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Developmental Roles of the Mi-2/NURD-Associated Protein p66 in Drosophila

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ABSTRACT

The NURD and Sin3 histone deacetylase complexes are involved in transcriptional repression through global deacetylation of chromatin. Both complexes contain many different components that may control how histone deacetylase complexes are regulated and interact with other transcription factors. In a genetic screen for modifiers of wingless signaling in the Drosophila eye, we isolated mutations in the Drosophila homolog of *p66*, a protein previously purified as part of the Xenopus NURD/Mi-2 complex. *p66* encodes a highly conserved nuclear zinc-finger protein that is required for development and we propose that the p66 protein acts as a regulatory component of the NURD complex. Animals homozygous mutant for *p66* display defects during metamorphosis possibly caused by misregulation of ecdysone-regulated expression. Although heterozygosity for *p66* enhances a *wingless* phenotype in the eye, loss-of-function clones in the wing and the eye discs do not have any detectable phenotype, possibly due to redundancy with the Sin3 complex. Overexpression of *p66*, on the other hand, can repress *wingless*-dependent phenotypes. Furthermore, *p66* expression can repress multiple reporters in a cell culture assay, including a Wnt-responsive TCF reporter construct, implicating the NURD complex in repression of Wnt target genes. By co-immunoprecipitation, p66 associates with dMi-2, a known NURD complex member.

A key event in most signal transduction pathways is the activation or repression of target genes in the nucleus by transcriptional regulators. In recent years, it has become evident that these transcription factors interact with chromatin and that regulation of chromatin structure plays an important role in controlling gene expression. One important mechanism for regulating chromatin structure involves histone acetylation/deacetylation. Histone acetylases are implicated in transcriptional activation while histone deacetylases are involved in repression (Kadosh and Struhl 1998; Kuo *et al.* 1998; Wang *et al.* 1998).

One of the major histone deacetylase (HDAC) complexes is the NURD complex (reviewed in Ahringer 2000). The complex has been purified from mammalian and Xenopus cells and homologs have been identified in *Caenorhabditis elegans*, Arabidopsis, and Drosophila, suggesting that the complex is conserved throughout plants and animals (Tong *et al.* 1998; Wade *et al.* 1998; Xue *et al.* 1998; Zhang *et al.* 1998, 1999; Ahringer 2000). The NURD complex is ~2 MD in size and is composed of eight proteins (reviewed in Ahringer 2000; Ng and Bird 2000). The histone deacetylases HDAC1 and HDAC2 and two histone-binding proteins, RbAp48 and RbAp46, are found in the NURD complex as well as in the other predominant cellular HDAC complex, the Sin3 complex. In addition to these proteins, the other proposed members of the NURD complex are Mi-2, MBD3, MTA1, and p66 (Tong *et al.* 1998; Wade *et al.* 1998, 1999; Xue *et al.* 1998; Zhang *et al.* 1998, 1999; Brackertz *et al.* 2002; Feng *et al.* 2002).

Recently, there has been evidence that the NURD complex is involved in a variety of developmental functions. These include embryonic patterning in Drosophila, *C. elegans*, and mice, repression by Polycomb proteins in Drosophila, and repression in mouse T-cell development (Ahringer 2000). The NURD complex as well as the Sin3 complex has also been implicated in repression by unliganded nuclear hormone receptors (Heinzel *et al.* 1997; Xue *et al.* 1998). In chromatin immunoprecipitation assays, both the Sin3 and the NURD complex were found to be associated with the Xenopus thyroid hormone receptor (TR)β A gene promoter region, suggesting that they could function redundantly to repress transcription (Li *et al.* 2002). In Drosophila, *dSin3A* regulates transcription mediated by the ecdysone nuclear hormone receptor (*EcR*), but there are no data implicating the NURD complex in regulation of ecdysone responses (Tsai *et al.* 1999).

The NURD complex has also been implicated in Wnt signaling in Drosophila where it may be involved in

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repression by TCF. dMi-2 is found in a complex with the histone deacetylase Rpd3, which in turn interacts with Groucho, a corepressor for many proteins, including the TCF/LEF-1 family of proteins (Cavallo *et al.* 1998; Chen *et al.* 1999; Brehm *et al.* 2000). In the absence of a Wnt signal, TCF is associated with the corepressors Groucho and CtBP and represses transcription (Cavallo *et al.* 1998; Valenta *et al.* 2003). When the Wnt signal is received, the downstream effect is the stabilization of β-catenin protein, which can then translocate into the nucleus where it forms a complex with TCF to activate transcription of target genes (Brunner *et al.* 1997; van de Wetering *et al.* 1997). Presumably, β-catenin disrupts the association of TCF with its corepressors so that it can now activate transcription. The mechanism of activation by TCF and β-catenin is not well understood, but recent evidence suggests that histone acetylation is important for this process (Wade *et al.* 1999; Billin *et al.* 2000; Hecht *et al.* 2000; Takemaru and Moon 2000). In addition, mutations in the *C. elegans* NURD component *egl-27* cause Wnt-like phenotypes in *C. elegans* (Herman *et al.* 1999), providing another link between Wnt signaling and the NURD complex.

In this study we report the identification and characterization of the Drosophila homolog of p66, a protein associated with the NURD complex. p66 is a nuclear protein that represses *wingless* (Wg) target genes and plays an essential role in development through the regulation of ecdysone-responsive genes. These results highlight the requirements for p66 and the NURD complex in regulating developmental decisions.

MATERIALS AND METHODS

Fly stocks: The UAS-p66 construct was made by inserting the *Apa*I (blunted)-*Sma*I insert from LD18074 into the *Bgl*II (blunted) site in the UAS vector. Transgenic lines were established using standard methods. For the screen, the following stocks were used: P[*sev-wg*^{ts}] (Cadigan *et al.* 2002), P[*sev-ras1*^{V12}] (Karim *et al.* 1996), and P[*sev-ras1*^{N17}] (Allard *et al.* 1996). The following lines were used for the rescue experiment and for overexpression studies: arm-GAL4 (Sanson *et al.* 1996), hs-GAL4 (Kraus and Lis 1994), da-GAL4 (Wodarz *et al.* 1995), ptc-GAL4 (Ingham and Fietz 1995), TM3 P[Kr-GFP] (Casso *et al.* 1999), UAS-p66, and UAS-lacZ (Brand and Perrimon 1993). For germline clone mosaics, mutants were recombined onto P[FRT]2A chromosomes. For somatic mosaic clones, mutants were recombined onto either P[FRT]2A chromosomes or P[FRT]80B chromosomes. Imprecise excisions were generated from P l(3)01814[j4A5] (from L. and Y. Jan collection) using standard methods.

To test the phase during which p66 absence is lethal, we crossed *p66* alleles A and L/TM3, *sb* to *sb*/TM3, *Ser*, actin-GFP. Sibling crosses were made from adults carrying the Ser marker and expanded into stocks. GFP-positive or -negative second instar larvae were collected from embryo collections of *p66*/TM3, *Ser*, and actin-GFP. Forty larvae were placed into each of three small vials containing food and allowed to develop. The number of pupae in each vial was counted ~9 days later. Dead pupae were dissected from their pupal cases and examined under a dissecting microscope.

Genetic screen and scanning electron microscopy pictures: The genetic screen was performed as previously described (Cadigan *et al.* 2002). Flies were prepared for scanning electron microscopy as previously described (Cadigan and Nusse 1996). The samples were viewed with an AMR1000 SEM and photographed using Polapan 400 film (Kodak).

Germline mosaics: Germline mosaics were generated using the autosomal FLP-dominant female sterile technique as previously described (Chou and Perrimon 1996). w; *p66*^{A} P[FRT]2A or w; *p66*^{J} P[FRT]2A females were crossed to ywP[FLP]22; P[ovoD^{1}]3L P[FRT]2A males. The progeny from this cross were heat-shocked at late third instar for 1 hr at 37°. Mosaic mothers were crossed to w; *p66*^{7}/TM3 P[Kr-GFP] males.

Generation and staining of somatic mosaic clones: These mosaics were generated with the FLP-FRT system as previously described (Xu and Rubin 1993). p66^{-} P[FRT]2A or p66^{-} P[FRT]80 flies were crossed to hs-FLP; P[pi-myc], P[FRT80B], or hs-FLP; P[pi-myc] P [FRT]2A. Clones were induced by heat shock of 24- to 48-hr after-egg-laying progeny at 37° for 1 hr. For Ac and Dll staining, *p66*^{16} P[FRT]80B stock was used. For examination of phenotypes in adult wings and legs, the stocks used were *p66*^{J} P[FRT]2A, *p66*^{A} P[FRT]2A, and p66^{16} P[FRT]80B.

Overexpression of p66: UAS-p6618 were crossed to Ptc-GAL4 flies. Discs were dissected and fixed as above. Adult flies were raised at 25° and 29°.

***In situ* hybridization and whole-mount immunostaining:** *p66 in situ* hybridization and staining was done as previously described (Cadigan and Nusse 1996), as was staining of imaginal discs (Cadigan *et al.* 1998). The primary antibodies were used at the following dilutions: rat anti-Wg, 1:200; rabbit anti-Wg, 1:8; rabbit anti-lacZ (Cappel), 1:250; and anti-myc, 1:5 (S. Blair, University of Wisconsin); anti-Ac, 1:4 (Developmental Studies Hybridoma Bank); anti-Dll, 1:150 (G. Panganiban); anti-rat p66, 1:50; and anti-rabbit p66, 1:50. For fluorescence microscopy, antibodies were used at the following dilutions: donkey Alexa anti-mouse, 1:1000 (Molecular Probes, Eugene, OR), and donkey Cy3 anti-rabbit, 1:200 (Jackson Immunochemicals). Confocal images were collected with a Bio-Rad (Richmond, CA) MRC 1000 confocal laser setup attached to a Zeiss Axioscope microscope.

Generation of anti-p66 antibody: p66-C protein (amino acids 873–917) was generated as follows: LD18074 was digested with *Xho*I and RI. The 250-bp fragment corresponding to amino acids 873–917 was ligated into the *Xho*I/RI sites of PGEX4-T3 (Pharmacia). p66-N protein (amino acids 116–303) was generated by digesting LD18074 with *Bam*HI and *Not*I. The 0.5-kb fragment corresponding to amino acids 116–303 was ligated into the *Bam*HI/*Not*I sites of PGEX4-T3 (Pharmacia). GST-fusion proteins were expressed in BL-21 bacteria and bound to glutathione sepharose beads. After washing, proteins were eluted in elution buffer (0.1% Triton, 200 mm NaCl, 50 mm Tris, pH 9.0). The p66-C protein was injected into rabbits and the p66-N protein was injected into rats (Josman Labs).

Cloning of the *p66* gene: The analysis of the *p66* gene and the subsequent isolation of its cDNA were performed using standard molecular biological techniques. The *p66* cDNA was isolated from a 0 to 5-hr cDNA library from wild-type embryos (Brown and Kafatos 1988) and is identical in sequence to EST LD18074 (Berkeley *Drosophila* Genome Project).

Rescue of the larval lethal phenotype: w; P[UAS-p66]/P[UAS-p66]; *p66*^{10}/TM6, Tb was crossed to w; arm-gal4/arm-gal4; *p66*^{7}/TM6, Tb. The progeny were reared at 18° and scored for the presence of non-Tubby pupae. As a control, w; +/+; *p66*^{10}/TM6, Tb was crossed to w; arm-gal4/arm-gal4; *p66*^{7}/TM6, Tb. Two independent transgenic lines were used. Significancy was tested by means of the χ^2 test.

RNA isolation and Northern blot: Larvae were staged as previously described (Andres and Thummel 1994). RNA was collected using Trizol following the manufacturer's protocol (Invitrogen, San Diego). Northern blot was performed using standard methods. The presence of equal amounts of ribosomal RNA, visualized by ethidium bromide staining, served as loading control.

Immunoprecipitation: An overnight collection of embryos was dechorionated then resuspended in 10× volume of TNT buffer (1% Triton, 50 mm Tris, pH 7.5, 150 mm NaCl). Embryos were then homogenized using a glass douncer and centrifuged at 14,000 rpm for 5 min. A total of 100 μl of the supernatant was first precleared by the addition of protein-G sepharose for 1 hr. Subsequently, 3 μl of affinity-purified anti-rabbit antibody (p66-C) was added to the precleared supernatant and rotated at 4° for 4 hr. A total of 20 μl of a 1:1 protein G sepharose/TNT slurry was added and rotated for an additional 2 hr. The complex was spun down and washed three times in TNT. Immunoprecipitates were then analyzed by Western blotting. For Western, anti-dMi-2N was used at a concentration of 1:10,000 and anti-p66 rat (p66-N) was used at 1:1000.

Plasmids: pp66myc was generated by cloning the *p66* gene in frame with myc in pBSmyc. LD18074 was digested with *Xba*I and *Stu*I. The fragment containing the coding region was ligated into pBSmyc, which had been digested with *Eco*RI, treated with Klenow, and then digested with *Xba*I. The resulting plasmid, pp66myc, was then digested with *Xba*I and *Hin*dIII. The fragment containing the *p66* gene was then ligated into the *Hin*dIII and *Xba*I sites of prk5SK to generate p66mycprk5.

Cell culture and luciferase assays: A total 293 cells were maintained in Dulbecco minimal essential medium + 10% fetal bovine serum (Gemini) supplemented with penicillin and streptomycin. Cells were passaged every 2–3 days. For luciferase assays, 3×10^5 cells were plated in 60-mm wells and 1 μg of total DNA was transfected using Superfect (QIAGEN, Chatsworth, CA) according to protocol. For β-catenin activation assays, transfections consisted of 0.1 μg of optimized top flash reporter (OT) (Klymkowsky *et al.* 1999) and 0.1 μg of EF1α-βgal (gift from G. Crabtree) and may have included 0.2 μg of EF1α-β-catenin, as well as varying amounts of p66mycprk5. CMV-gfp was added as necessary to keep the total amount of DNA in each transfection constant. Cells were lysed 48 hr after transfection, and luciferase and β-galactosidase assays were carried out according to protocol (Tropix, Promega, Madison, WI). For SRE reporter assays, transfections consisted of 0.1 μg of the SRE reporter, 0.2 μg of M1 receptor, 0.1 μg of EF1α-βgal, and if p66 was included, 0.6 μg of p66myprk5. CMV-gfp was added as necessary to keep the total amount of DNA in each transfection constant. Following transfection using Superfect (QIAGEN), cells were kept in DMEM + 0.5% FBS. Twenty-four hours post-transfection, cells were stimulated with carbachol (5 μm, as indicated) for 6 hr and lysed according to protocol for assays. For the NF-AT reporter assay, cells were transfected with 0.2 μg of NF-AT luciferase reporter, 0.1 μg of EF1α-βgal, and if p66 was included, 0.6 μg of p66mycprk5. CMV-gfp was added as necessary to keep the total amount of DNA in each transfection constant. Twenty-four hours after transfection, cells were stimulated with ionomycin (1 μm), phorbol 12-myristate 13-acetate (PMA, 25 ng/ml), and calcium chloride (10 mm). After 24 hr, cells were lysed for assays. Data shown are from one representative experiment. All measurements were done in duplicate.

RESULTS

***p66* enhances the P[*sev-wg*^{ts}] phenotype in the adult eye:** *wg* is required during many steps in Drosophila development and controls a wide range of patterning events (reviewed in Klingensmith and Nusse 1994). To search for novel genes interacting with *wg*, we performed a genetic screen to isolate dominant modifiers of a bristle phenotype caused by ectopic expression of *wg* in the eye. This screen has been described in more detail elsewhere (Cadigan *et al.* 2002), but briefly, we expressed a temperature-sensitive allele of *wg*, *wg*^{IL114}, under the control of the *sevenless* promoter (*sev-wg*^{ts}) at an intermediate temperature (17.6°) that allows partial *wg* function (Figure 1, A and B). Whereas a wild-type *wg* allele expressed using this promoter almost completely blocks bristle formation, expression of the temperature-sensitive allele at an intermediate temperature allows formation of approximately one-third of the wild-type number of bristles (Figure 1B) (Cadigan *et al.* 2002).

We identified two dominant enhancers on the third chromosome that formed a lethal complementation group, which we named *p66*, due to its homology with p66 of the Mi-2/NURD complex (see below). The majority of P[*sev-wg*^{ts}]/+ eyes contained 100–150 bristles. In contrast, flies that are additionally heterozygous for either of two independent *p66* mutations, *p66*^{10} and *p66*^{7}, have a reduced number of bristles (<100). These eyes otherwise appeared normal (Figure 1, C–E).

To determine if the mutations affected the *sevenless* promoter rather than *wg* function, we tested whether the loss of one copy of *p66* modified two other transgenes under the control of the *sevenless* promoter, a dominant negative *ras* (*sev-Ras1*^{N17}) and an activated *ras* (*sev-Ras1*^{V12}). *sev-Ras1*^{V12} expression results in a rough eye caused by extra R7 cells (Karim *et al.* 1996). Similarly, *sev-Ras1*^{N17} also has a rough eye phenotype; however, it is due to loss of R7 cells (Allard *et al.* 1996). Removal of one copy of *p66* did not have an effect on either the *sev-Ras1*^{V12} or the *sev-Ras1*^{N17} phenotype (data not shown). This suggests that *p66* does not affect the expression of the transgene through the *sevenless* promoter, but rather affects Wg signaling or Wg targets. Because p66 was identified as a copurifying protein with the Mi-2/NURD complex (see below), we also tested whether *dMi-2* (Kehle *et al.* 1998) has an effect on the *sev-wg*^{ts} phenotype. We found that loss of one copy of *dMi-2* (*dMi-2*^{6}) also causes a reduction in the number of bristles in a *sev-wg*^{ts} (Figure 1E).

More alleles of *p66* and phenotypes in homozygous mutant flies: We found that the deficiency Df(3L)Lxd6 did not complement the *p66*^{10} or the *p66*^{7} mutation. In addition, *P* element [j4A5], which maps within this deficiency, also failed to complement either *p66* allele. Precise excision of the *P* element reverted the lethality, demonstrating that the *P* element was responsible for the phenotype. Using imprecise excision, we created a new allele, *p66*^{16}. In addition, by screening for noncomplementing lethality over existing *p66* alleles, we isolated five new alleles using EMS, *p66*^{A}, *p66*^{H}, *p66*^{J}, *p66*^{L}, and *p66*^{S}. To determine the strength of each allele, we conducted complementation crosses and ordered the al-

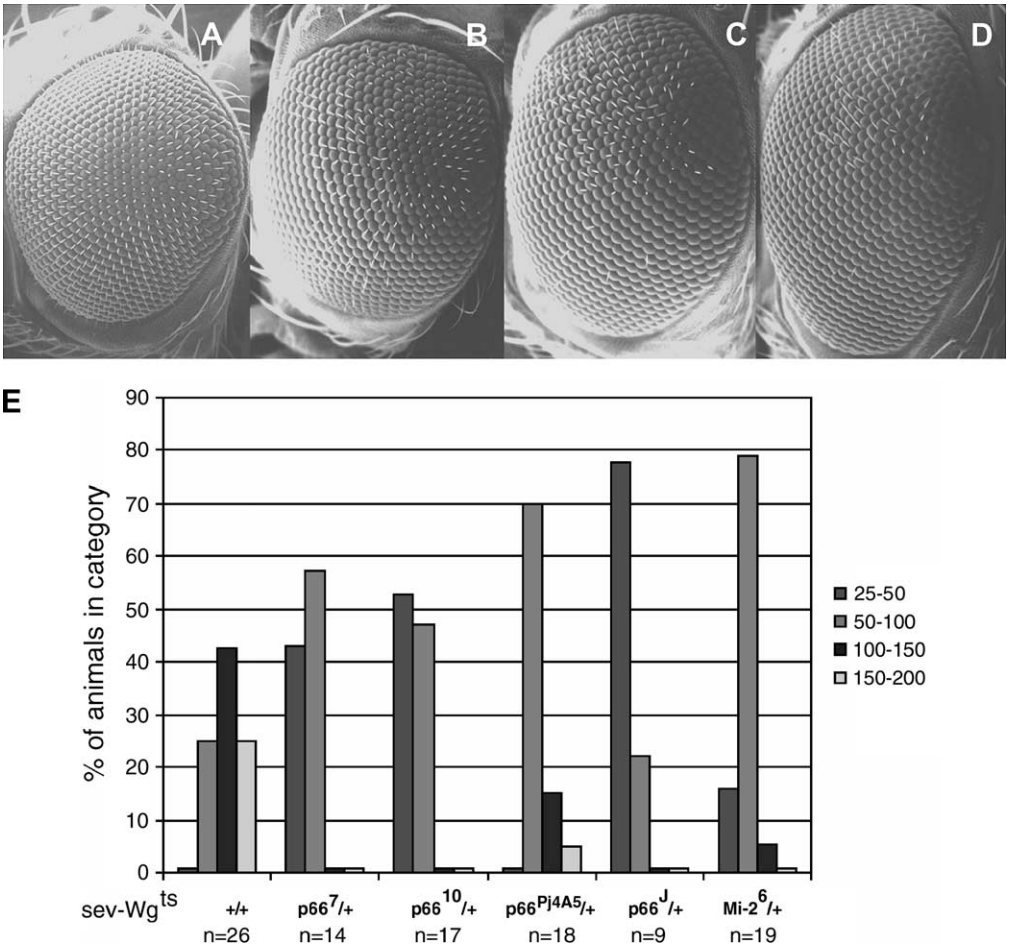


FIGURE 1.—*p66* mutations enhance *sev-wg*^{ts}. Scanning EM images of (A) wild type, (B) P[*sev-wg*^{ts}]/+, (C) P[*sev-wg*^{ts}]/+; *p66*^{10}/+, and (D) P[*sev-wg*^{ts}]/+; *p66*^{7}/+ eyes. All flies were grown at 17.6°. Anterior is to the left; dorsal side is at the top. A wild-type *wg* allele expressed using the *sevenless* promoter represses all bristle formation. P[*sev-wg*^{ts}] animals reared at 17.6° have a reduced number. P[*sev-wg*^{ts}] flies in the background of either *p66* mutation have fewer bristles than P[*sev-wg*^{ts}]. (E) *p66* and *dMi-2* mutations enhance the *sev-wg*^{ts} phenotype. The majority of *sev-wg*^{ts} flies in a +/+ background have 100–150 bristles. Removal of one copy of *p66* gene enhances the phenotype; note a shift to lower bristle numbers. Removal of one copy of *dMi-2* also enhances the phenotype.

leles on the basis of progression of development. On the basis of these results, the order of alleles is (from weak to strong): *p66*^{16}, *p66*^{7}, *p66*^{L}, *p66*^{S}, *p66*^{H}, *p66*^{J} = *p66*^{10} = *p66*^{A} = *p66*^{Lxd6}.

In all heteroallelic crosses, we observed survival through embryogenesis into larval stages. Strong allele combinations, *p66*^{J}/*p66*^{A} or *p66*^{J}/*p66*^{Lxd6}, died during second or third instar larval stages. In weaker allele combinations, mutants developed to the prepupal stage, but did not progress to the pupal stage. Numbers ranged from 30 of 124 (total) larvae reaching pupal stages for weak alleles (using GFP balancers and counting larvae that crawled out of the food) to 0 of 101 (total) for strong alleles. *Trans*-heterozygotes of weak alleles, such as *p66*^{16}/*p66*^{7} or *p66*^{L}/*p66*^{16}, survived to late pupal stages. Upon examination, the pupae appeared almost wild type but displayed a variety of phenotypes. Mutant prepupae develop to stage P3 where the dorsal air pocket forms; however, the prepupae do not progress to stage P4 as no withdrawal of pupae to the posterior end is evident. These mutant pupae have defects including shortened or bent legs, bristle defects, a split notum, and wing abnormalities (Figure 2, A–E). Some mutants exhibit necrosis near the head region (Figure 2, G and H). Other mutants lacked pigmentation (Figure 2, C, E, and G). In addition, the cases surrounding the prepupae were filled with white fluid, possibly due to incomplete histolysis of the fat bodies (Figure 2I). In some mutants, accumulation of white fluid caused the pharate adult to stick to the pupal case, possibly preventing the otherwise normal adult from eclosing (not shown).

Some of the *p66* mutants displayed phenotypes similar to defects in ecdysone responses such as *ultraspiracle* and *ftz-f1* (HENRICH *et al.* 1994; BROADUS *et al.* 1999). Moreover, mutations in *bonus*, a gene encoding a homolog of the vertebrate TIF1 transcriptional cofactor, lead to defects in leg elongation, bristle development, and pigmentation. The bonus protein can bind to nuclear receptor proteins. Likewise, mutations in *crooked leg*, a gene induced by ecdysone, lead to leg defects similar to those seen in p66 (D'AVINO and THUMMEL 1998). To follow up on a possible connection between *p66* and ecdysone signaling, we examined ecdysone-regulated gene expression in the mutants.

Metamorphosis is initiated by a pulse of ecdysone, which activates a hierarchy of gene expression (reviewed in RIDDIFORD 1993). One of the genes induced in the early puff is *E74*, an ETS domain transcription factor

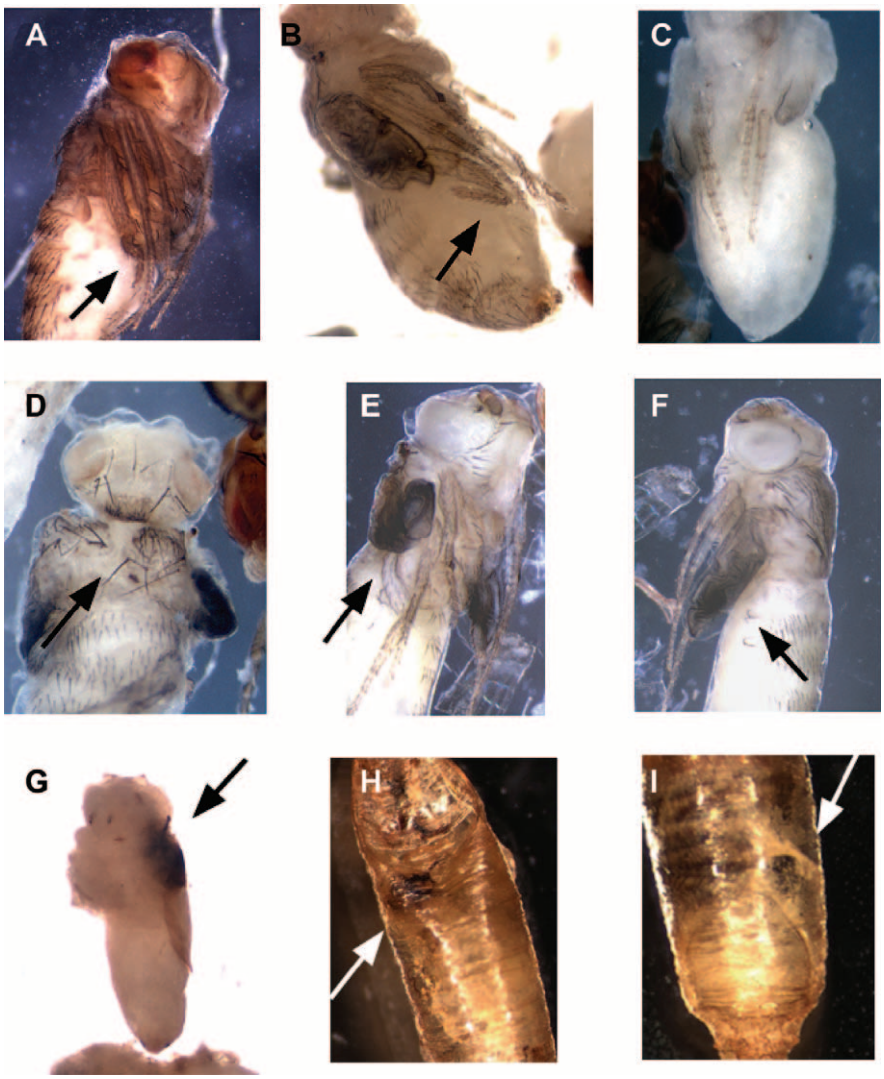


FIGURE 2.—*p66* mutants exhibit defects during pupal stage. Defects are indicated by arrows in A–I. (A) $p66^{L}/p66^{16}$ pupa has short third leg. (B) $p66^{16}/dp66^{7}$ pupa has third leg bent back. (C) $p66^{16}/p66^{7}$ pupa has well-formed legs, and wings have bristles and are darkened. In contrast, the abdominal region lacks pigmentation and bristles. (D) $p66^{16}/p66^{7}$ pupa has a split notum. (E) $p66^{16}/p66^{7}$ pupa has a folded right wing; compare to F. (F) Left wing of mutant in E appears normal. (G) $p66^{A}/p66^{J}$ pupa; note necrosis near head region. (H) $p66^{A}/p66^{L}$ pupa; note necrosis near head region. (I) $p66^{7}/p66^{S}$ pupa; note white fluid in pupal case.

(ASHBURNER *et al.* 1974; BURTIS *et al.* 1990). In wild-type animals, *E74* was induced normally at the larval/prepupal transition. In contrast, we did not detect any E74 expression in the mutant animals (Figure 3). We also examined the expression of DHR3, an orphan receptor that is also induced directly by ecdysone (KOELLE *et al.* 1992; HORNER *et al.* 1995). DHR3 expression peaks at the prepupal stage just as early genes such as E74 are repressed (KOELLE *et al.* 1992). Similar to E74 expression, DHR3 was expressed at lower levels in mutant animals, indicating a defect in activation of genes in response to ecdysone (Figure 3).

p66 appears to be maternally contributed because a *p66* transcript is detected in 0- to 2-hr embryos on a Northern blot and in early embryos by *in situ* hybridization (Northern blot not shown; *in situ* hybridization in Figure 7A). However, *p66* does not appear to be required for embryogenesis, because *p66* mutant germline clone embryos that lack both maternal and zygotic p66 function survive to larval or pupal stages. To examine adult phenotypes of *p66*, we made homozygous loss-of-function clones in imaginal discs. Mutant clones for all alleles appeared to be roughly equal in size to their twin spots, indicating that *p66* is not essential for cell viability or cell growth. This is shown for two alleles, $p66^{16}$, a weak allele, and $p66^{10}$, a strong allele (Figure 4, C and F). Figure 4G also shows that clones mutant for $p66^{10}$ lack staining for P66 protein.

Because we hypothesized that *p66* repressed downstream targets of *wingless* (Wg) signaling, we examined two targets of Wg in the wing disc, Distal-less (Dll) and Achaete (Ac) in $p66^{16}$ clones. Removal of *p66* did not affect expression of either gene (shown for Dll in Figure 4, A–E). In addition, mutant clones of the strong alleles $p66^{J}$ and $p66^{A}$ did not display any phenotype in adult wings or eyes (data not shown). Thus, we have been unable to find a loss-of-function phenotype in discs, which we attribute to possible redundancy with other histone deacetylase complexes (see DISCUSSION).

Cloning of the *p66* gene: The $p66^{10}$ and $p66^{7}$ mutations were mapped by recombination and deficiency complementation to 67E-F. Three lethal *P*-element lines

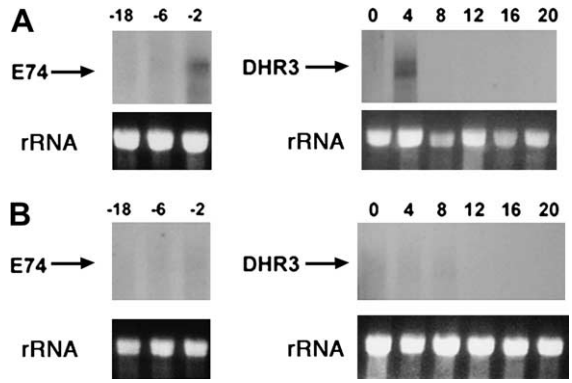


FIGURE 3.—Northern blot analysis of gene expression. Total RNA was collected from staged wild-type and $p66^{7}/p66^{A}$ animals and analyzed by Northern blot. Numbers at the top are hours pre- and postpuparation at 22°. In wild-type animals, *E74* expression is induced at the larval/prepupal transition (−2) and DHR3 levels are highest at the 4-hr time point (A). In *p66* mutant animals (B), expression of E74 and DHR3 are reduced when compared with wild type. Total RNA visualized by ethidium bromide staining was used as loading control (bottom).

also mapped to 67E-F and did not complement $p66^{10}$ (Figure 5A). Using the genomic DNA flanking these *P* elements, we detected 6.4- and 4.7-kb transcripts on a Northern blot (data not shown) and isolated a 3.5-kb cDNA that contained an open reading frame of 2751 nucleotides (CG32067). We sequenced the gene corresponding to the cDNA in the $p66^{L}$ mutant and detected a mutation (G > A) in a conserved exon/intron boundary. The mutant transcript would result in a truncated protein that lacks the last 80 amino acids (Figure 5, A and B).

The cDNA showed significant sequence similarity to Xenopus p66, a protein associated with the NURD complex (WADE *et al.* 1999). Homologs are also present in human, Xenopus, mouse, and *C. elegans* (Figure 5B) (FENG *et al.* 2002). All of the p66 proteins have a conserved zinc-finger motif that is similar to the zinc fingers of GATA transcription factors (ORKIN 1992). Immediately adjacent to the zinc finger are a conserved proline-rich region and a conserved basic region (Figure 5B).

To demonstrate that this cDNA encodes the functional p66 protein, we tested whether its expression could rescue *p66* mutants using the UAS/GAL4 system (BRAND and PERRIMON 1993). We expressed *p66* in heteroallelic homozygous $p66^{10}/p66^{7}$ mutants and scored for survival to pupal stages. In the absence of transgenic *p66* expression, ~5% of $p66^{10}/p66^{7}$ mutants survived to the pupal stage. However, expression of *p66* almost completely rescued this mutant combination (Figure 5C). This result was confirmed using two independent transgenic lines, providing additional evidence that the *p66* gene corresponds to the cDNA that we cloned.

Overexpression of *p66*: To explore *p66* function further, we overexpressed the gene using the UAS-GAL4 system. When we overexpressed *p66* using Daughterless-GAL4, a strong embryonic driver, the animals survived

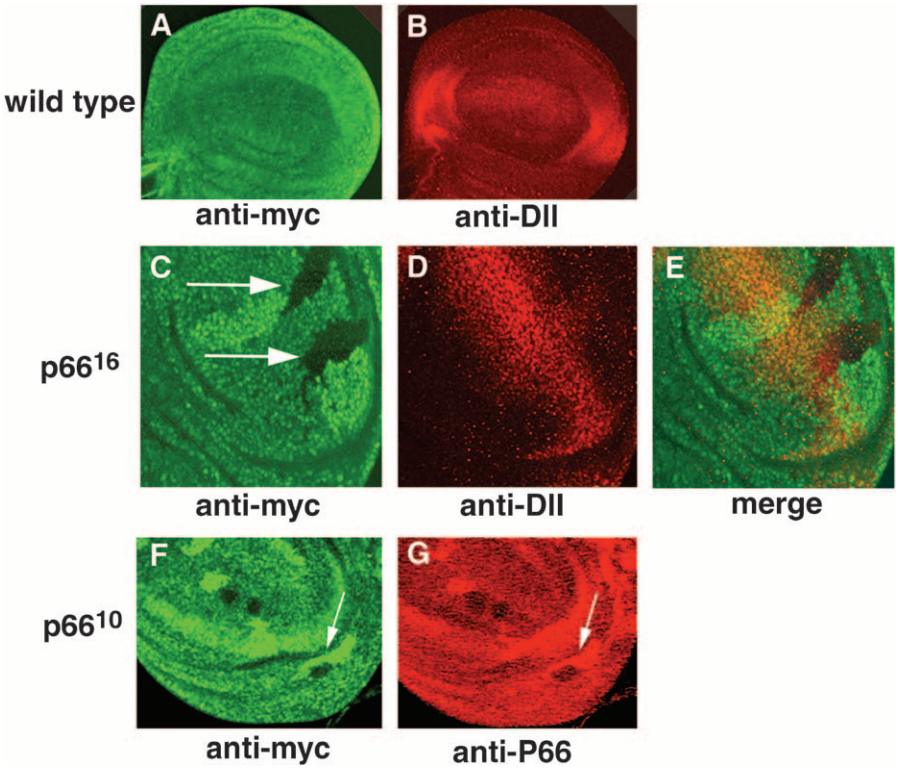
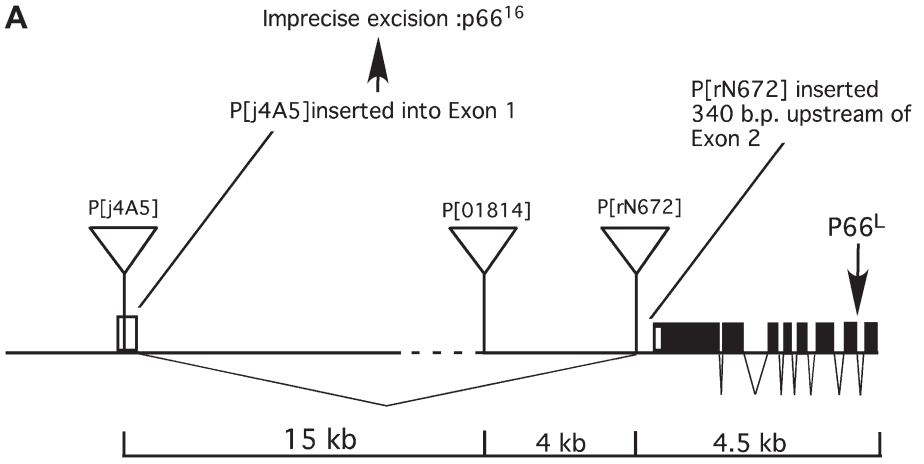


FIGURE 4.—Mosaic analysis of *p66* in imaginal discs. *p66* mutant clones were made using the $p66^{16}$ and $p66^{10}$ alleles in third instar wing imaginal discs. Discs were stained anti-myc antibody to mark homozygous mutant cells (A, C, and F) and either anti-Dll antibody (B and D) of anti-p66 antibody (G). Dll staining in mutant discs (D) is similar to Dll staining in wild-type discs (B). (E) Merge of myc and Dll stains in C and D. No growth difference between *p66* mutant cells (arrows) and wild-type cells (twin spot, bright green stain) is detected as the *p66* loss-of-function clones have sizes similar to the twin spots. (F and G) Homozygous $p66^{10}$ mutant clones in the imaginal wing disc. (F) Mutant clones are identified by lack of myc staining. (G) Clones were stained with anti-p66 carboxy terminal antibody. Arrow indicates a homozygous mutant clone that also lacks P66 protein.



B

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Dros         1 MAKMEVDDVVDLSLGSGRDPALTITPAPPPAALSNRDLRALTANNAGLTITPSASPATNN
Celegans     1 ------------------MAQVQQVPSSPMNGIPEK-------NGNGLSIEESASTAVAA

Dros        61 GTTSNSSNTNSNSSSTTTTTNNNNNSSSNTNSSSANNNNNNNSINASDGVTGATSGSIGG
Celegans    36 LAHIQNGAVLFHGTPTK-----------DLNGASTSIDTDDLQLSPSDLRRRSTRASALK

Dros       121 GQDENKVQRRVLRPRTEPKSYAEAPDIVLLPARTNGRQQNGNIDSETDDEEMPPYVPIKE
Celegans    85 AQEKIKLKDDIVQ----------GP------------QKRANDDDDMEDEELGDEINEQS

Dros       181 LSHAELKEREKSLRK--LRQDLR-NEETKLMLLKKLKQSQQVMKENLVVTPTSVSPNNPL
Celegans   123 APKKRRLENGKEFDQTCFRFGLRANNEGEVYAMTDESENSSIHESEMEVVRYHYEKMKNK

Dros       238 SAIPAALTSKGSLSVTPTSAVPLPAHSKGSRSSLSGSGGPAVSISATNSRTGSSSSAAAA
Celegans   183 EPDEEQLRERLNMRREAENQLREEEAKLLVLRKMKDSQTRAITKLAAETKAADLAEATSA

Dros       298 ASAIAAAAMSGQHRSGSNSILPPPRSSLPAGATLAAGTTITPATSSSHRSSNLPPRTNL-
Celegans   243 AYKPAVATNGKSVTNGKNSMIESNNKTMNGLKNLSIPQQLELLQKLSSQSPAAKQAYIMA

Dros       357 -PNLTITPSVTITPTSAPPSSLKPRNVPGTISNSVSITPALPLSQSHQNQHNDQKNDRST
Celegans   303 KKNPAQTTQLFQQLITINNQNLQKQKELSAAETNASASASPAVQQSQQAQQPQQAQITQS

Dros       416 PREDAQTPAQRQAAAKMALRKQLEKTLLQIPPPKPPPPEMHFIPNPSNTDFVYLLGLETV
Celegans   363 KLLNQQTPAQRIQAARLAFRAQADKQLTTIPTQKSQPHDITFLPNPNASAFLALHGLDLV

Dros       476 VDYLTVNKKANAVAAP--FRCAQCKIDFTPVWKWEKQTNKDPKVICEQCVTSNVKKALKA
Celegans   423 VQHVLKDRSNETPYAGPSYRCEECKTECAHTWKAIGSTQDDLHLYCENCVRSAQKRKNRT

Dros       534 EHTNRLKQAFVKALQQEQEIEQRLASQSSPSPVDNHGSSGGGNSLSVSAVSSNSSSSHMQ
Celegans   483 DQTALLKRAFQKITAQEKEFEKKIA--------------EG------------------

Dros       594 RERELQLQASHASAAAALVNLPPSVTVIPTKCQTPTPATPRPTPPPQSQATPPPSSGGSR
Celegans   510 ----QLEQYAEAKAAA----PATSQTIPTSSTATVSSIPL-VPRLPQ----IPSSTGSST

Dros       654 SSRAAATAAAAAIAAQQAGILSPGPSPAHHHEPTTSSRSSRNDRLDHHHQSQQQQQRDQR
Celegans   557 PTQAVKT------------------STPIHSTPKSSSSSAK------------------

Dros       714 ERDQQQRDQQSQQQAQNYDKYSAATLAAAAHLSALGGMGGLNINSLASLGNLGNLGNLSQ
Celegans   580 -KTAAQLQQQSMQ--------------------------G--MNQLFSTAMLRNNPQMQQ
                                                        *
Dros       774 LGNLAALGGLGNFGGASTGSANPAAGLQGSSPAATAAAMQAFQQQLFRALGQGLGGNPQH
Celegans   611 M--LQMYQALAMGGGAANMANNQMAML--FQ----AQAMQAAQAQVARAQA----A--K-

Dros       834 MMQFAPLLYSYQMAMAQAAQVAAFNNNNKKSSSSSGSAKNSSSSNSMAEVQRAAELQRQY
Celegans   656 ---A-------QAAQAQAAQAQAQAQANREANQQSMLIQ-ALMSNGQVNVQALQQLQK--

Dros       894 LLEMIPPQQQSGASGSRQNNWKA
Celegans   703 ---LTPEQQKALIEVVKRQTRK-
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FIGURE 5.—*p66* cloning and sequence. (A) Genomic structure of the *p66* gene (CG 32067) with location of *P*-element alleles (67E-F). The open reading frame is denoted by solid boxes. Open boxes indicate noncoding regions of the transcript. The position of several *P*-element inserts is indicated as well as the position of the $p66^{L}$ allele. Imprecise excision of the P[J4A5] insert gave rise to the $p66^{16}$ allele but the exact boundaries of the excision are not known. (B) Alignment of Drosophila and *C. elegans* P66 proteins: identical residues are red; similar residues are blue. Three conserved domains: proline-rich region (green box), GATA-like zinc finger (red box), and conserved basic region (black box). Asterisk at amino acid 822 denotes truncation in $p66^{L}$ mutation. (C) Rescue of *p66* mutants. Expression of *p66* cDNA using Arm-Gal4/UAS-*p66* rescues $p66^{10}/p66^{7}$ mutant animals. Without *p66* cDNA, 6–6.5% of mutant animals survive to pupal stage. With *p66* cDNA expression, 26.9–28.5% of mutant animals survive to pupal stage. For UAS-p66 line 9, control *n* (total) = 367; with transgene, *n* (total) = 360. For line 23, control *n* (total) = 1226; with transgene, *n* (total) = 207.

embryogenesis, but died during first instar larval stage. We also overexpressed *p66* using the Ptc-GAL4 driver, which is expressed in the wing, leg, and notum (Figure 6, B, D, H, and I). Ptc-GAL4; UAS-*p66* flies lacked scutellar notum bristles (Figure 6, A and C). The formation of these bristles is *wg* dependent (PHILLIPS and WHITTLE 1993); however, *wg* expression in these animals is similar to the wild-type pattern (Figure 6, B, D, H, and I; we

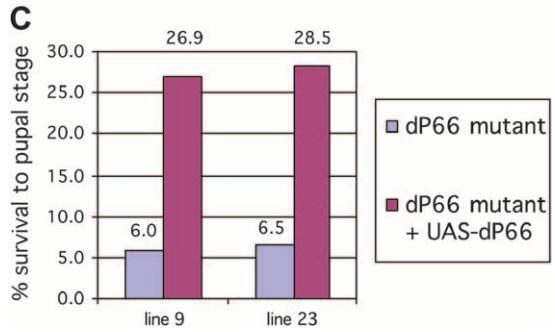


FIGURE 5.—*Continued*.

do, however, note that the high intensity of staining for P66 protein in the red channel leads to signal "bleeding" into the green channel used to detect Wg protein). Therefore, p66 does not repress *wg* itself, but likely represses downstream *wg* targets in the notum. The adults resulting from overexpression of *p66* in imaginal discs had normally patterned wings, but the wings were smaller in size (data not shown). In addition, the legs had elongation defects and often resembled stumps (Figure 6, E–G).

p66 is a ubiquitously expressed nuclear protein: At the RNA level, *p66* is ubiquitously expressed throughout embryogenesis. The transcripts were present by Northern blot in 0- to 2-hr embryos, suggesting that *p66* is maternally provided (data not shown). In early syncytial stages, we detected the transcript by *in situ* hybridization in agreement with our Northern data (Figure 7A). During stage 10, higher levels of the transcript are observed in the presumptive neuroectoderm (Figure 7B). After germband extension, *p66* is highly expressed in the central nervous system (Figure 7C).

In addition, we examined p66 protein distribution using antibodies generated to both the C terminus and the N terminus of p66. The distribution of p66 in the embryo, as detected by both antibodies, was ubiquitous, similar to the transcript pattern (Figure 7, D–F). p66 was also expressed in both follicle and nurse cells of the ovary and clearly localized to the nucleus, which is consistent with a role in transcriptional regulation (Figure 7G). Salivary gland cells also displayed p66 staining in the nucleus (Figure 7H). No staining was observed in homozygous *p66* mutant clones in the disc, providing further evidence that the *p66* cDNA corresponds to the *p66* gene as well as demonstrating specificity of the antibody (Figure 4, F and G).

p66 is associated with the NURD complex: To determine whether Drosophila p66 is associated with the Drosophila NURD complex, we tested for interaction between p66 and dMi-2. Mi-2 is a component that is specific to the NURD complex and not found in the Sin3 complex (reviewed in AHRINGER 2000).

We made Drosophila extracts of 0- to 24-hr embryos. We used either the p66 C-terminal antibody (p66-C)

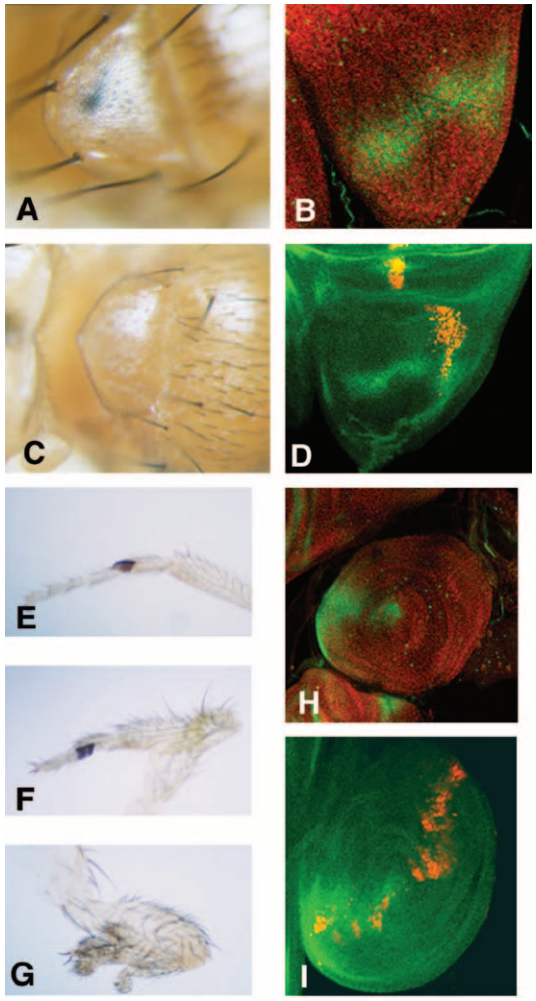


FIGURE 6.—*p66* overexpression phenotypes in imaginal discs. (A and B) Wild-type notum. (C and D) Ptc-GAL4;UAS-p66 notum. (B and D) Third instar imaginal discs stained with anti-Wg (green) and anti-p66C (red). p66 expression was very strong in Ptc stripe, and therefore endogenous p66 cannot be seen in D. Scutellar bristles are repressed by expression of p66 (C), although *wingless* expression is not affected. (E–I) Overexpression of p66 in leg imaginal discs. Wild-type (E) and Ptc-GAL4;UAS-p66 (F and G) legs. Note loss of distal structures in F and G when compared to E. Wild-type (H) and Ptc-GAL4;UAS-p66 (I) third instar imaginal discs stained with anti-Wg (green) and anti-p66C (red). p66 expression was very strong in the Ptc stripe, and therefore endogenous p66 cannot be seen in H. (I) Overexpression of p66 does not repress Wg expression.

or the dMi-2N antibody for immunoprecipitation from these embryonic extracts and analyzed the components by Western blot (Figure 8). The p66-C antibody immunoprecipitated both the p66 protein and dMi-2, while the preimmune serum failed to precipitate either protein. Similarly, using the dMi-2 antibody, we were able to immunoprecipitate p66 (Figure 8). From these experiments we conclude that p66 is in a complex with the Drosophila NURD complex.

p66 can repress a TCF-responsive reporter: BILLIN *et al.* (2000) have demonstrated that inhibition of histone

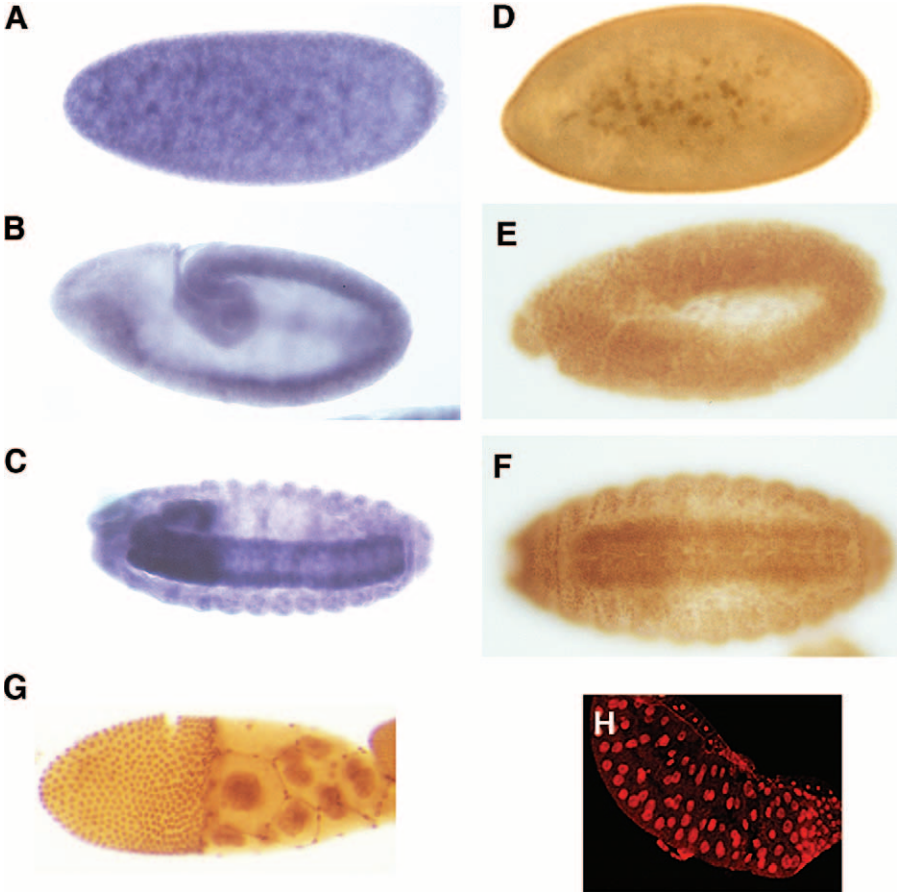


FIGURE 7.—p66 expression pattern. (A–C) The expression of the *p66* transcript was analyzed by *in situ* hybridization in embryos. (D–H) The expression of P66 protein was analyzed by antibody staining. Anterior is to the left. (A) Syncytial blastoderm embryo. (B) Stage 9 germband extended embryo. (C) Stage 15 embryo. (D) Cellular blastoderm embryo. (E) Stage 11 embryo germband retracting embryo. (F) Stage 15 embryo. The protein was ubiquitously expressed. (G) Detection of P66 protein in ovaries using the anti-p66 N antibody. The protein is localized to the nucleus of the nurse cells as well as the follicle cells. (H) P66 protein is present in the nuclei of salivary gland cells.

deacetylases causes derepression of Wnt-responsive promoters. In addition, they found that under repressive conditions, LEF-1 interacts with HDAC1 independently of mSin3A, suggesting that LEF-1 mediates repression through the NURD histone deacetylase complex. In agreement, we found that removal of *p66* enhanced the *sev-wg*^{ts} phenotype and overexpression repressed *wg*-dependent bristles without affecting *wg* expression. In combination with our finding that p66 associates with the NURD complex, these experiments suggest that *p66* could be involved in repression by TCF/LEF-1 family members.

To further investigate this possibility, we examined the effects of *p66* expression on the activation of the TCF-responsive OT (KLYMKOWSKY *et al.* 1999). We transfected 293 cells with the OT reporter and β-catenin. As expected, β-catenin efficiently activated the OT reporter. Coexpression of *p66* inhibited β-catenin activation of the OT reporter in a dose-dependent manner, maximally inhibiting activation by 70% (Figure 9A). The ability of *p66* to repress the OT plasmid was not restricted to 293 cells as we also observed repression of the OT reporter in NIH3T3 cells (data not shown).

To determine if *p66* is a specific repressor of the Wnt pathway or a more general repressor, we tested whether *p66* could repress other reporter genes. We found that *p66* could repress activation of the SRE reporter by the M1 receptor (Figure 9C). Additionally, coexpression of *p66* also inhibited activation of the *NF-AT* reporter by calcium signaling (Figure 9D). However, *p66* did not repress an albumin luciferase reporter, demonstrating that there is some specificity in its action (Figure 9B).

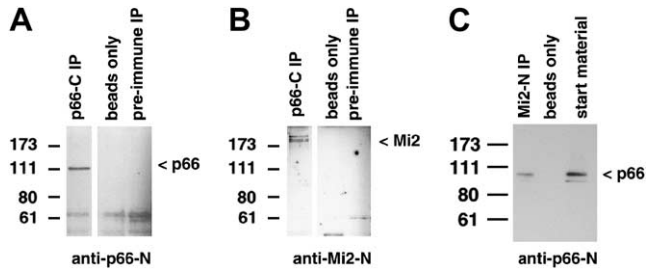
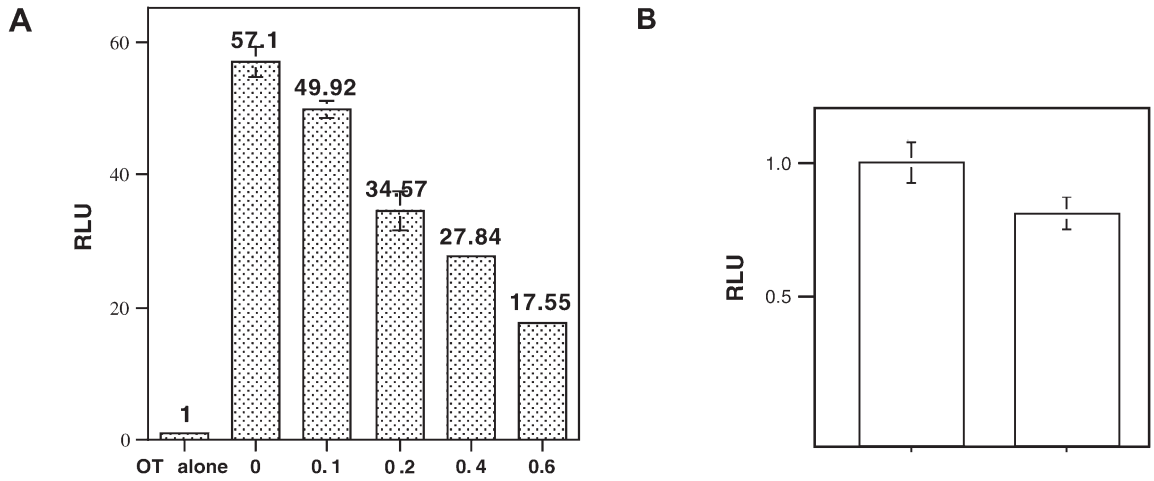
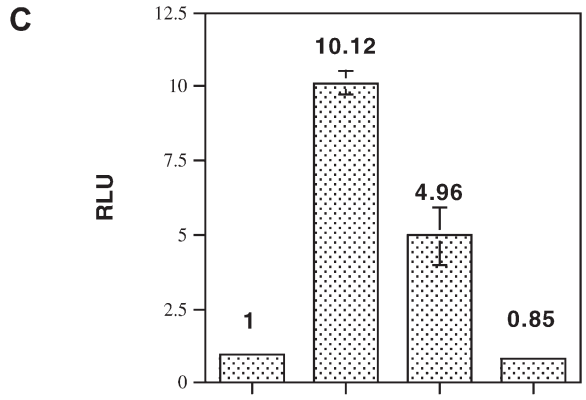


FIGURE 8.—P66 and dMi-2 form a complex *in vivo.* (A and B) Immunoprecipitation of embryonic extracts using the anti-P66 antibody. (Lane 1) Immunoprecipitates using the anti-P66-C antibody. (Lane 2) Control immunoprecipitates using the protein G sepharose beads only. (Lane 3) Control immunoprecipitates using the preimmune serum. Proteins were analyzed by SDS page and immunoblotted with anti-P66 N antibody (A) or dMi-2-N (B). The anti-P66 antibody precipitates both P66 and dMi-2. (C) Immunoprecipitation of embryonic extracts using the dMi-2 antibody (lane 1) Immunoprecipitates using the anti-dMi-2-N antibody. (Lane 2) Control immunoprecipitates using the protein G sepharose beads only (lane 3) embryonic extracts. Proteins were analyzed by SDS-page and immunoblotted with anti-P66N antibody. The anti-dMi-2N antibody precipitates P66.

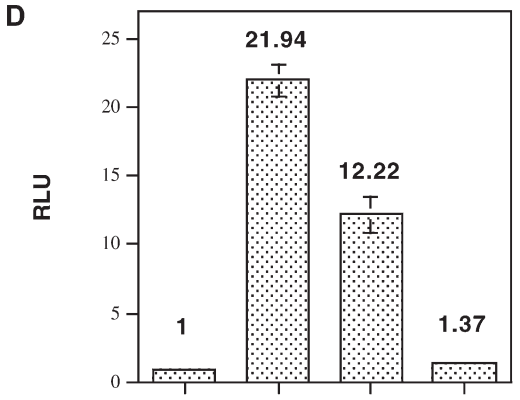


OT (μg)	0.1	0.1	0.1	0.1	0.1	0.1
βgal (μg)	0.1	0.1	0.1	0.1	0.1	0.1
β-catenin (μg)	0	0.2	0.2	0.2	0.2	0.2
P66 (μg)	0	0	0.1	0.2	0.4	0.6
GFP (μg)	0.8	0.6	0.5	0.4	0.2	0

Albumin luciferase	0.2	0.2
βgal	0.1	0.1
P66	0	0.4
GFP	0.4	0



SRE	+	+	+	+
M1	+	+	+	+
Carbachol	-	+	+	-
P66	-	-	+	+



NFAT	+	+	+	+
Ca^{2+}, PMA	-	+	+	-
P66	-	-	+	+

FIGURE 9.—*p66* overexpression represses multiple reporters. (A) *p66* inhibits β-catenin activation of OT. The effects of *p66* on β-catenin activation of the OT reporter were examined in 293 cells. The OT plasmid was cotransfected with β-catenin and increasing amounts of *p66*. Transfections also included a plasmid encoding EF1α-βgal to normalize for transfection efficiency by measurement of β-galactosidase activity. Measurements were performed in duplicate. Results are from a typical experiment and given in relative luciferase units. (B) *p66* overexpression does not affect albumin luciferase. Albumin-luciferase was cotransfected with *p66* (0.4 μg). Luciferase activity was measured 48 hr post-transfection. All transfections also included a plasmid encoding EF1-β-galactosidase to normalize for transfection efficiency. Although this experiment shows a slight decrease in expression, others have shown a slight increase in expression in the presence of *p66*. (C) *p66* inhibits activation of SRE reporter by M1 receptor. The SRE-luciferase plasmid was cotransfected with the M1 receptor and 0.6 μg of *p66*. After 36 hr, cells were stimulated with carbachol (5 μM) for 6 hr, and luciferase activity was measured. (D) *p66* inhibits activation of NF-AT reporter by Ca^{2+} and PMA. The NF-AT reporter plasmid was cotransfected with p66. Twenty-four hours after transfection, cells were stimulated with ionomycin (1 μM), phorbol 12-myristate 13-acetate (PMA, 25 ng/ml), and calcium chloride (10 mM). After 24 hr, cells were lysed for assays.

DISCUSSION

NURD complex in Wnt signaling: Through a genetic screen for modifiers of wingless signaling, we identified mutations in *p66*, the Drosophila homolog of p66, a protein that had been previously identified as part of the NURD chromatin-remodeling/histone deacetylase complex (WADE *et al.* 1999). Loss of *p66* enhanced the *sev-wg^{ts}* phenotype in the eye; *i.e.*, the resulting phenotype resembled increased wingless signaling. Since loss

of *p66* did not affect other transgenes expressed using the same promoter as *sev-wg^{ts}*, we believe that removal of *p66* does not affect *wingless* expression through the *sevenless* promoter, but instead represses *wingless* target genes involved in bristle formation. Our results are in agreement with previous experiments that have implicated the NURD complex in Wnt signaling. It has been reported that Lef-1 repression involves HDAC-1 function. This interaction takes place in the absence of mSin3A, leading to the hypothesis that Lef-1 repression involves recruitment of the NURD complex (Billin *et al.* 2000). Here, we present three pieces of evidence to further support this hypothesis. First, loss of *p66* enhances a *wg* overexpression phenotype. Second, overexpression of *p66* can repress activation of a TCF reporter by β-catenin in tissue culture cells. Finally, overexpression of *p66 in vivo* represses the formation of *wingless*-dependent scutellar bristles without repressing *wingless* expression. Together, these experiments provide additional evidence that the NURD complex is involved in repression of Wnt target genes.

***p66* and the ecdysone pathway:** The lethality of *p66* mutants is caused by misregulation of ecdysone-regulated genes during larval stages. If *p66* was involved in repression of ecdysone-induced targets such as *E74* and *DHR3*, then loss of *p66* should lead to ectopic gene expression. In contrast, we have found that ecdysone-induced genes are not activated, suggesting that the role of *p66* is more complex and indirect. In microarray studies of ecdysone response, 44% of genes that changed expression were repressed (White *et al.* 1999). We speculate that repression of one or more of these genes is required for ecdysone-induced expression and that *p66* is required for this process.

An additional connection between the NURD complex and ecdysone response is made through *Bonus* (*bon*), the Drosophila homolog of TIF1. TIF1 was identified as a protein that interacts with HP1, a heterochromatin-associated protein (Le Douarin *et al.* 1996). The NURD complex may be involved in histone modification to allow HP1 binding (Nishioka *et al.* 2002; Zegerman *et al.* 2002). Similar to *p66* mutants, *bon* mutants die during pupal stages due to misregulation of ecdysone-induced genes. Furthermore, E74 expression is also reduced in *bon* mutants (Beckstead *et al.* 2001). Thus, we speculate that p66 and the NURD complex may be involved in regulation of ecdysone response through HP1-mediated repression.

p66 functions as a repressor: *p66* mutations can affect both *wg* and ecdysone-induced gene expression. Furthermore, in cell culture reporter assays, we found that expression of *p66* inhibits activation of a TCF reporter by β-catenin, of a SRE reporter by the M1 receptor, and of an NF-AT reporter by high levels of intracellular calcium. In addition, two human p66 homologs, hp66α and hp66β, function as transcriptional repressors when tethered to a promoter, suggesting that transcriptional repression is a shared activity of p66 proteins (Brackertz *et al.* 2002; Feng *et al.* 2002). However, p66 did not repress an *albumin* luciferase reporter, implying that expression of *p66* does not cause a defect in general transcription. Therefore, we conclude that p66 can repress multiple signaling pathways, and we hypothesize that this repression is mediated by recruitment of the NURD histone deacetylase complex. This conclusion is also supported by previous reports that expression of p66 can change the localization of MBD3, a component of the NURD complex (Feng *et al.* 2002).

Although overexpression of *p66* can repress multiple signaling pathways, we have been unable to detect loss-of-function phenotypes that would be consistent with this hypothesis. In Drosophila, relatively mild and tissue-specific phenotypes for repressors have also been found for *Pangolin/dTCF* (Schweizer *et al.* 2003) and *naked cuticle* (Rousset *et al.* 2001). With respect to *Pangolin/dTCF*, this could be due to its dual role as both a repressor and an activator (Schweizer *et al.* 2003), but in other cases, a lack of phenotypes is possibly due to redundancy among parallel pathways (not necessarily among related genes). This suggestion is in analogy to the SynMuv genes in *C. elegans*. Animals mutant in either a synMuvA or a synMuvB gene alone have a normal vulva; animals mutant for both a synMuvA gene and a synMuvB gene have a multi-vulval (Muv) phenotype (Horvitz and Sulston 1980; Ferguson and Horvitz 1989). Indeed, in the *C. elegans* vulva, p66 functions in a redundant pathway (G. Poulin, F. Solari and J. Ahringer, personal communication).

p66 and other components of the NURD complex: Initial preparations of the NURD complex from mammalian cells did not contain p66 (Tong *et al.* 1998; Xue *et al.* 1998; Zhang *et al.* 1998), but p66 was present when the complex was purified from Xenopus oocytes (Wade *et al.* 1999). Subsequently, p66 was found to be associated with the NURD complex in mammalian cells in association with the methyl DNA-binding protein MBD2, as part of the MeCP1 complex. Is p66 a component of the NURD complex or an accessory factor? We co-immunoprecipitated dMi-2 and p66, suggesting that they form a complex. However, if all of the complex members participate in the same processes, then the corresponding mutants should also have the same phenotypes. To ascertain whether *p66* and *rpd3* (HDAC homolog) function together is difficult since *rpd3* also participates in the Sin3 complex (reviewed in Ahringer 2000). Thus, it is likely that *rpd3* will display a wider range of phenotypes than a mutant of the NURD complex alone.

However, we can compare the *p66* mutant to the other NURD complex mutant characterized in Drosophila, *dMi-2*. *dMi-2* is required for oogenesis and for cell viability (Kehle *et al.* 1998). *rpd3* is also likely important for oogenesis, as a hypomorphic *rpd3* allele produces very

few eggs (Mannervik and Levine 1999). In contrast, *p66* mutant germlines produce normal embryos, and *p66* mutant clones survive in third instar imaginal discs. *dMi-2* zygotic mutants die during larval stages, which coincides with the lethality of strong allele combination of *p66* mutants (Kehle *et al.* 1998). It is possible that *dMi-2* may also be required for ecdysone response in larval stages. Since the p66 mutant phenotype does not completely mimic Mi-2 mutant phenotypes, we suggest that p66 is not a core component of the NURD complex, but could have a regulatory function. However, it is equally possible that differences in allelic strength or perdurance of maternal contributions obscures the full range of phenotypes on the several components.

Our results are further supported by experiments demonstrating that dMi-2 and the Drosophila MBD2/3 protein do not colocalize in nuclei. dMi-2 is distributed ubiquitously in embryonic nuclei, while Drosophila MBD2/3 is localized in a speckled pattern (Marhold *et al.* 2004). This result suggests that Drosophila MBD2/3 is not an integral component of all dMi-2 complexes.

p66 function: How might p66 function? In mammalian cells, human p66 protein is associated only with the NURD complex as part of the MeCP1 complex, which additionally contains methylated DNA-binding activity through the MBD2 protein (Hendrich and Bird 1998; Feng and Zhang 2001; Feng *et al.* 2002). Similarly, the Xenopus NURD complex, which copurifies with p66, in contrast to mammalian NURD complexes, also has methylated DNA-binding activity (Wade *et al.* 1999). The NURD complex, through Mi-2, interacts with the zinc-finger proteins Hunchback, Tramtrak, and Ikaros (Kehle *et al.* 1998; Kim *et al.* 1999; Murawsky *et al.* 2001). We hypothesize that p66, also a zinc-finger-containing protein, functions similarly to these transcription factors to recruit the NURD complex to methylated DNA.

This hypothesis is supported by experiments that demonstrate that human p66 interacts with MBD2 and MBD3 *in vitro*. Furthermore, overexpression of p66 can change localization of MBD3 (Feng and Zhang 2001; Brackertz *et al.* 2002). On the basis of these results, we hypothesize that p66 may function as a link between the NURD complex and the methylated DNA-binding proteins MBD2 and MBD3. We propose that p66 recruits the NURD complex to mediate methylation-mediated silencing. Since both DNA methyl transferases and DNA-methylated binding proteins exist in Drosophila, it is likely that there is some vestige of a methylation system in Drosophila, although the function is unknown at this time (Tweedie *et al.* 1999; Gowher *et al.* 2000; Lyko *et al.* 2000).

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