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Distinct Contributions of Conserved Modules to Runt Transcription Factor Activity

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Runx proteins play vital roles in regulating transcription in numerous developmental pathways throughout the animal kingdom. Two Runx protein hallmarks are the DNA-binding Runt domain and a C-terminal VWRPY motif that mediates interaction with TLE/Gro corepressor proteins. A phylogenetic analysis of Runt, the founding Runx family member, identifies four distinct regions C-terminal to the Runt domain that are conserved in *Drosophila* and other insects. We used a series of previously described ectopic expression assays to investigate the functions of these different conserved regions in regulating gene expression during embryogenesis and in controlling axonal projections in the developing eye. The results indicate each conserved region is required for a different subset of activities and identify distinct regions that participate in the transcriptional activation and repression of the segmentation gene *sloppy-paired-1* (*slp1*). Interestingly, the C-terminal VWRPY-containing region is not required for repression but instead plays a role in *slp1* activation. Genetic experiments indicating that *Groucho* (*Gro*) does not participate in *slp1* regulation further suggest that Runt's conserved C-terminus interacts with other factors to promote transcriptional activation. These results provide a foundation for further studies on the molecular interactions that contribute to the context-dependent properties of Runx proteins as developmental regulators.

INTRODUCTION

The Runx transcription factors comprise a family of vital developmental regulators that participate in multiple pathways extending from pattern formation and sex determination in *Drosophila* to blood, bone, neural, and stomach development in mammals (Duffy and Gergen, 1994; Komori, 2002, 2003; Lian *et al.,* 2003; de Bruijn and Speck, 2004; Enomoto *et al.,* 2004; Ito, 2004). *Runx* genes have been identified in all animals, with single gene family members in basal metazoans such as sponges and sea anemones, as well as in nematodes, the spider *Cupiennius salei* and in sea urchins (Damen *et al.,* 2000; Robertson *et al.,* 2002; Sullivan *et al.,* 2008). Most vertebrates contain three Runx family members (Levanon and Groner, 2004; Ito, 2008), whereas four family members are conserved in the genomes of arthropod insects such as *Drosophila*, mosquitoes, beetles, bees, and wasps (Bao and Friedrich, 2008).

The hallmark of the Runx proteins is the highly conserved DNA-binding Runt domain (Kagoshima *et al.,* 1993). In addition to interacting with DNA, this domain also mediates interaction with a conserved and unrelated partner protein, referred to as CBFβ/Bro. The CBFβ/Bro proteins do not bind DNA or make DNA contacts as a component of the resulting heterodimeric complex, but instead stabilize a conformation of the Runt domain that has enhanced DNA-binding affinity (Tang *et al.,* 2000; Bravo *et al.,* 2001; Zhang *et al.,* 2003). The Runt domain has also been shown to mediate functional interactions with a variety of factors that are involved in transcription regulation, including other sequence specific DNA-binding proteins as well as non-DNA–binding cofactors (for review see Ito, 2004).

A second conserved characteristic shared among Runx proteins is a C-terminal pentapeptide VWRPY motif that mediates interactions with the TLE/Gro family of corepressor proteins (Aronson *et al.,* 1997; Levanon *et al.,* 1998; Javed *et al.,* 2000, 2001) In *Drosophila*, the Runt VWRPY motif is required for repressing specific stripes of expression of the pair-rule genes *hairy* (*h*) and *even-skipped* (*eve*; Aronson *et al.,* 1997). The VWRPY motif and Gro both also participate in the maintenance, but are not required for the initial establishment of Runt-dependent repression of the *en* segment-polarity gene (Wheeler *et al.,* 2002). In mammals, the VWRPY-TLE interaction is similarly involved in a subset of Runx protein functions. Although a Runx1 derivative lacking the VWRPY motif supports the development of hematopoietic progenitors from either embryonic stem cells or from fetal liver cells, in both cases there are defects in CD4 silencing and abnormal thymocyte development (Nishimura *et al.,* 2004; Kawazu *et al.,* 2005). Likewise, although the VWRPY motif is not required for the Runx3-dependent development of either sensory neurons or dendritic cells, there is a failure to properly regulate dendritic cell maturation in Runx3 [ΔVWRPY] mice (Yarmus *et al.,* 2006). The C-terminal VWRPY motif is conserved in basal metazoans, including

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the sea anemone *Nematostella vectensis* and the freshwater hydra *Hydra magnipapillata* (Sullivan *et al.*, 2008), indicating this is an ancient aspect of Runx protein function.

Functional studies with all three mammalian Runx proteins have identified regions outside of the Runt domain and the VWRPY motif that contribute to the regulatory activities of these proteins. This includes regions that mediate interactions with a number of other transcription factors and different co-activators and corepressors (for review see Ito, 2004). All three mammalian Runx proteins contain regions C-terminal to the Runt domain that contribute to transactivation in cell-based transcription assays (Kanno *et al.*, 1998; Thirunavukkarasu *et al.*, 1998; Ito, 1999; Pande *et al.*, 2008). Two other notable properties shared by these C-terminal regions are the unique ability to interact with both BMP-responsive as well as TGFβ-responsive SMAD proteins (Miyazono *et al.*, 2004), and a conserved nuclear matrix–targeting signal (NMTS; Zeng *et al.*, 1997). Interestingly, the Runx1-dependent repression of CD4 in thymocytes requires the NMTS, but appears to be independent of interactions with the Sin3 or Groucho/TLE corepressors (Telfer *et al.*, 2004). The NMTS is also important for the activity of the Runx2 and Runx3 proteins (Zaidi *et al.*, 2001; Pande *et al.*, 2008).

The goal of this work is to identify regions that contribute to the regulatory properties of the *Drosophila* Runt protein, the founding Runx family member. The *runt* gene was initially identified based on the pair-rule segmentation defects in mutant embryos (Nusslein-Volhard and Wieschaus, 1980) and was subsequently found to participate in other developmental pathways in the fly, including sex determination and neurogenesis (Duffy and Gergen, 1991; Duffy *et al.*, 1991; Kaminker *et al.*, 2002). As found for other Runx proteins, Runt is capable of either activating or repressing gene transcription in a context-dependent manner. These dual regulatory properties are exemplified by the parasegment-specific regulation of the *slp1* segmentation gene. Activation of *slp1* in the two posterior-most cells of each odd-numbered parasegment in the late blastoderm embryo requires Runt in concert with the Zn-finger transcription factor encoded by the pair-rule gene *odd-paired* (*opa*). These same two transcription factors are also expressed in adjacent cells that comprise the anterior half of the even-numbered parasegments at this stage, but in these cells the presence of the homeodomain transcription factor Fushi-tarazu (Ftz) converts Runt from an activator to a repressor of *slp1* transcription (Swantek and Gergen, 2004). Although these combinatorial rules are well established and can indeed be used to manipulate *slp1* expression in all cells in the late blastoderm embryo in a Runt-dependent manner, the specific molecular requirements for Runt-dependent activation versus repression have yet to be elucidated.

Regions outside of the conserved Runt domain clearly contribute to the specificity of Runt function. Neither the mammalian Runx1 protein, nor Lozenge, a second *Drosophila* Runx family member are effective in altering the expression of different Runt target genes in ectopic expression assays in the early *Drosophila* embryo (Pepling and Gergen, 1995; Tracey *et al.*, 2000). However, a chimeric protein that contains the Runt domain of mammalian Runx1 in the context of the flanking N- and C-terminal regions of the *Drosophila* protein mimics Runt function in these assays (Pepling and Gergen, 1995). Taken together, these observations indicate that regions of Runt outside of the Runt domain are distinct from those of these two other Runx family members and that these regions are functionally relevant.

A previous alignment of *runt* sequences from *Drosophila melanogaster*, *D. pseudoobscura*, and *D. virilis* identified eight regions of high sequence homology that are likely to contribute to the regulatory functions of Runt, including of course the Runt domain and the C-terminal VWRPY motif (Pepling and Gergen, 1995). In this article, an extended phylogenetic analysis reveals that these eight homology regions are maintained in other drosophilid species, but that conservation in some regions dissipates when the comparison is widened to include other insects. We investigate the importance of four of the most well-conserved regions for Runt function during *Drosophila* development. We find that deletion derivatives lacking these different conserved regions all retain activity in vivo, but with differential effects on different activities of Runt. Indeed, the results indicate that Runt's conserved C-terminus contributes to the activation, rather than the repression of *slp1* and identify a distinct conserved module that is required for repression of this target. These findings provide compelling evidence for the modular architecture of the Runt transcription factor and lay groundwork for identifying the molecular interactions that contribute to the context-dependent regulatory properties of this protein.

MATERIALS AND METHODS

Drosophila *Mutations and Runt Deletion Transgene Lines*

The maternally expressed *P{GAL4-nos.NGT}* Gal4-drivers have been described previously (Tracey *et al.*, 2000; Wheeler *et al.*, 2002), as have the *P{UAS-runt.T}232*, *P{UAS-runt.T}15*, *P{UAS-runt[CK].L}77*, *P{UAS-runt[Δ8].S}$^{4-3}$*, *P{UAS-opa.VZ}10*, *P{UAS-opa.VZ}12*, and *P{UAS-opa.VZ}14* transgenic lines (Li and Gergen, 1999; Tracey *et al.*, 2000; Wheeler *et al.*, 2002; Swantek and Gergen, 2004). *P{UAS-ftz}^{263}* is a third chromosome-linked transgene obtained from Leslie Pick (University of Maryland) that is comparable in activity to the previously described second chromosome-linked *P{UAS-ftz}^{261}* (Swantek and Gergen, 2004; Lohr and Pick, 2005). The *Gro^{BX22}*, *Gro^{E48}*, and *Rpd3^{04456}* mutations are also as described previously (Wheeler *et al.*, 2002).

Excite PCR was performed with primers to generate in-frame deletions of different conserved regions of Runt essentially as described for the previously generated Runt[CK], Runt[FLAG] and Runt[FLAGΔ8] expression constructs (Kramer *et al.*, 1999; Wheeler *et al.*, 2000). pB:Runt[FLAG-Δ3] was created using the primers 5′-GCCAAGTCCTCGGCCTCC-3′ and 5′-TCTTGGCTCCCGTGGCCCGTC-3′. pB:Runt[FLAG-Δ6] was created using the primers 5′-GTGGCGGATTACAAGGATGACG-3′ and 5′-CTGGGTGGGCGAGGAGCTGG-3′. (Bases that transcribe the FLAG epitope-tag are underlined.) pB:Runt[FLAG-Δ7] was created using the primers 5′-GGTCCTGGAGCGGTAGCC-3′ and 5′-CTTATCGTCGTCATCCTTGTAATC-3′. The products lack amino acids S^{233}-L^{284}, H^{410}-D^{453}, and P^{456}-S^{476} of the normal protein, respectively. The deletions were confirmed by sequencing the plasmid and a StyI/BstEII fragment (Runt [Δ3]) or a BbsI/ApaI fragment (Runt [Δ6], Runt [Δ7]) spanning the deletion was cloned into pB:ED(Bam-8ΔKS)Runt[FLAG] and digested accordingly. The p[UAS:Runt[FLAGΔ3]], p[UAS:Runt[FLAGΔ6]], and p[UAS:Runt[FLAGΔ7]] germline transformation constructs were generated by insertion of BamHI fragments from the appropriate pB:Runt[FLAG] construct into BglII-linearized pUAS:T vector and sequenced to confirm the orientation. Transformant lines were recovered by standard P-element germline transformation. The *UAS-Runt[Δ8]^{79}* and *UAS-Runt[Δ8]^{49}* lines were obtained by P-element–mediated mobilization of the previously described and weaker *UAS-Runt[Δ8]$^{4-3}$* line (Wheeler *et al.*, 2000).

Immunofluorescence Detection of Protein Expression and Nuclear Localization

Salivary gland expression of the different Runt deletions was obtained by mating the appropriate *UAS* constructs with the salivary gland GAL4 driver *P{w[+mc] = Sgs3-GAL4.PD}TP1* (Tweedie *et al.*, 2009). Larvae were grown at 18°C. Flat-bottom wells were blocked with PBT (phosphate-buffered saline [PBS] and 10.1% Tween) and 2% BSA an hour before larval salivary gland dissections in PBS at 4°C. The glands were fixed in PBS, 2%BSA, and 3% formaldehyde for 5 min; washed in PBT and 2% BSA; incubated with anti-FLAG M2 antibody (Sigma, St. Louis, MO) for 30 min; washed in PBT and 2% BSA; incubated with FITC-conjugated anti-mouse antibody (Invitrogen, Carlsbad, CA) for 30 min; washed in PBT; and mounted in PBS, 50% glycerol, and 2% *n*-propyl gallate.

Viability Assay

Females homozygous for *NGT11 or NGT40* were crossed to males heterozygous for each transformed *UAS-Runt*, *UAS-Runt[CK]*, UAS-*Runt[FLAG]*, *UAS-Runt[Δ3]*, *UAS-Runt[Δ6]*, *UAS-Runt[Δ7]*, and *UAS-Runt[Δ8]* line. Viability measurements for transgenes on chromosome II were determined using males heterozygous for the *UAS* transgene and the *CyO* balancer. Viability measurements with transgenes on chromosome III were determined using males heterozygous for the *UAS* transgene and a chromosome carrying the dominant

Pr and *Dr* mutations due to the reduced fitness of flies heterozygous for either the *TM3* or *TM6* third chromosome balancers. The relative viability is the percent of progeny that inherit the *UAS* transgene relative to their *CyO* (or *Pr Dr*) sibs, rounded to the nearest decile, except for values between 0 and 10%, which are rounded to the nearest fifth percentile.

Axonal Redirection

The MT14-Gal4 driver was used to drive expression of different *UAS-Runt* constructs in third-instar larval photoreceptor neurons R2 and R5 as described previously (Kaminker *et al.*, 2002). Homozygous MT14-Gal4 virgin females were mated to males homozygous for the different *UAS-Runt* transgenes, and larvae were grown at 18°C before dissection of third-instar eye imaginal disk optic lobe preparations. Axonal projections within the optic lobes were detected using a 1:50 dilution of 24B10 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), an mAb that recognizes the photoreceptor membrane protein chaoptin (Van Vactor *et al.*, 1988). The biotinylated secondary antibody was blocked with an acetone-washed powder of ground larvae. The signal was amplified and visualized using the Vectastain DAB kit (Vector Laboratories, Burlingame, CA).

Embryo Manipulation and In Situ Hybridization

Ectopic expression of the different *UAS-Runt* deletion derivatives was obtained using the maternally expressed *NGT* GAL4 drivers. Experiments investigating *en* repression involved matings between homozygous *NGT40* females and the different *UAS-Runt* males. Initial experiments to screen for the ability of the different Runt deletions to perturb *slp1* expression were carried out using females homozygous for both *NGT40* and *NGTA* that produce slightly higher levels of ectopic expression. Experiments investigating the maternal dose-dependent effects of *Gro* and *Rpd3* mutations on *slp1* repression and activation involved matings between females heterozygous for these different mutations that were also heterozygous for *NGT40* and *NGTA* to homozygous *UAS-Runt*^{15}; *UAS-Ftz*^{263} males or to homozygous *UAS-Runt*^{15} *UAS-Opa*^{14} males, respectively. In this case the increased potency of *UAS-Runt*^{15} compensates for the reduced levels of maternally provided GAL4, giving a level of ectopic Runt expression that gives clear effects on *slp1* expression while remaining in a range that is sensitive to changes in the levels of *runt* activity (Swantek and Gergen, 2004). Embryos were collected and processed for in situ hybridization with digoxigenin-labeled (Boehringer Mannheim, Indianapolis, IN) anti-sense RNA probes for *en* and *slp1* as described previously (Swantek and Gergen, 2004).

RESULTS

Evolutionary Conservation of Runt in Drosophila

A comparison of *runt* sequences of *D. melanogaster*, *D. pseudoobscura*, and *D. virilis* previously identified eight blocks of high sequence homology that were separated by nonconserved spacers (Pepling and Gergen, 1995). This observation was interpreted to reflect a modular architecture.

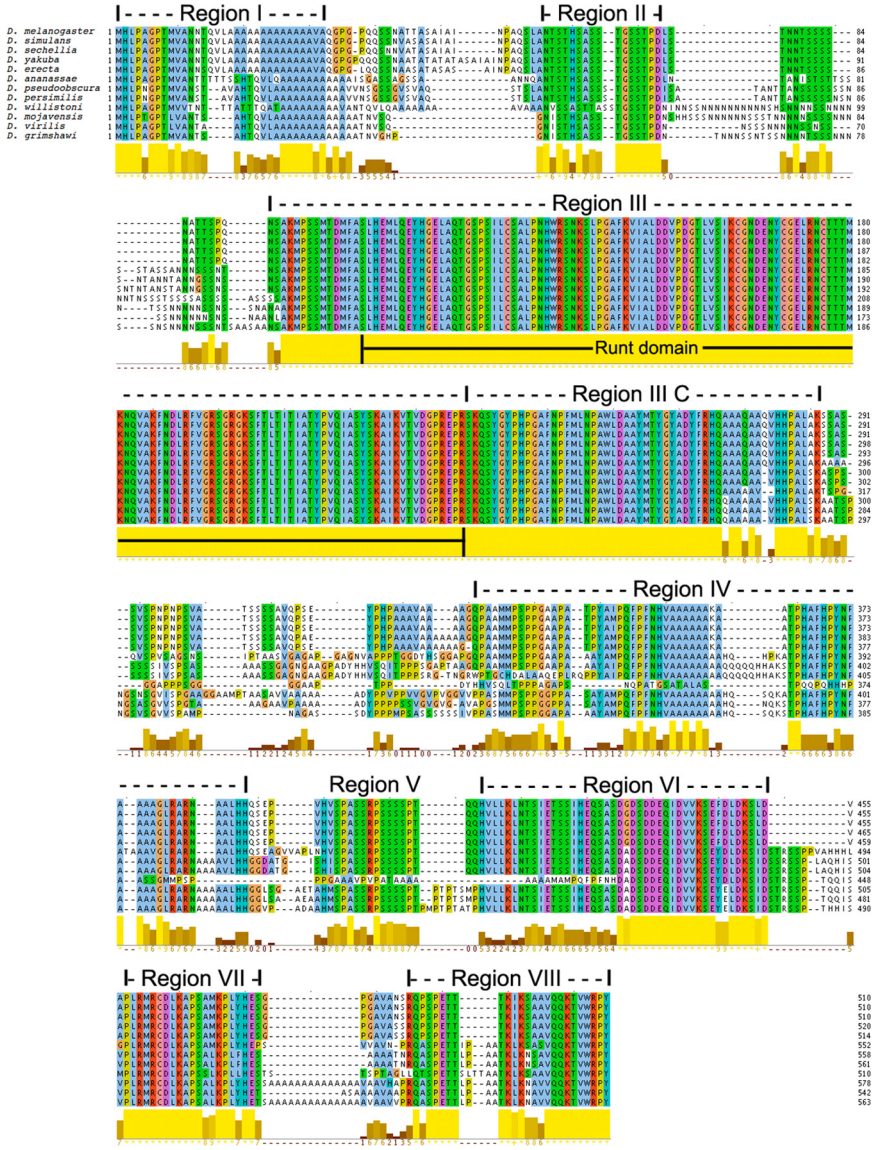

Figure 1. Conservation of the Runt protein in *Drosophila*. The figure shows a ClustalW2-generated alignment of Runt protein sequences from 12 different *Drosophila* species. The *D. melanogaster* amino acid sequence (single -letter code) is given at the top of each segment of the alignment with the other species listed in the order of their increasing divergence from *D. melanogaster*. The top five species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba* and *D. erecta*) comprise the melanogaster subgroup. The melanogaster group includes these five plus *D. ananassae*. The color-coding of conserved regions in the alignment is as provided by ClustalW2: Hydrophobic (A, F, L, M, V, W), light blue; Basic (K, R), red; Acidic (D, E), purple; Polar (N, Q, S, T), green; C, pink; G, salmon; and H and Y, blue. The limits of the eight conserved regions identified in the initial three-way alignment are indicated above the *D. melanogaster* sequence. These initial limits were used to guide the generation of the deletion constructs used to investigate the in vivo functions of the different conserved regions. A ClustalW2-generated plot of sequence conservation is provided across the bottom for each of the different sequence segments. Positions that are conserved with sequence identity in all species are indicated in yellow in this plot, with an asterisk (*) below the amino acid position. The limits of the Runt domain are indicated within the extended block of sequence conservation revealed in the plot for region III. The region I alignment shown in the figure fails to identify a conserved pentapeptide motif (S/T)QVL(Q/A) that precedes a homopolymeric run of eight (*D. willistoni*) to 12 (all of the others except *D. ananassae*, *D. psuedoobscura*, and *D. persimilis*) alanine residues.

Alignment of Runt sequences from 12 different *Drosophila* species reveals that the eight previously identified regions are present and for the most part intact in all of the species except for a clear divergence of regions IV and V and the N-terminal half of region VI in *D. willistoni* (Figure 1). Region III is the largest conserved block and encompasses the entire 128-amino acid Runt domain with conserved N-terminal and C-terminal extensions of 14 and 54 amino acids, respectively. The Runt domain sequence is identical in all 12 species, with perfect identity extending contiguously for 12 residues to the N-terminus and 39 residues to the C-terminus (Figure 1). The little variation that is observed in region III is consistent with the generally accepted phylogenetic relationships of these species. The entire 196-amino acid region is identical in all six species within the melanogaster group. The two species within the obscura group, *D. pseudoobscura* and *D. persimilis* are nearly the same but share the deletion of a single glutamine residue in the C-terminal extension. This same deletion is shared by the more distantly related species *D. mojavenesis*, *D. virilis,* and *D. grimshawi*, all three of which also share a nearby alanine-to-glutamine substitution.

Three of the regions outside of the Runt domain that were identified in the initial three-way alignment are not intact in *D. willistoni.* The 12-way alignment also reveals that four of the regions outside of the Runt domain are subject to a sequence interruption (Table 1). The one region identified in the initial alignment that clearly does not survive as a discrete functional module in the 12-way alignment is region IV. This region corresponds to a 57-residue, alanine- and proline-rich region of the *D. melanogaster* protein that also contained a single site of sequence disruption in the initial three-way alignment. Even excluding *D. willistoni* from the alignment, this region is disrupted by sequence breaks at four different positions that identify five distinctive smaller regions of homology, the largest of which is an alanine-rich sequence that is identical at 16 of 17 positions (excluding *D. willistoni*). Based on this divergence, region IV does not correspond to a single functional module that is under high selective pressure. In contrast, the conservation observed across the Drosophilidae for other regions, especially the contiguous blocks of very high sequence homology identifies regions of the Runt protein that are under selective pressure and thus likely to be functionally important.

Conservation of Runt in Nondrosophilid Insects

Runt protein sequences are available for nondrosophilid insects including the mosquitoes *Aedes aegypti* and *Anopheles gambia*, the silkworm *Bombyx mori*, the flour beetle *Tribolium castaneum*, the bee *Apis mellifera* and the wasp *Nasonia vitripennis*. We compared these sequences to the *D. melanogaster* sequence in order to determine whether the conserved regions observed in the Drosophilidae extended to other insects. There are substitutions at a total of 42 different positions in the Runt domain relative to that in *Drosophila* and even an amino acid insertion in *Tribolium* (not shown). There are seven amino acids that are conserved within the Runt domain of these other insects that differ from a residue in *Drosophila*. Five of these seven substitutions involve a replacement also found in the vertebrate Runx proteins.

None of the regions N-terminal to the Runt domain appear to be conserved in these other insects. Indeed, the nondrosophilid proteins are smaller than those in *Drosophila* and contain only short (6–25 amino acid) regions N-terminal to the Runt domain. Except for *A. aegypti*, the N-termini of the insect Runt proteins start with MHLP (data not shown). It is interesting to note that a similar sequence, MRIP is found at the N-terminus of the vertebrate Runx proteins.

There is evidence of sequence conservation C-terminal to the Runt domain. The two most prominent regions of conservation are the C-terminal extension of the Runt domain and the hallmark VWRPY motif. The wasp sequence is unusual in that there are an additional two amino acids that follow the VWRPY sequence. In addition to the VWRPY motif there are several other residues from region VIII that are conserved in other insects, including a lysine and a somewhat further upstream SP-TK(I/L) sequence (Figure 2). The conservation of the C-terminal extension to the Runt domain is more extensive spanning a region of 37–44 amino acids. The nine-residue segment immediately C-terminal to the Runt domain includes two amino acids that are identical in all of these species with conservative substitutions at most of the other positions (Figure 2). Perhaps even more striking is a 15-amino acid region that begins 13 amino acids C-terminal to the *Drosophila* Runt domain. This segment is demarcated by conserved tyrosine and phenylalanine residues and includes four other absolutely conserved positions (Figure 2). A similar architecture, with two short conserved motifs located immediately adjacent and some 20 amino acids C-terminal to the Runt domain is found in several vertebrate Runx proteins and is thought to modulate

Table 1. Conserved regions of Runt proteins in *Drosophila* and other insects

Original region	Size[a]	Sequence breaks[b]	Contiguous blocks of homology (identity) in all 12 *Drosophila*	Homology in other insects	Comments
I	25	1	10 (8); 14 (11)	No	N-terminus, poly-Ala
II	16	1	9 (3); 7 (7)	No	Ser, Thr rich
III	196	1	187 (182); 8 (7)	Runt domain and C-terminal extension	Runt domain with 14-amino acid (N-terminus) and 54-amino acid (C-terminus) extensions
IV	62	4	—	No	2 Ala rich regions, His/Pro
V	15	0	—	Mosquito	Ser (7), Pro (3) rich
VI	44	0	23 (19)	Yes	Acidic patch
VII	20	0	20 (17)	Yes	RCDLKAP motif
VIII	25	1	8 (6); 17 (13)	Yes	VWRPY motif

[a] In *D. melanogaster*; number of residues.

[b] The sequence breaks are for an alignment that excludes *D. willistoni* because regions IV and V and the N-terminal half of region VI are not intact in this species.

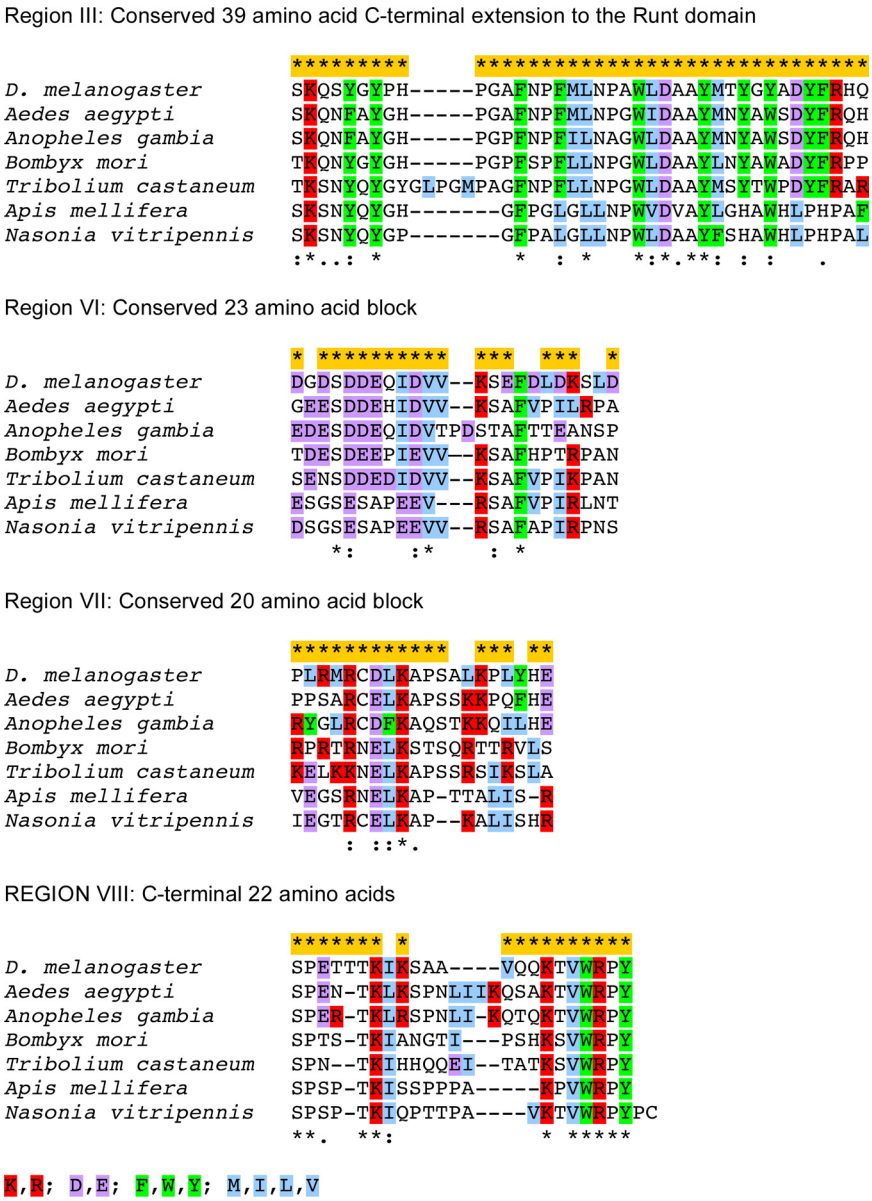

Figure 2. Conservation of RUNT C-terminal modules in other insects. The figure shows an alignment of the regions of Runt proteins from six nondrosophilid insects with corresponding intervals from conserved regions III, VI, VII, and VIII of the *Drosophila* proteins. The top line of sequence information in each segment is from *D. melanogaster*, with residues that are conserved with identity in all 12 *Drosophila* species indicated by a yellow bar and an asterisk (*) above the sequence. Species identification is provided to the left of each of the other sequence segments. Residues that are conserved with identity in all of the sequences in this alignment are indicated below the alignment with an asterisk, conserved substitutions are indicated with a colon, and similarities are indicated with a period. A legend for the color coding used to identify basic, acidic, aromatic, and hydrophobic amino acids is provided at the bottom of the figure.

DNA-binding activity (Ito, 1999). However there is no apparent sequence homology between the C-terminal Runt domain extensions of the vertebrate and insect proteins. In vertebrates this region is rich in basic and acidic residues, whereas the corresponding conserved regions of the insect proteins are predominantly hydrophobic, including aromatic residues at six positions and include only single charged lysine and aspartic acid residues.

All of the insect proteins have sizable regions (from 134 to 238 amino acids) located between the region III and region VIII homologies noted above. The three subregions of region IV that are conserved in several *Drosophila* species are not evident in these other insects, although all of the proteins are rich in proline in the region downstream of region III (data not shown). The homology to region V is evident in the more closely related mosquito sequences, but not in the bee and the wasp, although all of the proteins have serine- and proline-rich regions N-terminal to a block of homology with region VI (data not shown). The homology to region VI is comprised of several acidic residues followed by a hydrophobic patch and a basic lysine or arginine, a feature of the block of sequence identity shared by the *Drosophila* Runt proteins (Figure 2). Region VII is less well conserved, although there is homology centered on the 12-amino acid block that is identical in the *Drosophila* proteins (Figure 2). Although the sequence divergence is greater when the analysis is extended to these other insects, the conservation that is observed for the C-terminal extension of the Runt domain as well as for regions VI, VII, and VIII provides further evidence that these regions are functionally important.

In Vivo Activity of Runt Derivatives Lacking Conserved Modules Outside the Runt Domain

Deletion derivatives were generated to investigate the functional importance of the four regions that showed evidence of conservation in nondrosophilid insects. Not surprisingly, deletion of the Runt domain eliminates in vivo function (Kramer *et al.*, 1999). Indeed, a mutation in the Runt domain that abrogates the interaction with the Bro protein fully eliminates Runt activity in a number of different in vivo

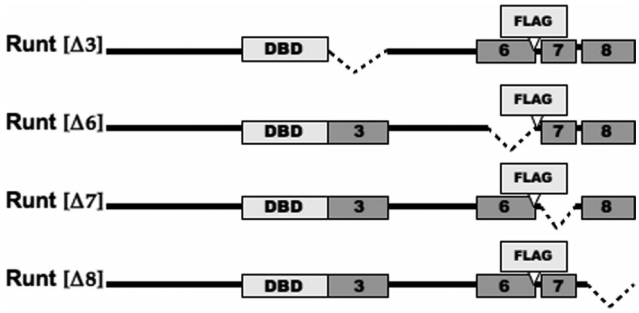

Figure 3. Runt deletion constructs. Schematic diagram indicating the regions removed in different *UAS-Runt* deletion constructs and the location of the FLAG epitope tag between conserved regions VI and VII. The solid horizontal line represents the Runt protein, with boxed regions on this line indicating the relative locations of different conserved regions. Regions removed in different deletion constructs are indicated by dashed lines that connect the regions flanking the deletions. Runt[Δ3] removes the segment of conserved region III that is immediately C-terminal to the Runt domain (DBD).

assays (Li and Gergen, 1999). In contrast, the Runt[CK] derivative, which contains two point mutations in the Runt domain (C127S, K199A) that perturb DNA-binding (Kramer *et al.*, 1999), retains the ability to establish repression of the segment-polarity gene *en* (Vander Zwan *et al.*, 2003), indicating DNA-binding independent activities of Runt. The role of the conserved C-terminal extension of the Runt domain was not tested in these previous experiments. Therefore we generated a deletion derivative, termed Runt[Δ3] that retains the Runt domain but that lacks this conserved C-terminal extension, from amino acids S233 to L284 inclusive (Figure 3). To test the functional importance of regions VI, VII, and VIII, we also generated deletions that remove the full extent of each of these conserved regions. All of the different deletion derivatives were generated in the context of a *UAS-Runt* expression construct that also contains a FLAG epitope tag inserted between amino acids A455 and P456, i.e., in the linker region between conserved regions VI and VII (Figure 3). Therefore we also generated a construct containing the FLAG epitope tag inserted in this same position in the full-length Runt protein as a control. These several different Runt derivatives were all inserted into the *pUAS-T* germline transformation vector, and transgenic lines were generated for each of the *UAS-Runt* constructs. The activities of the different deletions were examined in a number of different in vivo assays and compared with results obtained with the full-length wild-type protein as well as the DNA-binding defective Runt[CK] derivative.

We used a GAL4 driver that is expressed in larval salivary glands to examine the effects of the different deletions on protein expression and subcellular localization. The Runt-FLAG protein and all four of the different deletions show accumulation within nuclei, indicating that none of the deletions dramatically affect protein stability or nuclear localization (Figure 4). The Runt-FLAG, Runt[Δ6], and Runt[Δ8] derivatives all show a punctate pattern within the nucleus similar to that described for the nuclear matrix-associated mammalian Runx proteins (Zeng *et al.*, 1997; Zaidi *et al.*, 2001). In contrast, the Runt[Δ3] and Runt[Δ7] proteins show more uniform expression throughout the nucleus (Figure 4, C and E). This result suggests these two regions contribute to subnuclear localization, potentially mediating association with the nuclear matrix. We have not examined whether the punctate expression observed in these salivary gland preparations corresponds to bona fide association with the nuclear matrix and further note that there is no obvious homology of either region III or region VII with the conserved NMTS of the vertebrate Runx proteins.

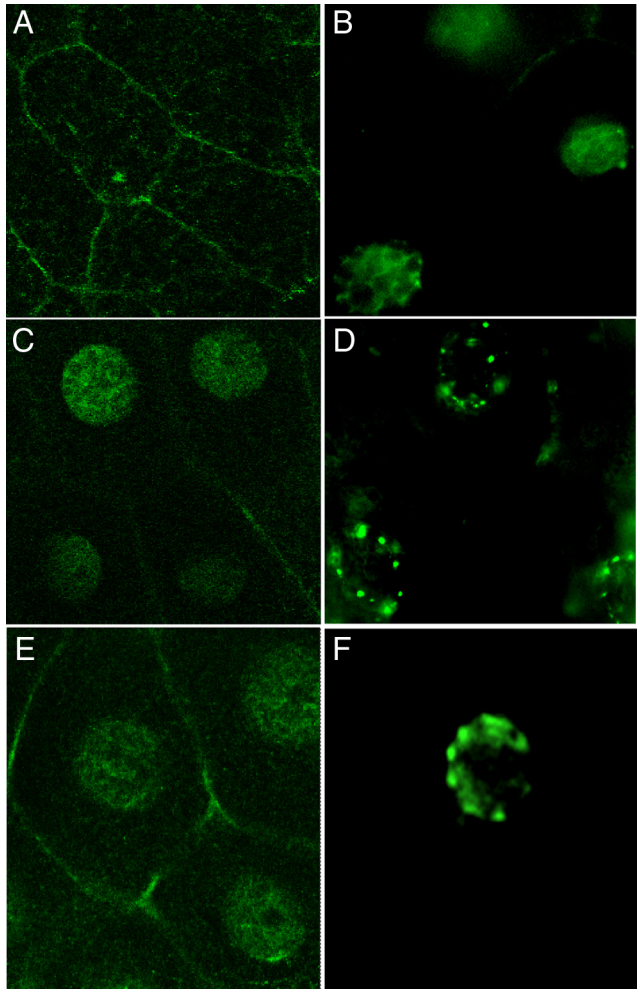

Figure 4. Expression and nuclear localization of the Runt deletion derivatives. Expression of different Runt deletion constructs in larval salivary glands as detected by the anti-FLAG M2 mAb. (A–F) Background antibody control of salivary glands that are not expressing a FLAG-tagged protein (A), FLAG-tagged full-length Runt (B), Runt[Δ3] (C), Runt[Δ6] (D), Runt[Δ7] (E), and Runt[Δ8] (F). The images in A, C, and E have higher background fluorescence due to differences in the antibody used for detection and the imaging instrumentation when the experiment was extended to include Runt[Δ3]. Images for Runt[Δ7] were acquired using both sets of conditions and indicate the difference is due to background, and not differences in expression levels of the Runt deletion derivatives. The images with higher background are used for the control in A and the Runt[Δ3] and Runt[Δ7] proteins as this best demonstrates their similarity in this assay.

We used ectopic expression assays to investigate the in vivo functional activity of the different deletion derivatives. As an initial test that also provides information on the relative strength of different insertions of the same *UAS* transgene constructs, we measured the lethality produced by ectopic expression at the blastoderm stage in response to maternally expressed GAL4 (Tracey *et al.*, 2000). All of the different *UAS-Runt* transgene insertions for every construct show evidence of lethality using the strong *NGT40* maternal GAL4 driver (Table 2), indicating that all of the different deletion derivatives retain activity in vivo. As expected,

Table 2. Lethality produced by blastoderm stage expression of Runt[Δ] derivatives

Transgene construct	Insertion line (chromosome)	Relative viability (% female:% male) ×NGT11	×NGT40
UAS-Runt	**232 (II)**	**10:00**	**00:00**
UAS-Runt[*CK*]	**77 (II)**	**100:10**	**00:00**
UAS-Runt-FLAG	1–2 (II)	100:100	50:30
	1–3 (III)	**30:00**	**00:00**
UAS-Runt[Δ*3*]	63–2 (III)	100:40	00:00
	43–1 (II)	90:00	00:00
	46–1 (III)	**05:00**	**00:00**
UAS-Runt[Δ*6*]	17–1 (II)	100:05	00:00
	16–2 (II)	30:00	00:00
	3–1 (III)	**05:00**	**00:00**
UAS-Runt[Δ*7*]	21–3 (III)	100:90	00:00
	45–2 (II)	**05:00**	**00:00**
UAS-Runt[Δ*8*]	4–3 (II)	100:60	00:00
	79 (III)	100:10	00:00
	49 (III)	**50:00**	**00:00**

The representative lines used in subsequent experiments are indicated by boldface.

there is less lethality in crosses using the *NGT11* driver, which has ~40% of the activity of *NGT40* (Tracey *et al.*, 2000), and males are more sensitive to the lethality associated with ectopic Runt expression. The differences in lethality obtained with different insertions of the same construct provide an indication of the relative expression levels and allowed us to identify strong representative lines for each construct for use in subsequent studies (bold in Table 2). In each case these representative lines are completely lethal when expressed using the *NGT40* driver but produce escapers in crosses with *NGT11*.

Distinct Requirements for Runt-dependent Axonal Pathfinding

Ectopic expression assays have been previously used to demonstrate a role for Runt in regulating the targeting of photoreceptor axonal projections during development of the *Drosophila* eye. Adult flies have ~800 ommatidiae in each eye, with each ommatidia containing eight photoreceptor neurons, R1–R8. During the third-instar larval stage these photoreceptors differentiate from epithelial cells of the eye imaginal disk and extend axons into the optic lobe. The axons of photoreceptors R1 through R6 terminate in the optic lobe within a layer referred to as the lamina plexus (Figure 5A), a structure that can be visualized based on expression of the membrane associated chaoptin (Figure 5B). In contrast, the axons of the Runt-expressing R7 and R8 photoreceptors project past the lamina plexus and terminate in the medulla. Interestingly, ectopic expression of Runt in photoreceptors R2 and R5 is sufficient to redirect all photoreceptor axons to extend beyond the lamina into the medulla via a process that does not involve changes in expression of cell-specific developmental markers (Kaminker *et al.*, 2002). We used this ectopic expression assay to investigate the functional requirements for Runt in this process. The phenotypic consequence of ectopic Runt is disappearance of the lamina plexus due to extension of all photoreceptor axons into the medulla (Figure 5C). A similar loss of the lamina plexus is produced by ectopic expression of Runt[CK] and Runt[Δ7] (Figure 5, D and G), but not by the Runt[Δ3], Runt[Δ6], and Runt[Δ8] derivatives (Figure 5, E, F, and H). The ability of the Runt[CK] protein to redirect axonal projections provides another example of a DNA-binding–independent activity of Runt. The observation that conserved region VII is not required for axonal redirection, whereas the flanking regions VI and VIII contribute to function in this assay provides a clear indication of the distinct functional activities of these different conserved regions.

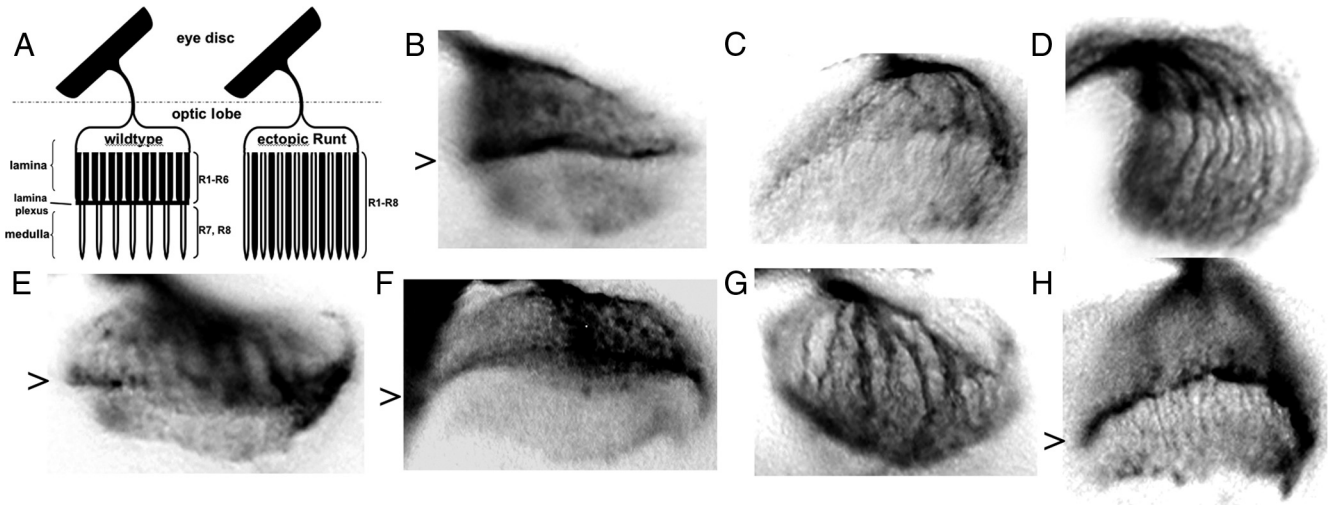

Figure 5. Functional specificity of Runt-dependent axonal pathfinding. (A) Schematic of eye disk and optic lobe in *Drosophila* third-instar larvae. Axons of wild-type photoreceptor neurons R1–R6 terminate between the lamina and medulla of the optic lobe forming the lamina plexus. Axons of photoreceptors R7 and R8 that normally express Runt terminate in the medulla. (B) Optic lobe of wild-type larvae with axonal projections revealed by immunodetection of photoreceptor membrane-associated chaoptin. The arrowhead indicates the lamina plexus. (C) *MT14* GAL4-driven *UAS-Runt* expression in R2 and R5 redirects all axons to terminate in the medulla leading to elimination of the lamina plexus. (D–H) Axonal projections in optic lobes of larvae with *MT14* driven expression of *UAS-Runt[CK]*^{77} (D), *UAS-Runt[Δ3]*$^{46\text{-}1}$ (E), *UAS-Runt[Δ6]*$^{3\text{-}1}$ (F), *UAS-Runt[Δ7]*$^{45\text{-}2}$ (G), and *UAS-Runt[Δ8]*^{49} (H), with arrowheads indicating preparations that retain a lamina plexus.

Distinct Contributions of the Conserved Modules to Transcription Regulation by Runt

The two most well-characterized targets for transcriptional regulation by Runt are the segmentation genes *en* and *slp1.* Expression of *en* in odd-numbered parasegments is extremely sensitive to the *NGT*-driven expression of Runt in blastoderm stage embryos and is repressed by ectopic expression levels that do not affect expression of other pair-rule and segment-polarity genes (Wheeler *et al.,* 2002). As described for wild-type Runt, *NGT*-driven expression of the epitope-tagged Runt[FLAG] protein is effective at repressing the odd-numbered *en* stripes in a gastrula stage embryo (Figure 6C) and maintains this repression during germband extension (Figure 6D). A similar result is obtained in embryos ectopically expressing the Runt[Δ6] (Figure 6, I and J) and Runt[Δ7] (Figure 6, K and L) proteins, indicating that these conserved regions are not required for *en* repression. Previous studies demonstrated that the Runt-dependent repression of *en* is separable into two distinct steps: establishment and maintenance (Wheeler *et al.,* 2002). As expected from this previous work, the Runt[CK] and Runt[Δ8] proteins are capable of establishing repression (Figure 6, E and M) but do not maintain this repression during germband extension (Figure 6, F and N). This same phenotype is observed in embryos expressing the Runt[Δ3] protein (Figure 6, G and H). The failure of Runt[CK] and Runt[Δ8] to maintain repression was interpreted to indicate the importance of DNA-binding and the recruitment of Gro in this process (Wheeler *et al.,* 2002). The observation that Runt[Δ3] is impaired in maintaining *en* repression indicates that this conserved region may contribute to one or both of these molecular interactions. It is notable that all of the Runt deletions tested in these experiments retained the ability to establish the initial repression of *en*. This initial Runt-dependent repression involves genetic interactions with the transcriptional repressor Tramtrack (Ttk; Wheeler *et al.,* 2002). The results presented above indicate that none of the conserved regions of Runt tested in these assays is critically involved in this interaction.

Runt acts as both an activator and a repressor of *slp1* (Swantek and Gergen, 2004). A striking observation from this previous work was the ability of Runt to activate *slp1* in the anterior head region when it is coexpressed with the Zn-finger transcription factor Opa. This anterior activation of *slp1* is unique among the segmentation genes and occurs in cells that normally do not express Runt and the other pair-rule transcription factors. This anterior activation assay thus provides a means for investigating the ability of the different Runt deletion constructs to activate *slp1* independent of activity from the endogenous Runt protein. The anterior activation of *slp1* that is evident in embryos with *NGT*-driven expression of wild-type Runt and Opa (Figure 7A) is also observed with the Runt[Δ6] and Runt[Δ7] derivatives (Figure 7, D and E), indicating that these two conserved regions are not required for Runt-dependent *slp1* activation. In contrast, activation of *slp1* is reduced in embryos expressing the Runt[CK], Runt[Δ3], and Runt[Δ8] proteins (Figure 7, B, C, and F). It is interesting to note that the functional requirements for *slp1* activation match the requirements for maintenance of *en* repression, i.e., regions VI and VII are not required whereas the DNA-binding activity of Runt, the region immediately C-terminal to the Runt domain and the VWRPY-containing C-terminus of the protein contribute to both the activation of *slp1* at the gastrula stage and the maintenance of *en* repression during germband extension. This raises the possibility that maintenance of *en* repression during these later stages is due to the inappropriate prior activation of *slp1* in anterior odd-numbered parasegments, i.e., in cells that would normally express the odd-numbered *en* stripes. However, the levels of ectopic Runt needed to establish and maintain *en* repression are below those needed to activate *slp1,* indicating that the maintenance of *en* repression and the activation of *slp1* are two distinct regulatory targets of Runt.

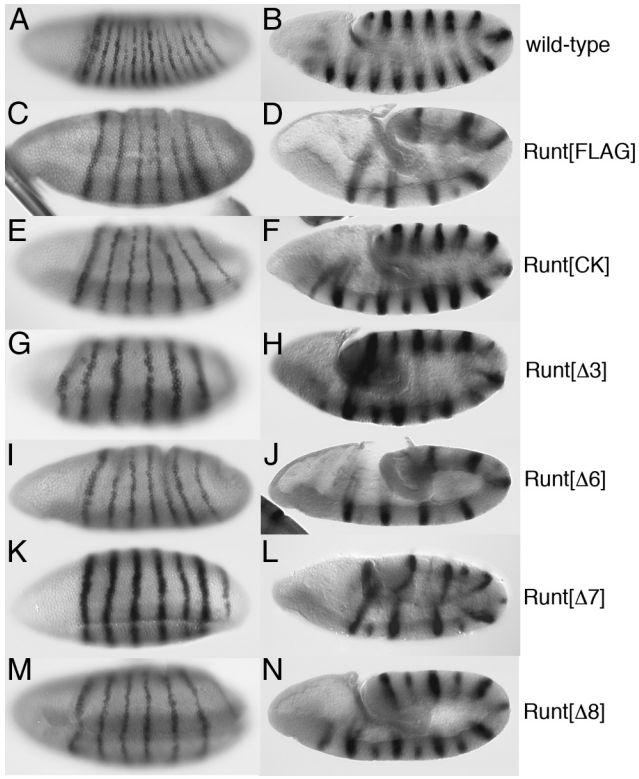

Figure 6. Differential requirements in establishing and maintaining *en* repression. Expression of *en* mRNA as revealed by in situ hybridization in gastrula stage (left column) and germband extension stage (right column) *Drosophila* embryos. (A and B) Wild-type expression at these two stages. (C–N) The response of *en* to *NGT*-driven expression of *UAS-Runt[FLAG]*$^{1-3}$ (C and D), *UAS-Runt[CK]*^{77} (E and F), *UAS-Runt[Δ3]*$^{46-1}$ (G and H), *UAS-Runt[Δ6]*$^{3-1}$ (I and J), *UAS-Runt[Δ7]*$^{45-2}$ (K and L), and *UAS-Runt[Δ8]*^{49} (M and N). Embryos with ectopic Runt expression were generated by mating homozygous *NGT40* females to males carrying the pertinent *UAS-Runt* transgene for all crosses except for those with the Runt[Δ8], which were done with homozygous *NGT40* + *NGTA* females, which produce ~1.5× the level of *NGT*-driven expression as *NGT40*. The three Runt derivatives that maintain *en* repression, Runt[FLAG], Runt[Δ6] and Runt[Δ7], all give completely penetrant repression in gastrula and early germband extension stage embryos that is stably maintained in 45 of 46 stage 10 and 11 embryos with Runt[FLAG], 13 of 26 embryos generated in crosses with *UAS-Runt[Δ6]*$^{3-1}$*/TM3* males, and in 19 of 21 Runt[Δ7]-expressing embryos. The inability of Runt[CK] and Runt[Δ8] to maintain *en* repression has been described previously (Wheeler *et al.,* 2002). A similar loss of this activity is observed for Runt[Δ3], which in this experiment produced fully penetrant early repression (29 of 29 gastrula and early germband extension stage embryos) that was not maintained later (43 of 43 stage 10 and 11 embryos express the odd-numbered *en* stripes, with 20 of 43 showing equal levels of expression of even and odd stripes). The de-repression of odd-numbered *en* stripes in these later stages is also observed for the Runt[CK], Runt[Δ3], and Runt[Δ8] (as shown in N) at the higher ectopic expression levels obtained using homozygous *NGT40* + *NGTA* females.

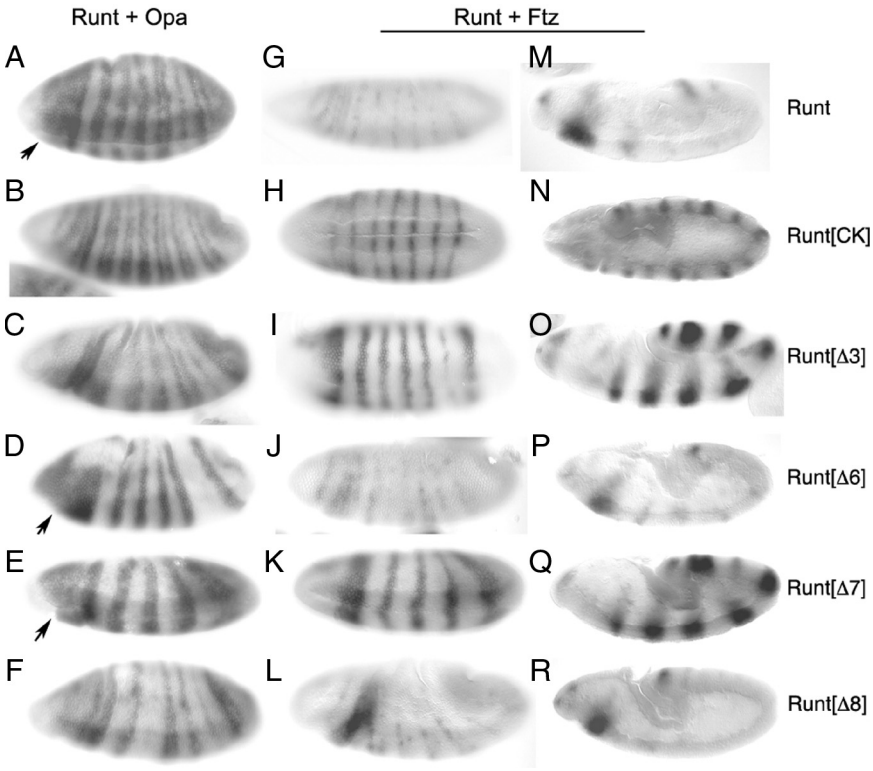

Figure 7. Differential requirements for *slp1* activation and repression. Expression of *slp1* mRNA as revealed by in situ hybridization. (A–F) gastrula stage expression of *slp1* in response to *NGT*-driven coexpression of Opa and different Runt deletion derivatives. In all cases ectopic expression was obtained using females homozygous for both the *NGT40* and *NGTA* GAL4 drivers. (A) *UAS-Runt*^{232} and *UAS-Opa*^{12}, 60 of 65 gastrula stage embryos scored in this experiment showed ectopic anterior *slp1* activation comparable or stronger than that shown in this panel. The remaining five embryos had weaker anterior activation with incomplete fusion of stripes within the segmented region of the embryo. (B) *UAS-Runt[CK]*^{77} and *UAS-Opa*^{12}, 32 of 39 gastrula stage embryos showed incomplete fusion of *slp1* stripes similar to that depicted in this panel, and six of seven the remaining embryos showed evidence of weak anterior activation, with one embryo showing clear evidence of ectopic anterior activation. (C) *UAS-Runt[Δ3]*$^{46\text{-}1}$ and *UAS-Opa*^{14}, 34 of 37 embryos showed incomplete fusion as depicted, with the other three showing evidence of weak anterior activation. (D) *UAS-Runt[Δ6]*$^{3\text{-}1}$*/TM3* and *UAS-Opa*^{14}, 13 of 25 gastrula stage embryos showed strong anterior activation similar to that shown in this panel. As expected in a cross with males heterozygous for the *UAS-Runt[Δ6]* construct, 12 of 25 showed minor alterations in the spacing of *slp1* stripes produced by *NGT*-driven expression of Opa alone. (E) *UAS-Runt[Δ7]*$^{21\text{-}3}$*/TM3* and *UAS-Opa*^{14}, 13 of 28 gastrula stage embryos in crosses with these heterozygous males showed strong anterior activation. (F) *UAS-Runt[Δ8]*^{49} and *UAS-Opa*^{10}, 28 of 38 gastrula stage embryos showed abnormal spacing of *slp1* stripes, whereas 10 of 38 showed loss of specific stripes similar to that shown, presumably due to repression by Runt[Δ8] (see below). None of the embryos in this cross showed strong anterior activation. Arrows indicate regions of anterior *slp1* activation in response to Runt, Runt[Δ6], and Runt[Δ7]. The potent activity of Runt[Δ7] in *slp1* activation is underscored by the use of the weaker *UAS-Runt[Δ7]*$^{21\text{-}3}$ line in this coexpression assay. Similarly, the inability of the Runt[Δ8] derivative to activate *slp1* is underscored by the use of *UAS-Opa*^{10} as this line is stronger than the *UAS-Opa* lines used for the other Runt constructs (Swantek and Gergen, 2004). The *slp1* response to NGT-driven coexpression of Ftz and these different Runt deletion derivatives in gastrula (G–L) and germband extension stages (M–R). In all cases ectopic expression was obtained by mating females homozygous for both *NGT40* and *NGTA* to males homozygous for *UAS-Ftz*^{263} and the pertinent Runt transgene: (G and M) *UAS-Runt*^{232}, 15 of 22 gastrula stage embryos show partial to complete repression of the even-numbered *slp1* stripes; (H and N) *UAS-Runt[CK]*^{77}, 0 of 23 gastrula stage embryos showed any evidence of repression of the even-numbered stripes; (I and O) *UAS-Runt[Δ3]*$^{46\text{-}1}$, 0 of 8 gastrula stage embryos show repression of all of the even-numbered stripes, although there is a region specific reduction in stripe 10 expression in several embryos; (J and P) *UAS-Runt[Δ6]*$^{16\text{-}2}$, 10 of 10 gastrula stage embryos show evidence of repression¤ with four of these having nearly complete repression similar to that shown in this panel. Note that the Runt[Δ6] line used in this experiment is slightly weaker than the line used in the *slp1* activation assay described above; (K and Q) *UAS-Runt[Δ7]*$^{45\text{-}2}$, 0 of 17 gastrula stage embryos show repression of all even-numbered stripes, although expression of stripe 10 is reduced in 16 of these 17 embryos; (L and R) *UAS-Runt[Δ8]*^{49}*/TM3*, 17 of 33 gastrula and early germband extension stage embryos scored in the cross with these heterozygous males showed partial to complete repression of the even-numbered stripes. The odd-numbered *slp1* stripes are repressed in all of the embryos in these different crosses due to ectopic Ftz expression in cells expressing endogenous Runt.

Runt is converted from an activator to a repressor of *slp1* by the homeodomain transcription factor Ftz (Swantek and Gergen, 2004). Endogenous Runt and Ftz are expressed in the anterior half of the even-numbered parasegments where both are required to prevent *slp1* expression. Ectopic expression of Ftz alone leads to repression of *slp1* in the posterior half of the odd-parasegments where endogenous Runt is also present. More important for this work is the observation that *NGT*-driven coexpression of Runt and Ftz also leads to repression of *slp1* in the posterior half of the even-numbered parasegments, resulting in the elimination of expression throughout the presegmental region of the embryo (Figure 7G). This coexpression assay thus provides an approach to investigating the ability of different Runt deletion constructs to repress *slp1* independent of the activity of endogenous Runt protein. Two of the deletions, Runt[Δ6] and Runt[Δ8], retain activity as repressors (Figure 7, J and L), whereas the Runt[CK], Runt[Δ3], and Runt[Δ7] proteins fail to repress the even-numbered *slp1* stripes in this assay (Figure 7, H, I, and K). The observation that these last three Runt derivatives fail to repress *slp1*, yet are competent in the initial establishment of *en* repression at this stage (Figure 6, E, G, and K) indicates a clear distinction in the molecular requirements for Runt-dependent repression of these two targets. It was noted above that the Runt[Δ8] protein is effective at establishing *en* repression at the gastrula stage, but this repression is not maintained during germband extension (Figure 6, M and N). In contrast, the Runt[Δ8]-dependent repression of *slp1* is maintained during these later stages (Figure 7R). This observation provides a further indication for differences in the Runt-dependent repression of *en* and *slp1*. There are some differences in early versus late *slp1* expression in embryos that coexpress Ftz and the Runt[CK], Runt[Δ3], and Runt[Δ7] proteins (Figure 7, N, O, and Q). However, it is difficult to interpret these changes without examining the response of other Runt targets in these embryos.

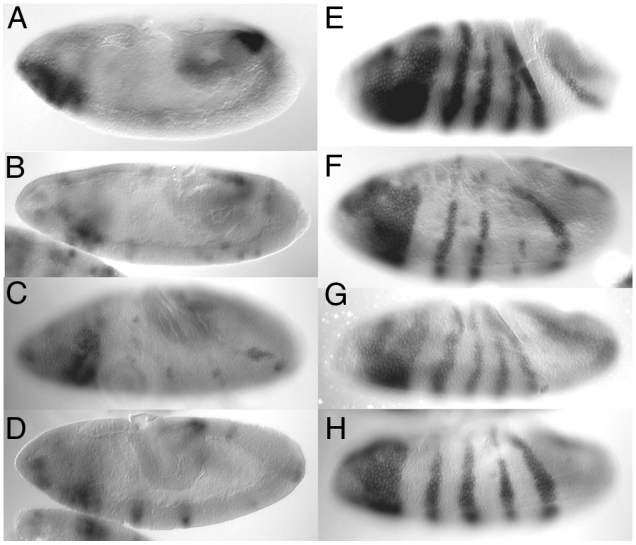

Figure 8. Runt-dependent *slp1* regulation is insensitive to *Gro* and *Rpd3* dosage. Expression of *slp1* mRNA in response to *NGT*-driven coexpression of *UAS-Runt*^{15} and *UAS-Ftz*^{263} (A–D) or *UAS-Runt*^{15} and *UAS-Opa*^{14} (E–H) transgenes. (A) Sixteen of 20 gastrula and early germband extension stage embryos from crosses involving females heterozygous for both the *NGT40* and *NGTA* drivers that are wild-type for *Gro* and *Rpd3* show partial to complete repression of *slp1.* Reducing the strength of either the *NGT* driver or the *UAS-Runt* line reduces the efficiency of the repression of *slp1* observed in response to coexpression of Runt and Ftz (Swantek and Gergen, 2004). (B) Nineteen of 23 embryos from females heterozygous for *NT40, NGTA,* and the *Gro*^{BX22} mutation show repression of *slp1*. (C) Nineteen of 25 embryos from heterozygous *NGT40, NGTA* females that are also heterozygous for *Gro*^{E48} show *slp1* repression. (D) Thirty of 36 embryos from females heterozygous for *NGT40, NGTA,* and the *Rpd3*^{04556} mutation show evidence of repression. (E) Anterior activation in response to of Runt and Opa is observed in 57 of 58 embryos from females heterozygous for *NGT40* and *NGTA* that are otherwise wild type. Strong anterior activation is also observed in crosses with females that are also heterozygous for (F) *Gro*^{BX22}, 20 of 23 embryos; (G) *Gro*^{E48}, 30 of 39 embryos; and (H) *Rpd3*^{04556}, 46 of 55 gastrula and early germband extension stage embryos.

The Role of Groucho and Rpd3 in slp1 *Regulation*

The results presented above indicating that Runt's conserved C-terminal region VIII has no apparent role in *slp1* repression, but instead contributes to Runt-dependent *slp1* activation are somewhat surprising. The Runt[Δ8] protein lacks the C-terminal VWRPY motif that mediates interaction with the corepressor protein Gro (Aronson *et al.,* 1997). The maintenance of Runt-dependent *en* repression is sensitive to the maternal dosage of Gro and the Gro-interacting histone deacetylase Rpd3 (Wheeler *et al.,* 2002). We used assays similar to those used in this previous work to investigate whether Gro and Rpd3 have roles in Runt-dependent *slp1* regulation. *NGT*-driven coexpression of Runt and Ftz is as effective at repressing *slp1* in embryos from females heterozygous for either the *Gro*^{BX22} or *Gro*^{E48} mutations as in embryos with wild-type *Gro* dosage (Figure 8, A–C). This result is consistent with observations above indicating that the Gro-interacting C-terminus is not required for *slp1* repression. The maternal dosage of *Rpd3* also has no effect on *slp1* repression (Figure 8D).

The more interesting question is whether the requirement for region VIII in *slp1* activation reflects a role for Gro in this process. We used the anterior activation assay described above to investigate to investigate whether the dosage of either *Gro* or *Rpd3* influences Runt-dependent *slp1* activation. In this case the activation of *slp1* in anterior head regions in response to *NGT*-driven coexpression of Runt and Opa is not reduced in embryos from females that are heterozygous for mutations in either *Gro* or *Rpd3* (Figure 8, E–H). The extent of anterior activation obtained with the specific combination of *NGT* drivers and *UAS-Runt* and *UAS-Opa* lines used for these experiments is strong, but not maximal. Importantly, slight reductions (less than twofold) in the level of ectopic Runt activity results in significantly weaker anterior activation (Swantek and Gergen, 2004). These results thus provide strong evidence that the Runt-dependent activation of *slp1* is not sensitive to *Gro* dosage. On the basis of these observations, we conclude that the requirement for region VIII in *slp1* activation does not involve interactions with Gro but instead should involve interactions of Runt's conserved C-terminus with other factors that are involved in transcriptional activation.

DISCUSSION

Functionally Distinct Roles for Different Conserved Regions of Runt

The experiments presented above use several different assays to investigate the functional contributions of different conserved regions of Runt. Each of the four deletion derivatives affects a different set of properties (Table 3). The functional specificity demonstrated by the different deletions is consistent with the notion that different conserved regions correspond to functional modules that participate in

Table 3. Functional specificity of Runt's conserved regions

	Runt derivatives				
Functional assay	Runt[CK]	Runt[Δ3]	Runt[Δ6]	Runt[Δ7]	Runt[Δ8]
Nuclear localization	n.d.	Diffuse	Punctate	Diffuse	Punctate
Embryo lethality	+	+	+	+	+
Axonal redirection	+	−	−	+	−
Repress *en* (est.)	+	+	+	+	+
Repress *en* (maint.)	−	−	+	+	−
Activate *slp1*	−	−	+	+	−
Repress *slp1*	−	−	+	−	+

n.d., not determined.

distinct molecular interactions of the Runt protein. It is interesting to note that similar patterns of functional requirements for Runt are observed for two different pairs of assays. The observations that *NGT*-driven expression of all of the different Runt constructs is lethal and that all of the constructs also retain the ability to establish *en* repression are consistent with the idea that this initial repression of *en* is the principal basis for the lethality associated with ectopic Runt expression. Perhaps more interesting are the common functional deficits of the Runt[CK], Runt[Δ3], and Runt[Δ8] proteins in both *slp1* activation and the maintenance of *en* repression (Table 3). As mentioned above, the levels of Runt needed to maintain *en* repression in anterior odd parasegments are less than those needed for activation of *slp1* in these same cells, indicating that these are two distinct targets that have coincidental requirements for DNA-binding by Runt, the conserved region C-terminal to the Runt domain and the VWRPY-containing C-terminus.

The inactivity of Runt[CK] in maintaining *en* repression and in both the activation and repression of *slp1* strongly suggests that DNA binding by Runt is critical for these three aspects of Runt function. Runt[Δ3], which is also defective for these same three functions lacks a conserved region that is located just C-terminal of the DNA-interacting "tail region" loop of the Runt domain (Bravo *et al.*, 2001). Intramolecular interactions with regions C-terminal to the Runt domain of Runx1 modulate DNA binding in vitro (Kagoshima *et al.*, 1996; Kanno *et al.*, 1998) and can influence cooperative interactions with other DNA-binding factors (Gu *et al.*, 2000). Our results are consistent with the idea that the region C-terminal to the Runt domain makes important contributions to the in vivo DNA-binding activity of the Runt transcription factor.

Runt[Δ8] is similar to Runt[CK] and Runt[Δ3] in that it is defective in maintaining *en* repression and in activating *slp1*. A key difference between these three is the ability of the Runt[Δ8] to repress *slp1.* This result indicates that the C-terminal VWRPY motif is not required for *slp1* repression, a finding also consistent with the results of our genetic experiments indicating that Runt-dependent *slp1* repression is insensitive to *Gro* dosage. The observation that conserved region VIII contributes to *slp1* activation is somewhat surprising, especially given the previously documented physical interaction between the C-terminal VWRPY motif and the Gro corepressor (Aronson *et al.*, 1997). On the basis of these results, we propose that conserved region VIII also mediates interactions with a separate factor that participates in transcription activation. One issue is whether this proposed interaction also involves the VWRPY motif. Conserved region VIII contains two contiguous blocks of sequence conservation separated by a variable linker, suggesting that the proposed interaction with an activator may involve these other conserved amino acids. In any event, the relatively compact size of region VIII makes it likely that interactions with Gro and the proposed cofactor involved in Runt-dependent activation will be mutually exclusive.

The unique activities of Runt[Δ6] and Runt[Δ7] underscore the modular architecture and context-dependent activities of Runt. These two conserved regions are separated by two amino acids in the five species that comprise the melanogaster subgroup (Figure 1). In the set of assays we used, the only activity that is disrupted by deletion of region VI is the redirection of axonal projections in the developing eye (Table 3). Region VII is not required for this activity of Runt, but appears to contribute to the punctate subnuclear localization of Runt and is required for *slp1* repression. These last two properties are shared with Runt[Δ3]. Nuclear matrix association of Runx1 is important for CD4 repression (Telfer *et al.*, 2004), suggesting a potential parallel in the mechanisms of *slp1* repression by Runt and the repression of *CD4* by Runx1. The repression of *slp1* by Runt also requires the activity of the homeodomain protein Ftz as well as the Ftz-interacting orphan nuclear receptor protein Ftz-F1 (Swantek and Gergen, 2004; Hou *et al.*, 2009). It is reasonable to propose that regions III and VII are involved in molecular interactions with Ftz, Ftz-F1 and/or the nuclear matrix that are involved in converting Runt from an activator to a repressor of *slp1* transcription. Taken all together, our results provide compelling evidence for the functional modularity of Runt and lay groundwork for identifying molecular interactions that contribute to the regulatory properties of this conserved family of transcriptional regulators.

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