Hairless is a cofactor for Runt-dependent transcriptional regulation

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ABSTRACT **Runt is a vital transcriptional regulator in the developmental pathway responsible for segmentation in the *Drosophila* embryo. Runt activates or represses transcription in a manner that is dependent on both cellular context and the specific downstream target. Here we identify Hairless (H) as a Runt-interacting molecule that functions during segmentation. We find that H is important for maintenance of *engrailed (en)* repression as was previously demonstrated for Groucho (Gro), Rpd3, and CtBP. H also contributes to the Runt-dependent repression of *sloppy-paired-1 (slp1)*, a role that is not shared with these other corepressors. We further find distinct roles for these different corepressors in the regulation of other Runt targets in the early *Drosophila* embryo. These findings, coupled with observations on the distinct functional requirements for Runt in regulating these several different targets, indicate that Runt-dependent regulation in the *Drosophila* blastoderm embryo relies on unique, target-gene-specific molecular interactions.**

Monitoring Editor
Julie A. Brill
The Hospital for Sick Children

Received: Jun 3, 2010
Revised: Feb 1, 2011
Accepted: Feb 3, 2011

INTRODUCTION

Eukaryotic transcription involves binding of transcription factors to *cis*-regulatory elements that then communicate to promoters to influence transcription. These factors can have both positive and negative regulatory effects. Models for communication can involve direct contacts with components of basal machinery, as well as recruitment of chromatin-modifying enzymes and/or chromatin-remodeling machinery (Cramer *et al.*, 2000; Wan *et al.*, 2001). An important class of molecules involved in these processes are cofactor proteins, either coactivators such as CBP (CREB binding protein) or corepressors (e.g., Sin3, Gro, CtBP) that do not bind DNA but are frequently found in transcriptional regulatory complexes that are associated with histone-modifying activities (Nibu *et al.*, 1998; Chen *et al.*, 1999; Phippen *et al.*, 2000; Barolo *et al.*, 2002). The complexity of understanding the importance of the interactions between different transcription factors and these various cofactors in controlling the transcriptional output is underscored by findings that many transcription factors appear to have dual roles and can function as both activators as well as repressors of transcription.

The Runx family of developmental regulators provides an example of transcription factors that have such dual regulatory properties. These proteins are characterized by the Runt Domain, a highly conserved 128–amino acid region that mediates interaction with the Beta partner protein and resultant binding to DNA (Kagoshima *et al.*, 1993; Golling *et al.*, 1996; Bushweller, 2000; de Bruijn and Speck, 2004). Each family member also contains a C-terminal VWRPY amino acid pentamer that mediates interaction with the TLE/Gro family of corepressors (Aronson *et al.*, 1997; Ito, 1997; Soderhall *et al.*, 2003). Runx proteins in mammalian systems activate or repress target genes in a context-dependent manner (Collins *et al.*, 2009). In *Drosophila*, the Runt Domain protein Lozenge simultaneously activates *prospero* and *D-Pax2* while repressing *Deadpan* during the process of cone cell differentiation in the developing eye imaginal disk (Canon and Banerjee, 2000). Runt, the founding member of this transcription factor family, was originally identified based on its role as a pair-rule gene during *Drosophila* segmentation (Gergen and Butler, 1988). The central function of the pair-rule genes is to establish the metameric expression patterns of segment-polarity genes, such as *en, wingless* (*wg*), and *slp1*, in the late-blastoderm-stage embryo. Runt functions as both an activator and a repressor of these three targets in a manner that depends on the presence or absence of other specific pair-rule transcription factors (Tracey *et al.*, 2000; Wheeler *et al.*, 2000; Swantek and Gergen, 2004).

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10-06-0483) on February 16, 2011.

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Abbreviations used: CtBP, C-terminal binding protein; TLE, transducin-like enhancer-of-split.

The distinctive effects of Runt on these different downstream targets are likely to involve regulated interactions between Runt and different coactivators and corepressors. Three proteins that directly interact with Runt and participate in transcription regulation have been identified to date. Brother (Bro) and Big-brother (Bgb), the two *Drosophila* homologues of mammalian CBFβ, interact with the Runt Domain to enhance DNA binding (Golling *et al.*, 1996). This protein–protein interaction appears to be essential for all functions of Runt as a Runt derivative containing a point mutation in the Runt Domain that disrupts this interaction is inactive in a number of different in vivo assays (Li and Gergen, 1999). The third known Runt-interacting protein is the VWRPY-interacting Gro corepressor (Aronson *et al.*, 1997). The Runt:Gro interaction contributes to a subset of Runt's regulatory functions, including repression of specific stripes of the pair-rule genes *even-skipped* (*eve*) and *hairy* (*h*) and maintenance of repression of the odd-numbered *en* stripes, but is not involved in the initial establishment of Runt-dependent *engrailed* (*en*) repression in the blastoderm embryo (Wheeler *et al.*, 2002). Recent results indicate that Runt-dependent repression of *sloppy-paired-1* (*slp1*) does not require the C-terminal VWRPY (Walrad *et al.*, 2010), suggesting that repression of this target also does not involve interactions between Runt and Gro.

It is notable that the Runt:Gro interaction is not detected in a yeast two-hybrid assay that uses full-length Runt, and removal of the Runt Domain was necessary to observe this protein–protein interaction (Aronson *et al.*, 1997). Intramolecular interactions between the Runt Domain and flanking N- and C-terminal residues have been documented for the mammalian Runx proteins (Kim *et al.*, 1999; Gu *et al.*, 2000; Inman *et al.*, 2005), suggesting that the presence of the Runt Domain could potentially interfere with the identification of cofactors that interact with other regions of these proteins. On the basis of this observation, we conducted a yeast two-hybrid screen aimed at identifying other Runt-interacting proteins from an embryonic cDNA library using as bait a Runt protein construct that lacks the Runt Domain. As expected, this screen led to recovery of cDNA clones for the Gro corepressor. Among the other Runt-interacting candidates identified by this screen was the protein encoded by *Hairless* (*H*), a well-characterized antagonist of the Notch signaling pathway (Maier, 2006) that also interacts with the corepressors Groucho and CtBP (Barolo *et al.*, 2002; Nagel *et al.*, 2005). Additional yeast two-hybrid experiments identify two conserved regions of Runt that contribute to the interactions with both Gro and H that are also required for a subset of Runt's regulatory properties in vivo (Walrad *et al.*, 2010). To investigate the role of H in Runt-dependent gene regulation, we examined the effects of reduced maternal H levels on the response of different downstream targets to ectopic Runt. We find that maintenance, but not establishment of Runt-dependent *en* repression, is compromised in embryos with reduced H in a manner similar to that obtained by reductions in levels of Gro, CtBP, or Rpd3. In contrast to this common role, we find that the Runt-dependent repression of *slp1* is sensitive to the level of H but not to the levels of Gro or CtBP. Chromatin immunoprecipitation experiments demonstrate that H protein associates with *cis*-regulatory regions of the *slp1* locus that mediate regulation in response to Runt, and the results of coimmunoprecipitation experiments provide evidence that Runt and H are components of a common complex in the early embryo. Additional genetic experiments indicate that the dosage of H had no effect on the Runt-dependent repression of *eve* and *hairy*. This provides a second functional distinction between the roles of H and Gro corepressors and demonstrates that Runt's properties as a transcriptional regulator involve distinct interactions with multiple cofactors in a manner that depends on both the target gene and developmental context.

RESULTS

Identification of Hairless as a Runt-interacting protein

A yeast two-hybrid screen carried out previously to identify Runt-interacting proteins resulted in the isolation of several cDNA clones for Bro and Bgb, the *Drosophila* homologues of the Runt Domain-interacting CBFβ protein (Golling *et al.*, 1996). This previous screen failed to identify cDNAs for Gro, a corepressor protein that interacts with the C-terminal VWRPY motif that is conserved in all Runx proteins (Ito, 2004). Indeed, the Runt:Gro interaction was not detected in directed yeast two-hybrid assays that used the full-length Runt protein (Aronson *et al.*, 1997). Additional biochemical and two-hybrid experiments indicate that the VWRPY motif is sufficient for mediating a weak interaction with Gro and further reveal that the Runt Domain itself interferes, either directly or indirectly, with the ability to detect the VWRPY:Gro interaction in a yeast two-hybrid assay. On the basis of these results, we undertook a second yeast two-hybrid screen for Runt-interacting proteins using as bait an internal deletion construct, RuntΔRH, that lacks most of the Runt Domain. A screen of >250,000 yeast colonies transformed with two-hybrid clones from a 0–6 h embryonic cDNA library resulted in the isolation of 35 clones that gave reproducible and specific two-hybrid signals with the RuntΔRH bait plasmid.

Sequence analysis indicated that this collection included 2 different cDNAs for Gro, one of which was recovered twice, and 18 additional potential interactors. The portions of Gro contained in these clones map the Runt-interacting region to the C-terminal half of the protein, a region that encompasses the 6 WD repeats (Figure 1A). This result is consistent with the finding that the WD-repeats of the human TLE proteins are required for interactions with Runx proteins (McLarren *et al.*, 2000; Buscarlet *et al.*, 2008). Among the other putative Runt interactors identified in this screen was H, an antagonist of the Notch-signaling pathway that interacts with and blocks the function of the Suppressor of Hairless (Su(H) transcription factor [Maier, 2006]). Interestingly, H interacts directly with Gro and CtBP (Barolo *et al.*, 2002; Nagel *et al.*, 2005), two factors that share a common role in maintaining Runt-dependent repression of the segment-polarity gene *en* (Wheeler *et al.*, 2002). The two-hybrid clone recovered in our screen encodes the C-terminal 406 amino acids of the H protein and contains the region that mediates the CtBP interaction but not the Su(H) interacting domain (Figure 1B). Although there is no evidence that Notch signaling plays any role in segmentation at the blastoderm stage, *H* is expressed maternally (Maier, 2006) and thus, like Gro and CtBP, is a potential cofactor for Runt at this stage.

We used yeast two-hybrid assays to identify regions of Runt that may be involved in mediating interactions with Gro and H. These experiments were guided by recent work demonstrating the modular architecture of Runt. Phylogenetic studies identify eight regions outside of the Runt Domain that are well conserved in other *Drosophila* species, four of which show evidence of conservation in other insects. Functional studies further reveal distinct requirements for each of these four most conserved regions in different aspects of Runt's regulatory properties (Walrad *et al.*, 2010). We generated a panel of deletion derivatives, each containing an internal in-frame deletion of one of these well-conserved regions, in the backbone of both full-length Runt and RuntΔRH two-hybrid expression constructs (Figure 1C). We also generated one additional deletion that removes the N terminus as this region shows some homology to the N terminus of mammalian Runx proteins. Yeast two-hybrid assays

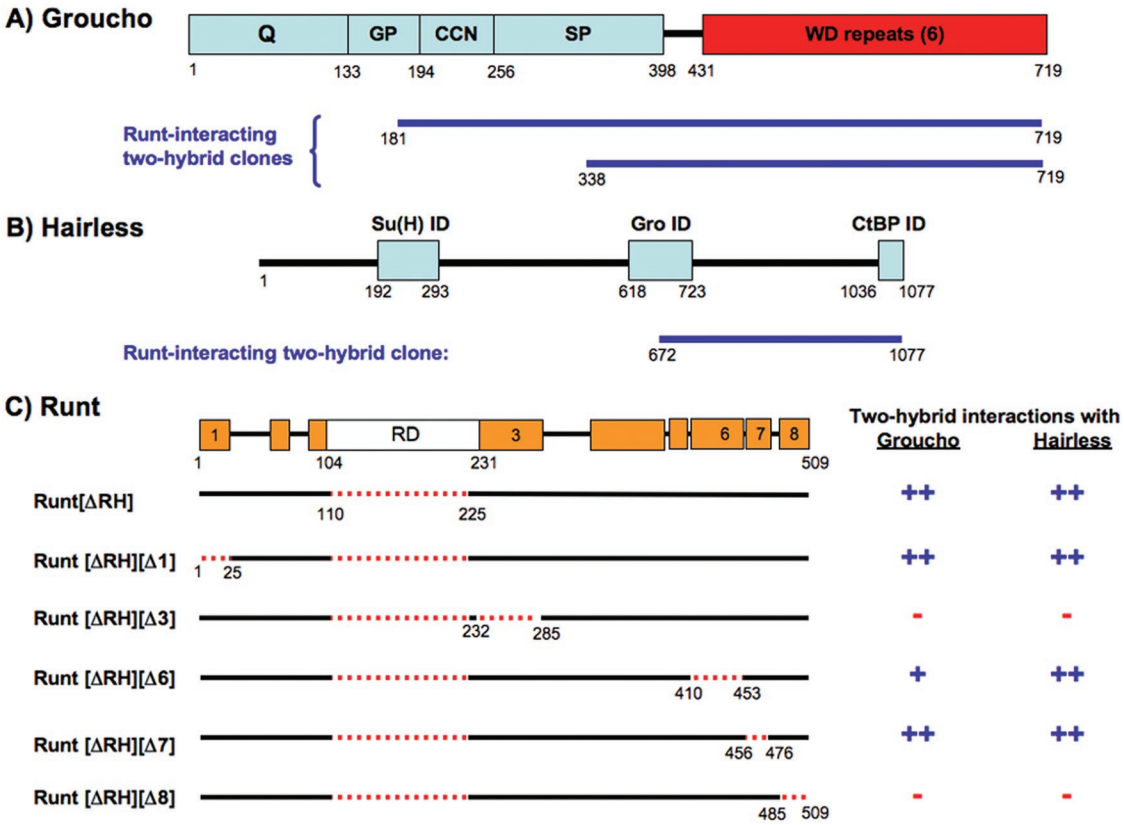

FIGURE 1: Two-hybrid interactions between Runt and the Gro and H corepressors. (A) Schematic diagram of Gro showing the location of different protein motifs. The conserved N-terminal glutamine-rich (Q) domain and C-terminal WD-repeat domains are separated by nonconserved glycine/proline (GP)- and serine/proline (SP)-rich regions and a loosely conserved CCN motif that contains putative phosphorylation sites for Cdc2 and Casein kinase II as well as a nuclear localization signal. The segments of Gro contained within the two clones recovered in the two-hybrid screen translate into protein fragments (blue lines) initiating at either amino acid Leu-181 or Pro-338 and extending to the C terminus. The longer clone overlaps the CCN, SP, and WD-repeat domains, whereas the shorter fragment contains only a portion of the SP motif and the WD-repeats. (B) One clone from the *H* gene that extends from Ser-672 to the C terminus was identified. This fragment overlaps a significant portion of the Groucho-interacting domain (ID) of Hairless (though not the eh1 domain) and the entire CtBP-ID. It does not contain the Su(H)-ID near the N terminus of H protein. (C) Schematic of Runt with boxes indicating each of the eight conserved regions. The largest conserved region contains the entire Runt Domain (RD), which mediates interaction with DNA and the Bro protein, as well as N- and C-terminal extensions of 14 and 54 amino acids, respectively. The segments removed in the Runt deletion constructs used in yeast two-hybrid experiments are indicated in the schematics below (red stippled regions), with amino acid coordinates provided for each deletion. Note that Runt[Δ3] removes the highly conserved 54–amino acid C-terminal extension (Ser-232 to Lys-285, inclusive), and not the conserved N-terminal extension of the Runt Domain. The strength of the yeast two-hybrid interaction for each deletion derivative with Gro and H is provided to the right of each schematic. ++, a strong two-hybrid signal (clear *lacZ* signal in <2 h); +. a positive but weak signal (*lacZ* activity detected between 2 and 6 h of incubation); – indicates the absence of any detectable interaction.

were used to examine the ability of each of these different deletion derivatives to interact with the Gro and H clones recovered in our two-hybrid screen. As found previously for Gro, the interaction with H is not detected using Runt two-hybrid constructs that have the fully intact Runt Domain (not shown). Results obtained with the Runt[ΔRH] constructs confirmed the importance of the VWRPY-containing C-terminal region 8 for the Gro interaction and further revealed that this same region also contributes to the interaction with H in this assay (Figure 1C). These experiments also indicated that the 52–amino acid region immediately C-terminal to the Runt Domain that is extremely well-conserved in other *Drosophila* species is important for interactions with both Gro and H in this two-hybrid assay. In contrast, the Runt[ΔRH][Δ1], -[Δ6], and -[Δ7] proteins all gave positive two-hybrid signals with both Gro and H clones, although the Runt[ΔRH][Δ6] derivative showed a reduced strength of an interaction signal with Gro (Figure 1C). As the C-terminal VWRPY motif is sufficient for mediating a weak interaction with Gro (Aronson *et al.*, 1997), the lack of a Gro-interaction signal with Runt[ΔRH][Δ3] may indicate that this deletion derivative is unstable in yeast. These results suggest that Gro and H recognize similar functional attributes of Runt, although further work will be needed to identify regions that are directly involved in mediating interaction with the H protein.

Common roles for Gro, H, and CtBP in Runt-dependent *en* repression

We used ectopic expression assays to investigate the importance of H in Runt-dependent transcriptional regulation. These

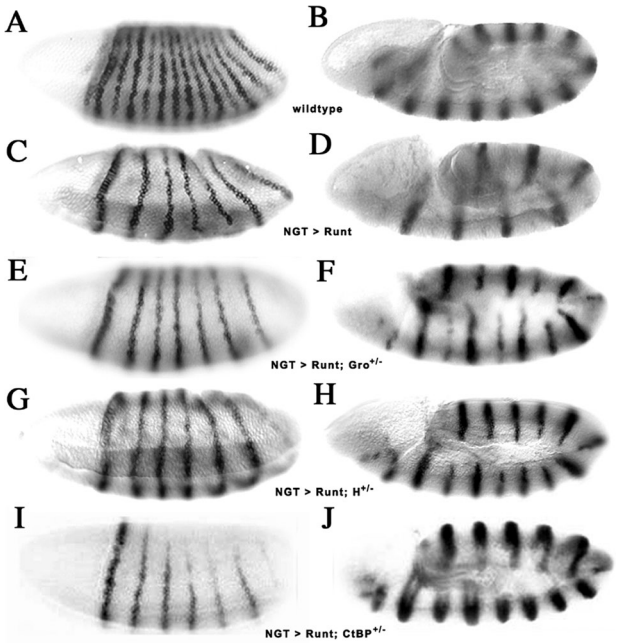

FIGURE 2: H participates in the maintenance of Runt-dependent *en* repression. In situ hybridization shows *en* expression in gastrula stage (left column) and germband extension stage (right column) embryos. Embryos in this and other figures are shown anterior toward the left, dorsal side upwards. (A, B) Wild-type (C, D) expression in progeny of females heterozygous for both the *NGT40* and *NGTA* GAL4-drivers crossed to males homozygous for the *UAS-runt[232]* transgene. This level of *NGT*-driven Runt blocks expression of the odd-numbered *en* stripes in all embryos at both stages. (E, F) Embryos from a cross between *NGT40; NGTA* heterozygous females that are also heterozygous for *Gro[BX22]* with *UAS-runt[232]* males. Although the odd-numbered *en* stripes are fully repressed early, expression is restored to intermediate levels similar to that shown in >50% of germband extension stage embryos in such crosses, with ~10% showing expression that is indistinguishable from the wild-type pattern. (G, H) Embryos from a parallel cross but using *NGT40; NGTA* heterozygous females that are heterozygous for *H[E31]*. The fully penetrant repression observed in gastrula-stage embryos is partially relieved in more than half of the germband extension stage embryos. For *H[E31]*, the full derepression as shown is found in 6% (n = 747) of the progeny in this cross. A similar proportion, (7% of 905 germband extended embryos) show full derepression in experiments with the *H[P81]* allele. (I, J) A parallel cross with females heterozygous for *NGT40*, *NGTA*, and the CtBP*[03463]* mutation yields a similar proportion of germband extension stage embryos with full derepression of *en*.

experiments take advantage of a GAL4-driven expression system that allows for quantitative manipulation of gene expression in all cells of a blastoderm-stage embryo (Tracey *et al.*, 2000). The segmentation gene most sensitive to GAL4-driven Runt at this stage is *en*, specifically the odd-numbered stripes, which are fully repressed by levels of Runt that do not alter the expression of other segmentation genes. Previous studies using this system revealed that the Runt-dependent repression of *en* could be separated into two distinct temporal phases: establishment and maintenance. Gro, CtBP, and the histone deacetylase Rpd3 are not required for the initial establishment of *en* repression, but reduction of the maternally provided levels of any of these three factors compromises the maintenance of this repression during germband extension (Wheeler *et al.*, 2002). We used similar experiments to test the role of *H* in *en* repression. As found previously for *Gro* and *CtBP*, the maternal dosage of *H* does not affect the initial establishment of Runt-dependent *en* repression (Figure 2, E, G, and I). However, as also previously found for *Gro* and *CtBP*, the Runt-dependent repression of *en* is not maintained in embryos from females heterozygous for mutations in *H* (Figure 2, F, H, and J). The parallel effects of reducing maternal dosage of Gro, CtBP, and H on this repression are consistent with the idea that a common corepressor complex involving all three factors (Barolo *et al.*, 2002) plays a role in maintaining Runt-dependent *en* repression.

Distinct roles for Gro and H in Runt-dependent pair-rule gene repression

Previous experiments using the heat-shock promoter to drive ectopic Runt revealed differential requirements for Gro in the repression of different targets of Runt (Aronson *et al.*, 1997; Tsai *et al.*, 1998). The stripe-specific repression of the pair-rule genes *eve* and *hairy* by ectopic Runt requires the VWRPY motif and is relieved by a reduction in maternal *Gro* dosage (Aronson *et al.*, 1997). Experiments using *NGT*-driven Runt expression confirm these results as the repression of *eve* stripe 2 (Figure 3C) and *hairy* stripes 1 and 6 (Figure 3D) is relieved by reducing the maternal dosage of *Gro* (Figure 3, E and F, respectively). We used the same assay system to investigate the role of *H* in the repression of these two pair-rule targets. In contrast to the findings with *Gro*, the Runt-dependent repression of *eve* and *hairy* is not relieved by a reduction in maternal *H* dosage (Figure 3G, 3H). Thus, although Gro and H share roles in maintaining Runt-dependent *en* repression, these factors have distinct roles in the repression of *eve* and *h*.

A distinct role for H but not Gro in Runt-dependent *slp1* repression

The segmentation gene that is second-most sensitive to ectopic Runt is *slp1* (Swantek and Gergen, 2004). Runt activates and represses *slp1* in a parasegment-specific manner that involves combinatorial interactions with other pair-rule transcription factors. The combination of Runt and the Zn-finger transcription factor Odd-paired (Opa) is required for *slp1* activation in odd-numbered parasegments. In contrast, in even-numbered parasegments, Runt is converted from an activator to a repressor of *slp1* due to the presence of the homeodomain transcription factor Fushi-tarazu (Ftz). Indeed, it is possible to uniformly repress *slp1* in all somatic cells of an early gastrula-stage embryo through *NGT*-driven coexpression of Runt and Ftz. We used this coexpression assay to investigate the role of different components of the common corepressor complex in Runt-dependent *slp1* repression.

The threshold coexpression levels of Runt and Ftz used for these experiments gave full *slp1* repression throughout the presegmental region in ~20% of gastrula-stage embryos. Most of the remaining embryos show weak, region-specific expression similar to that shown in Figure 4, B and C. Reducing the maternal dosage of *Gro* did not markedly effect the repression of *slp1* in response to *NGT*-driven coexpression of Runt and Ftz (Figure 4D). This result is consistent with the finding that *NGT*-driven coexpression of Ftz and a Runt derivative that is deleted for the C-terminal region that contains the Gro-interacting VWRPY motif results in repression of *slp1* (Walrad *et al.*, 2010). Taken together, these results indicate that Gro does not significantly participate in the Runt-dependent repression of *slp1*.

In contrast to the results with *Gro*, repression of *slp1* is substantially relieved in embryos from females that are heterozygous for *H*

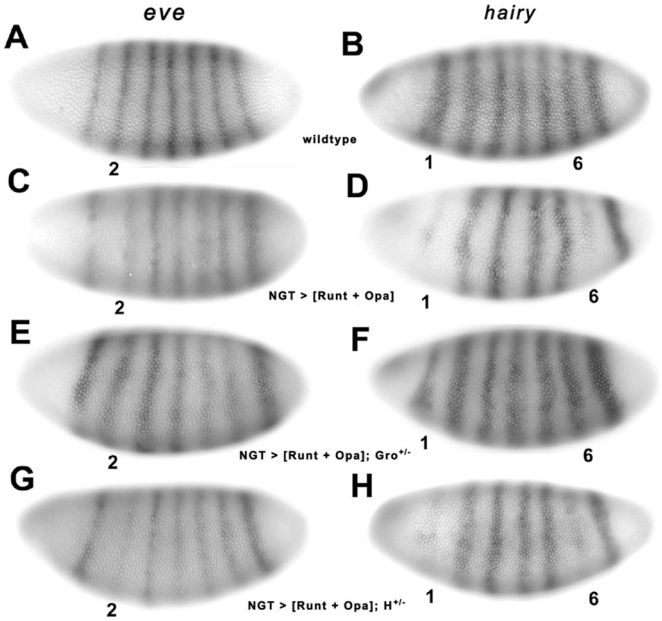

FIGURE 3: Distinct roles for Gro and H in the repression of *eve* and *hairy*. In situ hybridization showing the mRNA expression of *eve* and *hairy* in late-blastoderm-stage embryos. In wild-type embryos, both *eve* (A) and *hairy* (B) are expressed in seven stripes. The stripes that are subject to Runt-dependent repression are indicated by numbers. Note, *hairy* also is expressed in a dorsal domain of cells at the anterior end of the embryo. (C) Embryo showing partial repression of *eve* stripe 2 in response to ectopic Runt. This embryo is from a cross between *NGT40*; *NGTA* heterozygous females and *UAS-runt[15];UAS-opa[14]* homozygous males. At this level of ectopic Runt, ~50% of late blastoderm stage embryos show stronger, full repression of *eve* stripe 2. (D) Late blastoderm embryo from this same cross showing repression of *hairy* stripes 1 and 6. At this level of *NGT*-driven Runt, all mid- to late-cellular blastoderm-stage embryos show repression of these two *hairy* stripes. (E, F) expression of *eve* and *hairy* in embryos from a similar cross using *NGT40*; *NGTA* heterozygous females that are also heterozygous for *Gro[BX22]*. The reduction of maternal Gro dosage relieves the Runt-dependent, stripe-specific repression of these two pair-rule genes. In contrast, in parallel experiments with *NGT40;NGTA*, females that are also heterozygous for *H[1]*, *NGT*-driven Runt and Opa produces stripe-specific repression of both *eve* (G) and *hairy* (H) with penetrance similar to control crosses. Similar results were also obtained with *H[P8]* and *H[E31]*.

mutations (Figure 4E). In these crosses, none of the embryos show full repression, and more than half of the embryos show evidence of all 14 stripes, with some evidence of derepression in the anterior half of the even-numbered parasegments. These results indicate a dose-dependent role for H in the Runt-dependent repression of *slp1*, a role that is not shared with the Gro corepressor.

On the basis of these results, we also investigated the roles of *CtBP* and *Rpd3* in Runt-dependent *slp1* repression. Similar to the results obtained with *Gro*, reducing the maternal dosage of *CtBP* (Figure 4F) did not relieve Runt-dependent *slp1* repression. A slightly higher proportion of embryos from *Rpd3* heterozygous females showed evidence of a weak striped pattern (Figure 4G), suggesting that *Rpd3* may make a minor contribution to *slp1* repression at this stage of development. Yet in both cases the proportion of embryos that show complete *slp1* repression was the same as in experiments with control crosses. We conclude that these corepressors, like Gro, do not have a substantial role in Runt-dependent *slp1* repression.

H associates with Runt and Runt-binding regions of the *slp1* locus

The recent identification of two Runt-responsive early-stripe enhancer elements for *slp1* (Prazak *et al.*, 2010) makes this a particularly attractive model for investigating Runt-dependent regulation. A distal early stripe element (DESE) is capable of mediating both activation and repression by Runt, whereas a distinct proximal early stripe element (PESE) is only capable of mediating Runt-dependent repression. ChIP/chip assays with chromatin from early *Drosophila* embryos identify two predominant regions of Runt association within the *slp1* locus (MacArthur *et al.*, 2009) that correspond well to the DNA regions identified by these functional studies (Figure 5A). We used chromatin immunoprecipitation to investigate whether H also showed association with the *slp1* locus. Each of the four specific intervals tested in our assays gave a stronger ChIP signal with the anti-H serum than with control serum. Notably, the two strongest signals were obtained with the primer pairs centered on the region of Runt association within the DESE and PESE enhancers (Figure 5B). The weak ChIP signals detected for H at the *slp1* promoter and at −10 kb may be due to higher background with the anti-H serum, or reflect association of H throughout the *slp1* locus, but in either case the stronger ChIP signals observed within the DESE and PESE regions are consistent with the idea that H is directly involved in Runt-dependent *slp1* regulation. We extended this analysis to also examine H association in chromatin isolated from embryos in which all cells are repressing *slp1* in response to Runt and Ftz. Although comparable levels of association are observed with the −10-kb and PESE intervals, there are increases in the level of H association with the *slp1* promoter and the DESE region in chromatin from embryos that are uniformly repressing *slp1* in response to Runt and Ftz (Figure 5C). These results provide strong biochemical evidence that H participates in Runt-dependent *slp1* repression in the *Drosophila* embryo.

The genetic and biochemical experiments indicating that H participates in *slp1* regulation were prompted by the discovery of a yeast two-hybrid interaction between the Runt and H proteins. We used a coimmunoprecipitation assay to see whether we could detect interactions between these two proteins in extracts from *Drosophila* embryos. A protein with the anticipated molecular mass of 53 kDa is specifically detected with a cocktail of monoclonal antibodies against Runt (Duffy *et al.*, 1991) following immunoprecipitation with an anti-H serum (Figure 5D). As expected, this signal is enriched in an extract from a nuclear pellet compared with the soluble cell supernatant. Several specific bands of a molecular mass around 20 kDa, presumably degradation products, are also detected specifically in the H immunoprecipitates. It should be noted that Runx degradation products in a similar size range are also observed in mammalian cell extracts (Wang and Speck, 1992). Indeed, the extremely dynamic pattern of Runt protein accumulation during the early stages of *Drosophila* embryogenesis requires that the protein has a relatively short half-life.

Corepressors do not contribute to Runt-dependent *slp1* activation

The above results indicate that the participation of Gro and H in Runt-dependent transcriptional repression involves distinct, target-gene-specific interactions. As Runt is also involved in transcriptional activation, we wondered whether any of these factors might also contribute to Runt-dependent activation. We took advantage of the unique and relatively simple rules for *slp1* regulation to investigate this possibility. The combination of Runt and Opa is necessary and sufficient for *slp1* activation in all somatic blastoderm cells that do

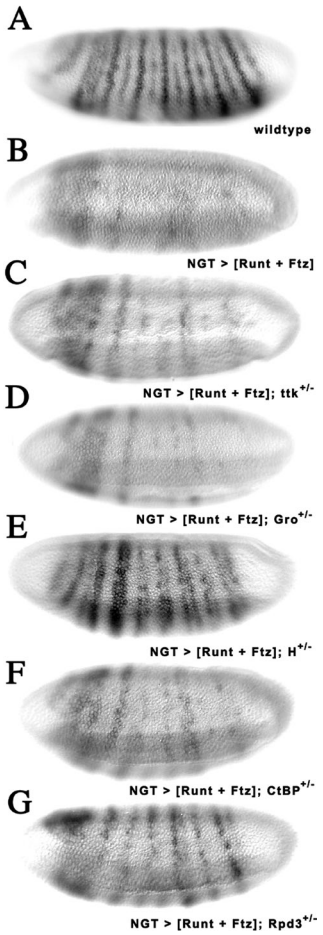

FIGURE 4: H, not Gro, contributes to *slp1* repression. In situ hybridization showing *slp1* mRNA expression in gastrula-stage embryos. For these experiments, *slp1* expression was scored in embryos that show evidence of mesodermal invagination but that have not yet undergone significant germband extension. This narrow developmental window of <15 min allows for a careful comparison of expression phenotypes that can change dynamically during development. (A) In wild-type embryos at this stage, *slp1* is expressed in 14 two-cell wide stripes, corresponding to the two posterior-most cells in parasegments 1–13 as well as a stripe 0 that is anterior to parasegment 1. There is also expression in a band of dorsal cells anterior to the presegmental region. The level of ectopic Runt and Ftz obtained in embryos from a cross between females heterozygous for both the *NGT40* and *NGTA* drivers and males homozygous for both *UAS-runt[15] and UAS-ftz[263]* is sufficient to fully repress *slp1* expression in 20% of the embryos. Most of the remaining embryos show weak expression in the head region with very weak (B) to weak (C) expression of a subset of stripes in the presegmental region. None of the embryos show evidence of all 14 *slp1* stripes in response to this level of *NGT*-driven coexpression of Runt and Ftz. The level of *NGT*-driven Runt in these embryos is approximately threefold greater than obtained in the cross-es with *UAS-runt[232]* that were used to examine *en* repression (Li and Gergen, 1999). Note that the embryo in (B) is from an experimental cross with *NGT40; NGTA* heterozygous females that are otherwise wild-type, whereas the embryo in (C) is from females that are also heterozygous for *ttk[1e11]*. These two maternal genotypes produce an identical range of *slp1* expression phenotypes, indicating that, unlike the Runt-dependent repression of *en*, Ttk does not make an important dose-dependent contribution to *slp1* repression. (D) Representative embryo from a cross with *NGT40; NGTA* heterozygous females that are also heterozygous for *Gro[BX22]* showing *slp1* repression in response to Runt and Ftz. Approximately half (35 of 67 = 52%) of the gastrula and early germband extension stage embryos in this cross show *slp1* repression at this level if not stronger, with 14 of 67 embryos (21%) showing some evidence of all 14 stripes. Similar results were obtained in crosses with *Gro[E48]*, with 54 of 97 (56%) embryos showing weak to no *slp1* expression and 22 of 97 embryos (23%) with evidence of all 14 stripes. (E) Representative embryo from a cross with *NGT40, NGTA* females that are also heterozygous for *H[E31]*. In this cross, only 14 of 119 (12%) gastrula and early germband extension stage embryos showed strong *slp1* repression, whereas 82 embryos (69%) showed evidence of all 14 stripes similar to that shown here. Similar results were obtained with *H[P81]*. (F) Representative embryo from an experimental cross using females heterozygous for *NGT40; NGTA* and *CtBP[03463]*. In this experiment, 11 (28%) of gastrula-stage embryos scored showed an even more severe, fully repressed phenotype. (G) Representative embryo from a cross with *NGT40; NGTA* heterozygous females that are also heterozygous for *Rpd3[04556]*. In this case, the most common phenotype at the gastrula stage (49%, n = 39) is faint expression of most of the stripes; full repression was still obtained in 20% of the embryos.

not have Ftz (Swantek and Gergen, 2004). Indeed *NGT*-driven coexpression of Runt and Opa is sufficient to drive *slp1* expression in the head region (Figure 6B). None of the other pair-rule or segment-polarity genes show this response. This coexpression assay thus provides a very useful platform for investigating the roles of other factors in Runt-dependent activation as the potential complications arising from cross-regulation between the different pair-rule transcription factors are eliminated. The anterior activation of *slp1* in response to ectopic coexpression of Runt and Opa is not overtly influenced by reductions in the maternal dosage of G*ro, H, CtBP* or *Rpd3* (Figure 6, C–F). This finding is consistent with the expectations that these corepressor molecules would not be involved in Runt-dependent activation.

Although the anterior activation is unchanged, there are distinctions in the *slp1* expression pattern within the segmented regions of embryos obtained from females heterozygous for mutations in the different cofactors. *NGT*-driven expression of Runt and Opa in embryos from females that are otherwise wild-type results in five stripes that are posterior to the domain of anterior expression (Figure 6B). This expression corresponds to the activation of *slp1* in cells from parasegments 3, 5, 7, 9, and 13. The larger size of the interstripe domains, as well as the elimination of expression in cells from what would be parasegment 11, is due to the expanded expression of *ftz* in response to Runt and Opa (Swantek and Gergen, 2004). The embryos from females that are heterozygous for *Gro* or *H* mutations show expression between stripes 9 and 13, with some evidence of a partial restoration of some of the even-numbered stripes (Figure 6, C and D). This spotty derepression is not observed in embryos from females that are heterozygous for *CtBP* or *Rpd3* mutations (Figure 6, E and F). In the case of *H*, this partial derepression could well be due to the role of *H* in Runt-dependent *slp1* repression. However, this explanation does not account for the effects of reducing maternal *Gro* dosage. To further investigate this phenomenon, we examined the expression of *ftz* in embryos of these same genotypes. The expanded expression and nearly complete fusion of *ftz* stripes 5 and 6 (corresponding to parasegments 10 and 12) that is produced by *NGT*-driven coexpression of Runt and Opa (Figure 6H) is diminished by reductions in the maternal dosage of either *Gro* or *H* (Figure 6, I and J). It is further notable that expression of other *ftz* stripes is also not as significantly expanded in these embryos. The reduced activation of *ftz* is not observed in embryos from females that are heterozygous for mutations in *CtBP* or *Rpd3* (Figure 6, K and L), indicating a

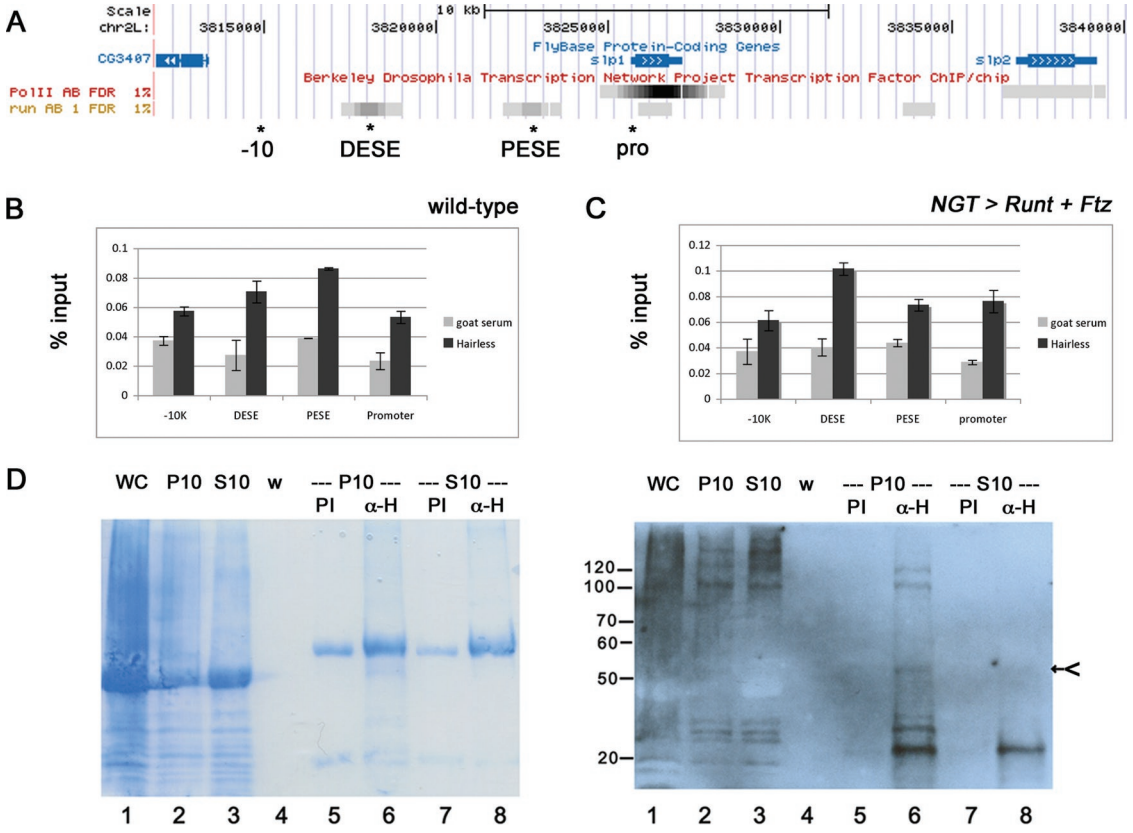

FIGURE 5: Hairless associates with Runt and *slp1* regulatory elements in vivo. (A) Screen shot of an ~25-kb interval spanning the *slp1* locus from UCSC genome browser showing the location of the *CG3407*, *slp1*, and *slp2* transcription units. The results of ChIP/chip assays for Runt and Pol II with chromatin from blastoderm-stage embryos obtained by the Berkeley Drosophila Transcription Network Project are provided below this map (MacArthur *et al.*, 2009). The two most prominent regions of Runt association correspond well to the minimal Runt-responsive early stripe elements identified from 8.1–7.2 kb and from 3.1–2.5 kb upstream of the *slp1* start site. Asterisks below this map indicate the location of PCR products generated with primer pairs located at 10 kb, 7.5 kb (DESE), 2.8 kb (PESE) upstream, and flanking the *slp1* promoter. (B) Results of ChIP assays with anti-Hairless and control goat serum using chromatin from 3–4 h *y w[67c23]*) embryos. All four intervals give a ChIP signal for H greater than obtained with the control goat serum, with the strongest association detected with the Runt-associated DESE and PESE intervals. The lower signals observed at −10 kb and at the promoter may be due to generally higher background with the anti-H serum, or reflect some level of H association across the entire locus. (C) Results of similar ChIP assays using chromatin from similarly staged embryos from a cross between females homozygous for both the *NGT40* and *NGTA* GAL4 drivers and males homozygous for *UAS-runt[15]* and *UAS-ftz[263]*. All gastrula-stage embryos in this cross show uniformly strong repression of *slp1* in all cells (Wang *et al.*, 2007). Increased H association is observed with the DESE interval, and potentially also at the promoter in these *slp1*-repressed embryos. (D) Results of coimmunoprecipitation assays using an anti-Hairless antibody and *Drosophila* embryo extracts. The left panel shows the Coomassie stained gel, and the right panel shows the results of a Western blot using a cocktail of anti-Runt monoclonal antibodies. The gel lanes are as labeled (1) whole cell (WC) lysate from 0–6 h embryos, (2) nuclei-enriched (P10) fraction, (3) nuclei-depleted (S10) fraction, (4) last wash (w) from the P10 anti-Hairless immunoprecipitation, (5) P10 immunoprecipitation with preimmune (PI) serum, (6) P10 anti-Hairless (α-H) immunoprecipitation, (7) S10 immunoprecipitation with preimmune (PI) serum, and (8) S10 anti-Hairless (α-H) immunoprecipitation. The anticipated migration of Runt at ~53 kDa is indicated by the arrow on the right. The bands detected between 20 and 25 kDa are presumed to be degradation products; the more slowly migrating bands detected near 100 kDa may reflect covalent modifications that alter mobility.

common role for Gro and H in this process that is not shared with CtBP and Rpd3. These results reveal a role for Gro and H in the activation of *ftz* but do not indicate whether these factors are directly involved in mediating activation as the effects could be an indirect effect, perhaps via the derepression of genes that contribute to *ftz* repression. These results do, however, identify a pathway that is sensitive to the dosage of both Gro and H, but not to the dosage of CtBP and Rpd3, yet another distinct combination of corepressor requirements in the regulation of segmentation gene targets in the *Drosophila* blastoderm embryo.

DISCUSSION

A principal conclusion from this work is that the H protein interacts with Runt and contributes to the regulation of a subset of Runt target genes in the early *Drosophila* embryo. H is most well characterized as an antagonist of the Notch-signaling pathway via its interaction with Su(H). Our results indicate a role for *H* during the initial establishment of the segmented body pattern, a process that does not involve the Notch-signaling pathway. Previous studies have supported Notch-independent roles for *H* at other developmental stages and in other tissues (Maier *et al.*, 1997, 1999; Furriols and

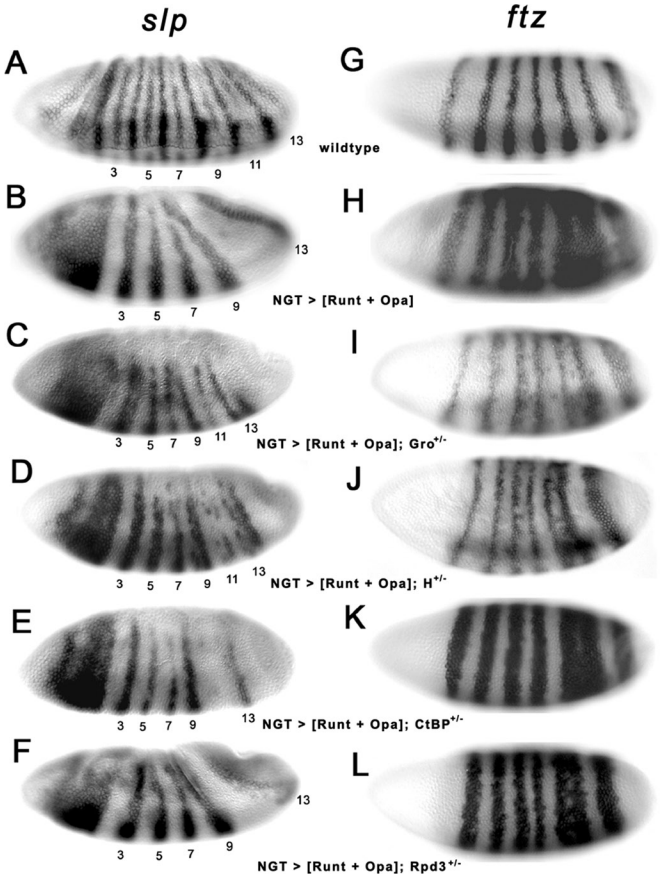

FIGURE 6: Gro and H have common effects on other Runt targets. In situ hybridization reveals expression of of *slp1* (A–F) and *ftz* (G–L) mRNAs in gastrula and cellular blastoderm stage embryos, respectively. (A) Wild-type gastrula-stage embryo shows 14 two-cell-wide stripes of *slp1*. Expression in odd-numbered parasegments appears later than in the even parasegments and is weaker at this stage. (B) Embryo from a cross between *NGT40; NGTA* heterozygous females that are otherwise wild-type and males that are homozygous for *UAS-Runt*[15] and *UAS-Opa[14]* showing *slp1* activation in the anterior head region with expanded expression domains in cells from odd-numbered parasegments that would normally express the homeodomain protein Eve and not Ftz. The exception is parasegment 11, where *slp1* fails to be expressed due to the expanded expression of Ftz in these cells (see below). (C, D) Anterior *slp1* activation in response to *NGT*-driven Runt and Opa is obtained in similar crosses using *NGT40; NGTA* heterozygous females that are also heterozygous for the *Gro[BX22]* and *H[E31]* mutations, respectively. Expression in parasegment 11 reappears in embryos from both of these crosses with the other stripes also showing a more mottled appearance. (E, F) Embryos from crosses involving *NGT40*; *NGTA* heterozygous females that are also heterozygous for the *CtBP[03463]* and *Rpd3[04566]* mutations, respectively, show expression similar to that found in embryos from *NGT40*; *NGTA* females that are otherwise wild-type, including loss of stripe 11. (G) Wild-type *ftz* expression in a late cellular blastoderm stage embryo consists of seven approximately four-cell-wide stripes. (H) The *ftz* stripes expand in embryos from a cross between *NGT40*; *NGTA* females and homozygous *UAS-Runt[15] UAS-Opa[14]* males, with nearly complete fusion of stripes 5 and 6 (representing expression in parasegments 10 and 12). (I, J) The activation of *ftz* in response to Runt and Opa is reduced in embryos from similar crosses with females that are also heterozygous for *Gro[BX22]* or *H[E31]*, respectively, with the loss of fusion of stripes 5 and 6. (K, L) In contrast, embryos from similar crosses with females that are heterozygous for *CtBP[03463]* or *Rpd3[04566]* have broadened *ftz* stripes with fusion of stripes 5 and 6.

Bray, 2000; Nagel *et al.*, 2000, 2005; Barolo *et al.*, 2002). A deletion construct of H that is unable to interact with Su(H) maintains activity during wing development, and the C-terminal region contained in this deletion construct that is required for activity was proposed to serve as a scaffold for interactions with other cofactors (Maier *et al.*, 1997). It is notable that the *H* clone isolated in our screen overlaps with this putative scaffold region.

There are several parallels in the interactions between Runt and the corepressor proteins H and Gro. The two-hybrid interaction of both proteins is not detected with the full-length Runt protein, and their interactions in the context of the Runt[ΔRH] derivative are disrupted by the additional removal of either the conserved C-terminal extension of the Runt Domain or the VWRPY-containing C terminus. Consideration has been given that the interaction between Runt and H in yeast cells could potentially be mediated by Tup1, the yeast homologue of Gro (Davie *et al.*, 2003). Were the Tup1 protein responsible for recruiting the Hairless clone to Runt in this assay, we would also have maybe expected to have isolated Rpd3 and CtBP clones in the same manner, which we did not. In addition, the presence of endogenous yeast Tup1, itself bound to multiple deacetylases and transcriptional repressors, on the *LexA* operon would likely block Gal4 activation domain function. We therefore believe the Runt:H interaction to be direct.

In vivo, both H and Gro have dose-dependent effects on the maintenance but not the establishment of Runt-dependent *en* repression. The maintenance of *en* repression also involves Rpd3 and CtBP (Wheeler *et al.*, 2002); Rpd3 is a histone deacetylase, and CtBP can recruit both a histone H3 lysine 9 methyltransferase and an H3 lysine 4 demethylase (Shi *et al.*, 2003, 2004), strongly suggesting that maintenance of *en* repression involves chromatin modifications within the *en* locus. The observation that Gro interacts directly with Rpd3 (Chen *et al.*, 1999; Winkler *et al.*, 2010) coupled with the finding that H interacts with Gro as well as with CtBP (Chen *et al.*, 1999; Barolo *et al.*, 2002; Nagel *et al.*, 2005) is consistent with the idea that these four cofactors may comprise a common corepressor complex that is recruited by Runt to establish stable *en* repression.

Although maintenance of *en* repression involves both Gro and H and potentially a common corepressor complex, our results indicate distinct roles for these two cofactors in the Runt-dependent repression of other targets. The repression of *eve* stripe 2 and *h* stripes 1 and 6 is sensitive to the level of maternally provided Gro, but not to the levels of maternal H. In contrast, the repression of *slp1* by Runt and Ftz is sensitive to the level of maternal H but not the level of Gro. It is notable that Runt-dependent repression of *slp1* is also not significantly affected by the levels of maternally provided Rpd3 or CtBP. The last observation indicates the role of H in *slp1* repression is not likely to involve recruitment of CtBP and further suggests that the repression of this target in the blastoderm embryo does not involve chromatin modifications. This suggestion is consistent with results indicating that *slp1* regulation at this stage is not associated with changes in histone acetylation but instead involves the regulation of elongation by Pol II complexes that have initiated transcription and that are paused downstream of the promoter (Wang *et al.*, 2007). Recent studies on the *cis*-regulatory regions responsible for early *slp1* expression have led to a model whereby Runt and Ftz act to repress expression by blocking productive interactions between the DESE and PESE enhancers and the *slp1* promoter (Prazak *et al.*, 2010). The observation that H associates with both of these enhancer regions as well as with the promoter and that the level of association increases when *slp1* is fully repressed is consistent with the idea that productive interactions between these two enhancers

and the *slp1* promoter are blocked by H recruitment to these different regions of the *slp1* locus.

A comparison of the results described here with a recent functional dissection of Runt underscores the distinctive context-dependent requirements for the regulation of different targets. The conserved region immediately C-terminal to the Runt Domain, which contributes to interactions with both Gro and H in a yeast two-hybrid assay, is also required for both the maintenance of *en* repression as well as the early repression of *slp1* in response to *NGT*-driven coexpression of Runt and Ftz (Walrad *et al.*, 2010). In contrast, the C-terminal region containing the VWRPY motif, which also contributes to two-hybrid interactions with both Gro and H and which is required for maintenance of *en* repression, is somewhat surprisingly not required for Runt-dependent *slp1* repression. This result indicates that the structural requirements for interactions between Runt and H in a yeast two-hybrid assay are not identical to the requirements for the functional interactions between these two factors that are involved in regulating *slp1* expression in the *Drosophila* embryo. The fact that the regulation of different targets of Runt depends not only on distinct regions of the protein but also involves interactions with different set(s) of cofactors highlights the context-dependent activity of Runt and presumably other members of the Runx family of transcriptional regulators. This complexity presents formidable challenges for unraveling the molecular mechanisms of Runt-dependent transcription regulation. The information provided by these studies coupled with the tools available in the *Drosophila* system should continue to provide valuable opportunities for further investigating the regulation of gene expression by Runt and other transcription factors during animal development.

MATERIALS AND METHODS

DNA constructs

The generation of a panel of constructs with in-frame deletions of different conserved regions C-terminal to the Runt Domain in the context of a full-length Runt cDNA clone containing a FLAG epitope tag inserted between Val-454 and Ala-455 has been described previously (Walrad *et al.*, 2010). A construct deleted for the less conserved N-terminal region, pB:Runt[Δ1] was created using a similar strategy using primers 5′-GCGGTAGCCCAGGGTCCTG-3′ and 5′-ATGCATCTCGGATCCACTAGTTC-3′. PCR with this primer pair generates a deletion that lacks amino acids His-2–Ala-28 of the wild-type protein. A plasmid containing the desired deletion was identified by sequencing, and a *Xba*I/*Bst*EII fragment with the deletion was cloned into *Xba*I + *Bst*EII digested pB:Runt^{FLAG}. The pertinent *Bam*HI fragment from the resulting clone was inserted into the *Bgl*II site of the pUAS-T vector (Brand and Perrimon, 1993) to create pUAS-Runt[Δ1].

Yeast two-hybrid assays for protein–protein interaction were done using the LexA DNA-binding domain vector pSTT91 and the GAL4 activation domain vector pACT. These shuttle vectors carry the *TRP1* and *LEU2* markers, respectively (Sutton *et al.*, 2001; Connelly *et al.*, 2006). To create pSTT91-Runt^{wt}, a fragment from *Eco*RI digested pGBT9:Runt (Golling *et al.*, 1996) was inserted in frame into the *Eco*RI site of pSTT91. The pSTT91-Runt[ΔRH] construct, which contains an internal deletion removing amino acids 110–225, was generated by recloning the *Eco*RI fragment from pGBT9:Runt[ΔRD] (Aronson *et al.*, 1997) into the *Eco*RI site of pSTT91. To generate two-hybrid constructs that combine the Runt[ΔRH] deletion with other deletions in pSTT91, pertinent pUAS-Runt deletion constructs were digested with *Eco*RI, and resulting *Eco*RI fragments were cloned into *Eco*RI-digested pB:ED[Bam-8,ΔKS] (Walrad *et al.*, 2010). Each of these pBluescript-based constructs was then digested with *Pst*I and *Sal*I, treated with T4 DNA polymerase, and then religated to generate an internal in-frame deletion of 116 amino acids within the Runt Domain. These double-deletion fragments were then released via *Eco*RI digestion and ligated into *Eco*RI-digested pSTT91.

Yeast two-hybrid screen

A 0–6 h *Drosophila* embryonic library cDNA library in the pACT transformation vector (generous gift from L. Pick) was cotransformed with pSTT91Runt[ΔRH] into the yeast strain *L40* (*MATa, leu2, ade2, his3, trp1, LYS2::lexAop-HIS3, URA3::lexAop8-lacZ*) as described previously (Bartel *et al.*, 1993; Golling *et al.*, 1996; Aronson *et al.*, 1997). Approximately 263,000 colonies were screened for growth on His$^-$ Trp$^-$ Leu$^-$ media. Colonies that grew were patched onto His$^-$ Trp$^-$ Leu$^-$ media, transferred onto filter paper, frozen in liquid nitrogen, and screened for β-galactosidase (*lacZ*) activity production as described previously (Golling *et al.*, 1996; Aronson *et al.*, 1997). Positive colonies were restreaked twice onto His$^-$ Leu$^-$ Trp$^-$ media and retested for growth and *lacZ* activity. Positive candidates were grown on Leu$^-$ media and selected for Ade2 deficiency to cure cells of the pSTT91Runt[ΔRH] plasmid. False positives were identified by mating cured pACT containing yeast strains with a panel of *AMR70* (*MATα trp1 leu2 his3 URA3::lexAop-lacZ*) yeast strains carrying the different bait plasmids: pSTT91-Lamin, pSTT91-Sir1, pSTT91-Sir3, pSTT91-Runt, and pSTT91-Runt[ΔRH]. Successful matings between different *L40* (pACT carrying *MATa*) and *AMR70* (pSTT91 carrying *MATα*) cells were selected on Leu$^-$ Trp$^-$ media, and the mated colonies were assayed for *lacZ* activity. Positive candidate pACT plasmids that survived this specificity test were isolated from yeast, transformed into bacteria, and the cDNA inserts sequenced to identify the interacting protein clones.

Drosophila strains and transgenes

The Gal4-drivers *P(GAL4-nos.NGT)40 (NGT40)* and *P(GAL4-nos.NGTA) (NGTA)* have been described previously (Tracey et al., 2000; Wheeler et al., 2002). The *P(UAS-runt.T).232 (UAS–runt[232]), P(UAS-runt.T).15 (UAS-runt[15]), P(UAS-opa.VZ).14 (UAS-opa[14])*, and *P(UAS-ftz.UL).263 (UAS-ftz[263])* transgenic lines were described previously (Li, 1999; Tracey *et al.*, 2000; Wheeler *et al.*, 2002; Swantek and Gergen, 2004). The *y w[67c23]* strain used to generate all transgenic lines was used as the wild-type control strain for in situ hybridizations. Stocks carrying the *H[1], gro[E48], gro[BX22], ttk[1e11], CtBP[03463]*, and *Rpd3[04556]* mutations were obtained from the Bloomington Stock Center (Bloomington, IN). Stocks carrying the *H[E31], H[P81]*, and *H[P8]* mutations were obtained from D. Maier (University of Hohenheim, Germany). To examine the dose-dependent effects of these different mutations on Runt-regulated targets, females heterozygous for these various mutations that also carried single copies of both the *NGT40* and *NGTA* transgenes were mated to males homozygous for different combinations of *UAS-runt, UAS-ftz,* and *UAS-opa* transgenes described in the text. Embryos from these experimental crosses were collected and fixed as described (Tsai and Gergen, 1994; Swantek and Gergen, 2004). In situ hybridization with digoxigenin-labeled (Roche, Indianapolis, IN) antisense RNA probes for *en*, *slp1, ftz, eve,* and *h* was carried out as described previously (Walrad *et al.*, 2010). Embryo images were captured on a Zeiss Axio microscope using a 10×/0.3 lens.

Chromatin immunoprecipitation

ChIP experiments were conducted as described previously (Wang *et al.*, 2007) using 10 μg goat anti-Hairless antibody (dT-20; Santa Cruz Biotechnology, Santa Cruz, CA) with 300 μg cross-linked

chromatin from 3–4 h embryos. Chromatin used as the wild-type control was isolated from *y w[67c23]* embryos, whereas embryos from a cross between females homozygous for both the *NGT40* and *NGTA* GAL4-drivers and males homozygous for the *UAS-runt[15] and UAS-ft[263]* transgenes were used to isolate chromatin representing the *slp1*-repressed state. An equal amount of goat serum was used for the negative control. Quantitative PCR was done using primer pairs centered near peak regions of Runt association within two Runt-responsive early-stripe elements of the *slp1* gene (Prazak *et al.*, 2010), as well for a further upstream region and the *slp1* transcription start site.

Immunoprecipitation

Wild-type (Oregon R) 0–6 h embryos (courtesy of D. Finnegan) were dechorionated, washed, and stored at −20°C. Then 1 ml of embryos was homogenized for 4 min in IP Buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, protease inhibitor cocktail; Roche, Indainapolis, IN) in an ice-bath sonicator and examined at time points throughout to verify that the nuclei were intact. The cell extract was cleared by microcentrifugation (1000 × *g*, 4 min at 4°C) and centrifuged at 10,000 × *g* (15 min at 4°C) to yield the S10 supernatant and P10 pellet (nuclei-enriched) fractions. IP Buffer was added to equate the volume of the P10 fraction with that of the S10 fraction, NonidetP-40 was then added to 0.1% to both fractions, and the P10 fraction was further sonicated 5 min and examined to verify that the nuclei were disrupted. Both fractions were then preblocked with ProtG agarose beads (Sigma, St. Louis, MO) for 30 min at room temperature. For immunoprecipitation, P10 and S10 were equally divided and incubated overnight at 4°C with (1:200) of either anti-Hairless "A" antibody (courtesy of D. Maier) or preimmune serum. Prewashed ProtG agarose beads were added to each of the four IPs for 1 h at 4°C and pelleted by centrifugation at 10,000 × *g* for 4 min, discarding the flow-through. The beads were washed with IP lysis buffer six times, and bound proteins were extracted with boiling Laemmli sample buffer. IP samples were separated on a 10% SDS gel for 1.1 h at 120 mV. The proteins were transferred to nitrocellulose membrane using a semidry transfer apparatus for 1.5 h at 0.14 A. The membrane was blocked using phosphate-buffered saline (PBS) with 5% nonfat dry milk powder for 1 h at room temperature, incubated in anti-Runt monoclonal cocktail overnight at 4°C, washed in PBS, incubated with HRP-conjugated ProtA (Calbiochem, San Diego, CA) 2 h, and washed in PBS. Hairless-associating Runt protein was visualized using standard ECL (Invitrogen, Carlsbad, CA) detection protocols.

ACKNOWLEDGMENTS

We thank the laboratory of Rolf Sternglanz, particularly Jessica Connelly and Ann Sutton, for their assistance and materials for the yeast two-hybrid procedure. We thank David Finnegan and Suzanne McDermott for *Oregon R* flies and materials for the immunoprecipitation assay. We thank Dieter Maier, Anette Preiss, and Anja Nagel for Hairless reagents and discussion. This work was supported by grants from the NSF (MCB 0344486 and MCB 0721430) to J.P.G.

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