The Putzig-NURF Nucleosome Remodeling Complex Is Required for Ecdysone Receptor Signaling and Innate Immunity in *Drosophila melanogaster*

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Manuscript received February 15, 2011

Accepted for publication March 3, 2011

ABSTRACT

Putzig (Pzg) was originally identified as being an integral component of the TRF2/DREF complex in *Drosophila melanogaster*, thereby regulating the transcriptional activation of replication-related genes. In a DREF-independent manner, Pzg was shown to mediate Notch target gene activation. This function of Pzg entails an association with the nucleosome remodeling factor complex NURF, which directly binds the ecdysone receptor EcR and coregulates targets of the EcR via the NURF-specific subunit *Nurf-301*. In contrast, *Nurf-301* acts as a negative regulator of JAK/STAT signaling. Here, we provide evidence to show that Pzg is fundamental for these functions of NURF, apart from the regulation of Notch signaling activity. A jump-out mutagenesis provided us with a *pzg* null mutant displaying early larval lethality, defects in growth, and molting accompanied by aberrant feeding behavior. We show that Pzg is associated with EcR *in vivo* and required for the transcriptional induction of EcR target genes, whereas reduced ecdysteroid levels imply a NURF-independent function of Pzg. Moreover, *pzg* interferes with JAK/STAT-signaling activity by acting as a corepressor of Ken. Lamellocyte differentiation was consistently affected in a JAK/STAT mutant background and the expression level of defense response genes was elevated in *pzg* mutants, leading to the formation of melanotic tumors. Our results suggest that Pzg acts as an important partner of NURF in the regulation of EcR and JAK/STAT signaling.

THE *putzig* (*pzg*) gene is located near the centromere on the left arm of the third chromosome. It encodes a zinc finger protein with a molecular weight of about 160 kDa (EGGERT *et al.* 2004; KUGLER and NAGEL 2007). Pzg was identified as p160, being an integral component of the TATA-binding protein-related factor 2 (TRF2)/DNA replication-related element binding factor (DREF) multiprotein complex (HOCHHEIMER *et al.* 2002). This complex activates the transcription of several replication-related genes (HOCHHEIMER *et al.* 2002).

The downregulation of *pzg* gene activity by RNA interference (*pzg*-RNAi) revealed the fact that Pzg is essential for the function of the TRF2/DREF complex, which regulates cell cycling and growth during *Drosophila* development (KUGLER and NAGEL 2007). The ubiquitous

induction of *pzg*-RNAi is associated with a developmental delay and leads to loss of tissue due to reduced proliferation (KUGLER and NAGEL 2007). Pzg was shown to have a dual input on proliferation processes during development. Aside from its role in the TRF2/DREF complex, Pzg positively influences Notch (N) signaling (KUGLER and NAGEL 2007). The impact of Pzg on N activity is independent of DREF, as only Pzg, and not DREF, can be detected at the promoters of different N target genes. Furthermore, it was demonstrated that Pzg activates N signaling by chromatin activation. In this context, we showed that Pzg is associated with the nucleosome remodeling factor (NURF), thus entailing Notch target gene activation (KUGLER and NAGEL 2010).

The NURF complex contains four subunits, Iswi, Nurf-38, Nurf-55, and Nurf-301 (XIAO *et al.* 2001). The Nurf-301 subunit is the only subunit specific to the NURF complex, whereas the other three NURF components also appear in other chromatin remodeling complexes, for example, the TRF2/DREF complex (VIGNALI *et al.* 2000; XIAO *et al.* 2001; HOCHHEIMER *et al.* 2002; CORONA and TAMKUN 2004). NURF remodels chromatin by catalyzing energy-dependent nucleosome sliding (TSUKIYAMA and WU 1995; XIAO *et al.* 2001). Nurf-301 contains two AT-hook peptide motifs and an acid

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.111.127795/DC1>.

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domain with high similarity to the high mobility group A (HMGA) proteins (XIAO *et al.* 2001). Both domains participate in DNA–protein and protein–protein interactions (REEVES and BECKERBAUER 2001). It was shown that NURF binds different transcription factors to promote transcriptional activation or repression of target genes, depending on the gene context (*e.g.*, XIAO *et al.* 2001; BADENHORST *et al.* 2005; KWON *et al.* 2008). Whole-genome expression analyses revealed an important function of NURF for ecdysone receptor (EcR) signaling (BADENHORST *et al.* 2005). *In vitro*, NURF binds EcR in the presence of ecdysone, implying that it acts as a coactivator of EcR on ecdysone-responsive promoters (BADENHORST *et al.* 2005). The *Nurf-301* mutants are characterized by a developmental delay and the failure to pupariate (BADENHORST *et al.* 2005). This phenotype is due to impaired EcR signaling, as most of the known ecdysone targets were significantly downregulated in *Nurf-301* mutants (BADENHORST *et al.* 2005).

In contrast to NURF's function as a coactivator, NURF has been implicated in the transcriptional repression of genes that are downstream of the JAK/STAT pathway (KWON *et al.* 2008). The NURF mutants display melanotic tumors, which also occur after dysregulated activation of JAK/STAT signaling (LUO *et al.* 1995; BADENHORST *et al.* 2002). NURF physically and genetically interacts with the JAK/STAT repressor Ken and it is localized to promoters containing Ken binding sites. A large proportion of defense response genes contain overlapping Ken and STAT target sequences, suggesting that NURF is recruited by Ken to repress STAT target genes (KWON *et al.* 2008). Consistent with this, a common set of defense response genes is significantly upregulated in the NURF loss and JAK/STAT gain-of-function mutants (KWON *et al.* 2008).

We recently showed that Pzgf forms a complex with NURF and that this association is quintessential for the epigenetic activation of Notch target genes (KUGLER and NAGEL 2007; KUGLER and NAGEL 2010). Pzgf associates with all four members of NURF and the entire Pzgf–NURF complex is found on N target gene promoters (KUGLER and NAGEL 2010).

In this report, we show that Pzgf is also required for metamorphosis and innate immunity in *Drosophila melanogaster*, apart from its role in Notch target gene activation. We generated a null mutant allele of *pzgf* (*pzgf*⁶⁶) that displays a range of phenotypes reminiscent of those observed in mutants with an impaired ecdysone-signaling cascade. The *pzgf*⁶⁶ homozygotes are early larval lethal with defects and delays in larval development, growth, feeding, and molting. Pzgf is located at the regulatory region of well-defined EcR target genes with a downregulated expression in *pzgf*⁶⁶ mutants, suggesting a coregulator function of *pzgf* with respect to EcR nuclear activity. Intriguingly, ecdysteroid levels are perturbed in *pzgf*^{66/66} larvae, implying an additional NURF-independent influence on EcR-signaling activity. Finally, the *pzgf*⁶⁶

mutant flies evolve melanotic tumors and show an up-regulation of immune response genes. Immunoprecipitation experiments revealed that Pzgf can be detected in a complex with the transcriptional repressor Ken, indicating a corepressor activity of Pzgf in the JAK/STAT pathway. We suggest that Pzgf is an essential cofactor of NURF in the regulation of these pathways, implying a deep interdependence of these two in many developmental processes of *Drosophila melanogaster*.

MATERIALS AND METHODS

Drosophila strains and fly work: If not stated otherwise, flies were raised at 25° on standard cornmeal fly food seeded with baker's yeast. The following stocks were obtained from the Bloomington stock center: the *P{SUPor-P}/KG04911* (BL13616) line gained in the Berkeley Drosophila Genome Project disruption project (BELLEN *et al.* 2004); the deficiencies *Df(3L)Pc/TM3Sb* (BL3002), *Df(3L)Pc-MK/TM2* (BL4430), and *Df(3L)Pc-2q/TM2* (BL3068) all uncovering the *pzgf* locus; the Gal4 lines *cg-Gal4.A2* (BL7011; ASHA *et al.* 2003) and *Hml-Gal4G.6-4* (BL6396; GOTO *et al.* 2003); the UAS-lines *UAS-EcR.A* (BL6470), *UAS-EcR.B1* (BL6468), *UAS-lacZ* (BL8529), and the mutant strains *y¹v¹hop^{Tum-1}/FM7c* (BL8492) and *yw; Ki¹ry⁵⁰⁶ Δ2-3* (BL4368). The other stocks used in this study were: *da-Gal4* (WODARZ *et al.* 1995), *en-Gal4* (BRAND and PERRIMON 1993), *enGFP-Gal4* (NEUFELD and EDGAR 1998), *phantom-Gal4* (gift from M. O'Connor, University of Minnesota, MN), *P0206-Gal4* (WARREN *et al.* 2004) both lines kindly provided from C. Mirth, University of Washington (Seattle, WA); *UAS-pzgf-RNAi* (KUGLER and NAGEL 2007), *Nurf301²/TM6B* (BADENHORST *et al.* 2005), *STAT92E-GFP* (BACH *et al.* 2007), and *yw; e⁴tx* (PREISS *et al.* 1988).

pUAST-pzgf was cloned by shuttling the *pzgf* cDNA via *EcoRI*/*XhoI* into the pUAST vector. The *pzgf* full-length cDNA clone (LD15904) was obtained from Open Biosystems (Heidelberg, Germany). Several transgenic lines were generated by injecting *yw^{67c}* embryos using established methods (ASHBURNER 1989) and compared for their expression level. For further experiments, transgenes located on the second chromosome were used.

Generation and verification of the *pzgf*⁶⁶ mutant allele: We used imprecise *P*-element excision to generate *pzgf* mutant alleles. The starting *P*-element *P{SUPor-P}/KG04911* was inserted 20 bp upstream of the *pzgf* transcription start site and harbored two marker genes, *white* (*w*⁺) in the 5' region and *yellow* (*y*⁺) in the 3' region (see scheme in Figure 1A). Therefore, we were able to perform a site-directed screening for flies that lost the marker *w*⁺, located toward the *pzgf* transcription start site, but that still retained the *y*⁺ marker. The *yw; Ki¹ry⁵⁰⁶ Δ2-3* virgin females, providing the transposase, were mated to *KG04911* males. The F₁ males (*yw; Ki¹ry⁵⁰⁶ Δ2-3/KG04911*) were then crossed with *yw; e⁴tx* virgins, and the F₂ progeny was screened for site-directed *P*-element excision by the loss of the eye color marker *w*⁺. 74 *white*-eyed and *y*⁺ flies were collected and individually balanced to establish stocks for further investigations.

Mapping of breakpoints of the *pzgf*⁶⁶ deletion mutant: As the *y*⁺ body color was still present in the *pzgf* mutant candidates, we designed an upper primer within the *y* body enhancer and used a set of 3' lower primers, which bind at different regions within the *pzgf* gene region (see supporting information, Figure S1A and Table S1). The respective PCR products were gel purified and sequenced from both ends (Figure S1B). Most of the potential *pzgf* mutants showed an internal deletion within

the *P* element and did not affect the *pzg* gene. As *pzg*⁶⁶ mutants are homozygous lethal, the allele was maintained in stocks heterozygous for *TM6B* or for facile selection of homozygous animals balanced over *TM6B ubi-GFP* (*Df(3L)Ly/TM6Bubi GFP*; BL4887, obtained from the Bloomington stock center).

Southern blot analysis: To verify the deletion in the *pzg*⁶⁶ allele, we performed Southern blot analysis according to standard protocols (SAMBROOK *et al.* 1989). Genomic DNA from the wild-type (Oregon R), *KG04911 P*-element starter line, as well as *pzg*⁶⁶/*TM6B*, was digested with *Bgl*II or *Nco*I, electrophoresed on a 0.7% agarose gel, blotted on a nitrocellulose membrane, and probed with a radiolabeled genomic probe comprising the deleted region. The predicted restriction fragments and the corresponding bands are shown in Figure S1, A and C, and the details are given in the legend to Figure S1.

Semi-quantitative RT-PCR analysis: High-purity mRNA was isolated from 100-mg larvae 90–100 hr after egg laying (AEL) of the indicated genotype by using the PolyA Tract magnetic selection kit from Promega (Madison, WI). The mRNA was reversely transcribed using the Photoscript II RT-PCR kit from New England Biolabs (Ipswich, MA) at 42° according to the manufacturer's protocol. The PCR was performed for 35 cycles. The primer sequences are listed in Table S1.

Immunoprecipitation, cross-linked chromatin immunoprecipitation (XChIP), and Western blot analysis: Immunoprecipitations were performed according to NAGEL *et al.* (2005) using protein extracts from 100 first-instar larvae. For precipitations we used guinea pig anti-Pzg antibodies at a 1:100 dilution (KUGLER and NAGEL 2007), and for detection we used rat anti-Pzg (1:500), mouse anti-Ken, and Barbie (1:100, Abcam, Cambridge, UK), mouse anti-EcR_A (1:50), mouse anti-EcR_{B1} (1:50), and mouse anti-EcR_{common} (1:50); all three mouse antibodies were developed by C. Thummel and D. Hogness, and were obtained from the Developmental Studies Hybridoma Bank (DSHB), developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA).

For chromatin immunoprecipitation of third-instar larvae (96–112 hr AEL, including the 20-hydroxyecdysone, 20-HE, peak), we used the ChIP Assay Kit according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). For precipitation, guinea pig anti-Pzg (1:100) and guinea pig pre-immune sera (1:25, mock control) were used, and 1.5% of the precipitated DNA was used per PCR reaction. There were 35 cycles, and samples were taken every two cycles from the 31st to the 35th cycle to show a linear amplification range. Signals were quantified using the histogram function of ImageJ software (<http://rsb.info.nih.gov/ij/>). As negative controls, we used primer sets within the open reading frame of the analyzed genes. The primer sets for the amplification process are listed in Table S1.

The Pzg protein levels in *pzg*^{66/66} mutants were measured by Western blot experiments. Protein extracts from 100 first instars from either wild-type or homozygous *pzg*⁶⁶ mutants (hand picked by the loss of the GFP-balancer marker under a fluorescence microscope) were homogenized in 50 µl RIPA buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; protease inhibitor cocktail from Roche Diagnostics, Basel, Switzerland) and after 10 min centrifugation 25 µl SDS-loading buffer (250 mM Tris-HCl, pH 7.6, 0.001% bromophenol blue, 5% [vol/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 40% [vol/vol] glycerol) was added and immediately boiled for 5 min. Then, 15 µl of the supernatant per lane was loaded onto a 10% polyacrylamide gel and separated, followed by electrical blotting on a nitrocellulose membrane. The Pzg protein was detected on the blots by

using guinea pig anti-Pzg (1:5000) antibodies and mouse anti-β-Tubulin (1:50; DSHB) antibodies. Secondary antibodies, coupled to alkaline phosphatase (1:500), were obtained from Jackson Laboratories (Dianova, Hamburg, Germany).

Immunofluorescence staining of tissues: *Drosophila* hemocytes from 15 third-instar larvae were suspended in 200 µl of Shields and Sang M3 medium with 20% fetal calf serum (FCS) and a protease inhibitor cocktail (Roche Diagnostics). The hemocytes were pelleted after a 10-min centrifugation step at 5000 rpm. The supernatant was discarded and the hemocytes were resuspended in 100 µl of Shields and Sang M3 medium with 20% FCS. Fixation of the hemocytes and antibody staining was performed according to KWON *et al.* (2008). The cells were stained with α-Mys-specific antibodies (CF6G11; DSHB) and rhodamine-coupled phalloidin (Invitrogen molecular probes, Carlsbad, CA); nuclei were stained with DAPI (Roche Diagnostics).

Antibody staining of larval wing disks was performed according to MÜLLER *et al.* (2005), using guinea pig anti-Pzg antibodies (1:1000). Secondary antibodies coupled to Cy3 were purchased from Jackson Laboratories (Dianova, Hamburg, Germany). The ring-gland-specific induction of *UAS-pzg-RNAi* was analyzed with the help of *phantom-Gal4*, *UAS mCD::GFP/TM6B Tb*, and *P0206-Gal4*, *UAS mCD::GFP*, visualizing the prothoracic gland with the help of GFP. Rhodamine-coupled phalloidin was used to stain the boundaries of the cells and guinea pig anti-Pzg antibodies were used to verify the reduction in Pzg activity.

Lethal-phase analysis: Eggs were collected from *pzg*⁶⁶/*TM6Bubi-GFP* flies during a 1-hr interval on apple juice plates with fresh yeast paste. Homozygous *pzg*⁶⁶ first-instar larvae (*n* > 145) were selected by their lack of GFP expression. These larvae were placed onto fresh plates and the number of living larvae was determined every 5 hr. For comparison, the same procedure was performed with wild-type larvae. All flies were incubated at 25° and larval instars were distinguished by spiracle and mouth-hook development (BATE and MARTINEZ ARIAS 1993).

Da-Gal4 rescue experiments: The following strains were established for the rescue experiments: *da-Gal4* was recombined with *pzg*⁶⁶ mutants to generate the *da-Gal4 pzg*⁶⁶/*TM6B* strain. *UAS-pzg* was combined with *da-Gal4 pzg*⁶⁶/*TM6B* to generate *UAS-pzg/UAS-pzg; da-Gal4 pzg*⁶⁶/*TM6B* flies or with *pzg*⁶⁶/*TM6B* to establish *UAS-pzg/UAS-pzg; pzg*⁶⁶/*TM6B* strains. The different strains were mated with the aim of obtaining *pzg*⁶⁶ homozygous flies with either one or two copies of *da-Gal4* and/or *UAS-pzg*. The rescue was assessed at third-instar larval, pupal, and adult stages by screening for individuals lacking the balancer chromosome. At least 250 animals were analyzed per genotype. The correct genotype of the rescued flies was further verified by PCR (Table S1 and Figure S2).

Ecdysone feeding assay: To mimic the pulses of ecdysone, staged larvae (*pzg*^{66/66} *vs.* wild type) were periodically transferred between food lacking and food containing the activated form of ecdysone, 20-HE (Sigma, St. Louis, MO). The experiment was performed according to FLUEGEL *et al.* (2006), whereby larvae were fed for 8 hr on standard food immediately after a molt and then moved to food with ecdysone until the next molt. The 20-HE was mixed with baker's yeast (1 mg 20-HE dissolved in 42 µl 100% ethanol, added to 958 µl water and 0.5 g dry yeast). This mixture was evenly spread over apple juice plates. The lethal phase was then noted over the course of development.

Feeding response: To analyze feeding behavior, a blue-colored yeast paste was offered to first- and second-instar larvae as a food source to follow food uptake within the gut.

Mouth hook contraction studies: The relative frequency of mouth-hook contraction of the larvae is directly correlated with the ingested amount of food (GUTIERREZ *et al.* 2007).

FIGURE 1.—Schematic of the genomic region of *pzg* and verification of the *pzg*^{66/66} mutant. (A) Scheme of the *pzg* genomic region at 78C5–78C6. The 5' region of the *pzg* gene is enlarged below. Rectangles indicate the *pzg* transcript with introns (interrupted rectangles) and the Pz coding region (shaded). The *P* element in strain *KG04911* (triangle) is oriented in such a way that the *white* marker gene is adjacent to the *pzg* gene. The two breakpoints in the *pzg*^{66/66} deletion are indicated by two arrows with asterisks; the deletion is depicted by dashed lines. Small arrows underneath show the position of primer pair *pzg1* used for RT-PCR. (B) Semiquantitative RT-PCR analysis of *pzg* and β -tubulin transcripts in wild-type and *pzg*^{66/66} larvae. Note the absence of a *pzg* PCR product in homozygous *pzg*^{66/66} mutant larvae. For control, reactions were performed with (+) and without (–) reverse transcriptase. (C) Loss of Pz protein in *pzg*^{66/66} mutants compared to the wild type is shown in a Western blot. Anti- β -tubulin was used as the loading control.

Therefore, mouth-hook contractions were counted in 30-sec intervals for first- and second-instar *pzg*^{66/66} mutant larvae and were statistically compared with the numbers in wild-type larvae of the same age.

Feeding behavior studies: First-instar larvae were placed onto the edges of apple juice plates harboring fresh yeast paste as a food source in the middle. According to GUTIERREZ *et al.* (2007), wild-type larvae are attracted by the yeast source and wander toward the middle of the dish. Every 15 min we counted how many larvae of the respective genotype (wild type compared with *pzg*^{66/66}) had reached the source and statistically documented the results.

Documentation of phenotypes: Pictures of whole larvae were documented using a Wild stereomicroscope equipped with a Pixera camera (Optronics, Goleta, CA) using the Pixera Viewfinder, version 2.0, software. Confocal pictures were taken with a Zeiss Axioskop linked to a Bio-Rad MRC1024 scanhead using Bio-Rad Laser Sharp 3.1 software. The figures were arranged using Corel Photo Paint, GIMP, and Corel Draw software. Hemocyte pictures were taken in the Biosensorik-Department, Institute of Physiology (University of Hohenheim) with the Zeiss ApoTome, using AxioVision LE Rel. 4.5 software. Wing size was determined using ImageJ software for pixel measurements and repeated at least twice under identical conditions. Statistical significance was verified according to Student's *t*-test (<http://www.physics.csbsju.edu/stats/t-test.html>).

RESULTS

Generation and verification of a *pzg* mutant in *D. melanogaster*: Depletion of *pzg* by RNA interference results in an 80% reduction in Pz protein levels (KUGLER and NAGEL 2007). To further study the biological role of *pzg* during the development of *Drosophila*,

we generated a *pzg* null mutant by imprecise *P*-element excision (for a detailed description see MATERIALS AND METHODS). As *pzg* is crucial for cell proliferation and development (KUGLER and NAGEL 2007), we expected that *pzg* mutants should be lethal. The *P*-element jump-out mutagenesis provided us with 74 *pzg* mutant “candidates” displaying only heterozygous adult viability. From each of these stocks, genomic DNA from about 200 flies was extracted and analyzed by Southern blot and PCR analyses for the presence of *pzg* sequences (see MATERIALS AND METHODS). The boundaries of the *pzg*^{66/66} deletion were mapped by Southern blot analysis and specified by sequence analysis (Figure S1 and data not shown). The *pzg*^{66/66} mutant allele carried a deletion of 7083 bp within the *P* element and a deletion of 839 bp within the *pzg* gene, including transcription and translation start sites (Figure 1A and Figure S1A), suggesting that it was a null allele. This is in line with our molecular data, where we did not detect the *pzg*-specific transcript by RT-PCR analysis or the Pz protein on Western blots using a Pz-specific antibody in *pzg*^{66/66} homozygotes (Figure 1, B and C). Finally, the *pzg*^{66/66} mutant chromosome was tested in *trans* to three deficiencies *Df(3L)Pc/TM3Sb*, *Df(3L)Pc-MK/TM2*, and *Df(3L)Pc-2q/TM2*, all known to uncover the *pzg* locus: *pzg*^{66/66} failed to complement the lethal phenotype of all three deletions tested.

pzg^{66/66} mutants show severe developmental defects:

The downregulation of *pzg* gene activity by RNA interference caused an extensive reduction in tissue size and significantly delayed larval development (KUGLER and NAGEL 2007). Thus, we expected the *pzg*^{66/66} null mutant to be characterized by proliferation and growth defects. The embryonic development of homozygous *pzg*^{66/66} mutants was not affected, presumably due to the large amount of maternal Pz protein that we detected in *pzg*^{66/66} mutant embryos using a Pz-specific antibody (data not shown). The *pzg*^{66/66} larvae displayed a strong developmental delay and early lethality. The *pzg*^{66/66} homozygotes were smaller and thinner than the wild-type larvae (Figure 2A). The *pzg*^{66/66} larvae showed an almost linear mortality rate with increasing age, and none of the larvae survived more than 150 hr (Figure 2B). During this time they molted only once, reaching the second larval stage, but then there was no further increase in size. In summary, the *pzg*^{66/66} mutants were developmentally delayed and died as tiny larvae in the second larval stage.

Rescue of *pzg*^{66/66} mutants: To ensure that the phenotypes observed in *pzg*^{66/66} resulted from the loss of *pzg* gene activity, we performed rescue experiments. We made use of the Gal4/UAS system (BRAND and PERRIMON 1993) to ectopically express *pzg* in *pzg*^{66/66} mutants with the aim of restoring viability. We built a fly stock that comprised the ubiquitous driver *da-Gal4*, the *UAS-pzg* construct containing the *pzg* full-length cDNA, as well as the heterozygous *pzg*^{66/66} mutant allele. The stock was kept over a TM6B *Tb* balancer

FIGURE 2.—Developmental delay and lethal phase of *pzg^{66/66}* mutants. (A) Comparison of the size of wild-type (WT, left), homozygous *pzg^{66/66}* (*pzg^{66/66}*, center), and “rescue” larvae (*UAS-pzg/UAS-pzg; da-Gal4 pzg^{66/66}/da-Gal4 pzg^{66/66}*, right). Pictures were taken 97 hr AEL for each genotype. As determined by mouth-hook morphology, the wild-type and rescue larvae have reached the third-instar stage, whereas the *pzg^{66/66}* mutant is still in the second larval instar, displaying a small and slim phenotype. (B) The lethal phase of *pzg^{66/66}* homozygous larvae is nearly linear with increasing age. Fifty percent of the analyzed animals died within 93 hr after egg deposition (dashed line) and none of the animals survived for more than 150 hr. Note that they remained in the second larval instar. Wild type *n* = 146, *pzg^{66/66}* *n* = 148 larvae analyzed. (C) Rescue of *pzg* mutants. *pzg^{66/66}* homozygotes (*da-Gal4 pzg^{66/66}* recombined line) all died at second larval stage (L2). Ectopic *pzg* expression pushed the lethal phase beyond the second molt into the third larval stage (L3). The presence of one copy of *da-Gal4* allowed a small percentage to form pre-pupae (PP) (genotypes were *UAS-pzg/2; da-Gal4 pzg^{66/66}/pzg^{66/66}* or *UAS-pzg/UAS-pzg; da-Gal4 pzg^{66/66}/pzg^{66/66}*), whereas even some adults (A) were obtained in the presence of two *da-Gal4* copies (*UAS-pzg/2; da-Gal4 pzg^{66/66}/da-Gal4 pzg^{66/66}*). Here, a somewhat stronger rescue is observed with two *UAS-pzg* copies (*UAS-pzg/UAS-pzg; da-Gal4 pzg^{66/66}/da-Gal4 pzg^{66/66}*). For each genotype at least 250 animals were analyzed.

chromosome to easily visualize the genotypes. The correct genotype of rescued *pzg^{66/66}* mutants was confirmed by PCR analysis on single animals (Figure S2). We distinguished the endogenous *pzg* gene copy of the balancer chromosome from the *UAS-pzg* construct with a primer pair spanning a 60-bp intron (Figure S2). While combining the fly stock, we observed a rescue effect. Some of the *pzg^{66/66}* mutants that carried one copy each of *da-Gal4* and *UAS-pzg* survived to the third-instar larval stage, whereas *pzg^{66/66}* larvae died as second instars (Figure 2C). By increasing the number of copies of both the Gal4 driver and the *UAS-pzg* construct, the lifetime of the mutant animals was extended even further, allowing pupariation and even metamorphosis into adults (Figure 2C). The rescued animals showed no apparent phenotype and regained a size comparable to the wild-type control that was already beginning the larval stages (Figure 2A). These data provide definitive evidence that only the *pzg* gene is affected in the *pzg^{66/66}* mutant and that the *pzg^{66/66}* mutant phenotype specifically results from a lack of the Pzg protein.

Pzg acts as a cofactor of NURF in EcR signaling: The developmental delay observed in *pzg^{66/66}* mutants agrees well with the defects observed in *Nurf-301* mutants, the latter playing a well-established role in metamorphosis mediated by ecdysone receptor signaling (BADENHORST *et al.* 2005). As the NURF complex functions as a direct coactivator of the ecdysone receptor itself (BADENHORST *et al.* 2005), it is very conceivable that Pzg is also necessary for this function of NURF. In this case, Pzg should be present in a common complex together with NURF and EcR. This was confirmed by co-immunoprecipitation with an anti-Pzg antibody using extracts from wild-type third-instar larvae. Indeed, we detected EcR.A and EcR.B in association with Pzg (Figure 3A).

Ecdysone-ligated EcR binds to ecdysone response elements (EcRE) in the promoters of EcR-responsive genes (CHERBAS *et al.* 1991). As Pzg was present in a complex with EcR *in vivo*, we expected Pzg at EcRE as well. Via chromatin immunoprecipitation experiments (ChIPs) we verified the presence of Pzg on the promoters of two EcR target genes, *Eig71Ea* and *ImpE2*,

FIGURE 3.—Pzg activity is required for nuclear EcR activity. (A) Pzg associates with EcR *in vivo*. Proteins immunoprecipitated (IP) from larval protein extracts by anti-Pzg antibodies were probed to detect different EcR forms (EcR.A, EcR.B). The input lane contained 25% of the larval extract used for the IP. Pre-immune serum was used as mock control to demonstrate specificity. (B) Chromatin immunoprecipitation from wild-type larvae using Pzg-specific antibodies; DNA enrichment after the 31st, 33rd, and 35th cycles is shown. Pzg is recruited to the *hsp27* promoter that contains eclosion response elements (EcRE), and binds the regulatory regions of the EcR target genes *Eig71Ea* and *ImpE2*, however, not at the EcR itself. Relative enrichment was estimated

for the 33rd PCR cycle sample from the ratio between Pzg IP and mock signals. Mean values and standard deviations of at least three independent experiments were calculated. PCR amplification of regions within the ORF of the respective genes was performed as an unrelated control. (C) Semiquantitative RT-PCR analysis of *EcR* and EcR target gene expression relative to β -tubulin (*tub*). In *pzg^{66/66}* mutants, *EcR* levels are unchanged, whereas expression of EcR targets *Eig71Ea* and *ImpE2* is strongly reduced in comparison to wild type (WT). As a control, reactions were performed with (+) and without (−) reverse transcriptase. (D) Rescue of *pzg-RNAi*-induced tissue loss by different EcR isoforms. Adult wings of *enGFP-Gal4 UAS-pzg-RNAi/UAS-lacZ* (red) are shown superimposed over wings where the EcR isoform EcR.A (blue) or EcR.B1 (green) was co-overexpressed.

as well as on the EcRE of the well-defined *hsp27* target gene (Figure 3B). However, Pzg was absent from the regulatory region of the EcR gene itself, which supports the assumption that Pzg acts as a coactivator of EcR rather than influencing *EcR* gene activity (Figure 3B).

The role of NURF as a cofactor of EcR predicts a positive role for Pzg in the transcriptional activation of EcR target genes. To this end, we examined the transcript levels of *Eig71Ea* and *ImpE2*, as well as of *EcR* itself, in wild-type *vs.* homozygous *pzg^{66/66}* larvae 90–100 hr AEL by semiquantitative RT-PCR analyses. As shown in Figure 3C, expression of the EcR target genes *Eig71Ea* and *ImpE2* was strongly reduced or even abolished, whereas the transcript levels of *EcR* and of β -tubulin were not altered. BADENHORST *et al.* (2005) have already shown that expression levels of the EcR target genes *Eig71Ea* and *ImpE2* are diminished in *Nurf-301* mutants whereas the transcript level of *EcR* itself was not altered.

To address a further functional interplay between EcR signaling and *pzg* we tested for genetic interactions between *pzg* and *EcR*. For technical reasons, we used RNA interference of *pzg* as the 80% reduction in Pzg protein levels results in distinct phenotypes that can be documented in the adult fly (KUGLER and NAGEL 2007). Increasing the activity of EcR signaling by overexpressing different isoforms of the receptor significantly suppressed the small wing phenotype caused by

the induction of *pzg-RNAi* (Figure 3D). Altogether, these data strongly indicate that Pzg acts together with NURF in activating EcR target genes.

***pzg^{66/66}* mutants show further signs of impaired growth and metamorphosis:** In contrast to the early lethality of *pzg^{66/66}* mutants, null alleles of *Nurf-301* can develop further and fail to undergo larval to pupal metamorphosis (BADENHORST *et al.* 2005). The developmental arrest and small body size of *pzg^{66/66}* mutants led us to investigate whether or not the animals can take up food at all. A feeding experiment with blue-colored yeast paste as the food source revealed that *pzg^{66/66}* mutants were able to grab the offered yeast paste, as visualized by the colored gut; however, this gave no conclusion as to whether the amount of absorbed food was in the wild-type range or not (Figure 4A). The reduced mouth-hook contractions observed in *pzg^{66/66}* mutants would rather suggest a reduction in food intake (Figure 4B). Although we observed a slight increase in body weight of the *pzg^{66/66}* mutants with increasing age, we must assume that the *pzg* mutation affected food uptake and/or metabolism as well (Figure 4C). While performing the feeding assay we discovered a defective locomotive behavior in *pzg^{66/66}* mutant larvae that stayed dispersed on the plates, whereas the wild type went straight to the yeast (Figure 4, D–E). These defects in locomotive behavior have already been described for larvae with lowered endogenous 20-HE titers and result from a depression in

FIGURE 4.—*pzg^{66/66}* mutants show growth disadvantages and signs of reduced ecdysteroid titers. (A) *pzg^{66/66}* mutants do not uptake food, as visualized by the light-blue-colored yeast in the larval gut. (B) *pzg^{66/66}* larvae (red bars) perform significantly fewer mouth-hook contractions per time interval relative to the control larvae (WT, blue bars) at first (L1) and second (L2) instars. (C) Larval weight relative to larval age. In comparison to wild type (WT, blue bars), homozygous *pzg^{66/66}* mutant (red bars) larvae weigh less, but still increase their weight with increasing age. (D–E) Mutant *pzg^{66/66}* larvae display defective locomotive behavior. (D) In contrast to wild-type larvae (left), which quickly moved to the central yeast source, the *pzg^{66/66}* animals were more sluggish and remained dispersed. (E) Number of larvae that reached the food source over time. All wild-type larvae reached the yeast within 1 hr (yellow curve), but only 40% of *pzg^{66/66}* larvae did (red curve). (F) Ecdysone feeding assay. Relative survival rate of control (wild type) was set as 100%, blue bars) and homozygous *pzg^{66/66}* larvae (red bars) on food containing (+) or lacking (–) 20-HE.

synaptic transmission (Li *et al.* 2001). In line with the prolonged larval instars and the failure of a second molt, this locomotive problem might originate from a reduced ecdysteroid titer during larval development in *pzg^{66/66}* mutants. To test this possibility, we attempted to rescue these defects by feeding ecdysteroids to *pzg^{66/66}* first-instar larvae. Such an approach was shown to efficiently rescue phenotypes associated with ecdysone-deficient mutations in *Drosophila* (FREEMAN *et al.* 1999; BIALECKI *et al.* 2002; FLUEGEL *et al.* 2006). On food-lacking 20-HE, about 60% of the *pzg^{66/66}* mutants passed the first larval instar, but then died in the second instar (Figure 4F). The addition of 20-HE to the diet had a tremendous impact on the survival rate of homozygous *pzg^{66/66}* larvae. Almost 90% of *pzg^{66/66}* mutants survived to the second larval instar and nearly all of them reached the third instar (Figure 4F).

In *Drosophila*, ecdysteroids are synthesized in the prothoracic glands (PG) of the larval ring gland and then released in the hemolymph and converted by peripheral tissues to the active form 20-HE (summarized in GILBERT *et al.* 2002). The obvious failure to achieve correct ecdysteroid titers could reflect problems in ecdysteroid synthesis and/or release or structural defects in the ring gland of *pzg^{66/66}* mutants. To analyze these pos-

sibilities, we used the Gal4/UAS system to target *pzg-RNAi* in the PG by using *phantom-Gal4* or *P0206-Gal4*; the latter drives additional expression in the corpora allata. As previously shown, a reduced ecdysteroid titer, induced, for example, by knockdown of the sumoylation gene *smt3* in the PG, produces animals arrested in their development at the third instar, followed by additional 3-week persistence at this larval stage (TALAMILLO *et al.* 2008). In contrast, no appropriate phenotype was observed when *pzg-RNAi* was induced in the PG and the progeny hatched without any visible defects (data not shown). The external morphology of the gland in *pzg-RNAi*-induced larvae did not exhibit obvious changes when compared with the wild type. Finally, no definite changes in size or morphology of PG cells subjected to *pzg-RNAi* was found, suggesting that *pzg* has no essential function for their survival and development (data not shown).

Pzg is involved in innate immunity: Besides being an activator of gene transcription, NURF antagonizes JAK/STAT signaling by repressing several STAT-dependent genes involved in innate immunity (KWON *et al.* 2008). To investigate the requirement of *pzg* in this process, we first looked for the appearance of melanotic tumors in *pzg* mutants as a typical indicator of a dysregulated immune

system (MINAKHINA and STEWARD 2006). In *Drosophila*, the immune response is sustained by specialized blood cells called hemocytes (plasmotocytes, lamellocytes, crystal cells) and by the fat body that secretes antimicrobial peptides (LEMAITRE and HOFFMANN 2007). The induction of *pzg-RNAi* by *cgGal4A.2* in hemocytes and the fat body (ASHA *et al.* 2003) induced melanotic tumors in larvae, pupae, and adults, implicating *pzg* in the innate immune function (Figure 5A). Comparable effects can be observed using the *Hml-Gal4* driver line (GOTO *et al.* 2003), which is expressed in a subpopulation of plasmotocytes implying that the melanotic tumor formation after *pzg* reduction is not exclusively derived from its induction in the fat body (Figure 5A). Melanotic tumors are also found in animals lacking the NURF-specific subunit *Nurf-301*, and the loss of one copy of *Nurf-301* enhanced tumor incidence in the *hop* gain-of-function mutant *hop^{Tum-l}*. As *hop* encodes for the *Drosophila* janus kinase JAK, these findings illustrate the negative role of NURF in JAK/STAT signaling (BADENHORST *et al.* 2002; BADENHORST *et al.* 2005; KWON *et al.* 2008). We observed a similar enhancement of tumor formation in *hop^{Tum-l}* mutants in the presence of only one *pzg* gene copy, demonstrating the requirement of Pzg for NURF activity with respect to JAK/STAT regulation (Figure 5B). Tumor frequency was increased in the *trans*-heterozygous *Nurf-301² +/+ pzg⁶⁶* combination, reflecting the synergistic impact of the two on tumor formation (Figure 5B).

These melanotic tumors result from increased lamellocyte production due to an overactivation of JAK/STAT-signaling activity that triggers lamellocyte differentiation (MEISTER 2004). In line with reports for *Nurf-301* mutants, we expected excess lamellocytes in *pzg^{66/66}* mutants (KWON *et al.* 2008). Unfortunately, the early larval lethality of *pzg^{66/66}* mutants prevented us from isolating circulating hemocytes from third-instar larvae. Instead, we performed antibody staining on hemolymph preparations from *hop^{Tum-l/+}; pzg^{66/+}* doubly heterozygous larvae compared to the single heterozygous mutant and wild-type animals. Lamellocytes were distinguished by their large size from the smaller plasmotocytes. Wild-type and *pzg⁶⁶* heterozygotes exhibit circulating lamellocytes very rarely: less than 1% of the total hemocytes corresponded to this cell type (Figure 5, C and D). Aggregated plasmotocytes are typically observed in *hop^{Tum-l}* mutants, resulting from increased expression levels of β -integrin subunits (KWON *et al.* 2008). As expected, numerous lamellocytes (>3%) were detected in *hop^{Tum-l}* preparations (RIZKI 1957; Figure 5, C and D). Lamellocyte incidence in *hop^{Tum-l/+}; pzg^{66/+}* larvae was significantly increased to >7%, demonstrating the requirement of Pzg for the restriction of JAK activity (Figure 5, C and D). As mutant *pzg⁶⁶* heterozygotes enhance *hop^{Tum-l}* tumor phenotypes, we further analyzed the influence of *pzg* on JAK/STAT signaling.

Inactivation of *pzg* leads to precocious activation of JAK/STAT activity: The interaction of loss-of-function *pzg⁶⁶* mutants and gain-of-function *hop^{Tum-l}* mutants supports the idea that Pzg acts together with NURF to prevent ectopic activation of JAK/STAT signaling. *Nurf-301* has been shown to repress STAT target gene activation, since *Nurf-301* mutants show increased expression of several immune response genes that are also upregulated in *hop^{Tum-l}* mutants (KWON *et al.* 2008). If Pzg is involved in the NURF-mediated repression of JAK/STAT targets, loss-of-function of *pzg* should result in ectopic activation of STAT targets as well. To test this, we first made use of the *STAT92E-GFP* reporter line. This line contains *Stat92E* binding sites upstream of the *GFP* that are derived from the *Socs36E* gene and reflects activity of the JAK/STAT pathway *in vivo* (BACH *et al.* 2007). In control wing imaginal disks, *STAT92E-GFP* is expressed in a broad ring surrounding the wing pouch as described by BACH *et al.* (2007) (Figure 6A, top). Down-regulation of Pzg activity by means of *pzg-RNAi*, for example in the posterior half of the wing disk, resulted in a strong ectopic activation of the *STAT92E-GFP* reporter within the affected cells (Figure 6A, bottom, arrows). This is consistent with our hypothesis that Pzg acts as cofactor of NURF in the repression of *STAT* target genes. We therefore addressed the expression levels of two different STAT-dependent defense response genes, *Dox-A3* and *IM23*, and of *ϵ Try* encoding a peptidase that is upregulated in *Nurf-301* mutants as well (KWON *et al.* 2008). Our semiquantitative RT-PCR analyses revealed an increase in the transcript levels of all three genes in *pzg^{66/66}* mutant larval extracts compared to the wild type (Figure 6B).

Pzg interacts with Ken in the repression of JAK/STAT signaling: JAK/STAT signaling is antagonized by a repressor complex consisting of Ken and NURF that competes with STAT for the binding of STAT target genes. In accordance, *Nurf-301* interacts with Ken at the genetic and molecular level (KWON *et al.* 2008). Our data so far indicate that Pzg is required for NURF repression of JAK/STAT-signaling output as well. In this case, we expected Pzg as an additional component of the Ken–NURF repressor complex. We were able to co-immunoprecipitate Ken with Pzg antibodies from extracts of third-instar wild-type larvae, demonstrating the presence of Pzg in the Ken–NURF repressor complex (Figure 6C).

Finally, we addressed the question of whether Pzg is present on the promoters of genes that are repressed by the Ken–NURF complex. In addition to the immune responsive genes *Dox-A3* and *ϵ Try*, we included *CG5791* in our analysis, the function of which is not yet known (KWON *et al.* 2008). The *CG5791* gene contains overlapping STAT and Ken binding sequences in its promoter region and is transcriptionally upregulated in *Nurf-301* mutants, indicating that it is a direct target of NURF as well as of STAT (KWON *et al.* 2008). Our

FIGURE 5.—Loss of *pzg* causes tumor formation. (A) *pzg*-RNAi induction in the fat body and/or hemocytes induces the formation of melanotic tumors, as seen in larvae, pupae, and adults. Genotypes: *cg-Gal4A.2/cg-Gal4A.2; UAS-pzg-RNAi/UAS-pzg-RNAi* (left) and *Hml-Gal4G.6-4/Hml-Gal4G.6-4; UAS-pzg-RNAi/UAS-pzg-RNAi* (right). (B) Loss of one copy of either *Nurf-301* or *pzg* enhances tumor incidence in *hop^{Tum1}/+* adult females. A synergistic effect was observed after a concurrent reduction of *pzg* and *Nurf-301* activity. $N > 150$ females were scored for each genotype. (C–D) Halving the *pzg* gene dose increases the amount of lamellocytes in *hop^{Tum1}/+* larvae. (C) Lamellocyte frequency in the respective genotypes was determined relative to the total number of hemocytes in a constant field. Cells were counterstained with phalloidin; total cell number was at least 350 in each count. (D) Hemolymph from wild type (WT), *pzg⁶⁶/+* (both negative controls), *hop^{Tum1}/+* (positive control), and *hop^{Tum1}/+; pzg⁶⁶/+* mutant larvae was isolated and the hemocytes were immunostained with antibodies specific for Mys (green). Cell morphology is revealed by staining with rhodamine-coupled phalloidin (red); nuclei are stained with DAPI (blue). Lamellocytes are recognized by their huge size relative to the smaller plasmatocytes. Whereas nearly no lamellocytes can be observed in the controls (top two rows), the incidence of lamellocytes is significantly increased in *hop^{Tum1}/+; pzg⁶⁶/+* transheterozygotes compared to *hop^{Tum1}/+* animals. Arrows indicate large lamellocytes, surrounded by small plasmatocytes, which tend to aggregate in *hop^{Tum1}/+* and *hop^{Tum1}/+; pzg⁶⁶/+* mutants (asterisks).

ChIP experiments showed the localization of Pzg at the respective promoter regions (Figure 6D). Taken together, our results demonstrate a requirement of Pzg in the Ken–NURF repressor complex, thereby regulating immune responsive genes that are controlled by the JAK/STAT-signaling output.

DISCUSSION

We know from our earlier work that Pzg is involved in the activation of Notch target genes and that this process entails the physical association of Pzg with NURF (KUGLER and NAGEL 2010). To extend our knowledge of *pzg* function during the development of *Drosophila*, we created a loss-of-function mutation in the *pzg* gene. We found that *pzg^{66/66}* null mutants die early in larval development, showing various defects in molting, growth, metamorphosis, and larval immunity. Our work on the *pzg^{66/66}* null allele provided evidence to show that Pzg is required for a much broader range of NURF-dependent developmental processes, includ-

ing the regulation of metamorphosis and innate immunity in the fly.

Pzg and its role in EcR signaling: More than strictly NURF-dependent?: The observation that a large set of ecdysone responsive target genes is impaired in *Nurf-301* mutants was one of the key findings triggering the idea that NURF is a coactivator of the EcR, allowing the progression from larval to pupal development (BADENHORST *et al.* 2005). Here, we showed that Pzg can physically associate with the EcR and that it is recruited to ecdysone responsive promoters *in vivo*, the expression of which is lost in a *pzg^{66/66}* mutant background. This correlates well with the conception of Pzg being an essential and critical cofactor of NURF-mediated influences on EcR nuclear activity. In contrast to this synergistic effect, we found that *pzg* null mutants do not exactly phenocopy the defects observed in the *Nurf301* mutants, but rather show more severe defects with respect to developmental delay and early larval lethality. This might be due to the fact that Pzg is not just part of the NURF complex but it also coregulates the expression of replication-related genes required for cell survival in a TRF2/DREF-dependent

FIGURE 6.—Pzg antagonizes JAK/STAT-signaling activity. (A) *pzg*-RNAi induction in the posterior compartment of the wing disk increases the expression of a STAT92E-GFP reporter (arrows). Control in top row: *en::Gal4::UAS-lacZ/STAT92E-GFP*; bottom row: *en::Gal4::UAS-pzg-RNAi/STAT92E-GFP*. Wing disks were stained with anti-Pzg antibodies (red). Posterior is to the right, dorsal is up. (B) The transcript levels of STAT responsive genes *Dox-A3*, *εTry*, and *IM23* were determined by semi-quantitative RT-PCR. The levels were normalized to *β-tubulin* (*tub*). As a control, reactions were performed with (+) and without (–) reverse transcriptase. (C) Pzg can be detected in a complex with the repressor Ken *in vivo*. Proteins immunoprecipitated (IP) from larval protein extracts by anti-Pzg antibodies were probed to detect Ken proteins. The input lane contained 25% of the larval extract used for the IP. A mock control was performed with preimmune serum to demonstrate specificity. (D) XChIP analyses showing that Pzg can be localized at the promoter regions of *Dox-A3*, *εTry*, and *CG5791*, but it is absent from the respective coding regions. Primer sets span regulatory regions that contain predicted Ken and NURF binding sites. Samples of the 31st, 33rd, and 35th PCR amplification cycles are shown. Relative enrichment was estimated for the 33rd PCR cycle sample from the ratio between Pzg IP and mock signals. The mean values and standard deviations of at least three independent experiments were calculated. PCR amplification of regions within the ORF of *Dox-A3*, *εTry*, and *CG5791* were performed as an unrelated control.

manner (HOCHHEIMER *et al.* 2002; KUGLER and NAGEL 2007). The observation that *pzg*^{66/66} mutants can molt to the third instar when fed ecdysteroids otherwise suggests that a reduced ecdysteroid level might be an additional

consequence of the *pzg* lesion. The production of ecdysteroids in arthropods is a process that is not yet completely characterized, involving numerous enzymes needed for the stepwise synthesis of 20-HE from cholesterol (GILBERT 2004; LAFONT *et al.* 2004; GILBERT and WARREN, 2005). While microarray data showed that the expression level of known ecdysone synthetic enzymes is unchanged in *Nurf-301* mutants (BADENHORST *et al.* 2005), a detailed analyses of their transcript levels in a *pzg*^{66/66} background awaits further investigation to decide whether *pzg* might influence EcR signaling at the level of ecdysteroid biosynthesis as well. Such a “multilevel” control of EcR-signaling activity was recently described for members of the histone acetyltransferase complex dATAC in *Drosophila*, emphasizing the importance of chromatin modifying factors in the timely and accurate coordination of metamorphosis control (PANKOTAI *et al.* 2010).

The apparent reduction in ecdysteroid titers in *pzg*^{66/66} larvae could alternatively be caused by impaired growth and/or differentiation of the hormone-producing tissues. So far, only a small number of genes are known to be required for ecdysone production without encoding synthetic enzymes. One example is the *molting defective* (*mld*) gene, whose mutants are developmentally arrested in the first-instar larvae harboring enlarged ring glands. This was interpreted as a consequence of their failure to produce enough hormones and a lack of feedback downregulation of their size (NEUBUESER *et al.* 2005). Like *mld*, *without children* mutants are characterized by an enlargement of the ring gland cells and both genes encode predicted transcription factors with a spectrum of target genes as yet unexplored (WARREN *et al.* 2001; NEUBUESER *et al.* 2005). In contrast, *pzg*-RNAi induction, specifically in the ring gland tissue, had no obvious consequences, neither on the amount nor on the size of the cells studied. However, as the Pzg protein can be detected in the nuclei of wild-type ring gland cells and since a low abundance of *pzg* activity is still detectable after *pzg*-RNAi depletion (data not shown; KUGLER and NAGEL 2007), we cannot completely exclude a subtle function of *pzg* in this context.

As *pzg*^{66/66} mutant larvae display a sluggish and retarded behavior in food uptake we instead favor rather indirect reasons for the impaired ecdysteroid levels. As a sterol auxotrophic organism, *Drosophila* synthesizes ecdysone from dietary sterols (GILBERT *et al.* 2002). Therefore, if food or food uptake is limited too much, the initial trigger for the chain reaction leading to ecdysteroid synthesis might be hampered. Notably, it was recently shown that low-nutrition conditions reduce the activity of the target of rapamycin (TOR) in the prothoracic gland. Consequently, lowered TOR signal activity suppresses ecdysone secretion, a defect that can be rescued either by a reinduction of TOR activity or ecdysone-supplied nutrition (LAYALLE *et al.* 2008). Therefore, further experiments will be required to clarify whether TOR-signaling activity is lowered in *pzg*^{66/66}

mutants, which might explain the “hunger-like” phenotype followed by a reduced ecdysteroid titer.

Pzg and NURF: antagonists of JAK/STAT activity and hematopoietic tumor formation: As a misregulation of JAK/STAT activity is associated with various diseases, including immune disorders and tumorigenesis, the knowledge of its spatial and temporal regulation is of the utmost importance. Consistent with its role in vertebrates, a number of mutant phenotypes in *Drosophila* that imply a developmental role for the JAK/STAT pathway during cellular proliferation have been described. These include hemocyte overproliferation, which can be observed in the dominant gain-of-function JAK allele *hop^{Tum-1}* (HANRATTY and DEAROLF 1993). As a consequence, the differentiation of a specialized class of hemocytes, the lamellocytes, is induced and melanotic tumors are formed (LUO *et al.* 1995). NURF was recently shown to act as an inhibitor of JAK/STAT-signaling activity, thereby antagonizing its tumor-inducing function during hematopoietic development and immune response (KWON *et al.* 2008). In a genome-wide RNAi screening aimed at identifying modulators of JAK/STAT activity in cultured *Drosophila* cells, *pzg*, formerly known as *CG7752*, was already mentioned as being a negative regulator verified by a significant increase of JAK/STAT-signaling activity after *pzg* knockdown (BAEG *et al.* 2005). Here, we provide the molecular evidence to show that Pzg, with NURF, acts as a corepressor of Ken with respect to STAT responsive genes, thereby preventing an immune-mediated inflammatory syndrome, *i.e.*, melanotic tumor formation. The Pzg protein physically interacts with Ken and is present at STAT responsive promoters, as well as at the promoter of a gene (*CG5791*) that is bound by both Ken and NURF alike (KWON *et al.* 2008). In an attempt to visualize increased JAK/STAT activity, particularly in hemocytes, we tried to monitor the expression of the *STAT92E-GFP* reporter in a *hop^{Tum-1}*-sensitized background; however, we failed to detect a specific activity, which went beyond the normal background staining in the wild type (data not shown). Although this reporter was demonstrated to accurately reflect JAK/STAT activity in a variety of tissues (BACH *et al.* 2007), hemocyte-specific induction is obviously more complex to follow. Therefore, we switched our analyses toward the wing disk of third instars, where STAT-GFP expression is known to overlap with the activating ligand *unpaired* that properly surrounds the wing pouch (BACH *et al.* 2007). Using this test system, we obtained an ectopic activation of STAT-GFP in the cells where *pzg-RNAi* was induced (Figure 6A). Although this result is consistent with our idea of *pzg* being a negative regulator of JAK/STAT-signaling activity, how can we explain that an increase in JAK/STAT activity is, in this context, tantamount to a loss of proliferation rather than causing the more expected pro-proliferative effect? This obvious caveat was nicely resolved by the observation that a functional switch of JAK/STAT activity occurs during wing imaginal disk development. In the early larval

stages JAK/STAT activity promotes proliferation, but it also acts as an anti-proliferative factor at later larval stages. This anti-proliferative role is mediated by a yet unidentified noncanonical JAK/STAT pathway (MUKHERJEE *et al.* 2005).

Interestingly, but not unexpectedly, the Pzg–NURF complex can function in the activation as well as in the repression of genes. For example, different ISWI-containing complexes have been published as coactivators and corepressors as well (CORONA *et al.* 2007; BURGIO *et al.* 2008), suggesting that the function of a chromatin complex depends on other factors given in the particular developmental context.

Melanotic tumor formation and innate immunity: A consequence of lowered EcR signaling in *pzg* mutants?: Ample evidence suggests that hormones and nuclear hormone receptors modulate innate immunity in both vertebrates and invertebrates (reviewed in WEBSTER *et al.* 2002; PASCUAL and GLASS 2006; FLATT *et al.* 2008). In insects, most investigations into the hormonal regulation of innate immunity were performed in *Drosophila*, leading to a quite complex and ambivalent picture of their relationship. In *Drosophila* Schneider 2 cells EcR-signaling activity promotes humoral immunity by potentiating the production of antimicrobial peptides such as Diptericin and Drosomycin (FLATT *et al.* 2008). This was further corroborated in the tumorous blood cell line *mbn-2* and in larvae where 20-HE renders the cells and tissue competent for the transcriptional induction of *dipthericin* and *drosomycin* genes (MEISTER and RICHARDS 1996; DIMARCQ *et al.* 1997; SILVERMAN *et al.* 2000). EcR-signaling activity plays a further role in the regulation of hematopoiesis and cellular immunity. In genetic backgrounds where ecdysone signaling is compromised, hemocyte proliferation and differentiation is impaired, resulting in a lowered immune capacity of third-instar larvae (SORRENTINO *et al.* 2002). In accordance, injection of 20-HE in third-instar larvae increases the phagocytic activity of plasmatocytes and thereby their cellular immune response (LANOT *et al.* 2001). In contrast, genome-wide microarray studies performed on *Drosophila* and the silkworm *Bombyx mori* revealed that several immune responsive genes were downregulated by 20-HE in an *EcR*-dependent manner (BECKSTEAD *et al.* 2005; TIAN *et al.* 2010). These observations led to the conclusion that immunity-related genes are part of the EcR-signaling network, presumably positively regulated at the onset of metamorphosis and coordinately downregulated at the larval to pupal transition (BECKSTEAD *et al.* 2005). If indeed the increase in JAK/STAT activity is a direct consequence of the reduced EcR signaling observed in the *pzg* mutant background, we would expect an ectopic activation of ecdysone signaling to reverse this effect. To this end, we concomitantly overexpressed the EcR in a *pzg-RNAi* mutant during wing disk development; however, this did not change the ectopic induction of the

STAT-GFP reporter (Figure S3A). Moreover, feeding *hop^{Tum-1/+}; pzg^{66/+}* mutant larvae with 20-HE did not change the observed incidence of lamellocytes, as would have been predicted if the reduced EcR-signaling activity is the basic cause of this effect (Figure S3B). Therefore, we have no experimental evidence to show that impaired EcR-signaling activity directly provokes tumor formation in *pzg* mutants.

We thank P. Badenhorst, C. Mirth, the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank (DSHB) for flies and antibodies. We are indebted to M. Mezger for the *UAS-pzg* transgenics and A. Preiss, D. Maier, and C. Protzer for their help during jump-out mutagenesis. We appreciate the excellent technical assistance of H. Mastel, T. Stösser, and I. Wech. We thank A. Huber (Department of Biosensorik) for kindly letting us use the ApoTome microscope. We are grateful to A. Preiss for critical comments on the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft through a grant to A.C.N. (NA 427/2-1).

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Communicating editor: T. SCHUPBACH

GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.111.127795/DC1>

The Putzig-NURF Nucleosome Remodeling Complex Is Required for Ecdysone Receptor Signaling and Innate Immunity in *Drosophila melanogaster*

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DOI: 10.1534/genetics.111.127795

FIGURE S1.—Generation and verification of the *pzg*^{66/66} mutant. (A) For description of the graph see Figure 1A. For the verification of potential *pzg* deletion mutants PCR analyses were performed, using the primer set of KGbodyUP and either lower primer LPIII, LPIV or LPV (small arrows). For Southern blot analyses, a *Bgl*III restriction digest was performed (sites are numbered 1-5 in red), the probe spans the *pzg*⁶⁶ deletion (green). (B) Results of two different approaches (1, 2) of PCR amplification on *pzg*⁶⁶. PCR using LPIII produced no amplificate, because it lies within the *pzg*⁶⁶ deletion. PCR amplification with LPIV and LPV primers resulted in DNA fragments of 0.95 kb and 2.3 kb, respectively, which were subsequently sequenced for exact break point analysis. (C) Southern Blot analysis, compare with A). *Bgl*III digestion produced a 4.9 kb fragment (1-5) in wild-type (WT) that is also detected in *pzg*⁶⁶ heterozygotes (*pzg*⁶⁶/+). In the *P* element line *P{SUPor-P}KG04911* (KG), two DNA fragments of 1.7 kb (1-2) and 8.8 kb (4-5) appeared. The smaller fragment (1-2, 1.7 kb) also occurred in the heterozygous *pzg*⁶⁶ line (*pzg*⁶⁶/+), as neither the upstream region of the *pzg* gene nor the 3' region of the *P* element were affected. However, the deletion in the *pzg*⁶⁶ line caused a loss of *Bgl*III restriction sites 3 and 4, resulting in the novel fragment 2-5 (6.7 kb).

FIGURE S2.—Genotyping of rescued *pzg^{66/66}* animals. (A) For description of the graph see Figure 1A. Position of the *pzg2* primer pair is indicated by arrows. *pzg2UP* lies within the 5' region deleted in the *pzg⁶⁶* allele; *pzg2LP* anneals 3' of the first intron within the *pzg* gene. Hence, the amplification product in the mutant and the wild-type provides a fragment that is 60 bp larger than the transgenic construct, which was derived from cDNA. (B, C) Genomic DNA of single animals was extracted and used for PCR analysis. (B) Wild-type (WT) and *da-Gal4 pzg⁶⁶/TM6B* larvae were used as controls: as expected, a 0.35 kb DNA fragment was obtained in a PCR reaction with *pzg2* primers. An example of a homozygous mutant rescued by transgene expression is shown (*UAS-pzg/UAS-pzg; da-Gal4 pzg⁶⁶/da-Gal4 pzg⁶⁶*): here, a 0.29 kb DNA fragment is produced only from the cDNA-derived transgene. (C) Examples of heterozygous (htz, TM6B *Tb*) and homozygous mutant (hz) larvae, prepupae and pupae are shown. The latter are rescued by the transgene. Genotyping by PCR clearly confirmed classification. The genotype is each *UAS-pzg/UAS-pzg; da-Gal4 pzg⁶⁶/TM6B*.

FIGURE S3.—Increased JAK/STAT activity is not a direct consequence of reduced EcR signaling in *pzg* mutant tissues. (A) Concomitant induction of EcR activity and *pzg*-RNAi in the posterior half of the wing disk does not affect the induction of the *STAT92E*-GFP reporter depicting JAK/STAT signaling activity. Genotypes shown: *en-Gal4/STAT92E-GFP; UAS-lacZ/+* (first row), *en-Gal4/STAT92E-GFP; UAS-EcR/+* (second row), *en-Gal4/STAT92E-GFP; UAS-pzg-RNAi/+* (third row), *en-Gal4/STAT92E-GFP; UAS-EcR/UAS-pzg-RNAi* (fourth row). *STAT92E*-GFP shown in green, anti-EcR in red, anti-Pzg in blue. (B) Lamellocyte incidence in *hop^{Tum¹}/+; pzg⁶⁶/+* hemocytes did not change after feeding the larvae with 20-HE. Arrows point to large lamellocytes. Cells are visualized with rhodamine-coupled Phalloidin (red); nuclei are stained with DAPI (blue).

TABLE S1
Primer pairs used in this study

RT-PCR primer pairs	sequence 5' → 3'	Ann. temp.	PCR product length
Dox-A3UP	TCGAGTGTCAACATTCCGTTTCGAGCGC	63.9°C	390bp
Dox-A3LP	ACCTCGCGAAGCTTCTCGGTGCGGT		
eTryUP	TGTCCACTCCGTTTCGCTCCTTC	60.8°C	535bp
eTryLP	ATATCAATGCTGATTCAATTGCGATTATTT		
EcRUP	TTGTAAGAATCCCGCGTATATGATCTATTATT	55.4°C	533bp
EcRLP	AATGACCACTTCGCCGAGCTCT		
Eig71EaUP	CATTTATGCATTAGTGGATAACTATTAAGTA	53.0°C	345bp
Eig71EaLP	AGAGTTTAAACTACAATAATGCGCC		
IM23UP	TGCCTGATTCTGTCTTTTGAATT	58.2°C	494bp
IM23LP	CAGATTTTTTAAAGAATTATAAATTATGCCCG		
ImpE2UP	GGCAATCAGTTCCAGAACACCCGTGTCCAGC	63.1°C	415bp
ImpE2LP	CGCCGGCATCGTCTGTTGACCACAATA		
pzg1UP	ACAAGAATGCCATTAACGAGGA	60.2°C	515bp
pzg1LP	TACATTGGTAGCGTGATTCTGTCTC		
Tub56DUP	GAACCTACCACGGTGACAGCGA	65.2°C	300bp
Tub56DLP	GGAAGCCAAGCAGGCAGTCGCA		
XChIP primer pairs	sequence 5' → 3'	Ann. temp.	PCR product length
CG5791-5'-UP	GCCATTCATGTTTACGTCTGG	51.4°C	206bp
CG5791-5'-LP	AGCTTTTCGATTGTATTTGAACAA		
CG5791-ORF-UP	CCGTTGGCTTTAATTCCGACTCT	57.3°C	342bp
CG5791-ORF-LP	ACCCCTCCGGTAAATGGTGC		
Dox-A3-5'-UP	AGTTAATACCCAACCTCGAGCGTCATGT	59.1°C	433bp
Dox-A3-5'-LP	GGAGTATCCGCGCTCAGATTCAA		
eTry-5'-UP	ATGGCCTTGATGGTGGAGCTGAA	60.5°C	224bp
eTry-5'-LP	TACTCCGCCAGGGTGATCGTGA		
eTry-ORF-UP	TACTTCTTTCCGTTTTTGGCCTGCG	61.2°C	414bp
eTry-ORF-LP	CACGAGGGTTGGAGTCGGCAATA		

EcR-5'-UP	TGGTAAATGTTGTAACTTTTCGGT	50.8°C	432bp
EcR-5'-LP	CAATTTCTCAGAGTGGA AAAACTG		
EcR-ORF-UP	CTTCATGCGCCTACCGGAGGAGT	61.9°C	413bp
EcR-ORF-LP	AGTTGATGAGGCCGTGGCCATTC		
Eig71Ea-5'-UP	TCCTTGAGCCCAAACATGAGCTTTTATAAA	53.5°C	291bp
Eig71Ea-5'-LP	CTCACTACAGAAGTTTCCGATCCA ACTGG		
Eig71Ea-ORF-UP	CTAAGTTATTTTATTTGTGCATACAATTCAA	51.4°C	330bp
Eig71Ea-ORF-LP	TGTCGTATGATTATCACAATGAAAAC		
hsp27EcRE-UP	GCAACAGACTGGCAATGATTT	52.6°C	187bp
hsp27EcRE-LP	TCAGAGTGCAACAGAGCTTGTAT		
(BADENHORST <i>et al.</i> 2005)			
Other primer pairs	sequence 5' → 3'	Ann. temp.	PCR product length*
KGbodyUP	GGCTTATTAAAAGACACAC	52.5°C	
LPIII	TCCACATCGTTTTCCAGGC		8,246kb (224bp)
LPIV	TCCGTTGACTTGA ACTGGCA		8,966kb (944bp)
LPV	TGTATTTGCTCGGGATCTTG		10,346kb (2,324kb)
pzg2UP	CTGGTGAACTACTACGACCGCCTGGA	59.3°C	cDNA: 288bp
pzg2LP	TTGGACTGTCTGGGTGCCTTGGAC		DNA: 348bp

* Length of amplificate refers to PCR of wild type or pzg^{66/66} mutant (brackets) tissue.
Ann. temp. = Annealing temperature

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