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Redundant function of Runt Domain binding partners, Big brother and Brother, during *Drosophila* development

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SUMMARY

The Core Binding Factor is a heterodimeric transcription factor complex in vertebrates that is composed of a DNA binding α-subunit and a non-DNA binding β-subunit. The α-subunit is encoded by members of the Runt Domain family of proteins and the β-subunit is encoded by the CBFβ gene. In *Drosophila,* two genes encoding α-subunits, *runt* and *lozenge,* and two genes encoding β-subunits, *Big brother* and *Brother,* have been previously identified. Here, a sensitized genetic screen was used to isolate mutant alleles of the *Big brother* gene. Expression studies show that Big brother is a nuclear protein that co-localizes with both Lozenge and Runt in the eye imaginal disc. The nuclear localization and stability of Big brother protein is mediated through the formation of heterodimeric complexes between Big brother and either Lozenge or Runt. Big brother functions with Lozenge during cell fate specification in the eye, and is also required for the development of the embryonic PNS. ds-RNA-mediated genetic interference experiments show that Brother and Big brother are redundant and function together with Runt during segmentation of the embryo. These studies highlight a mechanism for transcriptional control by a Runt Domain protein and a redundant pair of partners in the specification of cell fate during development.

Key words: *Big brother*, *Brother*, *lozenge*, *runt*, Runx, Eye development, Ommatidium, CBFβ, RNAi

INTRODUCTION

A fundamental problem in biology is determining how different cell types are specified during the development of a multicellular organism. This process is usually initiated by extracellular cues that activate signal transduction cascades which produce a specific cellular response. Transcription factors such as the Core Binding Factor (CBF) play a vital role in enabling such signaling cascades to regulate a set of target genes (reviewed by Ito, 1999). CBF is a heterodimer composed of a DNA binding α-subunit and a non-DNA binding β-subunit. In mammals, three genes encoding the α-subunit, Runx1, Runx2 and Runx3, and a single gene encoding the β-subunit, CBFβ (also called PEBP2β), have been identified (Bae et al., 1993; Miyoshi et al., 1991; Ogawa et al., 1993a; Ogawa et al., 1993b; Wang et al., 1993). *Runx1*$^{-/-}$ and *CBFβ*$^{-/-}$ knock-out mice show a complete block of fetal liver blood cell development (Niki et al., 1997; Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a; Wang et al., 1996b). The Runx2 gene is essential for osteoblast differentiation and skeletal morphogenesis, and knock-out mice are completely deficient for bone development (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). The Runx proteins contain within their sequence a conserved 128 amino acid motif called the Runt Domain (RD) that mediates interactions with DNA and the β-subunit (Meyers et al., 1993; Ogawa et al., 1993a; Ogawa et al., 1993b; Wang et al., 1993). The β-subunit increases the affinity of Runx proteins for DNA by altering the conformation of the Runt Domain (Meyers et al., 1993; Ogawa et al., 1993a; Ogawa et al., 1993b; Tang et al., 2000; Wang et al., 1993).

In humans, the RUNX1 and CBFβ proteins have been extensively studied in the context of oncogenic forms that cause leukemia. The most frequent translocation [t(8;21)] associated with acute myeloid leukemia (AML) encodes a fusion product between RUNX1 and the protein ETO (reviewed by Downing, 1999). The fusion protein includes the RD, interacts with CBFβ, and functions as a dominant negative transcription factor. The leukemic phenotype presumably results from the accumulation of secondary mutations. Additionally, a chromosomal inversion [Inv(16)] that generates a fusion protein, CBFβ-SMMHC, between CBFβ and the smooth muscle myosin heavy chain protein, has been associated with AML (Liu et al., 1993). This fusion protein complexes with RUNX1 but is retained in the cytoplasm, and therefore disrupts transcription (Adya et al., 1998; Kanno et al., 1998; Liu et al., 1993).

The Runt Domain was named after *runt* (*run*), best known for its role as a primary pair-rule gene in *Drosophila* embryonic patterning and segmentation (Gergen and Wieschaus, 1986). *run* also plays an important role in sex determination by directly controlling the expression of the *sex lethal* gene (Kramer et al., 1999). Additionally, *run* function is required for

the development of the EL neurons in the embryonic CNS (Dormand and Brand, 1998; Duffy et al., 1991). A second protein containing the RD is Lozenge (Lz), which specifies both neuronal and non-neuronal cell types in the *Drosophila* eye (Daga et al., 1996; Flores et al., 1998). In the non-neuronal cone cells, Lz functions combinatorially with the transcription factors downstream of the Notch (N) and Epidermal growth factor receptor (EGFR) signaling pathways to activate the expression of *D-Pax2* (also known as *shaven*; Flores et al., 2000). Similar mechanisms also operate in the control of the *prospero (pros)* gene in the eye (Xu et al., 2000). Additionally, *lz* functions during the development of olfactory sensory organ precursors (Gupta et al., 1998) where it regulates the expression of the proneural gene *amos* (Goulding et al., 2000). Finally, studies have defined a role for Lz in *Drosophila* hematopoiesis in establishing a sub-population of blood cells called the crystal cells (Lebestky et al., 2000; Rizki and Rizki, 1980). It is interesting to note that both vertebrate and *Drosophila* RD proteins are involved in the control of blood cell fate.

In *Drosophila*, two genes encoding β-subunits, *Brother (bro)* and *Big brother (Bgb)*, were first identified through homology searches (Fujioka et al., 1996; Golling et al., 1996). These are expressed in distinct but overlapping patterns during embryogenesis (Fujioka et al., 1996; Golling et al., 1996). Furthermore, Bro and Bgb have been shown to physically interact with Run and increase its affinity for DNA (Fujioka et al., 1996; Golling et al., 1996). Overexpression experiments using either Bro or Bgb suggest that such complexes can form in vivo (Li and Gergen, 1999). A more complete functional analysis of the *Drosophila* partner proteins was lacking because mutations in these genes were unavailable at the time.

Here we report a loss-of-function analysis of the partner proteins. A genetic screen using a sensitized background isolated a mutation in *Bgb*, and ds-RNA-mediated interference strategies illustrate a redundant role for *Bro* and *Bgb* during development. We further show that Bgb functions during embryonic and eye development in the context of Run and Lz and is stabilized only in the presence of these proteins.

MATERIALS AND METHODS

Scanning electron microscopy

Adult flies were fastened to metal mounts with clear fingernail polish. Images were digitally acquired using the natural SEM mode of a Hitachi S-2460N scanning electron microscope with an attached Robinson detector.

Immunohistochemistry and ds-RNA experiments

A rabbit polyclonal antibody (α-Bgb) was generated against a C-terminal peptide of the predicted Bgb sequence (RDNRQDEMEAVR) (Fujioka et al., 1996; Golling et al., 1996). The antibody was affinity purified using the peptide conjugated to a Pierce Immunopure column. The purified antibody was preadsorbed against fixed cuticle from wild-type larvae and used at a final dilution of 1:150. Additionally, the following antibodies were used: α-Run (1:250; Dormand and Brand, 1998), αPros (1:10,000; Kauffmann et al., 1996), and DSHB antibodies α2B10 (1:20), αElav (1:400), αEngrailed (1:200), and mAb22C10 (1:200). Secondary antibodies (goat α-mouse-HRP, goat α-rabbit-HRP) were obtained from Jackson ImmunoResearch and used at a final dilution of 1:200. Eye discs were stained as described previously (Rogge et al., 1995).

ds-RNA experiments were performed essentially as described previously (Kennerdell and Carthew, 1998). The results from the injections are summarized in Table 1. For each experiment, the observed segmentation phenotype is shown in three independent examples in Fig. 5. A certain fraction of the injected embryos either hatched as wild type or were grossly and non-specifically affected as a result of the injection. Their numbers relative to the total injected was independent of the nature of the ds-RNA and are included in Table 1.

Genetics

EMS (methanesulfonic acid ethyl ester; 25 mM; Lewis and Bacher, 1968) was used to mutagenize Oregon-R male flies. The mutagenized males were crossed to lz^{ts1} virgin females and the progeny were reared at 25°C. The F_1 male progeny were screened for an eye phenotype. Mutations were balanced over either *SM6a* or *TM6b* chromosomes and the enhancement was mapped using standard mapping chromosomes.

Df(3L)Bgb^{K4} was generated by crossing *y w / Y; P[1556 ry*$^{+}$*] ry e / TM3* with *y w / y w; Δ2-3 Ki / Δ2-3 Ki*. Male progeny were mated to lz^{ts1} virgins. The progeny from this cross were reared at 25°C and the males were scored for an enhanced eye phenotype. Mosaic clones were generated by crossing *w / Y; Bgb*^{D} *FRT80B / TM6*b with *ey-flp w / ey-flp w*; *P[w*$^{+}$*] FRT80B / P[w*$^{+}$*] FRT80B*. Mutant tissue was identified by the absence of pigment.

Mosaic clones in adult eyes were fixed and sectioned as described previously (Coyle-Thompson and Banerjee, 1993). For rescue experiments, full length *Bgb* cDNA (Fujioka et al., 1996) was cloned into the *Xba*I and *Not*I site of *hsp70-pCaSpeR* and germline transformants were generated.

High resolution sequence analysis of the *Bgb*^{D} and *Bgb*^{9} mutants was performed using the ^{33}P-Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Transient transfections were performed using the *Drosophila* Expression System Kit (Invitrogen) and antibody staining was performed as described by Fehon (Fehon et al., 1990).

RESULTS

Isolation of dosage-sensitive enhancers

A temperature-sensitive allele of *lz*, lz^{ts1}, was used in a genetic screen to isolate mutations in interacting genes. At 25°C, lz^{ts1} flies have near wild-type eyes (Fig. 1B). However, when raised at 29°C these flies have an easily visible rough eye phenotype (Fig. 1C). Even at this non-permissive temperature, lz^{ts1} alleles have a milder eye phenotype than that due to a *lz* null allele (Fig. 1D). This led us to propose that the temperature-sensitive

Table 1. Summary of phenotypes obtained using ds-RNA-mediated genetic interference

	Buffer	3 μg/μl ds-*runt*	3 μg/μl ds-*Bro*	3 μg/μl ds-*Bgb*	1.5 μg/μl ds-*Bro* ds-*Bgb*
Number injected	480	240	240	240	480
Number survived	142	97	129	106	196
Number with segmentation phenotype	0 (0%)	84 (87%)	64 (50%)	0 (0%)	142 (72%)
Number with non-specific phenotype	6 (4%)	5 (5%)	9 (7%)	5 (5%)	2 (0.4%)
Number of wild-type phenotype	136 (96%)	8 (8%)	56 (43%)	101 (95%)	52 (26.6%)

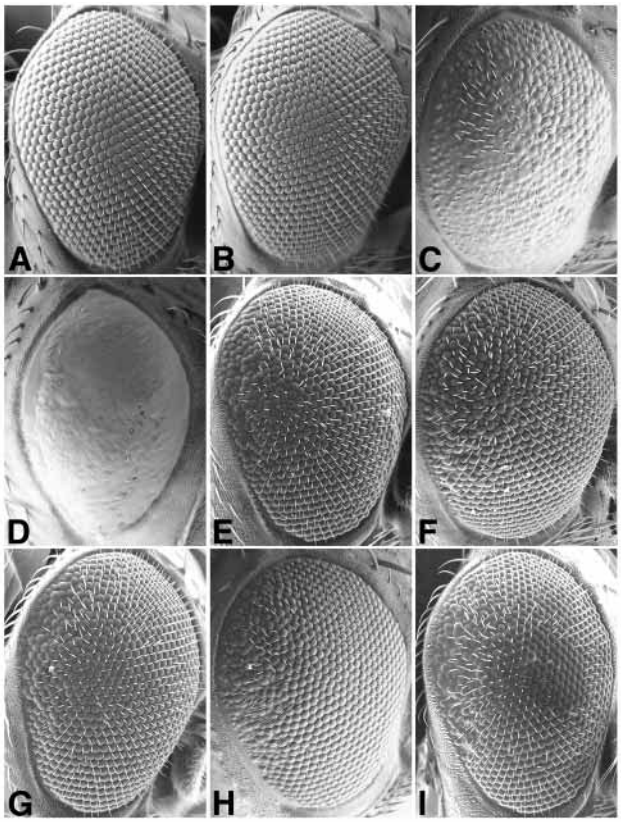

Fig. 1. A sensitized genetic screen for dosage-sensitive enhancers of lz^{ts1}. Scanning electron micrographs of adult eyes. Posterior is to the left and dorsal is up. All flies were reared at 25°C except for the one shown in C which was reared at 29°C. (A) The wild-type *Drosophila* eye has a regular array of ordered facets. (B) lz^{ts1} flies, when reared at 25°C, have wild-type eyes. (C) When lz^{ts1} flies are reared at 29°C, the eye appears rough and disorganized. (D) lz^{R1}, which is a null allele of *lz*, gives rise to a more severe eye phenotype than lz^{ts1} at any temperature. (E-I) Interacting mutations. When raised at 25°C, in a lz^{ts1} background, loss of one copy of each enhancer locus causes a slight roughening of the posterior area of the adult eye. Under a light microscope the eyes also appear to be slightly glossy, suggesting a defect in lens secretion. (E) lz^{ts1}; en(*lz*)3C/+. (F) lz^{ts1}; en(*lz*)Y/+. (G) lz^{ts1}; en(*lz*)D/+. (H) lz^{ts1}; en(*lz*)4G/+. (I) lz^{ts1}; en(*lz*)4F/+.

allele of *lz* functions at a threshold level such that any further reduction of the functional output of the Lz pathway would generate a mutant eye phenotype. In this scenario, at 25°C, a single copy loss-of-function mutation in a gene related to the Lz pathway is expected to generate an eye phenotype resembling that of a lz^{ts1} fly raised at a higher temperature.

Wild-type male flies were mutagenized with EMS and were mated with virgin lz^{ts1} females. Approximately 100,000 male progeny were reared at 25°C and scored for an eye phenotype. 10 flies were recovered that showed a disorganized eye phenotype. The mutations were crossed inter-se to generate 5 complementation groups: en(*lz*)4G/I, en(*lz*)3C/4C, en(*lz*)4F/4H, en(*lz*)Y, and en(*lz*)D/9 (Fig. 1E-I). These mutations are recessive embryonic lethal with the exception of en(*lz*)4G, which is homozygous viable with a disorganized eye. The en(*lz*)4G/I mutants were determined to be alleles of *D-Pax2* based on their failure to complement the eye-specific $D\text{-}Pax2^{spa\text{-}pol}$ allele (Fu and Noll, 1997). The enhancer called Y maps to the left arm of the second chromosome between *dp* (25A2) and *b*(34D5), is not uncovered by any known deletions, and was not analyzed further. The en(*lz*)D/9 mutants were determined to be mutations in *Bgb* and will be discussed in detail below. The en(*lz*)3C/4C mutations mapped to the left arm of the third chromosome and failed to complement *Df(3L)HR370*. Additional complementation analysis with known mutations within the breakpoints of this deletion determined that the en(*lz*)3C/4C mutations are alleles of *hsp83* (Hackett and Lis, 1983). The en(*lz*)4F/4H mutations mapped to the right arm of the third chromosome and failed to complement *Df(3R)RK8-21*. Complementation analysis with known mutations within the region established that en(*lz*)4F/4H are alleles of *osa*, also known as *eyelid (eld*) (Kennison and Tamkun, 1988; Treisman et al., 1997).

The en(*lz*)D/9 mutants were identified as alleles of *Bgb* based on several lines of evidence. First, we used a P-element excision strategy to generate a deletion belonging to the region because no chromosomal aberrations existed that eliminated this locus. A P-element located near *Bgb* was excised in a lz^{ts1} sensitized background. About 20,000 lines were screened and one excision allele, en(*lz*)K4, was identified as a dominant enhancer of lz^{ts1} at 25°C. This deletion completely eliminates the *Bgb* locus. The right breakpoint of this deletion maps within the transcribed region of an uncharacterized gene (Adams et al., 2000; Rubin et al., 2000) between *Bro* and *Bgb.* Thus, *Df(3L)K4* does not eliminate any part of the *Bro* gene. The EMS-induced mutants, en(*lz*)D/9, fail to complement the lethality of *Df(3L)K4* suggesting that they too carry a mutation in a gene uncovered by the deletion. Sequence analysis showed that en*(lz)*9 carries a mutation (A801C) in *Bgb* which results in a change from glutamic acid to aspartic acid in the Bgb sequence. Although non-conserved in the vertebrate protein, this amino acid is physically close to the segment that binds the α-subunit (Goger et al., 1999; Tang et al., 2000; Warren et al., 2000). More strikingly, the en*(lz)*D chromosome carries a mutation (G808A) in *Bgb* which results in a change from a conserved glycine to an arginine. This glycine in Bgb is conserved in the vertebrate CBFβ and *Drosophila* Bro proteins. This amino acid is also close to residues that physically interact with the Runt Domain (Goger et al., 1999; Tang et al., 2000; Warren et al., 2000). It is therefore likely that this mutation disrupts the stability of a Bgb/RD complex in vivo. Finally, flies carrying *hsp70-Bgb* constructs were generated using P-element mediated transformation (Rubin and Spradling, 1982) and assessed for rescue of the *Bgb* phenotype. Three independent *hsp70-Bgb* transformant lines were placed in homozygous en(*lz*)D and en(*lz*)9 genetic backgrounds. In each case, complete rescue to adult viability was obtained with the basal level of expression achieved without the application of a heat shock. Taken together, these data establish that en(*lz*)D and en(*lz*)9 carry mutations in *Bgb* and these lines were therefore renamed Bgb^{D} and Bgb^{9}. The *Df(3L)K4* was renamed $Df(3L)Bgb^{K4}$.

Expression studies

The *Drosophila* eye imaginal disc undergoes morphogenesis during the third larval instar. At this stage, a furrow sweeps across the disc from the posterior to the anterior. Cells entering the furrow undergo a synchronized round of cell division to give rise to a set of undifferentiated cells as well as pre-clusters

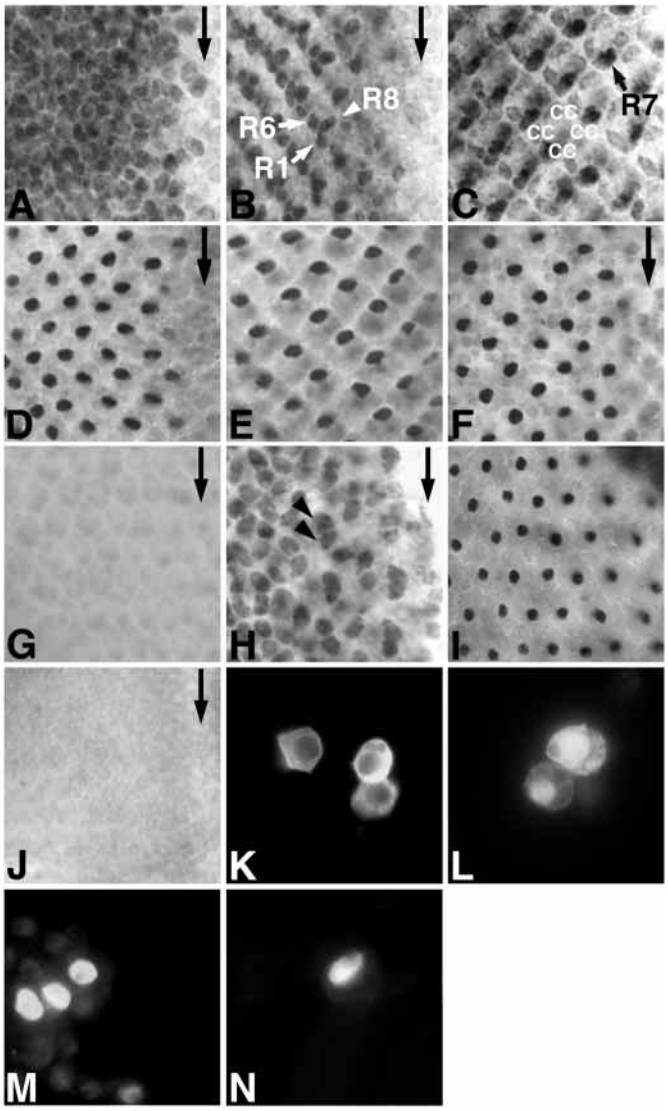

Fig. 2. Bgb and Run expression in the eye imaginal disc. Posterior is to the left. Arrow indicates the position of the furrow. (A-C) Wild-type eye disc stained with α-Bgb antibody. (A) At the basal level, nuclear staining is seen in the undifferentiated pool of cells. This staining begins immediately posterior to the furrow. (B) At a slightly more apical level, Bgb protein is seen in the nuclei of the R8, R1 and R6 cells. The expression of Bgb in the R8 cell initiates within 1 column of the furrow, while the expression in R1 and R6 initiates about 4 columns posterior to the furrow. (C) At the most apical focal plane, cone cells (cc) and the R7 cell express Bgb beginning about 7 columns posterior to the furrow. The R7 cell expresses Bgb at a much higher level than in any of the other cells. (D,E) Wild-type eye disc stained with α-Run antibody. (D) Run protein is detected in the nuclei of the R8 cell, initiating 1 column posterior to the furrow. Run expression was determined to be in R8 by the central location of this cell within an ommatidial cluster and through co-localization with the R8 specific marker, Boss (Kramer et al., 1991 and data not shown). (E) Run expression is seen in R7 about 7 columns posterior to the furrow. The timing of Run expression in R7 and R8 correlates well with the expression pattern of the Bgb protein in these cells. (F,G) Bgb expression in a lz^{77a7} background. lz^{77a7} is an eye-specific null allele of *lz* (Flores et al., 1998). (F) Bgb expression is limited to the R8 cell. (G) R7 expression of Bgb is absent. (H) Bgb expression in a lz^{sprite} background. With this gain-of-function allele, *lz* is misexpressed in R3 and R4 (Daga et al., 1996). Arrowheads indicate a corresponding ectopic expression in R3 and R4 of the Bgb protein. (I-J) Run expression in a lz^{77a7} background. (I) Run expression in lz^{77a7} is seen only in the R8 cell. (J) At the slightly higher R7 focal plane, no Run staining is observed in the R7 cell. (K,L) S2 cells stained with α-Bgb. (K) In cells transiently transfected with *Bgb*, the majority of the Bgb protein is found in the cytoplasm. (L) When *Bgb* and *lz* are co-transfected, the majority of Bgb protein moves to the nucleus. (M,N) S2 cells stained with α-Lz. (M) In cells transiently transfected with *lz* cDNA, Lz protein is seen in the nucleus. (N) Cotransfection of *Bgb* and *lz* does not affect the localization of Lz protein.

of five neurons that differentiate in a stereotypical developmental order: R8, R2/R5, then R3/R4 (for a review of eye development see Wolff and Ready, 1993). The remaining undifferentiated cells subsequently give rise to R1/R6, R7, and the non-neuronal cone and pigment cells.

In order to determine the expression pattern of Bgb during morphogenesis of the eye disc, we generated a rabbit polyclonal antibody against a C-terminal peptide of Bgb that is not homologous with any peptide stretch of Bro. Bgb expression is seen in the basal nuclei of the undifferentiated cells immediately posterior to the furrow (Fig. 2A). Among the differentiated cells, Bgb expression is first obvious in the R8 cell (Fig. 2B). Posteriorly, Bgb is seen in the R1/R6 cells (Fig. 2B), and the R7 and cone cells (Fig. 2C). The level of Bgb is much higher in the R7 cell than in any of the other cells in the eye disc.

The expression of Bgb closely corresponds to that of Lz with some notable differences. Like Bgb, Lz is also expressed in the undifferentiated cells behind the furrow as well as in the nuclei of the differentiated R1, R6, R7 and cone cells (Flores et al., 1998). Unlike Bgb, however, Lz is not expressed in R8, nor is it expressed at a higher level in R7 than in the other cells. We predicted that Bgb is likely to function with a different RD protein in R7 and R8, and therefore we studied the expression pattern of Run in the eye. Strikingly, Run is specifically expressed in the R7 and R8 cells of the eye disc (Fig. 2D,E). Taken together, the expression pattern of Bgb correlates nicely with the combined nuclear expression patterns of Run and Lz.

The correlation between Lz and Bgb staining patterns is functionally relevant. In a *lz* mutant background, Bgb expression is eliminated from all cells in which Lz is normally expressed (Fig. 2F,G). Additionally, when Lz is misexpressed in R3 and R4, Bgb protein is seen in the nuclei of these cells (Fig. 2H). This misexpression is not accompanied by an increase in *Bgb* transcripts (not shown) which suggests that Lz does not transcriptionally regulate *Bgb*. Instead, as Lz and Bgb are binding partners, it is likely that Lz stabilizes the Bgb protein in cells in which they are co-expressed. We also examined the relationship between Run and Lz proteins. The expression of the Run protein is genetically downstream of Lz in the eye. In a *lz* mutant background, Run expression is limited to the R8 cell (Fig. 2I) and is eliminated from the presumptive R7 cell (Fig. 2J). Thus, in a *lz* mutant background, the only cell that expresses a RD protein is R8. This is significant since in this background, R8 is also the only cell that expresses Bgb (Fig. 2F). This suggests once again that the α-subunit, in this case Run, is able to stabilize the β-protein.

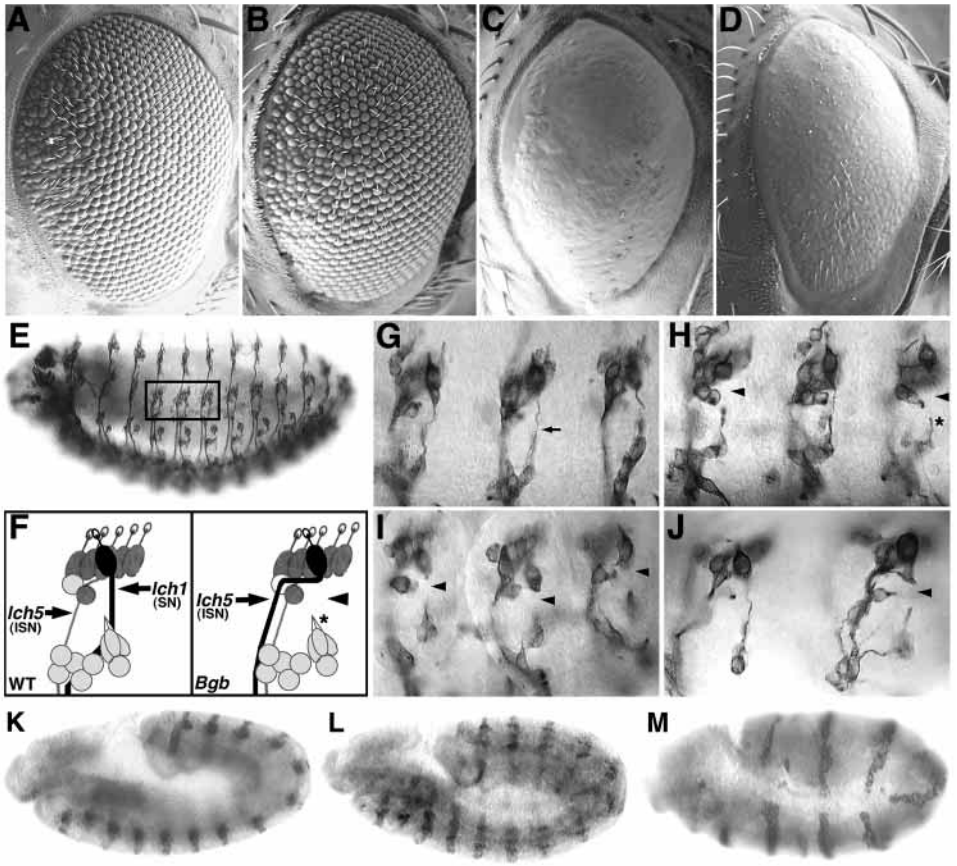

Fig. 3. *Bgb* phenotypes. (A-D) SEMs of adult eyes. Posterior is to the left and dorsal is up. All flies were reared at 25°C. (A) lz^{ts1}; Bgb^{D}/+. The Bgb^{D} mutant enhances lz^{ts1} on the posterior side of the eye (compare with Fig. 1B). (B) lz^{ts1}; $Df(3L)Bgb^{K4}$/+. The Bgb^{K4} deletion enhances the lz^{ts1} eye phenotype. (C) lz^{R1}. The *lz* null eye is very smooth and does not show any ommatidial organization. (D) lz^{ts1}; $Df(3L)Bgb^{K4}/Bgb^{D}$. Rare escapers of this genotype have a phenotype resembling a *lz* null eye. (E-M) Embryonic phenotype of *Bgb* and *run* mutants. Anterior is to the left and dorsal is up. Embryos were stained with mAb22C10 (E-J) or α-Engrailed antibody (K-M). (E) Wild-type stage-15 embryo. mAb22C10 recognizes neurons of the chordotonal organs (box). These are easily identified on the basis of their stereotypical arrangement and position in abdominal segments A1-A7. (F) A schematic diagram of the lateral chordotonal neurons based on that by Campos-Ortega and Hartenstein (1997). Within each wild-type cluster (WT panel) the axon of the single lateral chordotonal neuron, *lch1,* pioneers the segmental nerve (SN) to its target in the midline. The remaining five lateral chordotonal neurons, *lch5,* follow the intersegmental nerve (ISN) to their target in the midline. In *Bgb* mutant embryos (*Bgb* panel), the axon of the *lch1* neuron incorrectly follows the ISN to the midline. In this and subsequent panels an arrowhead indicates the absence of the *lch1* axon in its normal position. This axon does not join the SN, but instead aberrantly turns toward the ISN and follows this incorrect path to the midline. The ventral sensilla, *vp5,* has an apical projection (asterisk in F and H to distinguish this structure from the axon of the *lch1* neuron). (G,H) Lateral chordotonal neurons of (G) wild-type and (H) Bgb^{D}/Bgb^{D} stage-15 embryos. (G) The axons of the *lch1* neurons correctly follow the SN to the midline (arrow). (H) In the mutants, axons of the *lch1* neurons do not follow the SN (arrowheads), but incorrectly project towards the *lch5* and follow the ISN to the midline. In this genetic background the phenotype is not fully expressed and only 2 of the 3 lateral chordotonal clusters shown have this defect. (I) Lateral chordotonal neurons of $Bgb^{D}/Df(3L)Bgb^{K4}$ stage-15 embryo. In these mutants, the axons of the *lch1* neurons aberrantly project towards the *lch5* and do not follow the SN to the midline (arrowheads). This phenotype is similar to, but stronger than, that seen in (H). (J) Lateral chordotonal neurons of run^{YP17}/run^{YP17} stage-15 embryo. Only two sets of lateral chordotonal neurons are seen within the region corresponding to the previous panels because of a lack of segments in this mutant (Gergen and Wieschaus, 1986). The axon of one of the *lch1* neurons shown misprojects and does not follow the SN to the midline (arrowhead). (K) Wild-type stage-11 embryo. Engrailed protein is expressed as a 14 stripe pattern in the posterior compartment of each segment. (L) Bgb^{D}/Bgb^{D} stage-11 embryo. Engrailed expression is as seen in wild type. (M) run^{YP17}/run^{YP17} stage-11 embryo. These embryos show a segmentation phenotype, not seen in *Bgb* mutants.

A correlation of the in vivo results described above was also seen in S2 cells. The nuclear localization of Lz is constitutive and does not require Bgb protein (Fig. 2M,N). In contrast, when *Bgb* alone was expressed in S2 cells, nearly all of the Bgb protein remained cytoplasmic (Fig. 2K). However, when a construct expressing wild-type *lz* was co-transfected, a majority of the Bgb protein was seen in the nucleus (Fig. 2L). Thus, Bgb cannot be translocated to the nucleus in the absence of Lz. The cytoplasmic protein can be detected in S2 cells because of the high level of expression. Presumably, in the in vivo situation, the cytoplasmic Bgb protein is rapidly degraded in the absence of an α-subunit.

Phenotypic analysis

The identification of *Bgb* mutants as enhancers of lz^{ts1} provides genetic evidence that Bgb functions during eye development. The enhanced roughness produced by the loss of one copy of *Bgb* in this sensitized background is rather mild (Fig. 3A,B). However, rare escaper flies of $Df(3L)Bgb^{K4}/Bgb^{D}$ genotype can be generated in a lz^{ts1} background. These flies are extremely weak and rarely seen. They can only be rescued if they are dissected from their pupal cases. Such flies have a very strong adult eye phenotype (Fig. 3D) that is identical to that of the *lz* null allele (Fig. 3C).

Bgb mutants also show defects in the development of the embryonic peripheral nervous system (Fig. 3E-J) as illustrated by staining with mAb22C10 which recognizes neurons in the central and peripheral nervous systems (Fujita et al., 1982). The axons of lateral chordotonal neurons present in the abdominal segments of wild-type embryos follow two distinct paths to the midline. While the axon of the lateral monoscolopidial chordotonal organ (*lch1)* follows the

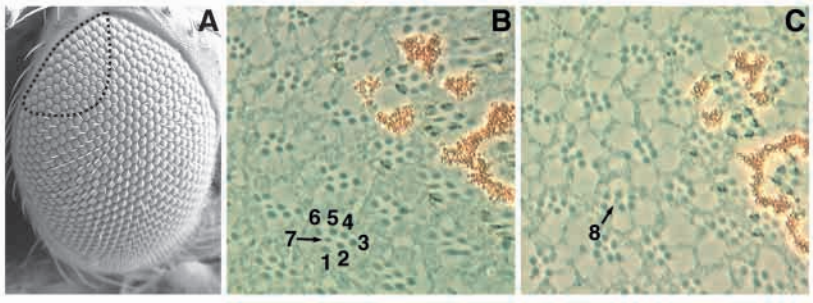

Fig. 4. Lack of *Bgb* eye phenotype in a lz^+ background. (A) Somatic clone of Bgb^D/Bgb^D tissue in the eye. The mutant tissue was first identified at low magnification using the difference in pigmentation between the mutant and the wild-type tissue. This boundary was subsequently marked in the SEM with the black dotted line. No defects are evident in the external structure of the facets within the clone. (B,C) Plastic section through a somatic clone of Bgb^D/Bgb^D tissue. (B) In this clone, marked by the lack of pigment granules, the R1-6 and R7 cells (1-7) appear completely normal. (C) In this deeper section of the same clone, R8 cells (8) are seen to develop normally.

segmental nerve (SN) to the midline, the axons of the lateral pentascolopidial chordotonal organ (*lch5*) follow the intersegmental nerve (ISN) (Fig. 3G). In Bgb^D/Bgb^D mutant embryos the *lch1* neuron turns toward the ISN and incorrectly follows this path to the midline (Fig. 3H). A similar phenotype with higher expressivity is seen in $Bgb^D/Df(3L)Bgb^{K4}$ embryos (Fig. 3I). The *hsp70-Bgb;* Bgb^D/Bgb^D rescued flies are completely viable and healthy flies, and as expected, do not show this PNS phenotype (not shown). PNS defects are also seen in run^{YP17}/run^{YP17} mutant embryos (Fig. 3J), however these defects are difficult to interpret because of the additional segmentation phenotypes of *run* (Fig. 3M), not seen in *Bgb* mutants (Fig. 3L). The lack of segmentation phenotypes in *Bgb* could be due to a maternal contribution of the *Bgb* gene product. Alternatively, this could reflect a functional redundancy between the two partner proteins (see below).

Bgb and *Bro* function redundantly during development

The results described in Fig. 3A-D establish a role for *Bgb* in eye development. However, when Bgb^D/Bgb^D clones were generated in an otherwise wild-type (lz^+) eye, the external phenotype of the facets (Fig. 4A) as well as the morphology of all retinal cells (Fig. 4B,C) appeared completely normal. Clones of Bgb^D were also examined in larval eye discs that were stained with antibodies directed against Elav, Cut and Pros. The expression pattern of these markers of neuronal and non-neuronal cells was wild type in all discs examined (not shown). Thus, all neuronal and non-neuronal cells in the eye are able to develop normally in the absence of the Bgb protein. The presence of a gene encoding the second partner protein, Bro, in the *Drosophila* genome, suggested to us the possibility that *Bro* and *Bgb* may function redundantly during development. This was investigated during embryonic development using the ds-RNA-mediated genetic interference method (Kennerdell and Carthew, 1998).

We injected embryos with ds-*Bgb*, ds-*bro*, or a mixture of ds-*Bgb* and ds-*bro*, and compared the resulting cuticular phenotypes with those resulting from loss of *run* function. *run* mutants are characterized by defects in segmental patterns which are visualized as deletions of denticle belts. *run* alleles can be ordered according to their strength by examining the denticles of the second and third abdominal segments (Gergen and Wieschaus, 1986). Weaker alleles of *run* tend to have only small deletions between these denticles (Fig. 5B), while stronger alleles have large deletions that are often accompanied by mirror-image duplications of denticle belts (Fig. 5C). As expected, injection of ds-*run* results in a cuticular phenotype (Fig. 5D-F) that is identical to the one seen with *run* null alleles (Fig. 5C).

Injection of ds-*Bgb* does not have any significant effect on the pattern of segmentation (Fig. 5G-I). In comparison, ds-*Bro* causes a segmentation phenotype (Fig. 5J-L) reminiscent of hypomorphic alleles of *run* (compare with Fig. 5B). Only a partial deletion of the naked cuticle between the second and third abdominal segments is apparent. Doubling the concentration of ds-*Bro* did not affect the severity of the phenotype (not shown). Strikingly, simultaneous injection of both ds-*Bgb* and ds-*Bro* resulted in embryos that have a much stronger segmentation phenotype (Fig. 5M-O), which is identical to that seen in a null allele of *run* (Fig. 5C), or in embryos injected with ds-*run* (Fig. 5D-F). These data show that although the disruption of *Bgb* does not generate a phenotype on its own, a role for *Bgb* in segmentation is revealed upon the disruption of both partner genes.

DISCUSSION

Sensitized genetic screens have proved to be powerful tools in identifying interacting proteins that participate in many different developmental pathways. A particularly impressive use of this technique in the *Drosophila* eye led to the identification of the mutations in the components of the RTK pathway (reviewed by Zipursky and Rubin, 1994). We have used such a screening technique to generate mutations in genes that function with *lz* during eye development. The identification of mutations in a direct transcriptional target of Lz, *D-Pax2* (Flores et al., 2000), and the gene encoding a binding partner of Lz, *Bgb*, suggests that this screen is able to detect proteins whose function is directly related to that of Lz.

In our screen, two alleles of *hsp83* were isolated as dominant enhancers of lz^{ts1}. *Drosophila* Hsp83 is a chaperone protein that has been shown to physically interact with Raf (van der Straten et al., 1997). Mutations in *hsp83* were identified as downstream modifiers of the *sevenless* and *EGFR* RTK pathways (Simon et al., 1991; van der Straten et al., 1997). Recent studies have indicated an extensive collaboration between RTK pathways and Lz in the regulation of direct target genes such as *D-Pax2* and *pros* (Flores et al., 2000; Xu et al., 2000). It is therefore likely that *hsp83* strengthens the RTK signal transduction cascade that functions with Lz in the regulation of target genes. In addition, *HSP90*, the mammalian homolog of *hsp83*, has been shown to associate with a variety

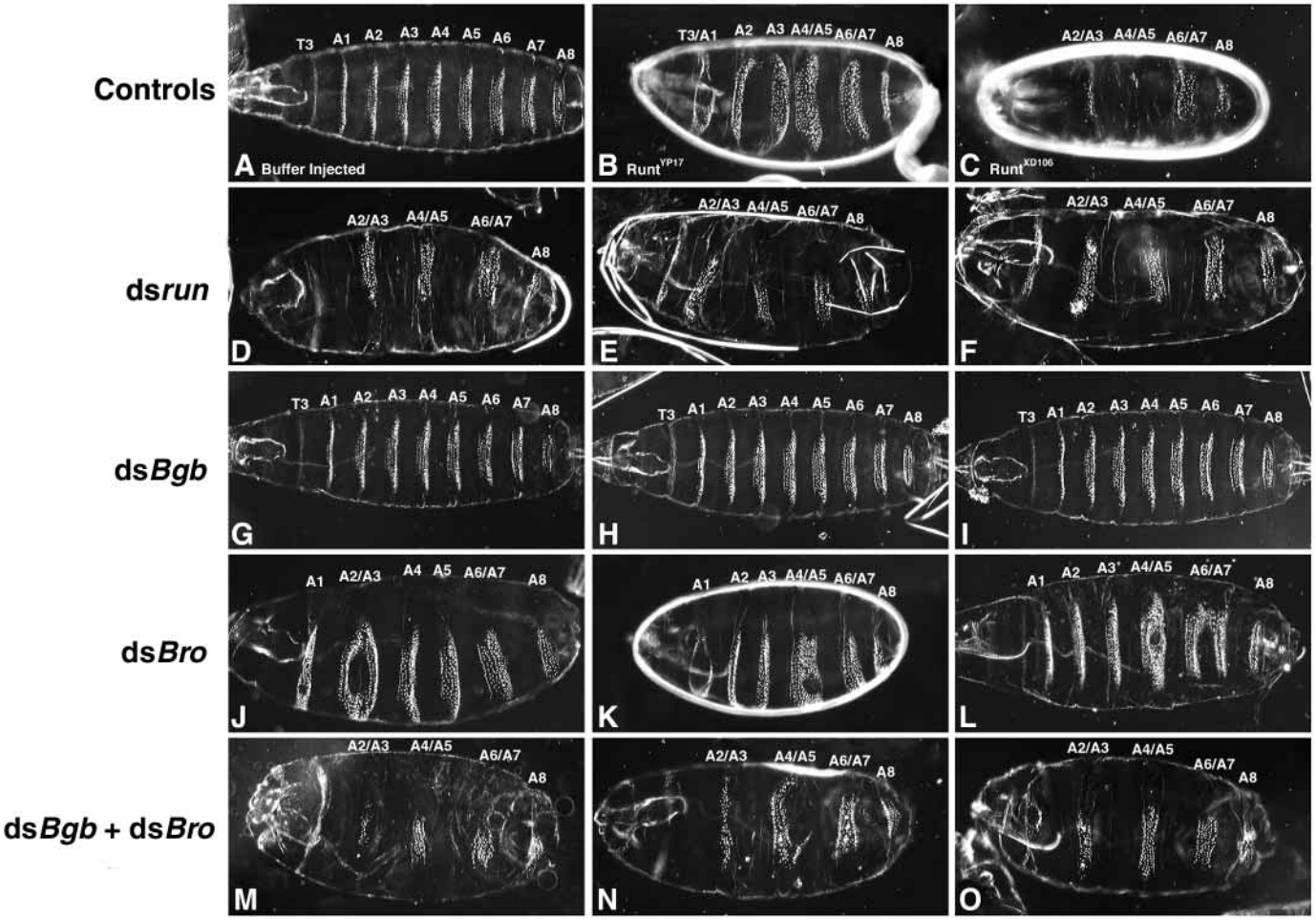

Fig. 5. ds-RNA-mediated genetic interference. Cuticle preparations showing denticle belts corresponding to abdominal segments A1-A8 and thoracic segment T3. Anterior is to the left. (A) Wild-type injected with buffer only. T3 and A1-A8 denticle belts are clearly visible. (B) *run*^{YP17}. This hypomorphic allele produces a weak segmentation phenotype. A deletion of the lateral edges between the A2/A3 denticle belts results in the curved appearance of these belts. Deletion of the naked cuticle between the A4/A5 and the A6/A7 denticles is also evident. (C) *run*^{XD106}. This null allele produces a severe segmentation phenotype. Large deletions between the A2/A3, A4/A5, and A6/A7 denticle belts are apparent. These deletions result in mirror image duplications of the corresponding denticles. (D-F) Three independent examples of wild-type embryos injected with double stranded RNA corresponding to the *run* gene (ds-*run*). In each example there is a complete deletion of the naked cuticle between A2/A3, A4/A5, and A6/A7 which results in a mirror image duplication of the remaining denticles. This is the same phenotype as that seen in null alleles of *run* (compare with C). (G-I) Three independent examples of wild-type embryos injected with ds-*Bgb*. No perceptible alteration of the segmentation pattern is evident. These animals are indistinguishable from those injected with buffer (compare with A). (J-L) Three independent examples of wild-type embryos injected with ds-*Bro*. Defects in the segmentation pattern are similar to those seen in hypomorphic *run* mutants (compare with B). (M-O) Three independent examples of wild-type embryos injected with a mixture of ds-*Bgb* and ds-*Bro*. An extremely strong segmentation phenotype identical to that seen with null alleles of *run* (compare with C) and wild-type embryos injected with ds-*run* (compare with D-E) can be seen. Thus, although injection of ds-*Bgb* did not affect segmentation, and injection of ds-*Bro* produced a mild segmentation defect, injection of a mixture of both ds-*Bgb* and ds-*Bro* had a synergistic effect on the pattern of segmentation.

of different transcription factors and has also been proposed to function in nuclear transport (reviewed by Helmbrecht et al., 2000). An analysis of the relationship between Hsp83 and Lz/Bgb might provide insight into the mechanism by which this transcription factor complex is translocated to the nucleus.

The screen also uncovered two alleles of *osa/eld* (Kennison and Tamkun, 1988; Treisman et al., 1997), a member of the *brahma* (*brm*) complex, involved in chromatin remodeling (Collins et al., 1999; Kennison and Tamkun, 1988; Vazquez et al., 1999). The identification of *osa* as a dominant enhancer suggests that Lz may have a function related to chromatin remodeling. This is not surprising as other Runx family members are thought to function in this manner. For example, Runx2 binding has been implicated in the remodeling of the rat osteocalcin promoter (Javed et al., 1999). Additionally, during myeloid differentiation, Runx1 has been shown to interact with p300/CBP (Kitabayashi et al., 1998), a protein involved in histone acetylation. Further, *Drosophila* Run has been shown to bend DNA and is likely involved in modifying the architecture of target enhancers (Golling et al., 1996). In the eye, Lz is essential for pre-patterning an undifferentiated population of cells and preparing them to activate different

target genes in response to signal transduction cascades. It is possible that this process involves remodeling of the individual enhancers through the mediation of an Osa/Lz complex. The identification of *osa* as a genetic modifier of *lz* suggests the need for future biochemical experiments to establish if such protein complexes are indeed formed during development.

In this paper we have focused on the function of the partner proteins since mutations in *Bgb* were identified as modifiers of *lz*. The similarity in the phenotype of lz^{ts1}; $Bgb^{D}/Df(3L)Bgb^{K4}$ mutants to the null allele of *lz* suggests an absolute functional requirement of the partner protein during eye development. Similarly, the ds-RNA interference results suggest that both partner proteins are able to function with Run during embryonic pattern formation.

It remains to be proved if the disorganization seen in the PNS of *Bgb* is attributable to Bgb function with the known RD proteins. Similar PNS defects are seen in *run* mutants, but these phenotypes are difficult to interpret because of the additional segmentation phenotypes that could indirectly affect PNS development. It remains possible that Bgb functions with an as yet uncharacterized RD protein in the PNS. Consistent with this explanation, a survey of the sequence of the *Drosophila* genome (Adams et al., 2000) reveals two additional RD proteins.

Our S2 cell expression data show that Bgb is only translocated to the nucleus in the presence of Lz. Although Bgb has a nuclear localization signal (NLS; Fujioka et al., 1996), these data suggest an additional requirement of Lz binding for its transport to the nucleus. Similar regulation of nuclear transport has been reported with Single-minded (Sim) and Tango (Tgo) heterodimers (Ward et al., 1998) as well as with Homothorax (Htx) and Extradenticle (Exd) heterodimers (Pai et al., 1998). In these examples, the localization to the nucleus of either Tgo or Exd, depends on the presence of Sim or Hth, respectively (Pai et al., 1998; Ward et al., 1998). Recent work has shown that Hth binding allows nuclear transport of Exd by simultaneously inhibiting its nuclear export signal (NES) while activating its NLS (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Bgb does not have a leucine-rich sequence typically associated with an NES (Fischer et al., 1995; Wen et al., 1995); co-localization into the nucleus in this case is likely to involve an unmasking of the NLS causing its exposure to the transport machinery. Obviously, nuclear localization of both the α- and the β-subunit is a prerequisite for activation of transcription. In fact, in human AML caused by Inv(16), the CBFβ fusion protein is exclusively retained within the cytoplasm (Adya et al., 1998; Kanno et al., 1998; Liu et al., 1993).

The Lz/Bgb complex provides an interesting example of post-translational stabilization of proteins through the formation of heterodimeric complexes. While we cannot rule out the possibility that low levels of Bgb protein remain in the cytoplasm of the cell in a *lz* mutant background, the likely explanation for the Bgb protein not being detectable in the absence of Lz or Run is that the β-subunit is degraded in the absence of the α-partner. Similar mechanisms involving degredation of a subunit operate in creating stable Exd/Hth and Sim/Tgo complexes. Tissue lacking Hth or Sim will cause degradation of Exd and Tgo, respectively (Pai et al., 1998; Ward et al., 1998). As an interesting contrast to our results, in mammalian systems it is the α-subunit, Runx1, that is stabilized by CBFβ. In this case, the absence of the β-partner causes a proteosome-mediated degradation of the α-subunit (Huang et al., 2001).

The initial cloning of *Bro* and *Bgb* raised the possibility that these genes might function redundantly during development. Although there is a stretch of 156 amino acids at the N terminus of Bgb that is not present in Bro (Fujioka et al., 1996), these proteins are 59% identical throughout the remainder of their sequence. Furthermore, *Bro* and *Bgb* have overlapping expression domains during embryogenesis (Fujioka et al., 1996; Golling et al., 1996). ds-RNA-mediated genetic interference experiments used here clearly show that Bro and Bgb function redundantly during development as heterodimeric partners of Run. A loss-of-function phenotype equivalent to a complete *run* null allele is only revealed in the absence of both Bro and Bgb.

The two partner proteins do not function redundantly in all tissues. This is highlighted by the fact that *Bgb* mutants have a PNS defect on their own. Thus, at least in this tissue, Bgb function is not redundant with that of Bro. This is different from redundant gene pairs such as *BarH1* and *BarH2* which are co-regulated in all tissues and always function together (Higashijima et al., 1992). It is also interesting to note that injection of ds-*Bro* generates a fairly strong segmentation phenotype, while injection of ds-*Bgb* does not affect segmentation patterning at all. Therefore, it is possible that in the wild-type fly, when both partners are present, Run preferentially functions with Bro. However, only in the absence of Bro, can compensation of Run function be achieved through its binding Bgb. A comparable situation exists in mice. The paralogs *Hoxa3* and *Hoxd3* are expressed in the same tissue, but clearly have distinct functional requirements (Chisaka and Capecchi, 1991; Condie and Capecchi, 1993). Yet, a compensating mechanism can be created in a background when one of the two genes is eliminated (Greer et al., 2000).

Redundancy is a classical and important problem in genetics. As vertebrate genomes are analyzed in increasing detail, it is becoming evident that single mutant phenotypes may be masked due to the function of an alternate redundant gene (Müller, 1999). The completion of the sequence of the *Drosophila* genome (Adams et al., 2000) has revealed many gene families that function redundantly. Indeed, the identification of the Rhomboid family of genes led to the observation that Rho1 and Rho3 function redundantly during *EGFR* signaling (Wasserman et al., 2000). Another example is *sloppy paired1 (slp1)* and *sloppy paired2 (slp2)* which were identified as functionally redundant genes based on the fact that they are expressed in the same tissue but at slightly different levels (Bellen et al., 1989; Grossniklaus et al., 1992; Wilson et al., 1989). In spite of these advances, detection of mutations in genes that function redundantly poses a difficult challenge to genetic analysis. Our data show that at least for the case in study, dosage-sensitive screens involving sensitized genetic backgrounds can be used for the purpose of identifying redundant genes. *Bro* and *Bgb* together can be considered to contribute 4 copies of the partner gene. Loss of 1 out of these 4 copies in a sensitized background (lz^{ts1}; Bgb^{-} Bro^{+}/Bgb^{+} Bro^{+}) gives rise to a detectable eye phenotype. Yet, loss of 2 copies in a wild-type background (lz^{+}; Bgb^{-} Bro^{+}/Bgb^{-} Bro^{+}) does not generate a mutant phenotype. This remarkable sensitivity to dosage suggests that properly sensitized genetic screens could be used in the detection of redundant gene function.

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