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.

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 corepressor 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 WWRPY 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).
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 (Bbg), 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 Bbg, 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
though the Runt\[\Delta RH\][\Delta 6] 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\[\Delta RH\][\Delta 3] 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 repression

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

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[\Delta RH] 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[\Delta RH][\Delta 1], -[\Delta 6], and -[\Delta 7] proteins all gave positive two-hybrid signals with both Gro and H clones, although the Runt[\Delta RH][\Delta 6] 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[\Delta RH][\Delta 3] 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.

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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 $H$.
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 kbp 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 communoprecipitation 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
Hairless is a cofactor for Runt half (35 of 67 = 52%) of the gastrula and early germband extension showing NGTA phenotypes, indicating that, unlike the Runt-dependent repression of maternal genotypes produce an identical range of from females that are also heterozygous for females that are otherwise wild-type, whereas the embryo in (C) is examine of the embryos show evidence of all 14 expression in 20% of the embryos. Most of the remaining embryos 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 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 Gro, 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
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; NGT4A 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; NGT4A 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; NGT4A 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; NGT4A 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; NGT4A 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[ARH] derivative are disrupted by the additional removal of either the conserved C-terminal extension of the Runt Domain or the WWRPY-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 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 to 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 WWRPY 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[A1] was created using a similar strategy using primers 5ʼ-GCGGTAGCCACAGGTTCTG-3ʼ and 5ʼ-ATGCACTCCGATCCACTGTCC-3ʼ. 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 generated using the LexA DNA-binding domain vector pSTT91 and the EcoRI-Runt deletion constructs were digested with EcoRI, which contains an internal deletion removing amino acids 110–225, and then ligated into EcoRI-digested pSTT91.

**Yeast two-hybrid screen**

A 0–6 h Drosophila embryonic 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::lexA-op-HIS3, ura3::lexA-op-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 restested 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 (MATa trp1 leu2 his3 ura3::lexA-op-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 MATa) cells were selected on Leu− 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 inserted sequenced to identify the interacting protein clones.

**Drosophila strains and transgenes**

The Gal4 drivers P(GAL4-nos.NGT)4 (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.V2).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[4E48], 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[IP8] 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 10x/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-hairless[263] transgenes were used to isolate chromatin representing the slp-1-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 slp-1 gene (Prazak et al., 2010), as well for a further upstream region and the slp-1 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, Indianapolis, 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 (10,000 × g, 4 min at 4°C) and centrifuged at 15,000 × g (15 min at 4°C) to yield the S1 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, 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.

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