss Axiophot (Carl Zeiss, Jena, Germany). Fly heads were pictured on a WILD stereomicroscope equipped with a Pixera camera (Optronics, Goleta, CA) by using the Pixera Viewfinder, version 2.0, software. Confocal images were acquired with a Zeiss Axioskop linked to a Bio-Rad MRC1024 scanhead by using Bio-Rad Laser Sharp 3.1 software (Bio-Rad, Hercules, CA). Figures were compiled using Corel Photo Paint and Corel Draw software (Corel, Ottawa, ON, Canada).

RESULTS

EP756 Acts as a Positive Regulator of Notch Signaling

In a genetic modifier screen, we identified *EP756* as suppressor of tissue loss caused by an overexpression of the Notch antagonist Hairless (H) during eye development (Müller *et al.*, 2005). This positive effect was not restricted to the eye, because it was likewise observed during wing development. Moreover, cell growth and proliferation induced by an enforced Notch signal was significantly enhanced (~20%) by a combined overexpression with *EP756*. Tissue specific overexpression of *EP756* caused a very mild enlargement of the respective tissues on its own. These data suggest a more general role of *EP756* in the control of cell proliferation as well as an intimate interaction with Notch signaling. They are shown in Supplemental Figure S1, A–P and W.

EP756 Drives the Expression of *putzig*, Which Is Essential for Developmental Growth

The *EP756* element is inserted ~0.1 kb upstream of transcription unit CG7752, and it drives its expression analyzed by in situ hybridization (data not shown). CG7752 encodes a Zn-finger protein. To investigate the function of CG7752 during *Drosophila* development, we designed an inverted RNAi-construct under UAS-control, allowing us to precisely ablate CG7752 function in a spatially and temporally controlled manner (Nagel *et al.*, 2002). Ubiquitous down-regulation of CG7752 activity, starting already during embryonic development, resulted in late embryonic/early larval death at 25°C. At 18°C, larval development was significantly delayed with respect to growth and developmental timing compared with control larvae. Eventually, the animals died at early pupal stages (Figure 1A). Owing to this influence on growth of the larvae, we renamed the CG7752 gene *putzig* (*pzg*), which is German for tiny/droll.

The requirement of *pzg* for normal organ growth during imaginal development was investigated in eye and wing anlagen: *pzg-RNAi* caused tremendous reduction of tissue size up to the total loss of the corresponding tissue (Figure 1, B and C). These effects could be reversed by the concurrent ectopic expression of *pzg* confirming that the defects are due to a specific loss of *pzg* function. To facilitate analyses, we raised polyclonal antibodies against Pzg protein that recognized a prevalent, nuclear antigen in all *Drosophila* tissues (Figure 1D). Pzg protein is strongly reduced in cells depleted for *pzg* activity by RNAi (Figure 1, D and E). On Western blots, the antibodies recognized a protein that runs at ~160 kDa, which is considerably higher than the predicted molecular weight of 110 kDa (Figure 1F). This is in agreement with other reports (e.g., Hochheimer *et al.*, 2002). In animals depleted for *pzg* by RNAi, we observed an ~80% reduction

Figure 1. Loss of *pzg* activity is associated with developmental delay and tissue loss. (A) Comparison of age-matched *da-Gal4 > UAS-pzg-RNAi* mutants (bottom) with control *da-Gal4 > UAS-lacZ* animals (top). Ubiquitous induction of *UAS-pzg-RNAi* caused a delay in development up to the early pupal stage, when the animals finally died. L1–L3, first to third instar; eP, early pupal stage. Ectopic expression of *UAS-pzg-RNAi* during early eye development (*eyeless-Gal4*) (B) or wing development (*scalloped-Gal4*) (C) resulted in reduced tissue size of eyes and wings, respectively (arrows). (D) Ubiquitous nuclear staining of Pzg protein in imaginal discs, e.g., the wing disk. (E–G) *pzg-RNAi* reduces Pzg protein levels. (E) Antibody staining reveals potent down-regulation of Pzg protein by RNAi in the central part of the wing (*omb^{hifid}-Gal4 > UAS-pzg-RNAi*). (F) Total protein extracts from 100 first instars of each genotype were used to quantify endogenous Pzg protein levels (arrows); actin was used as an internal standard. (G) Densitometric quantification of the Western blot revealed an 80% reduction of Pzg protein levels in the RNAi-treated animals relative to internal actin levels.

of endogenous protein levels compared with the wild-type, indicating that our RNAi-construct caused a strong hypomorphic situation (Figure 1, E–G).

pzg Is Involved in Cell Cycle Regulation

Pzg was recently coisolated with a protein complex including TRF2 and DREF, which is involved in the transcriptional activation of replication-related genes (Hochheimer *et al.*, 2002; Hyun *et al.*, 2005). Because a direct involvement of *pzg* in up-regulation of replication-related genes has not been demonstrated yet, we investigated whether *pzg* is required for cell cycle entry and progression. Indeed, depletion of *pzg* activity interfered strongly with DNA replication compared with untreated tissue (Figure 2, A–A'). Moreover, the number of cells entering mitosis was strongly reduced within the affected area (Figure 2, B–B'). In support of a rather direct requirement of Pzg for cell cycle control, expression of S phase-promoting factors such as *dE2F1* (Figure 2, C–C'), *Cyclin A* (Figure 2, D–D'), or *Cyclin D* (data not shown) was likewise diminished. These observations indicate that, in the absence of *pzg* activity, cells are hampered to enter the cell cycle.

If *pzg* is an essential factor for cell cycle entry, loss of Pzg might be overcome by ectopic expression of G1-S phase cell cycle components. Hence, we addressed possible genetic interactions between *pzg* and different cell cycle regulators by either increasing or decreasing their dose in *pzg* mutant wing tissue. Removal of one copy of the *Cyclin E* gene (*CycE^{TE35D}*), the *Cyclin D* gene (*CycD¹*), or of PCNA (*mus209^{k00704}*) enhanced the effects of *pzg* knockdown,

Figure 2. *pzg* is required for cell cycle progression. (A–D') Effects of Pzg depletion on the cell cycle in wing discs. RNAi of *pzg* is marked with green fluorescent protein (GFP) (green) or encircled with a dotted line. To avoid potential cell death within *pzg* depleted cells, the experiments were performed in a *p35* gain-of-function background (*UAS-p35, enGFP-Gal4 > UAS-pzg-RNAi*). (A and A') Replication, visualized by BrdU incorporation, is dramatically lower in *pzg* mutant cells (arrow). (B and B') The number of mitotic cells that are enriched in phospho-histone H3 (red, anti-PH3) is reduced in *pzg* mutant tissue (arrow). (C–D') Early cell cycle markers such as *dE2F1* (red in C and C') and *Cyclin A* (red in D and D') are strongly down-regulated upon Pzg depletion (arrows). (E–G) Effects of cell cycle factors on *pzg-RNAi* tissue loss. Shown are adult wings of *en-Gal4 > UAS-pzg-RNAi* (blue wing), superimposed with specimens where the given factor was reduced at the same time (red wing). Genotypes of the combinations are as follows: *en-Gal4 > UAS-pzg-RNAi/CycE^{TE35D}* (E); *en-Gal4 > UAS-pzg-RNAi/dap⁴* (F); and *en-Gal4 > UAS-pzg-RNAi/mus209^{k00704}* (G). (H) Quantification of wing size: at least 20 wings of each genotype were measured and the mean value was determined. The size of *en-Gal4 > UAS-lacZ* wings was taken as 100%, and the others were calculated as percentage of the reference. Error bars represent SD. **p* < 0.0005 by Student's *t* test; brackets indicate genotypes compared (1 with 2; 2 with 3–5). 1, *en-Gal4 > UAS-lacZ*; 2, *en-Gal4 > UAS-pzg-RNAi*; 3, *en-Gal4 > UAS-pzg-RNAi/CycE^{TE35D}*; 4, *en-Gal4 > UAS-pzg-RNAi/dap⁴*; and 5, *en-Gal4 > UAS-pzg-RNAi/mus209^{k00704}*.

whereas reduction of the Cyclin E-dependent kinase inhibitor *dacapo* (*dap⁴*) suppressed the effects. Accordingly, overexpression of *Cyclin E* or of *Cyclin D* restored the wing defects of *pzg-RNAi* (Figure 2, E–H and Supplemental Figure S1, Q–W). Moreover, the effects of *pzg-RNAi* on wing size do not seem limited to the *en-Gal4* domain, suggesting additionally nonautonomous effects. Together, these data strongly support the idea, that *pzg* is required for the activation of cell cycle genes, and eventually, for developmental growth and survival of the animal.

pzg Is Required for Notch Target Gene Activation

Originally, we had isolated *pzg* as a positive genetic modifier of Notch signaling. Accordingly, *pzg* could regulate Notch-

Figure 3. *pzg* is required for Notch but not for Hh signaling. (A and A') Adult wings of *N⁵⁴¹⁹/+* (A) and *N⁵⁴¹⁹/+; ptc-Gal4 > UAS-pzg-RNAi* (A') flies. *pzg-RNAi* increased the penetrance of this phenotype from 55 to 100%; moreover, the wing notches (arrows) were markedly enlarged. (B–B'') One copy of the hyperactive *Notch* allele *N^{AxM1}* is sufficient to restore the tissue size of *en-Gal4 > UAS-pzg-RNAi* wings. (C–C'') In agreement, halving the dose of the *Notch* antagonist *Hairless* (*H¹*) likewise rescues the *pzg-RNAi* effects (*en-Gal4 > UAS-pzg-RNAi; H¹/+*). The rescued wings are shown in red compared with the *pzg-RNAi* wings in blue. (D) Quantification of wing size was done with minimal 20 wings for each genotype. Mean values were determined using *en-Gal4 > UAS-lacZ* wings as reference (100%). Error bars show SD. **p* < 0.0005 by Student's *t* test; brackets indicate genotypes compared (1 with 2; 2 with 3 and 4). 1,

mediated growth control, supported by the observation that overexpression of *pzg* not only suppresses tissue loss caused by a reduction but also enhances tissue overgrowth caused by a gain of *Notch* signaling activity (Supplemental Figure S1, A–P, W). Moreover, *pzg-RNAi* strongly increased both the penetrance (from 55 to 100%) and the severity of the haploinsufficient wing phenotype of *N⁵⁴¹⁹/+* mutants (Figure 3, A–A'). If the role of *pzg* were to positively regulate *Notch* signaling, loss of *pzg* should be bypassed by an overactive *Notch* signal. Indeed, introducing either a gain-of-function *Notch* allele (*N^{AxM1}*) or a loss-of-function allele of the *Notch* antagonist *Hairless* (*H¹*), both rescued the growth defects caused by *pzg-RNAi* to near wild-type morphology and size (Figure 3, B–D). Thus, subtle enhancement of *Notch* signaling activity in a *pzg* mutant background yields a growth signal that is sufficient to overcome the respective cell cycle defects. These genetic experiments suggest that *pzg* acts upstream of *Notch*. In this case, we would expect a change of *Notch* activity in *pzg-RNAi* mutant tissues. Therefore, we examined the expression of several *Notch* target genes in *pzg-RNAi* mutant tissues. In wing imaginal discs, *cut*, *Enhancer of split m8*, and *vestigial* are expressed along the prospective wing margin, and their expression depends directly on *Notch* activity (Lecourtois and Schweisguth, 1995; Neumann and Cohen, 1996). We found that *cut*, *m8-lacZ*, and *vgBE-lacZ* expression are strongly reduced or even abolished in *pzg-RNAi* mutant cells (Figure 3, E–J'). In summary, Pzg positively regulates *Notch* signaling, and loss of *pzg* activity results in a lowered *Notch* signal.

The above-mentioned observations raise the possibility that *pzg* activates cell cycle genes indirectly through the activation of *Notch* signaling. Indeed, *Notch* overactivation is sufficient to induce transcription of G1-S cyclins *Cyclin D* and *Cyclin E* as well as of *string*, which regulates G2/M phase progression (Supplemental Figure S2, A–C). Hence, a *Notch* signal can drive cells throughout the entire cell cycle, causing massive overproliferation as observed previously (e.g., Go *et al.*, 1998; Giraldez and Cohen, 2003). These results are compatible with a model, whereby the major function of *pzg* is to up-regulate G1-S cyclins through the stimulation of *Notch* signaling. In this case, loss of tissue caused by an inhibition of *Notch* activity, e.g., through overexpression of the *Notch* antagonist *Hairless*, should be suppressed to the same degree no matter whether Pzg, *Cyclin D*, or *Cyclin E* is overexpressed. As noted above, *pzg* was isolated as a suppressor of *Hairless*-mediated tissue loss. We observed a significantly better rescue with Pzg compared with either *Cyclin D* or *Cyclin E*, and no rescue with *String*, indicating that the effects of Pzg on proliferation cannot be explained

en-Gal4 > UAS-lacZ; 2, *en-Gal4 > UAS-pzg-RNAi*; 3, *N^{AxM1}; en-Gal4 > UAS-pzg-RNAi*; and 4, *en-Gal4 > UAS-pzg-RNAi; H¹/+*. (E) Wild-type expression of *Cut* along the presumptive wing margin (arrow). (F, F') Reduction of *pzg* within the central domain of the wing disk (*omb^{hifid}-Gal4 > UAS-pzg-RNAi*; Pzg in red) resulted in a nearly complete down-regulation of *Cut* (green, open arrow). (G) Wild-type expression of *m8-lacZ* along the dorsoventral boundary and in proneural clusters of the wing disk (arrow). (H and H') *m8-lacZ* expression (green) is strongly reduced upon *pzg-RNAi* induction (Pzg in red) in the central domain of the wing disk (open arrow). (I) Expression of *vgBE-lacZ* at the dorsoventral boundary. (J and J') Depletion of *pzg* activity (red staining) results in a strong down-regulation of *vgBE-lacZ* expression (green, open arrow). (K and M) Wild-type expression of *Ci* and *dpp-lacZ*. (L, L' and N, N') Depletion of *pzg* (red) in the wing imaginal disk has no influence on *Ci* protein accumulation and localization (green; L, L') nor on *dpp-lacZ* activity (green; N, N').

solely through activation of Notch. The phenotypic enhancement of *pzg*-RNAi caused by the reduction of *Cyclin E* dose (Figure 2E) can be rescued by a concurrent reduction of *Hairless* gene dose (Supplemental Figure S2, D–K). These results can be explained by a dual input of Pzg, activation of Notch signaling as well as activation of cell cycle regulation.

Pzg Regulation of Notch Signaling Is Independent of DREF

Our data show that Pzg is required for proper expression of Notch target genes (Figure 3, E–J'). As a component of the TRF2/DREF complex, Pzg might exert its function by direct transcriptional activation. The TRF2/DREF complex is targeted to DNA via DRE elements located in the proximity of target promoters. There are potential, albeit variant DRE recognition sites in the promoter regions of *Notch*, *Su(H)*, members of the *E(spl)* complex as well as of *vestigial* (*vg*) (Supplemental Figure S3). To address a potential binding of DREF to these sites *in vivo*, we performed XChIP with anti-DREF and anti-Pzg as well as unrelated antibodies (as negative controls). As an additional control, we tested the 3' flanking region of *E(spl)m8* that lacks DRE-like sequences. We found no evidence for DREF binding on any of the potential DRE elements in our tested genes nor on the 3' UTR of *E(spl)m8* (Figure 4A and Supplemental Figure S3), whereas we could easily confirm DREF and Pzg presence on the promoter of *DNA-Polα* gene (Hirose *et al.*, 1993) (Figure 4A and Supplemental Figure S3). Interestingly, we were able to precipitate chromatin of the regulatory region of *vg* and of *E(spl)m8* with anti-Pzg antibody, in agreement with a DREF-independent binding of Pzg at the promoter regions. Apparently, Pzg is involved in the activation of these Notch target genes independently of DREF (Figure 4A and Supplemental Figure S3). To confirm this conclusion, we analyzed the expression levels of the Notch target genes *cut*, *E(spl)m8* and *vg* after DREF knockdown in the developing wing. We

made use of two *UAS-DREF-RNAi* lines of different origin that had been functionally analyzed earlier and that behaved largely identically in our assays (Yoshida *et al.*, 2004; Hyun *et al.*, 2005). In contrast to the lowered Notch target gene expression observed in *pzg* mutant tissues, we did not detect any changes in the DREF mutant territories (Figure 4, E and F). Moreover, we compared the distribution of DREF and Pzg on polytene chromosomes of salivary glands. Both, DREF and Pzg, are associated with hundreds of sites (Hart *et al.*, 1999; Eggert *et al.*, 2004), but they do not completely overlap. Overlap of Pzg and DREF was detected, e.g., at the *Polα* locus (Melov *et al.*, 1992; Hirose *et al.*, 1993; Hyun *et al.*, 2005), but not at the *E(spl)m8* or *vg* loci (Figure 4, B–D'), underscoring our hypothesis that Notch regulation is independent of DREF. No ectopic expression of Notch target genes was observed as a consequence of overexpression of Pzg during larval wing development. Therefore, we conclude that Pzg is required but not sufficient for the activation of Notch signaling (Supplemental Figure S4, A and B).

pzg May Regulate Notch Signaling Activity via Chromatin Activation

Three proteins within the TRF2/DREF complex are also components of the nucleosome remodeling factor NURF, which has been associated with chromatin activation (Tsukiyama and Wu, 1995; Xiao *et al.*, 2001; Badenhorn *et al.*, 2002; Hochheimer *et al.*, 2002). Several recent studies reveal a close functional connection between chromatin remodeling/modifying complexes and signal transduction pathways including Notch (Bray *et al.*, 2005; Eissenberg *et al.*, 2005; Müller *et al.*, 2005). Because *pzg* was copurified in the same complex, we reasoned that it might have a further role in chromatin activation, thereby positively regulating Notch signaling. In this case, loss of Pzg should interfere with hallmarks of activated chromatin, such as trimethylation of histone H3 on lysine 4 (H3K4me3), which is typically found

Figure 4. *pzg* regulates Notch independently of DREF. (A) XChIP analyses were performed on chromatin isolated from wild-type *Drosophila* embryos using anti-Pzg, anti-DREF, and guinea pig preimmune sera as mock control. Samples of the 31st, 33rd, and 35th PCR amplification cycle are shown. Relative enrichment was estimated for the 33rd PCR cycle sample from the ratio between DREF or Pzg immunoprecipitations and mock signals. Mean values of three independent experiments are given, including standard deviations. Loading scheme from left to right: 1% of input DNA from sonicated chromatin, mock XChIP, XChIP with Pzg, and XChIP with DREF antibodies. Sequences from the *Polα* promoter, the *vg*-boundary enhancer, the *E(spl)m8* promoter, and the 3'UTR of *E(spl)m8* as unrelated control were amplified by PCR from the purified DNA. (B–D'') Wild-type polytene chromosomes were immunostained with antibodies recognizing Pzg (green) or DREF (red). Colocalization of DREF and Pzg was found at the *DNA Polα-180* locus (3R, 93F2: arrow in top inset B–B'') but not at the *E(spl)m8* locus (3R, 96F10-13: arrow in C–C''). Likewise, no overlap was seen at the *vestigial* locus (2R, 49E1: arrow in D–D''). (E and F) Depletion of DREF activity does not interfere with expression of Notch target genes (arrow), e.g., *E(spl)m8-lacZ* (*en-Gal4* > *UAS-DREF-RNAi*) (E) or *vg-lacZ* (*omb^{bifid}-Gal4* > *UAS-pzg-RNAi*) (F).

Figure 5. *pzg* activates chromatin independently of DREF. (A) XChIP was done on chromatin isolated from either wild-type (top) or *da-Gal4 > UAS-pzg-RNAi* (bottom) first instars by using anti-H3K4me3 antibody. Rabbit preimmune sera served as mock control. Relative enrichment was estimated for the 33rd PCR cycle sample from the ratio between H3K4me3 immunoprecipitations and mock signals. Mean values and standard deviations of three independent experiments are shown. (B) Histone H3 trimethylated on lysine 4 (H3K4me3) is a hallmark of active chromatin, and it is found evenly in control wing discs (*en-Gal4 > UAS-lacZ*). (C) Depletion of DREF in the posterior wing compartment has no apparent effect on H3K4me3 accumulation (arrow; *en-Gal4 > UAS-DREF-RNAi*). (D and D') A likewise depletion of Pz (*en-Gal4 > UAS-pzg-RNAi*) results in a prominent reduction of H3K4me3 (arrow in D) within the expression domain of the *pzg*-RNAi construct shown in D'. Antibody staining against H3K4me3 (red; B–D) and Pz (green; D') is shown.

on transcriptionally active loci (Lachner and Jenuwein, 2002). In fact, *pzg* down-regulation caused a strong reduction of the H3K4me3 signal (Figure 5, B, D, and D'). This was independent of apoptosis, because H3K4me3 levels were unaltered in a DIAP1 gain-of-function background. Notably, we could not detect any changes in H3K4me3 distribution in *DREF*-RNAi mutant cells (Figure 5, B and C), arguing for a *DREF*-independent role of *pzg* in chromatin activation.

To investigate whether triple methylation of H3K4 occurs at Notch target genes in vivo, we examined the chromatin status of *E(spl)m8* and *vg* in wild-type first instars. Indeed, anti-H3K4me3 precipitated chromatin in the regulatory region of *vg* and *E(spl)m8* (Figure 5A). This epigenetic modification is dependent on Pz, because H3K4me3 was no longer detectable on the *vg* and *E(spl)m8* promoters in *pzg*-RNAi mutant larvae (Figure 5A) nor on the *Pola* promoter (data not shown). These results strongly argue for an impor-

tant role of Pz in chromatin activation at Notch target genes.

pzg Is Not Involved in Hh-mediated Cell Growth and Proliferation

Because our data suggest that *pzg* activates Notch signaling via chromatin activation, we might expect a positive influence on other signaling pathways as well. We decided to investigate Pz's requirement for the Hh signaling pathway. In *Drosophila*, Hh signaling can induce vast overproliferation during imaginal development, and it promotes the expression of *Cyclin E* and *Cyclin D* (Duman-Scheel *et al.*, 2002). Hence, Pz might as well act upstream of Hh signaling, similar to that seen for Notch. In this case, we would expect that removal of *pzg* activity interferes with the expression of *hh*, the activation of its downstream effector *Cubitus interruptus* (Ci), or the target gene *dpp*, which is not the case (Figure 3, K–N' and Supplemental Figure S4, H–H'). Alternatively, Pz might serve as cofactor for Ci-mediated growth regulation, as suggested from a large-scale yeast two-hybrid assay (Hermjakob *et al.*, 2004; <http://pim.hybrigenics.com>). However, we failed to confirm a direct protein interaction in vivo by coimmunoprecipitation experiments by using embryonic and larval protein extracts (data not shown). Although Pz seems not to be a molecular interaction partner of Ci, one might envisage scenarios in which Pz activity is still essential for Ci function, e.g., by enhancing Ci-effects on responsive promoters. In this case, loss of *pzg* might interfere with Ci-activity. Overexpression of Ci results in a considerable overproliferation, e.g., of the adult wing, which is largely independent of *pzg*. The observed overproliferation involves transcriptional up-regulation of both *Cyclin D* and *Cyclin E*, which, however, remained unaltered in a *pzg*-RNAi mutant background (Supplemental Figure S4, E–G). Hence, our results show that *pzg* activity is not required for Hh signal transduction and growth regulation.

DISCUSSION

Pz has been isolated as one component of a multiprotein complex that contains TRF2 and DREF (Hochheimer *et al.*, 2002). TRF2 allows transcription initiation from selected promoters independently of TFIID (Hochheimer *et al.*, 2002; Hochheimer and Tjian, 2003). DREF is a positive transcriptional regulator of cell cycle and replication-related genes, and it may guide TRF2 to the PCNA and DNA-polymerase α promoters (Hochheimer *et al.*, 2002). Assuming promoter recognition or binding requires Pz contained within the TRF2/DREF complex, depletion of Pz might destroy the complex or reduce its activity, easily explaining the dramatic proliferation defects. However, we note that only a subset of promoters containing DREF binding sites involves activation through TRF2, suggesting that DREF can act independently of TRF2 (Hochheimer *et al.*, 2002). Moreover, we find Pz activity independently of DREF, indicating that TRF2/DREF complex components can act either alone or in conjunction with other factors.

The TRF2/DREF complex contains several proteins involved in chromatin remodeling (Hochheimer *et al.*, 2002). Notably, Pz and one other TRF2/DREF component p190 are reminiscent of factors implicated in insulator function (Bell *et al.*, 2001). In accordance, Pz activity has been associated with position effect variegation and chromatin silencing (Eggert *et al.*, 2004). In contrast, our assays reveal an essential function of Pz in retaining robust K4-trimethylation of histone H3, which is directly associated with open chromatin structures (Lachner and Jenuwein, 2002). In ac-

cordance with our findings, *EP756* was recently identified as a suppressor of the *cut* allele *ct^K*. This *cut* mutation is caused by the insulator activity of a *gypsy* retrotransposon, which can be relieved by *EP756* overexpression (Krupp *et al.*, 2005). We show here that *EP756* drives Pzg expression in support of the notion that Pzg's epigenetic activity overcomes *gypsy* insulator function.

Three of the proteins found in the TRF2/DREF complex have been identified previously in the nucleosome-remodeling factor NURF, which consists in total of four subunits (Tsukiyama and Wu, 1995). NURF is associated with chromatin activation by facilitating transcription of chromatin in vivo (Tsukiyama and Wu, 1995; Xiao *et al.*, 2001). In fact, mutations in *Drosophila* ISWI, the catalytic subunit of NURF, and other nucleosome remodeling complexes caused phenotypes that are very reminiscent of *pzg*-RNAi-induced defects (Deuring *et al.*, 2000). Because DREF down-regulation has no effect on trimethylation of H3K4, it seems unlikely that the TRF2/DREF complex as a whole is involved in chromatin activation. Instead, Pzg may be part of a NURF-like chromatin-remodeling complex, depending on the developmental context.

Apart from a role in proliferation, we have uncovered an important role for Pzg as positive regulator of Notch signaling. Interestingly, we found that Pzg binds to chromatin in the regulatory region of the Notch target genes *E(spl)m8* and *vg*. This regulation is independent of DREF: albeit DREF binding sites are common to *Drosophila* promoters (Ohler *et al.*, 2002), neither Notch nor Notch target genes that we have investigated are transcriptional targets of DREF. Thus, reduced transcriptional activity of Notch target genes in *pzg*-RNAi mutant cells is due to a DREF-independent role of Pzg. Alternatively, Pzg could facilitate formation of the transcriptional activator complex that is assembled on Notch target promoters involving intracellular Notch itself (Schweisguth, 2004; Nam *et al.*, 2006; Wilson and Kovall, 2006). By using the yeast two-hybrid system, we have tested several Notch pathway members; however, we failed to detect any binding to Pzg (our unpublished data). We propose that Pzg has a dual function that is effected differently. On one hand, it activates proliferation-related genes in conjunction with TRF2/DREF, and on the other hand, it activates Notch signaling by chromatin activation independently of DREF.

Several lines of evidence support the idea that Notch signaling is particularly susceptible to chromatin remodeling. For example, Notch transcriptional activity requires the histone-modifying enzyme dBre1 that is indirectly required for K4-methylation of histone H3 (Bray *et al.*, 2005). Moreover, chromatin-modifiers were also shown to potentiate Notch activity during *Drosophila* wing development (Eissenberg *et al.*, 2005; Gause *et al.*, 2006). Finally, general transcriptional regulators and chromatin remodeling factors were found in several independent genetic screens to influence Notch signaling, indicating to a role of *pzg* in linking Notch to chromatin remodeling (Eissenberg *et al.*, 2005; Müller *et al.*, 2005). The bimodal activity of Pzg onto both cell cycle genes and Notch signaling provides further insight into the complex interplay between cell proliferation and differentiation in the fly.

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