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ARTICLES

Transient Requirements of YY1 Expression for PcG Transcriptional Repression and Phenotypic Rescue

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Abstract A hallmark of PcG transcriptional repression is stability of the repressed state, although the mechanism of this stability is unclear. The mammalian transcription factor YY1 can function as a PcG protein in *Drosophila* resulting in transcriptional repression and correction of phenotypic defects caused by mutation of its homologue, Pleiohomeotic (PHO). To discern the temporal requirements of YY1 expression for these functions, and to study its mechanism of stable repression in vivo, we used a *Drosophila* larval wing imaginal disc transcriptional repression system. We found that YY1 was needed transiently at day 3 or later of embryonic development to stably repress a reporter transgene at day 8 in wing imaginal discs. Stable transcriptional repression did not correlate with maintenance of YY1 or Polycomb DNA binding, but did correlate with persistence of histone H3 methylation on lysine 27. We also found that YY1 expressed during the first 7 days of development was sufficient for rescue of fly development (a 14 day process) in *pho* mutant flies. Therefore, YY1 was transiently required for correction of fly defects and was dispensable past the pharate adult stage. Possible mechanisms of repression by YY1 are discussed. J. Cell. Biochem. 96: 689–699, 2005. © 2005 Wiley-Liss, Inc.

Key words: YY1; transcription; PcG; development; chromatin; epigenetics

Polycomb group (PcG) proteins were first identified in *Drosophila* and function as high molecular weight complexes that maintain transcriptional repression patterns during embryogenesis [Pirrotta, 1998; Simon and Tamkun, 2002; Orlando, 2003]. Over a dozen *Drosophila* PcG proteins have been identified that show diverse effects at distinct developmental stages [Kennison, 1995; van Lohuizen, 1998]. Some PcG proteins result in embryonic lethality, while others exhibit less severe non-lethal defects [van Lohuizen, 1998; O'Carroll et al., 2001]. A variety of vertebrate PcG proteins have been identified that function similar to their *Drosophila* counterparts and regulate *hox* gene expression, skeletal development, hematopoiesis, neural development, cell growth control, chromatin remodeling, histone modification, and transcriptional repression [van der Lugt et al., 1994; Akasaka et al., 1997; Bel et al., 1998; van Lohuizen, 1998; Jacobs et al., 1999; Akasaka et al., 2001; Francis and Kingston, 2001; Orlando, 2003; Cao and Zhang, 2004]. Disruption of mammalian PcG proteins also contributes to breast, prostate, and lymphoid cancers, and to stem cell self renewal [Bea et al., 2001; Varambally et al., 2002; Kleer et al., 2003; Lessard and Sauvageau, 2003; Park et al., 2003; Valk-Lingbeek et al., 2004].

PcG proteins function as large multi-subunit complexes, and a number of complexes have been identified including the PRC1 and PRC2 (Esc/Ez) complexes [Francis and Kingston, 2001]. Additional complexes may exist to mediate specific functions on distinct gene systems [Satijn and Otte, 1999]. The PRC1 complex functions in chromatin remodeling, while the PRC2 complex contains enzymatic activities that result in histone deacetylation, and histone methylation (particularly on histone H3 lysines 9 and 27) [Shao et al., 1999; Cao et al., 2002;

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Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002; Fischle et al., 2003; Min et al., 2003]. Therefore, these complexes can induce epigenetic marks that may function to control gene expression.

Transcriptional repression function by PcG proteins in *Drosophila* apparently is mediated by PcG protein binding to regulatory elements termed Polycomb Response Elements (PREs), [Pirrotta, 1999; Satijn and Otte, 1999; Brock and van Lohuizen, 2001; Francis and Kingston, 2001]. Similar sequences were recently identified in mammals [Caretti et al., 2004; Kirmizis et al., 2004]. Although most PcG proteins do not possess sequence specific DNA binding activity, the *Drosophila* PcG proteins Pleiohomeotic (PHO) and Pleiohomeotic-like (PHOL), and their vertebrate homologue Yin Yang 1 (YY1) bind to specific DNA sequences in PREs and function to nucleate PcG complexes on DNA [Brown et al., 1998; Fritsch et al., 1999; Brown et al., 2003; Caretti et al., 2004; Srinivasan and Atchison, 2004; Wang et al., 2004].

YY1 is a multifunctional protein that can repress transcription, activate transcription, function as an initiator protein, and bind to the nuclear matrix [Basu et al., 1993; Usheva and Shenk, 1994; Bushmeyer et al., 1995; Galvin and Shi, 1997; Bushmeyer and Atchison, 1998; McNeil et al., 1998; Thomas and Seto, 1999]. We recently found that human YY1 can replace *Drosophila* PHO protein in flies to repress transcription, and to rescue phenotypic defects in *pho* mutants [Atchison et al., 2003]. YY1 DNA binding results in PcG protein recruitment and concomitant histone deacetylation and methylation [Srinivasan and Atchison, 2004]. Similarly, reduction of YY1 in mammals by RNAi knock-down reduces PcG DNA binding and histone methylation [Caretti et al., 2004]. Therefore, YY1 is a crucial factor for mediating PcG functions. Homozygous knock-out of the YY1 gene in mice results in peri-implantation lethality [Donohoe et al., 1999] demonstrating its importance for early development. However, it is not known whether YY1 is also crucial at later stages of development since conditional YY1 knock-outs have not been reported. The lack of conditional knock-outs in mice hampers study of mammalian YY1 function in different tissues and at different developmental stages. However, since mammalian YY1 can function in PcG repression and development in *Drosophila*, we chose to exploit this system because it enabled us to study temporal requirements of YY1 expression for in vivo functions.

We report here that YY1 expressed during the first 3 days of *Drosophila* development was sufficient to establish stable transcriptional repression that persisted for at least 5 days. Even a single pulse of YY1 expression at day 3 of development yielded repression at day 8 suggesting a stable epigenetic mechanism of repression. Repression correlated with presence of H3 methyl-lysine 27, but not continued DNA binding by YY1 or PC. We found that YY1 expressed through day 7 of development was also sufficient to yield phenotypically normal flies at day 14, indicating that YY1 is dispensable in adult organisms. Possible mechanisms of stable repression are discussed.

MATERIALS AND METHODS

Drosophila Lines and Crosses for Imaginal Disc Staining Experiments

hsp70-GalYY1 IDE-PRE-LacZ/CyO females were crossed with pho^1*/CID* mutant males to create *hsp70-GALYY1 IDE-PRE-LacZ/+; pho1/+* flies. CyO^- CID^- F1 offspring were crossed to each other to yield the recombinant chromosome with the *hsp70-GALYY1 IDE-PRE-LacZ* transgenes on a pho^1/pho^1 mutant background. Developing organisms were maintained at room temperature and subjected to various regimens of twice daily heat shock at 37°C for 45 min each. Resulting larvae were used for larval imaginal disc dissections followed by staining for LacZ expression. Eighteen percent of the dissected discs contained the recombinant chromosome on a pho^1/pho^1 genetic background. Total larvae dissected for each heat shock regimen are shown in Figure 2.

LacZ Staining of *Drosophila* Wing Imaginal Discs

Wing imaginal discs were dissected out from the third instar larvae and fixed for 30 min in PBS containing 1% glutaraldehyde, washed four times in 100 mM Tris, pH 7.5, 130 mM NaCl, 3 mM KCl, 5 mM sodium azide, 1 mM EGTA, and incubated in X-Gal solution. Wing discs were stained overnight at 37°C, mounted onto microscope slides, and photographed. Only a single of the two wing discs from each larvae was dissected to assure accurate staining percentages.

Phenotypic Correction Experiments

hsp70GaLYY1/CyO; Pho¹/CID flies were crossed and embryos were heat shocked for 45 min at 37°C twice a day on the specified days. *pho¹/pho¹* flies were identified by virtue of the absence of the CID wing marker. Genotypes were confirmed by PCR. Table I shows the heat-shock regimens that were followed to determine the temporal requirements of YY1 expression for rescue of the *pho¹/pho¹* mutant phenotype.

Fly DNA Preparation and PCR

Flies were homogenized in buffer containing 10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 μg/ml proteinase K and incubated at 37°C for 20 to 30 min. Proteinase K was inactivated by heating the samples at 85°C for 1–2 min and debris was removed by centrifugation at 14,000 rpm for 2 min. To genotype the flies, 1–2 μl of DNA was used for PCR against the *pho¹* allele and the YY1 transgene. Primers used for PCR are:

pho¹ mutant: TTTGGCATTGATGGCTTCACG
CCGGTGTTGCCCGTATGATTA
F*yy1* gene: GTGGTGCTCAGTTGTAGAATG
Rhsp703′: ATTAGACTCTTTGGCCTTAGTC-GAC

PCR amplification was for 35 cycles of 94°C 30 s, 59°C 30 s, and 68°C 6 min.

Western Blots

Fly line *103hsp70GalYY1* was heat-shocked for 45 min at 37°C. Flies were harvested at indicated times and homogenized in boiling electrophoresis sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.72 M β-mercaptoethanol, 10% glycerol. After boiling for 10 min and centrifugation, samples were subjected to the Western blot procedure with anti-GAL4 antibody or anti-MAPK antibody as a loading control (Santa Cruz Biotechnologies, Santa Cruz, CA). For westerns of imaginal disc proteins, 10 imaginal discs were homogenized in electrophoresis buffer and subjected to the Western blot procedure as above.

Imaginal Disc ChIPs

ChIP assays from wing imaginal discs were performed as previously described [Cao et al., 2002; Wang et al., 2004]. Briefly wing imaginal discs were dissected from late third instar

TABLE I. YY1 Must be Expressed in *pho¹/pho¹* Organisms During Early and Mid *Drosophila* Development for Phenotypic Rescue[a], but is Dispensable in Late Development

Heat-shock regimen	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	Flies examined	Viable *pho¹/pho¹* flies	Percent rescued by YY1	Percent maximal
1					*	*	*	*	*	*	*	*	*	19	0	0	0
2						*	*	*	*	*	*	*		90	1	1.1	6
3							*	*	*	*	*	*		102	0	0	0
4	*	*	*	*	*	*	*	*	*	*				74	4	5.4	30
5	*	*	*	*	*	*	*							44	8	18	100
6	*	*	*	*	*	*								218	16	7.3	41
7	*	*	*	*	*									184	4	2.1	12
8	*	*	*	*										151	4	2.6	14
9	*	*	*											129	0	0	0
10	*	*												156	0	0	0
11					*	*	*							48	0	0	0
12 No HS														144	0	0	0

*Days when heat-shocks were performed twice daily.
[a]Rescue was marked by survival of *pho¹/pho¹* mutant adult flies (confirmed by PCR genotyping) and correction of segmentation, antennal, and leg defects.

larvae in serum free SS3 medium, pooled, and stored on ice until fixation. Thirty discs were fixed with 1.1% formaldehyde in SS3 medium for 20 min. Following fixation, the discs were washed twice in ice cold phosphate buffered saline and sonicated in lysis buffer. Lysates were clarified by centrifugation and chromatin from the equivalent of 10 discs was immunoprecipitated with each antibody. Immunoprecipitations were done using the ChIP assay protocol described by Upstate Biotechnology. Immunoprecipitated DNA was dissolved in 50 μl of nuclease free water and 2 μl was used for quantitative PCR reaction using primers that amplify the Gal4 region in the *BGUZ* reporter [Srinivasan and Atchison, 2004]. Real time quantitative PCR was performed in duplicates using SYBR Green detection and Light Cycler System from Roche Molecular Biochemicals. Fold enrichment relative to mock immunoprecipitate and normalized to input values was calculated for each antibody with the formula $2^{\{(\text{CP mock}-\text{CP Input})-(\text{CP Ab}-\text{CP Input})\}}$, where CP is the threshold crossing point. Values were then normalized to daily heat-shock values defined as 100%. To ensure specific PCR amplification, every real time PCR run was followed by a melting curve analysis. Furthermore, the PCR products were also analyzed on agarose gels to ensure that they were of the expected molecular weight.

RESULTS

Temporal Requirements of YY1 Expression In Vivo for Transcriptional Repression in Wing Imaginal Discs

We previously showed that YY1 can repress transcription in vivo in a *Drosophila* larval wing imaginal disc transcriptional repression system [Atchison et al., 2003]. These studies used the *PBX-PRE-IDE-LacZ* reporter developed by Fritsch et al. [1999] (Fig. 1, top). This reporter gene is usually silent in wing imaginal discs because PHO nucleates PcG complexes over the PRE sequence [Fritsch et al., 1999]. In a pho^1/pho^1 mutant background, PHO is absent and no longer binds to the six PHO binding sites in the PRE. This results in lost repression and subsequent *LacZ* expression in a subset of wing disc cells [Fritsch et al., 1999]. However, if human YY1 is continually expressed from an *hsp70-GALYY1* transgene (by twice daily heat-shock), repression is restored in these cells resulting in no LacZ expression (Fig. 1) [Atchison et al., 2003]. Therefore, YY1 can repress transcription in vivo through a native PRE sequence.

While YY1 can repress transcription in wing imaginal discs through a PRE, the stability of this repression and the temporal requirements of YY1 expression are unknown. To determine the temporal requirements of YY1 expression for in vivo repression, we followed a similar

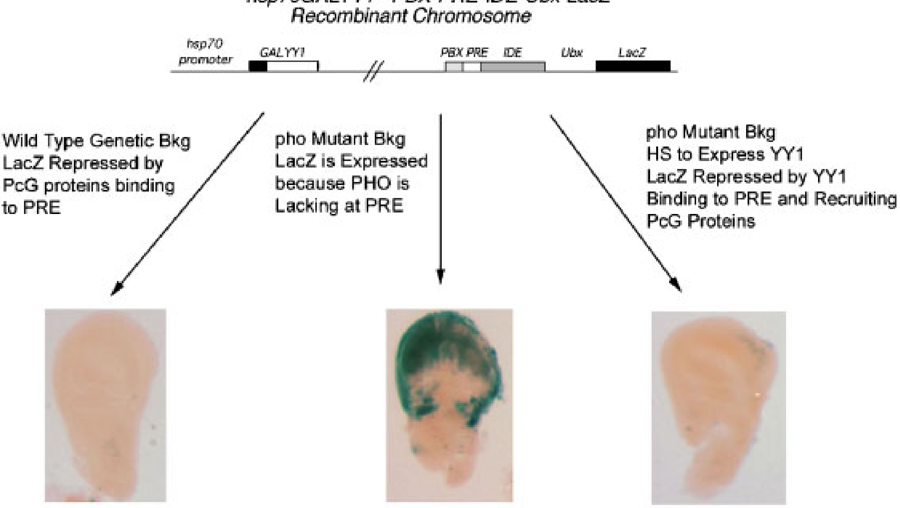

Fig. 1. GALYY1 can compensate for mutant *pho* in wing imaginal discs. A diagram of the recombinant chromosome with the *hsp70GALYY1* and *PBX-PRE-IDE-LacZ* transgenes is shown at the top. This recombinant chromosome was crossed into either wild type or pho^1/pho^1 mutant backgrounds and developing larvae were either untreated or heat-shocked twice daily. Wing imaginal discs stained for LacZ expression are shown below. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

experimental approach, except that heat shock was performed at various times to control the timing of YY1 expression. Subsequently, third instar larval wing discs were isolated at day 8 and processed for LacZ expression. As expected, in a *pho*^{1}/*pho*^{1} mutant background, PcG proteins cannot nucleate on the PRE sequence in many wing disc cells and the LacZ gene was expressed (Fig. 2, panel 1). However, wing discs from larvae that were heat-shocked daily for 6 days to express GALYY1 showed no LacZ expression (Fig. 2, panel 2). Similarly, heat shock on the first 4 days, or the first 3 days of development yielded transcriptional repression (panels 3 and 4). On the contrary, heat shock on only the first and second days, or on day 1 alone, did not result in transcriptional repression (panels 5 and 6). Therefore, YY1 must be expressed at least until day 3 of fly development in order to generate a stable repressive complex.

To determine whether shorter pulses of YY1 would be sufficient to generate stable transcriptional repression, we performed heat shocks on day 4 alone (panel 7), days 3 and 4 (panel 8), day 3 alone (panel 9), or day 5 alone (panel 10). All of these YY1 induction regimens led to stable transcriptional repression in wing imaginal discs. In summary, our results show that YY1 transcriptional repression in wing imaginal discs is remarkably stable. A single pulse of YY1 at day 3 of larval development results in stable transcriptional repression 5 days later on day 8.

YY1 Stability In Vivo

Our results above indicated that YY1 can cause repression of transcription a considerable time after induction of a single pulse of expression. To determine the relative levels of YY1 in vivo, flies were heat shocked at 37°C for 45 min, then harvested at various times. Protein extracts were analyzed by Western blot with anti-GAL4 antibody (Fig. 3A). We found that YY1 decreased with a half life of approximately 6 h (Fig. 3B), although this is a minimal estimate because the YY1 levels observed are a combination of synthesis plus degradation. By 24 h, the YY1 levels had decreased substantially (Fig. 3A, lane 6), trace levels were present at 48 and 72 h (lanes 7, 8), and YY1 was totally undetectable by 96 h (lane 9). Based on these results, we expected essentially no YY1 to be present in wing discs several days after heat shock. As anticipated, YY1 was easily detectable in wing discs immediately after heat shock (Fig. 3C, lane 4), but no YY1 was detected in samples that were heat shocked for either 3 or 4 days, then isolated on day 8 (Fig. 3C, lanes 2 and 3). This stands in striking contrast to the stable transcriptional repression observed 5 days after a single pulse of YY1 expression (Fig. 2, panel 9). Therefore, YY1 can initiate transcriptional repression that is stable apparently after YY1 protein levels have decayed.

Induction of a Stable Epigenetic Mark by YY1 Binding

Possible mechanisms for stable repression mediated by YY1 are that YY1 nucleates PcG complexes on DNA that are subsequently stable in absence of YY1, or that YY1 binding results in formation of an epigenetic mark sufficient for repression. To distinguish between these possibilities, we explored by chromatin immunoprecipitation (ChIP) assay the stability of PcG recruitment and H3 methylation induced by YY1 DNA binding. For these studies we were unable to use the *PBX-PRE-IDE-LacZ* reporter because endogenous PHO and PHOL proteins would bind the PRE and obscure effects due to YY1. To eliminate this problem, we used the *BGUZ* reporter transgene (Fig. 4A) we previously showed could bind to GALYY1 resulting in PcG recruitment and histone methylation [Srinivasan and Atchison, 2004]. This transgene vector could thus be used to measure the stability of protein recruitment and histone methylation because PC DNA binding and histone methylation are absent without YY1 induction (see below). We performed ChIP studies on wing imaginal discs isolated from *hsp70GALYY1 BGUZ* transgenic animals subjected to various heat-shock regimens, and assayed for GALYY1 binding, PC binding, and presence of H3 methyl-lysine 27 (Fig. 4B).

Wing discs from untreated larvae showed low levels of GALYY1 and PC binding, and low levels of methylation on H3 lysine 27 (Fig. 4B, open bars). As expected, daily heat shock led to high levels of GALYY1 binding to the reporter, as well as recruitment of PC and subsequent methylation of H3 lysine 27 (Fig. 4B, filled bars). If developing larvae were only heat shocked for 4 days, and discs were subsequently isolated at day 8, a distinct pattern was observed (Fig. 4B, shaded bars). First, as expected, by day 8 GALYY1 binding was dramatically reduced

Percent Maximal Stained (n)

1) NO HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
94 (77)

2) HS HS HS HS HS HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
0 (34)

3) HS HS HS HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
0 (85)

4) HS HS HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
0 (56)

5) HS HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
92 (79)

6) HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
100 (67)

7) HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
0 (61)

8) HS HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
0 (51)

9) HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
1 (132)

10) HS — Harvest
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8
0 (85)

Fig. 2.

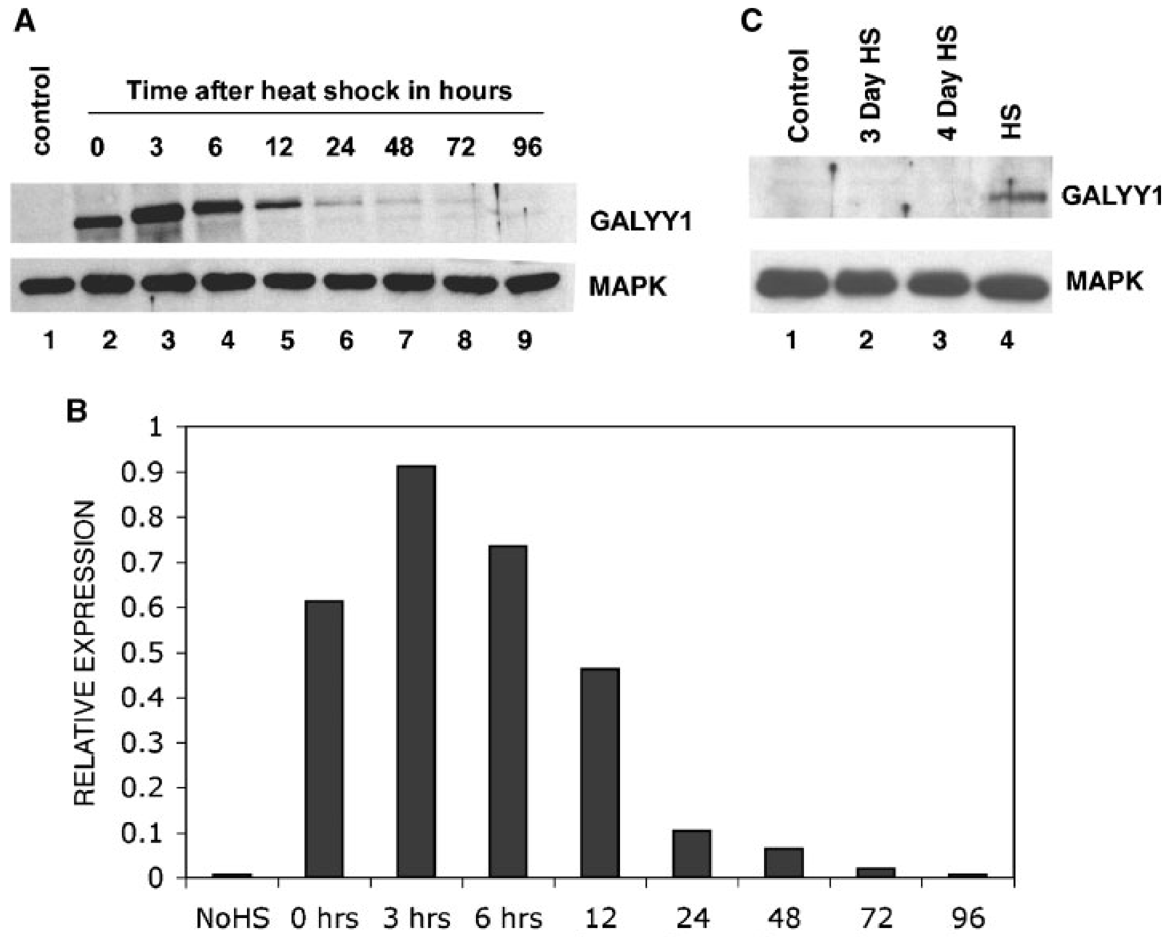

Fig. 3. YY1 is undetectable at times when transcriptional repression is maintained. **A**: Fly line 103hsp70GALYY1 was heat-shocked for 45 min at 37°C. Fly extracts were prepared at various times after heat-shock and subjected to the Western blot procedure with anti-GAL4 antibody. The filter was stripped and reprobed with antibody to MAPK to show comparable levels of protein extract in each lane. **B**: Quantitation of the YY1 data from (A) above. **C**: YY1 is undetectable in wing discs when transcriptional repression is maintained. Developing organisms were either untreated, or heat-shocked for 45 min at 37°C twice daily for the first 3 days or 4 days of development. Wing discs were then dissected on day 8 and processed for Western blot analysis with anti-GAL4 or MAPK antibody.

due to decay of the GALYY1 protein ($P < 0.05$ compared to daily heat-shock). Second, the level of PC binding dropped in parallel to the loss of GALYY1 ($P < 0.05$ compared to daily heat-shock). Therefore, YY1 binding did not result in formation of a PcG complex that was stable once YY1 expression decayed. On the contrary, H3 methyl-lysine 27 that was induced by transient GALYY1 expression remained at elevated levels (Fig. 4B; $P = 0.32$ compared to daily heat-shock). Thus, this epigenetic mark was more stable than YY1 and PC binding. The high range in the H3 methyl-lysine 27 data (Fig. 4B) was due to the fact that in one of the three experiments, H3 methyl-lysine 27 levels did drop to near background levels. This suggested that although H3 methylation persisted past the presence of YY1 and PC binding,

Fig. 2. YY1 expression on day 3 of development and beyond generates stable transcriptional repression. Developing organisms containing the *PBX-PRE-IDE-LacZ* reporter and *hsp70GALYY1* transgenes in a pho^1/pho^1 genotype were heat-shocked at various times to induce GALYY1 expression. Subsequently, wing discs were isolated on day 8 and processed for LacZ expression. The various heat shock regimens are shown in the **left panel** with arrowheads marked HS. Representative stained wing imaginal discs are shown at the **right**. 18% of the developing larvae will have a pho^1/pho^1 $hspGALYY1^+$ genotype. The percent wing discs expressing LacZ was determined then normalized to the maximal number of 18% defined as 100%. Numbers in parenthesis represent the number of larvae dissected. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

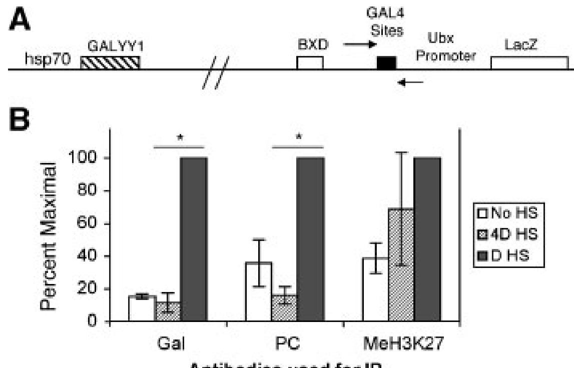

Fig. 4. Stable repression correlates with presence of H3 methyl-lysine 27, but not persistence of YY1 or PC DNA binding. **A**:Map of the *BGUZ* reporter transgene. **B**: YY1 and PC binding are transient but H3 methy-lysine 27 persists. Wing imaginal discs were isolated from *hsp70GALYY1 BGUZ* larvae that had been subjected to various heat shock regimens. ChIP assays were performed with anti-GAL, anti-PC, and anti-H3 methyl-K27 antibodies. Fold enrichment over control antibody was calculated by the $2^{\Delta\Delta CP}$ method, then replotted with daily heat-shock defined as 100% maximal. Error bars show the standard deviation from the mean. Data from three independent experiments are shown. Although GALYY1 and PC DNA binding fall to background levels by day 8 (shown by asterisks; $P < 0.05$ compared to daily heat-shock), H3 methyl-lysine 27 does not significantly drop compared to daily heat-shock ($P = 0.32$).

it was not present indefinitely. In summary, we conclude that PC DNA binding to this reporter is not stable in the absence of YY1, and that H3 methy lysine 27 persists longer than PC binding, but not indefinitely.

Phenotypic Correction of Pho Mutant Flies

The relative persistence of repression by transient YY1 expression suggested that YY1 might be only transiently required for correction of phenotypic defects caused by *pho* mutation. We previously showed that YY1 can correct phenotypic defects in pho^{1}/pho^{cv} mutant flies [Atchison et al., 2003]. These defects include segmentation defects, deformed antenna and arista structures, extra sex combs on 2nd and 3rd legs of male flies, and loss of claws on each leg. The mutant phenotype in pho^{1} homozygotes is even stronger and results in flies that die as pharate adults. To determine whether YY1 could correct this more severe *pho* mutant, and to study temporal requirements of potential YY1 correction, the *hspGAL-YY1* transgene was crossed into a pho^{1}/pho^{1} mutant background and developing samples were subjected to various heat shock regimens. Complete correction by YY1 would yield rescue in 18% of the flies (the percentage of flies carrying the pho^{1}/pho^{1} alleles and the YY1 transgene). Interestingly, we found that YY1 continuously expressed during the first 7 days of development was sufficient for complete rescue of fly development (Table I, regimen 5; 100% rescue of mutant flies). Expression during the first 6, 5, or 4 days (regimens 6–8) resulted in 41, 12, or 14% of the mutant flies corrected, respectively. Although these phenotypic corrections are below the maximum expected for complete correction, the ability to observe any correction with expression during only the first 4 days of development is impressive. On the other hand, no correction of mutant flies was observed if YY1 was induced only for the first 3, or the first 2 days of development (regimens 9 and 10). Induction for the first 10 days only yielded correction of 30% of the mutant flies perhaps due to toxic effects of multiple successive heat shocks. YY1 expression early in development however, was crucial for rescue because YY1 inductions beginning on days 5, 6, or 7 (regimens 1, 2, and 3) did not efficiently rescue fly development (although a single fly was rescued using regimen 2). In addition, induction on the consecutive days 5, 6, and 7 alone did not result in phenotypic correction (regimen 11). Thus, YY1 must be expressed during early and mid *Drosophila* development to rescue *pho* mutant flies, but is dispensable for late *Drosophila* development.

DISCUSSION

Our results indicate that YY1 can initiate transcriptional repression that is stable many days after YY1 expression ceases (Fig. 2). Several mechanisms of stable repression initiated by YY1 are possible. First, YY1 could generate PcG complexes on DNA that remain stably associated with the DNA after decay of YY1. Second, YY1 could induce epigenetic marks on histones surrounding the YY1 binding site, and these epigenetic marks would be crucial for the stable repression mechanism. Finally, YY1 might replace PHO at crucial developmental stages to initiate repression, and at subsequent stages the PHO relative, PHOL, might be sufficient to maintain PcG binding. Our results show that PC protein binding to *BGUZ* DNA mirrors the level of YY1 DNA binding (Fig. 4) indicating that PcG complexes do not remain associated with the DNA in the absence of YY1. Thus, YY1 is needed

to be continuously present in order for PC proteins to bind to the *BGUZ* reporter DNA. On the contrary, histone H3 methylation on lysine 27 persisted even after the decay of YY1. Thus, this epigenetic mark correlated with long-term transcriptional repression initiated by YY1. The mechanism for maintenance of this epigenetic mark in the absence of YY1 and PC binding is unknown. It is also not clear how long these epigenetic marks remain, although it is not likely that they persist indefinitely since in some experiments we observed substantial loss of H3 methylation on day 8 (Fig. 4B).

The PHO relative, PHOL, can bind to PRE sequences and might be expected to compensate for loss of PHO in pho^1 homozygous mutants [Brown et al., 2003; Wang et al., 2004]. However, the mutant phenotype of pho^1 homozygotes clearly show that PHOL cannot completely compensate for loss of PHO. Here, we show that transient expression of YY1 can rescue the *pho* mutant phenotype. While this rescue might be due to epigenetic mechanisms initiated by YY1, we cannot exclude the possibility that expression levels of PHOL are too low at certain stages of larval development, and that YY1 expression at these stages compensates for lack of PHO and the low levels of PHOL. At later stages, PHOL might be expressed at sufficient levels to compensate for decay of YY1 and absence of PHO. In this case, presence of adequate PHOL might maintain transcriptional repression rather than epigenetic marks. In any case, it is clear that YY1 expression need only be transiently induced to generate stable transcriptional repression and to phenotypically correct pho^1 homozygous mutants.

The stability of YY1 repression we observed is interesting. Other workers showed that deletion of PRE sequences after initiation of transcriptional repression results in lost repression and reactivation of gene expression [Busturia et al., 1997; Sengupta et al., 2004]. This indicates continuous presence of the PRE sequence is needed for stable repression. On the other hand, while deletion of PcG genes results in derepression of some *hox* genes [Beuchle et al., 2001], the timing of derepression is variable, and some PcG gene deletions have no effect on *hox* expression [Beuchle et al., 2001]. Our data indicate that persistence of H3 methyl-lysine 27 at the PRE location correlates with transcriptional repression. Deletion of the PRE would remove the regulatory DNA sequence, as well as the histone epigenetic mark, perhaps explaining the need for continuous PRE presence for stable repression [Busturia et al., 1997; Sengupta et al., 2004].

While a short pulse of YY1 expression on day 3 was sufficient for transcriptional repression on day 8, longer periods of YY1 expression were needed for phenotypic correction of *pho* mutant flies. This may not be surprising since phenotypic correction presumably requires regulation of multiple genes. In addition, phenotypic rescue might require non-PcG repression functions of YY1, or its activation function in other systems [Shi et al., 1997]. Here too, however, only transient YY1 expression was required. Expression was required during both early, and mid embryogenesis, but YY1 expression was dispensable after day 7. This time period corresponds to the transition from the third instar larval stage to the pupal stage. Thus, by mid development, YY1 can be lost with no apparent developmental consequences. Here, either YY1 is completely dispensable past the pharate adult stage, or, as described above, PHOL is able to compensate for lack of PHO and YY1 at these stages. Indeed, at the *Ubx* locus, PHOL apparently can recruit PcG proteins to DNA in the absence of PHO [Wang et al., 2004].

The third instar larval stage is a period of development when imaginal discs representing precursors to various body structures are being transformed into their adult structures. Flies mutant for *pho* have defects in antennae, leg, and claw structures (in addition to other homeotic transformations). Genes necessary for formation of these structures include *aristaless*, *bric-a-brac*, and *distalless*. Mammalian homologues of these genes exist suggesting possible target genes for YY1 during mammalian development.

In mice, homozygous mutation of the YY1 gene results in peri-implantation lethality (around gastrulation; Donohoe et al., 1999). This indicates that the YY1 relative, YY2, is unable to compensate for loss of YY1 in early embryonic development [Nguyen et al., 2004]. Our data suggest that YY1 may also be required for later developmental processes in mice, but these functions will require the generation of conditional knock-outs to be revealed. If a transient requirement for YY1 is found in mammalian systems, YY1 could be a useful target for therapies against diseases associated

with aberrant PcG function such as prostate cancer, breast cancer, and various lymphomas [Valk-Lingbeek et al., 2004]. However, the stability of repression, even in the absence of YY1, argues that such treatments may require extended periods of time to exert their effects.

In summary, we found that transient YY1 expression can result in stable transcriptional repression and phenotypic rescue of *pho* mutant flies. This repression may be due to epigenetic marks rather than stable PcG complexes associated with DNA, or may be due to YY1 providing a function within a developmental window when PHOL is insufficient.

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