A Novel Pzg-NURF Complex Regulates Notch Target Gene Activity

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Drosophila putzig was identified as a member of the TRF2–DREF complex that is involved in core promoter selection. Additionally, *putzig* regulates Notch signaling, however independently of DREF. Here, we show that Putzig associates with the NURF complex. Loss of any NURF component including the NURF-specific subunit *Nurf 301* impedes binding of Putzig to Notch target genes, suggesting that NURF recruits Putzig to these sites. Accordingly, Putzig can be copurified with any NURF member. Moreover, *Nurf 301* mutants show reduced Notch target gene activity and enhance *Notch* mutant phenotypes. These data suggest a novel Putzig–NURF chromatin complex required for epigenetic activation of Notch targets.

INTRODUCTION

Putzig (Pzg) is a component of a large multiprotein complex that includes the TATA-box-binding-protein–related factor 2 (TRF2) and the DNA-replication related element (DRE) binding factor DREF (Hochheimer *et al.,* 2002). The TRF2–DREF complex has been associated with the transcriptional regulation of replication-related genes that contain DREF binding sites (Hochheimer *et al.,* 2002). Accordingly, Pzg acts as a positive regulator of cell cycle and replication-related genes (Hochheimer *et al.,* 2002; Kugler and Nagel, 2007). In addition to this, we could show that Pzg is also required for Notch target gene activation in a DREF-independent manner (Kugler and Nagel, 2007). Presumably, Pzg functions at the level of chromatin activation, because the open chromatin structure typical of active Notch target genes is no longer detectable in a *pzg* mutant background (Kugler and Nagel, 2007).

The TRF2–DREF complex consists of more than a dozen of proteins and the biochemical function of most of them remains still elusive. Interestingly, it also contains three members of the nucleosome remodeling factor (NURF), imitation switch (ISWI), Nurf 55 and Nurf 38 (Hochheimer *et al.,* 2002). NURF is a multisubunit complex that has been associated with chromatin activation and repression (Badenhorst *et al.,* 2002, 2005; Kwon *et al.,* 2008). NURF triggers nucleosome sliding thereby provoking changes in the dynamic properties of the chromatin (Tsukiyama and Wu, 1995; Hamiche *et al.,* 1999; Kang *et al.,* 2002; reviewed in Bouazoune and Brehm, 2006). The subunit ISWI is a member of the SWI2/

SNF ATPase family and is thought to provide energy for nucleosome remodeling (Tsukiyama and Wu, 1995). Nurf 38 encodes an inorganic pyrophosphatase, which catalyzes the incorporation of nucleotides into a growing nucleic acid chain during transcription, replication, and DNA repair mechanisms (Gdula *et al.,* 1998). Nurf 55 harbors WD-40 repeats, which allow interaction with other proteins and protein complexes (Neer and Smith, 1996; Martinez-Balbas *et al.,* 1998). The fourth and largest subunit Nurf 301 is specific to the NURF complex, whereas all other members are shared with other chromatin modifying complexes (Xiao *et al.,* 2001; Barak *et al.,* 2003). Accordingly, Nurf 301 is not a component of the TRF2–DREF complex. Nurf 301 exhibits a number of protein motifs that typify transcription factors and other chromatin modifying proteins (Xiao *et al.,* 2001). In addition, the N-terminal region of Nurf 301 shows homology to the DNA-binding protein HMGA (high mobility group A) implying that Nurf 301 mediates the contact with the DNA or provides a platform to recruit other transcription factors (Reeves and Beckerbauer, 2001; Xiao *et al.,* 2001). In this context it has already been shown that Nurf 301 is required for the transcriptional activation for example of homeotic genes and notably of Ecdyson-receptor (EcR) and Wingless target genes (Badenhorst *et al.,* 2002; Badenhorst *et al.,* 2005; Song *et al.,* 2009).

The DREF independence of Pzg during the activation of Notch target genes raised the possibility that it may instead involve the NURF complex for chromatin activation. Here, we provide evidence for a functional interplay between Pzg and the NURF complex with regard to Notch target gene activation. Coimmunoprecipitations revealed that Pzg is present in protein complexes containing the known NURF subunits. Moreover, Pzg binding on Notch target genes is neither detectable in mutants of the NURF-specific subunit *Nurf301*, nor in mutants affecting other subunits of NURF. In addition, *Nurf301* is required for Notch target gene expression, which is impaired in *Nurf301* mutant cell clones. Consistent with this, *Nurf301* mutants enhance the *Notch* mutant wing phenotype, strongly arguing for an involvement of the NURF complex in Pzg-mediated epigenetic Notch target gene activation.

MATERIALS AND METHODS

Genetics, Fly Strains, and Documentation of Phenotypes

To generate *Nurf301* mutant clones using the Flp/FRT system, the *Nurf301²* null mutant allele was recombined with *FRT80B* and selected according to its gentamicin resistance and failure of complementation with other *Nurf* alleles. Flies of the genotype *ywhsflp; Nurf301²FRT80B/FRT80BM(3)67cubi-GFP* (Janody *et al.,* 2004, gift of J. Treisman, NYU Medical Center, NY) were exposed to a heat shock (37°C for 30 min) for 48–72 h after egg laying (AEL), and wing imaginal discs were prepared 120–144 h after AEL.

The following genotypes (fly stocks) were used for the experiments: Gal4/UAS system: *enGFP-Gal4* (Neufeld and Edgar, 1998), *Omb-Gal4/FM7a* (Lecuit *et al.,* 1996), *ptc-Gal4::UAS-pzg-RNAi* (Kugler and Nagel, 2007), *SD-Gal4* (Roy *et al.,* 1997; gift of K. Irvine, Rutgers, The State University of New York), *UAS-Nurf301-RNAi* (VDRC 24740), *UAS-Nurf55-RNAi* (VDRC 26455), *UAS-Nurf38-RNAi* (VDRC 3200) all obtained from Vienna Stock Center, *UAS-pzg-RNAi* (Kugler and Nagel, 2007); mutants: *Df(1)N⁵⁴¹⁹/FM7c*, *N⁵⁵ᵉ¹¹* (gift of A. Preiss, University of Hohenheim, Germany), *Iswi²/CyoGFP* (Deuring *et al.,* 2000; gift of P. Badenhorst, University of Birmingham, United Kingdom); *Nurf38¹/CyO* (BL12206); *Nurf301²/TM6GFP* (Badenhorst *et al.,* 2005); and reporter lines: *m8-lacZ* (Lecourtois and Schweisguth, 1995) and *vg-BElacZ* (Kim *et al.,* 1996). Generally, flies were raised at 25°C on standard fly food with the exception of the *Nurf301-RNAi* induction assays, which were performed at 25 and at 29°C.

Adult wings were embedded in Euparal (Roth, Karlsruhe, Germany), and at least 30 wings of each genotype were measured. Wing size was determined using ImageJ software for pixel measurements (http://rsb.info.nih.gov/ij/). The wing area was encircled with the Polygon tool. The Segmented Line tool was used to reconstruct the length of wing notches. To test statistical significance, p values were calculated according to Student's t test (http://www.physics.csbsju.edu/stats/t-test.html). Pictures were taken with Normarski optics on a Zeiss Axiophot (Carl Zeiss, Jena, Germany).

Immunhistochemistry, Immunoprecipitation, and Chromatin Immunoprecipitation

Wing imaginal discs were prepared from crawling third instar larvae of the respective genotype. The following antibodies were used: mouse anti-β-galactosidase (1:100; JIE7), mouse anti-Cut (1:20; 2B10), and mouse anti-Wg (1:25; 4D4), all obtained from the Developmental Studies Hybridoma Bank, University of Iowa (NICHD contract NO1-HD-7-3262), rabbit anti-cleaved caspase 3 (1:200, NEB Cell Signaling Technology, Beverly, MA), rat anti-Ci 2A1 at 1:2 dilutions (Motzny and Holmgren, 1995). Pictures of stained discs were taken with a Bio-Rad MRC1024 confocal microscope (Hercules, CA) on a Zeiss Axiophot.

Immunoprecipitations were performed according to Nagel *et al.* (2005) by using protein extracts from 100 first instar larvae. For precipitations we used guinea pig anti-Pzg antibodies (1:250; Kugler and Nagel, 2007) and rabbit anti-MOF antibodies (Matyunina *et al.,* 2008; gift of John Lucchesi, Emory University, Atlanta, GA). For detection, rabbit anti-ISWI (Tsukiyama and Wu, 1995), rabbit anti-Nurf301 (Kwon *et al.,* 2008), rabbit anti-Nurf55 (Martinez-Balbas *et al.,* 1998), rabbit anti-Nurf38 (Gdula *et al.,* 1998), and mouse anti-Notch intra (1:10; C17.9C6, DSHB) were used; all three Nurf antibodies were a gift from Hua Xiao (National Cancer Institute, Bethesda, MD).

Chromatin immunoprecipitations (ChIPs) were done with 300 first instar larvae each, using the ChIP Assay Kit from Upstate Biotechnology (Lake Placid, NY; Kugler and Nagel, 2007). The following primer sets were used: m8UP (5'-CAACAACCAAGGCGACCCGGCACGA) and m8LP (5'-TTTTT-GAAAAATTTGTATTCGGCTTGTTGCTG) for amplification of *Enhancer of split m8* [*E(spl)m8*] 5' regulatory region and vgdvUP (5'-GGGTGAATTCCG-CAACTCAATGTTGGCTT) and vgdvLP (5'-TATTTGGATCCGTTTAACTT-TAGGTTTCGGGACTGG) for amplification of the d/v regulatory region of the *vestigial* (*vg*) locus.

The following primer pairs for control reactions: Enhancer of split m8 (3') control: m83primUP (5'-AACAAGGGGTTAAGTGGCAGGAAG GTAAGG) and m83primLP (5'-C-AGTGTTGCATGCGCCAGTGTTAGGA); roX-1: UP (5'-GTCGAATTCGAAAAACACATTTACTAACAAATAA-3') and LP (5'-GTCGAATTCCCCAAAGAAATCCACATAACAT-3'); open reading frame (ORF) vg primer: vg ORF UP (5'-TTGTACTCCTCCTCGGGCGTC-3') and vg ORF LP (5'-GTGTGTGCGAGTGCGGGTG-3'); and ORF m8 primer: m8 ORF UP (5'-CACCAAGACCCAGATCTACCAG-3') and m8 ORF LP (5'-CGCTGT-GATATCCGGAGGA-3').

RESULTS

Pzg Does Not Directly Bind to the DNA of Notch Target Genes

We recently showed by cross-linking chromatin immunoprecipitation (XChIP) that Pzg is bound to the regulatory regions of Notch target genes, however, in a DREF-independent manner (Kugler and Nagel, 2007). Because *pzg* encodes

a Zn-finger protein, we investigated at first whether Pzg binds directly to Notch target gene promoters. We performed electromobility shift analyses with the sequences precipitated by Pzg in the XChIP, however, found no hints for a direct DNA binding of Pzg to these sites (data not shown). From this we conclude that Pzg requires at least one further partner for binding to Notch target gene promoters.

Pzg's Presence at Notch Target Genes Depends on the Presence of the NURF Complex

Pzg depletion is correlated with a reduction of open chromatin structure at different Notch target genes, assuming an important role of Pzg in chromatin activation at these Notch target sites (Kugler and Nagel, 2007). Because *Dref* mutant cells do not show this effect, Pzg must influence Notch signaling in a TRF2–DREF-independent manner (Kugler and Nagel, 2007). Three proteins of the TRF2–DREF complex are also components of the nucleosome remodeling factor NURF that has been involved in chromatin activation (reviewed in Bouazoune and Brehm, 2006). Hence, Pzg might act together with NURF in the context of Notch target gene activation. To investigate a possible association of Pzg and NURF in more detail, we first performed XChIPs with anti-Pzg antibodies in mutants of all four components of the NURF complex. This includes the NURF-specific subunit Nurf 301, which is not part of the TRF2–DREF complex (Xiao *et al.,* 2001; Hochheimer *et al.,* 2002; Barak *et al.,* 2003). Indeed, no Pzg protein was bound to the promoters of the Notch target genes *vg* and *E(spl)m8* in the absence of ISWI, Nurf 38, Nurf 55, or Nurf 301, whereas it was present in the wild type at these sites (Figure 1). This demonstrates that the NURF complex as a whole is involved in the Pzg-mediated epigenetic activation of Notch target genes. To further confirm our conclusion we compared the binding of Pzg to the *vg* and *E(spl)m8* loci on wild-type and *Nurf301* mutant salivary gland chromosomes. Consistent with the data gained in the XChIP, binding of Pzg is impaired at these loci in the absence of *Nurf301* in support of an important role for NURF in targeting Pzg to these sites (Supplemental Figure S1, A and B).

Pzg Associates with NURF Complex Members In Vivo. The failure of Pzg to bind to Notch target genes in NURF mutants—notably in the absence of the NURF specific subunit *Nurf301*—suggested an association of Pzg with NURF itself. To this end, we tested whether Pzg coprecipitated with NURF complex members using protein extracts from first instar larvae and Pzg antibodies. As expected for members of the TRF2–DREF complex, the three NURF components ISWI, Nurf 38, and Nurf 55 were found in a complex with Pzg (Figure 2). However, we also found Nurf 301 coprecipitating with Pzg, indicating that Pzg associates with the NURF complex as well (Figure 2). These data provide evidence that Pzg is present in at least two different protein complexes, TRF2–DREF and NURF.

NURF Promotes Expression of Notch Target Genes. The observation that Pzg associates with the NURF complex suggests that the influence of Pzg on Notch target genes involves chromatin activation by NURF. To further investigate this possibility, we first looked for genetic interactions between Notch and the NURF complex. To this end, we used the sensitized genetic background of heterozygous *N⁵⁴¹⁹/+* mutant animals and analyzed the haplo-insufficient *Notch* mutant wing phenotype, which is characterized by wing notches and thickening of the third longitudinal vein (Figure 3A).
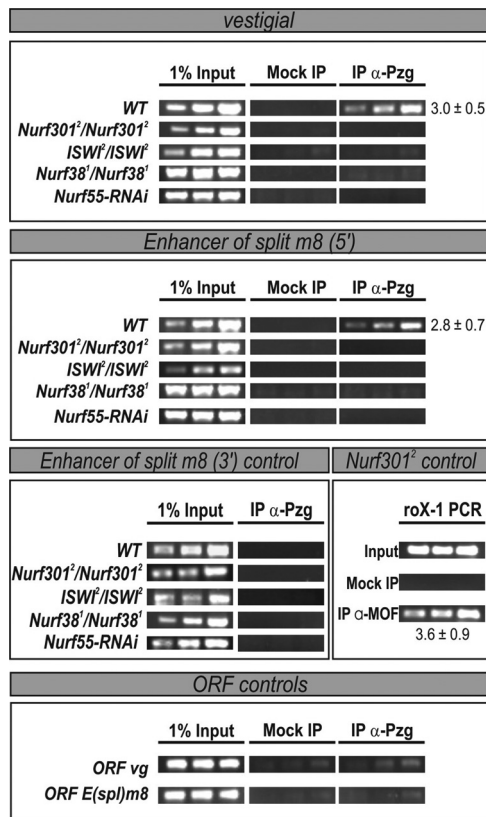
Figure 1. NURF complex is required for Pzg binding at Notch target genes. XChIP analyses were performed on chromatin isolated from either wild-type or *Nurf301²*, *Iswi²*, and *Nurf38¹* homozygous first instar larvae (as indicated). In the case of *Nurf55*, *da-Gal4::UAS Nurf55-RNAi* first instars were used. Anti-Pzg antibodies as well as guinea pig preimmune sera (mock control) and anti-MOF antibodies (positive control) were used for precipitations. Sequences from the *vestigial*-boundary enhancer (*vg*) and the *Enhancer-of-split m8* [*E(spl)m8*] promoter (m85′) were amplified. Samples of the 31st, 33rd and 35th amplification cycle are shown. Relative enrichment was estimated for the 33rd PCR cycle sample from the ratio between Pzg immunoprecipitations and mock controls. Mean values and standard deviations of at least three independent experiments were calculated. PCR-amplification of the *E(spl)m8* 3′UTR as well as a region in the ORF of *vg* and *E(spl)m8* is shown as unrelated control. As a positive control MOF binding was monitored at the *roX-1* locus in *Nurf301²* mutant chromatin.

Indeed, we noted an enhancement of the wing defects if the dose of either *NURF* gene, *Nurf38*, *Nurf55*, or *Nurf301* was reduced (Figure 3, A–G). Notably, decreasing the activity of *Nurf301* in a *Nurf301²* heterozygous mutant background strongly increased both, the penetrance and severity of wing margin defects (Figure 3, A and D). Similar results were obtained using another *N* allele, *N⁵⁵ᵉ¹¹*, confirming specificity of the genetic interaction data (Supplemental Figure S1, C–G). Because Nurf 301 is obligatory in the assembly of the NURF complex (Xiao *et al.*, 2001), the sensitivity of Notch toward the presence of Nurf 301 indicates that the NURF complex functions a positive regulator of Notch signaling in normal wing development (Figure 3, A–G).

To validate these genetic interaction results we analyzed also the expression of the well-defined Notch target genes *cut (ct)*, *Enhancer of split m8* [*E(spl)m8*], *wingless (wg)*, and *vestigial (vg)* in a *Nurf301* mutant background. In a first approach, we addressed this issue by using a *Nurf301-RNAi*
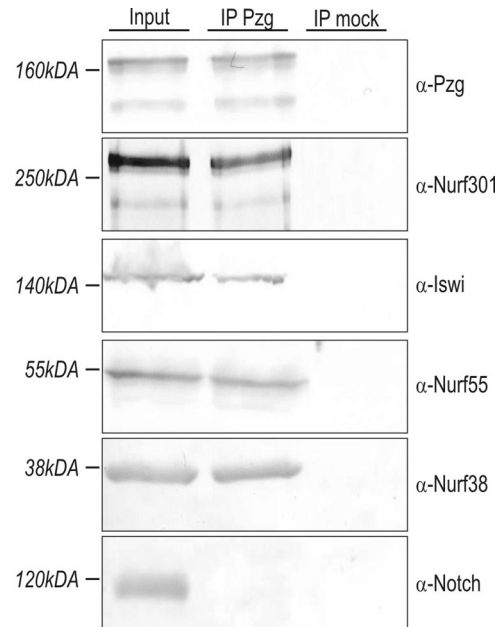

Figure 2. Pzg is associated with the whole NURF complex. Proteins immunoprecipitated (IP) from wild-type larval protein extracts by anti-Putzig antibodies were probed for Nurf 301, ISWI, Nurf 55, and Nurf 38 as indicated. The input column shows 25% of the extract used for the IP. IPs with guinea pig preimmune sera served as negative control (mock). Specificity of the used α-Pzg antibody was shown with the intracellular domain of Notch, which could not be copurified.

line under UAS control, allowing us to down-regulate the activity of *Nurf301* specifically in the wing anlagen. In addition, we took advantage of the temperature sensitivity of the system: strong *Nurf301* reduction was induced by raising the flies at 29°C, whereas intermediate levels were obtained at 25°C. However, the expression levels of all four target genes tested were affected, to a different degree: The expression of Cut, *E(spl)m8-lacZ* and *vg-lacZ* was strongly reduced or even abolished in *Nurf301* RNAi–treated cells at both temperature settings, whereas that of Wg was affected only at 29 but not at 25°C (Supplemental Figure S2). Because induction of RNAi reduces gene activity but does not necessarily abolish it, we wondered whether residual *Nurf301* activity supported the expression of the tested genes notwithstanding the RNAi treatment. To test this, we looked at cells that completely lacked *Nurf301* activity by inducing mutant cell clones using the null allele *Nurf301²* (Badenhorst *et al.*, 2005).

In a first attempt we failed to generate *Nurf301²* mutant clones, most probably because these cells have a growth disadvantage. Therefore, we generated *Nurf301²* mutant clones in a *Minute* mutant background, which allows growth of disadvantaged cells. Here, *Nurf301²* mutant clones exhibit a conspicuous reduction of the respective Notch target gene expression (Figure 4, A–C″). To exclude cell death as artificial trigger of the observed effects, levels of activated caspase 3 were examined but were unchanged in the mutant cells (Figure 4, D–D″).

To ensure that the *Nurf301²* mutation does not generally impede expression of developmentally regulated genes, we monitored Ci, which is an integral component of the Hedgehog signaling pathway known to be unaffected in a *pzg-RNAi* mutant background (Kugler and Nagel, 2007). As predicted, *Nurf301²* mutant cells have no visible effect on the
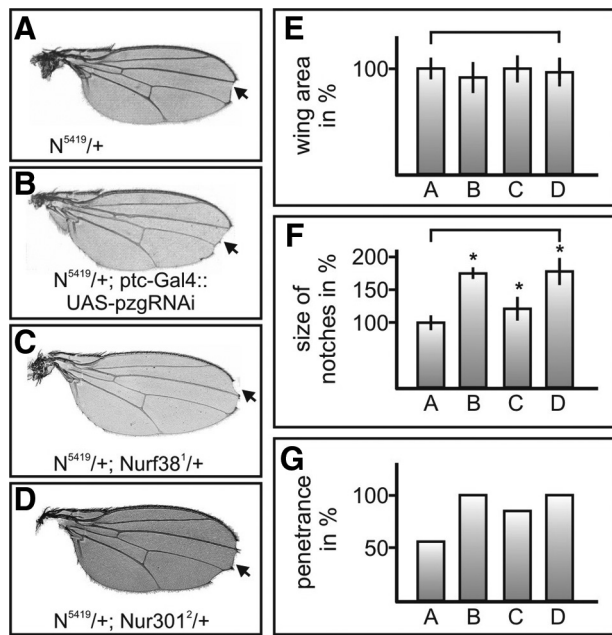
Figure 3. NURF genetically interacts with Notch. (A–D) The *Notch* mutant wing phenotype (marked by arrows) is enhanced by *pzg-RNAi* as well as by one mutant allele of different NURF complex members including the large subunit *Nurf301*. The genotypes were as followed: (A) $N^{5419}/+$, (B) $N^{5419}/+$; *ptc-Gal4::UAS-pzgRNAi*, (C) $N^{5419}/+$; *Nurf38[1]/+*, and (D) $N^{5419}/+$; *Nurf301[2]/+*. (E–G) The phenotypes from A through D were statistically analyzed. The labels A–D correspond to the genotypes shown in Figure 3, A–D. Mean values of at least 30 wings of each genotype were considered using $N^{5419}/+$ as reference (100%). Error bars, SD. *p < 0.0005 by Student's *t* test; brackets indicate phenotypes compared. (E) Quantification of the wing sizes. The wing sizes of the tested combinations do not statistically differ. (F) The length of the notches varies between the different combinations. A strong increase can be observed when *Nurf301* and *pzg* activity is reduced, whereas in combination with *Nurf38* the effect is not as strong. As the overall size of the wings is comparable, changes in the size of the notches are not due to a modified wing size. (G) Penetrance of wing notches was determined for the respective genotypes. Both, *Nurf38[1]* and *Nurf301[2]* mutants increased the penetrance of this phenotype.

expression of Ci, ruling out a global requirement of Nurf for the transcription of developmentally regulated genes (Figure 4, E–E").

Together, these results suggest that the NURF chromatin-remodelling complex is required for the activation of Notch target genes. To further confirm this conclusion, we analyzed the effects of a local down-regulation of *Nurf38* and *Nurf55* by RNAi on *vg* and on *E(spl)m8* expression using the Gal4/UAS system. Again, a strong reduction of Notch target gene activity was observed in the affected cells (Supplemental Figure S3). Together, these data indicate that Pzg interacts with NURF to promote transcription of Notch target genes.

DISCUSSION

Our work shows that Pzg is associated with at least two different types of protein complexes that are involved in transcriptional activation: the TRF2–DREF complex and the NURF complex. Interestingly, these two complexes share several members apart from Pzg despite their different roles in core promoter selection versus nucleosome sliding and

Nurf301[2] clones (GFP-)
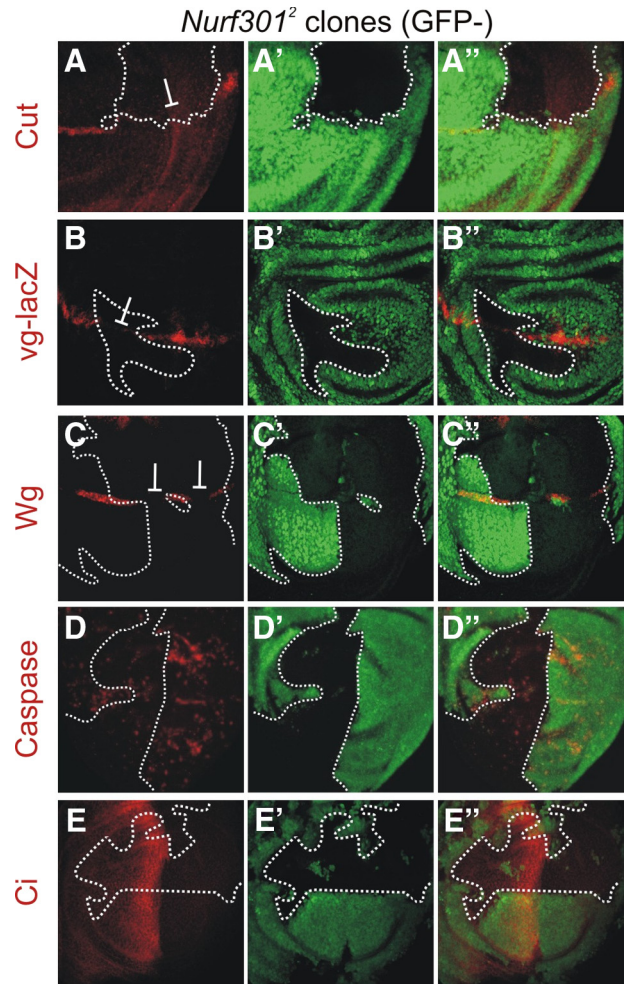
Figure 4. Notch target gene activity is reduced in *Nurf301[2]* mutant cell clones. (A–C") *Nurf301[2]* mutant clones in the wing imaginal disk marked by the absence of green fluorescent protein (GFP; outlined) reveal an autonomous reduction of the Notch target gene activity, including Cut (red in A and A"), vg-lacZ (red in B, B"), and Wg (red in C and C"). (D–D") No increase of apoptosis, detected by activated Caspase 3, was observed in *Nurf301[2]* mutant cells (loss of GFP) in comparison to the surrounding *Minute* GFP-positive cells. (E–E") *Nurf301* activity is not required for the expression of Ci (red in E and E").

chromatin activation (Martinez-Balbas *et al.*, 1998; Hochheimer *et al.*, 2002; Narlikar *et al.*, 2002). However, the specific role for Pzg in the promotion of Notch target gene transcription involves NURF and not the TRF2–DREF complex. Notably, NURF also promotes efficient expression of a subset of Wingless target genes (Song *et al.*, 2009). In this case, a direct interaction between ISWI and Armadillo, the major transcriptional coactivator of Wingless targets, was shown. We have no indication however, that *pzg* is involved in the regulation of *wg*, suggesting that the NURF complex recruits Pzg only onto specific promotors. Furthermore, the NURF subunit Nurf 301 contacts the Ecdysone receptor (EcR), thereby modulating the activity of ecdysone signaling during the larval and pupal stages of *Drosophila* development (Badenhorst *et al.*, 2005). How is NURF recruited to Notch target sites? Notch target gene activation involves a ternary complex containing the DNA-binding protein Suppressor of Hairless [Su(H)], intracellular Notch, and Mastermind, plus

other more general coactivators (reviewed in Bray, 2006; Kopan and Ilagan, 2009). We have no indication of a direct contact of Pzg to either Notch or Su(H), tested by coimmunoprecipitations as well as yeast two-hybrid assays (Figure 2 and data not shown). However, we cannot exclude contacts between the other components, notably Mastermind or ISWI. Mastermind has been shown to interact with several chromatin modifying proteins, for example, with the histone acetyltransferase p300 or with cyclin-dependent kinase 8 (Fryer *et al.*, 2004; Hansson *et al.*, 2009).

Several studies in *Drosophila* and vertebrates have shown that many Notch-responsive target genes are regulated by combinatorial signal inputs, which need the Notch ternary complex and additional cooperators bound to sites nearby. In contrast to cofactors within the transactivation complex, these other factors do not physically interact with the Notch ternary complex but instead synergize during transcriptional activation at Notch target gene promoters (e.g., Fu and Noll, 1997; Furriols and Bray, 2001; Lee *et al.*, 2007). It is conceivable, that a Pzg-NURF complex is likewise needed in conjunction with the Notch transactivator complex for full Notch target gene expression.

Gene Regulation Complexes Share Different Subunits

It is well established, that chromatin modification complexes share several components. For example, ISWI is not only contained in NURF and TRF2–DREF complexes but also in chromatin-remodeling and assembly factor (CHRAC) and ATP-utilizing chromatin-remodeling and assembly factor (ACF) in *Drosophila*, where it serves to increase the accessibility of nucleosomal DNA (Hochheimer *et al.*, 2002; reviewed in Narlikar *et al.*, 2002). Nurf 55, also known as CAF-1, forms a stable complex with *Drosophila* Myb and E2F2/RBf and regulates the transcription of several developmentally important genes (Martinez-Balbas *et al.*, 1998; Lewis *et al.*, 2004). Like ISWI and Nurf 55, also Nurf 38 is present in the TRF2–DREF complex (Hochheimer *et al.*, 2002). Pzg is contained within the TRF2–DREF and within the NURF complex serving the activation of proliferation related genes and N target genes, respectively (Hochheimer *et al.*, 2002; Kugler and Nagel, 2007). Not all NURF complexes, however, require *pzg*, for example, as during the activation of Wg target genes. Sharing components raises the question, how specificity of the different complexes is achieved. Obviously, specificity is mediated either by unique subunits or by certain combinations of shared subunits. These subunits may specifically modulate the activity of the ATPase subunit or, more likely, may help to target the remodeling complexes to particular promoters. Two members of the NURF complex, ISWI and Nurf 301, have been shown to directly target transcription factors (Badenhorst *et al.*, 2005; Song *et al.*, 2009). It is tempting to speculate, that Pzg might be a specific cofactor needed to realize some of the operation spectrum of NURF, notably during the epigenetic regulation of Notch target genes.

ACKNOWLEDGMENTS

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