The t(8;21) translocation converts AML1 into a constitutive transcriptional repressor

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Summary

The human translocation (t8;21) is associated with ~12% of the cases of acute myelogenous leukemia. Two genes, *AML1* and *ETO*, are fused together at the translocation breakpoint, resulting in the expression of a chimeric protein called AML1-ETO. AML1-ETO is thought to interfere with normal AML1 function, although the mechanism by which it does so is unclear. Here, we have used *Drosophila* genetics to investigate two models of AML1-ETO function. In the first model, AML1-ETO is a constitutive transcriptional repressor of AML1 target genes, regardless of whether they are normally activated or repressed by AML1. In the second model, AML1-ETO dominantly interferes with AML1 activity by, for example, competing for a common co-factor. To discriminate between these models, the effects of expressing AML1-ETO were characterized and compared with loss-of-function phenotypes of *lozenge* (*lz*), an *AML1* homolog expressed during *Drosophila* eye development. We also present results of genetic interaction experiments with AML1 co-factors that are not consistent with AML1-ETO behaving as a dominant-negative factor. Instead, our data suggest that AML1-ETO acts as a constitutive transcriptional repressor.

Key words: AML1 (RUNX1), AML1-ETO, MTG8, Lozenge, CBFβ, *Drosophila* eye, RUNX1T1

Introduction

The human disease acute myelogenous leukemia (AML) is characterized by the proliferation of undifferentiated hematopoietic precursor cells. The most common chromosomal abnormality associated with AML is a translocation (t8;21) that fuses the gene *ETO* (RUNX1T1 – Human Gene Nomenclature Database) to *AML1* (*RUNX1* – Human Gene Nomenclature Database) (Erickson et al., 1992; Nisson et al., 1992; Shimizu et al., 1992; Miyoshi et al., 1993; Look, 1997). The fusion gene resulting from this translocation encodes a chimeric protein, AML1-ETO. AML1-ETO contains the N terminus of AML1 and almost the entire ETO protein (Fig. 1A). AML1 (also known as Runx1) belongs to the Runt Domain (RD) family of transcription factors. Members of the RD family share a highly conserved DNA-binding domain (the eponymous RD) and a C-terminal VWRPY motif that is capable of recruiting transcriptional repressors, although AML1 also functions as a transcriptional activator (Fig. 1A) (reviewed by Peterson and Zhang, 2004). The RD also interacts with CBFβ, a co-factor that enhances the DNA-binding affinity of AML1 (Ogawa et al., 1993; Wang et al., 1993). In AML1-ETO, the entire AML1 RD is joined to ETO. ETO contains four conserved functional domains, so called Nervy Homology Regions (NHR) named after the *Drosophila* homolog of *ETO*, *nervy* (Fig. 1A) (Feinstein et al., 1995). Multiple regions in ETO interact with transcriptional repressors, including NHR4, which comprises two zinc fingers (reviewed in Peterson and Zhang, 2004; Hug and Lazar, 2004). These protein interactions suggest that ETO acts as a transcriptional repressor, further implying that AML1-ETO also represses transcription. Accordingly, AML1-ETO is able to repress transcription in vitro and, importantly, AML1-ETO is able to repress the expression of AML1 target genes. These findings suggest that AML1-ETO has the potential to regulate the expression of genes that are under the control of AML1 in vivo. A change in the regulation of AML1 target genes would be significant, given that *AML1* is expressed in differentiating hematopoietic cells and that *AML1* knockout results in a block of normal hematopoiesis (Okuda et al., 1996; Wang et al., 1996a).

These data have led to the proposal that AML1-ETO causes leukemia by interfering with either the function of endogenous AML1 or the transcriptional regulation of AML1 target genes (Lutterbach and Hiebert, 2000; Peterson and Zhang, 2004). Two classes of models can describe how AML1-ETO could interfere with normal AML1 activity (Fig. 1B). First, because AML1-ETO has the potential to interact with AML1 co-factors (such as CBFβ) through its RD, it could act as a dominant-negative molecule by competing with AML1 for these co-factors. Such a mechanism has been proposed for CBFβ::MYH11, a leukemogenic fusion that is thought to sequester the normally nuclear AML1 in the cytoplasm (Kanno et al., 1998; Adya et al., 1998; Li and Gergen, 1999). A second model is that AML1-ETO binds to AML1-binding sites via its RD and represses transcription of AML1 target genes. Unlike AML1, which can both activate and repress target genes, this model posits that AML1-ETO functions as a constitutive repressor. Although AML1-ETO has been shown to interact

with CBFβ and repress the expression of AML1-regulated genes in vitro and in cell culture, the available data do not distinguish between these two models. A knowledge of how this oncoprotein functions at a mechanistic level is important for understanding how AML1-ETO contributes to AML and for the design of possible therapies.

We used the *Drosophila* eye as an in vivo system to distinguish between these two models of AML1-ETO function. We chose the fly eye for these studies for two reasons. First, the eye is an experimentally accessible tissue that is well suited for analyzing gene function. Second, the role of the *AML1* homolog *lz* has been extensively studied in fly eye development (Daga et al., 1996; Batterham et al., 1996; Crew et al., 1997; Xu et al., 2000; Flores et al., 2000; Siddall et al., 2003; Canon and Banerjee, 2003). The *Drosophila* eye has also been used to analyze other human disease genes, including those that contribute to neurodegeneration such as spinocerebellar ataxia and huntingtin (Jackson et al., 1998; Kazantsev et al., 2002) (reviewed by Bonini and Fortini, 2002). In *Drosophila*, there are two characterized RD family members, *lz* and *runt* (*run*), and two uncharacterized genes that are predicted to encode RD transcription factors (Fig. 1A) (Rennert et al., 2003). There are also two well-conserved CBFβ homologs in flies, called *brother* (*bro*) and *big brother* (*bgb*), which are able to stimulate the ability of both fly and mammalian RD proteins to bind DNA (Golling et al., 1996; Li and Gergen, 1999; Kaminker et al., 2001). In vitro, RD factors from different species are able to recognize similar binding sites, suggesting that they may also recognize similar sites in vivo (Pepling and Gergen, 1995; Golling et al., 1996; Xu et al., 2000; Flores et al., 2000). Consistent with this molecular conservation, RD factors in different species also have analogous roles during development. For example, like *AML1* in humans, *lz* is expressed in the fly hematopoietic lineage, where it is necessary for the specification of a subset of hematopoietic cell types (Lebestky et al., 2000; Waltzer et al., 2003).

We found that the phenotypes resulting from AML1-ETO expression in the fly eye differ from those produced by expressing AML1 and from the lz^{null} phenotype, indicating that the effects of AML1-ETO on *Drosophila* eye development are distinct from these other genetic alterations. Furthermore, we show that expression of AML1-ETO represses the expression of *Drosophila Pax2* (*sv* – FlyBase) which is a directly activated target gene of Lz. These data suggest that AML1-ETO is able to repress the expression of RD targets in vivo. AML1-ETO is also able to block the expression of *deadpan* (*dpn*), a gene that is normally repressed by Lz. This result is particularly informative for distinguishing between the dominant-negative and constitutive repressor models, because we predict that a negatively regulated target would be de-repressed if AML1-ETO acts by dominantly interfering with Lz function, but would remain repressed if AML1-ETO behaves as a constitutive repressor. Finally, genetic interaction experiments with the RD co-factors Bro and Bgb are inconsistent with a dominant-negative model of AML1-ETO function. Together, these results support a constitutive repressor model of AML1-ETO function.

Materials and methods

Fly stocks

UAS-AML1-ETO flies were generously provided by B. Mathey-Prevot, who also supplied the *UAS-AML1* plasmid that was used to generate the *UAS-AML1* flies; lzr^{15}, a null allele of *lz*; *UAS-lz*; $Df(3L)bgb^{k4}$ (a small deficiency that removes *bgb*, referred to here as bgb^{null}); and *SME-lacZ* flies were kindly shared by the Banerjee laboratory; *UAS-run*, *UAS-bro* and *UAS-bgb* flies were gifts from the Gergen laboratory.

Immunofluorescence

Eye discs were dissected from 3rd instar larvae or pupae raised at 25°C. The discs were fixed in 4% formaldehyde in 1×PBS, incubated overnight in primary antibody diluted in 1×PBT (0.1% Triton X-100 in 1×PBS), washed and then incubated with fluorescently-conjugated secondary antibodies from Jackson ImmunoResearch. The following primary antibodies were used: mouse anti-β-gal (1/1000; Sigma), rabbit anti-βgal (1/1500; Cappell), rat anti-Elav 7E8A10 (1/50; Developmental Studies Hybridoma Bank), mouse anti-Cut (1/10; Developmental Studies Hybridoma Bank), rabbit anti-AML1 AP1651 (1/50; generously shared by P. Erickson) and rabbit anti-Dpn (1/200; kind gift of H. Vaessin).

UAS-NLS-AML1ΔETO, *UAS-AML1-ETOΔZF* and *UAS-lz-enR* plasmids

UAS-NLS-AML1ΔETO was created by PCR amplifying nucleotides 1-531 of *AML1-ETO* from an *AML1-ETO* cDNA template kindly supplied by B. Mathey-Prevot. Nucleotide 531 corresponds to the last nucleotide of the RD, which is also the last *AML1* nucleotide in *AML1-ETO*. The resulting *AML1ΔETO* PCR product was cloned into a modified *pUASt* vector that fuses an *NLS* in frame to 5′ of *AML1ΔETO*. *UAS-AML1-ETOΔZF* was generated using two *Xcm* restriction digest sites that flank the zinc fingers. This removes amino acids 591-698 of AML1-ETO. The digested *AML1-ETO* cDNA was incubated with T4 DNA polymerase to generate in-frame blunt ends that were then ligated together. *AML1-ETOΔZF* was sequenced and cloned in *pUASt*. *UAS-lz-enR* was created by substituting the *engrailed* repressor domain for nucleotides 1624-2259 of *lz* (*lz* cDNA gift of the Banerjee laboratory) and then cloning *lz-enR* into *pUASt*.

Results

Expression of AML1-ETO causes phenotypes that are distinct from other *RD* gene gain- and loss-of-function phenotypes

To study the function of AML1-ETO we used the Gal4-UAS system to express *AML1-ETO* in the fly eye (Brand and Perrimon, 1993). The eye was chosen to characterize AML1-ETO function in the fly for several reasons. First, expression of exogenous proteins during fly eye development typically does not result in lethality, allowing a thorough analysis of the resulting phenotypes. Second, the function and targets of the *Drosophila* RD gene *lz*, which is expressed in the eye, have been extensively characterized, thereby providing many valuable tools and markers with which to carry out this analysis (Daga et al., 1996; Crew et al., 1997; Xu et al., 2000; Flores et al., 2000; Canon and Banerjee, 2003). An underlying premise of this approach is that Lz target genes will respond to AML1-ETO in a similar manner as genes that are regulated by AML1 during human hematopoiesis. Although we focus here on effects in the eye, expression of AML1-ETO in the fly hematopoietic system also has dramatic phenotypic consequences (B. Mathey-Prevot, J.W. and R.S.M., unpublished). However, the lack of identified direct Lz targets in the fly hematopoietic system makes it less suitable to study the mechanism of AML1-ETO function.

A wild-type adult *Drosophila* eye has ~750 ommatidia

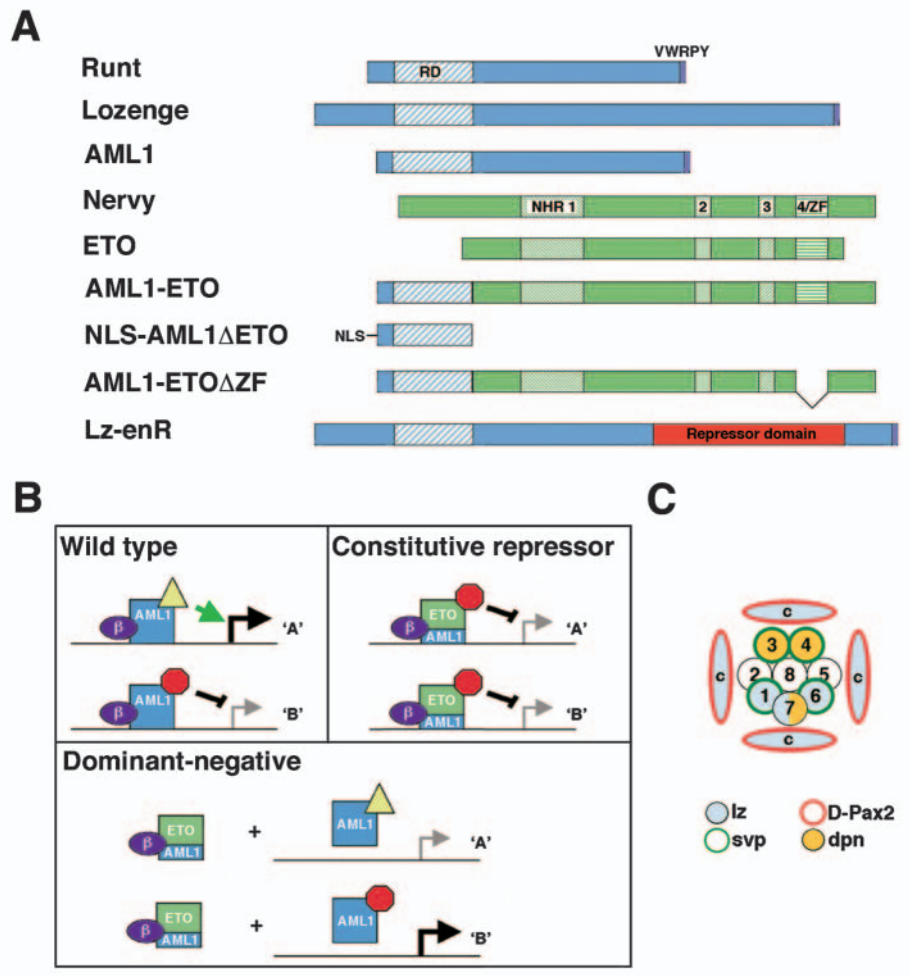

Fig. 1. Schematic of RD- and ETO-related proteins. (A) The RD proteins (blue) contain the RD, which binds DNA and interacts with CBFβ/Bro/Bgb, and a C-terminal VWRPY motif. ETO and Nervy (green) share four conserved domains [NHR 1-4; NHR 4 is the zinc-finger (ZF) domain]. enR (red) is from the Engrailed protein and includes its repressor domain. (B) Wild-type AML1 (blue) interacts with CBFβ (purple) and can bind either transcriptional activators (yellow triangle) or repressors (red hexagon) to regulate gene expression. There are two models to describe how AML1-ETO (blue+green) could be interfering with endogenous AML1 target gene expression to cause leukemia. First, AML1-ETO, which recruits transcriptional repressors through its C terminus, could repress the expression of all AML1 targets (constitutive-repressor model). Alternatively, AML1-ETO might titrate away AML1 co-factors, such as CBFβ, preventing AML1 from activating and repressing gene expression (dominant-negative model). In contrast to the predictions of the constitutive repressor model, negatively regulated targets would be de-repressed in the dominant-negative model. (C) A wild-type ommatidium contains eight photoreceptors (1-8; circles), four cone cells (c; ovals), eleven pigment cells (not shown) and three bristles (not shown). *lz* (blue), which expressed in photoreceptors 1, 6 and 7 and the cone cells, regulates the expression of *svp* (green), *Drosophila Pax2* (red) and *dpn* (yellow) as indicated.

organized in a regular array. Each ommatidium has eight photoreceptors (R1 to R8), four cone cells, eleven pigment cells and three interommatidial bristle cells (reviewed by Wolff and Ready, 1993). The eye forms from the eye imaginal disc, which is a monolayer epithelium. In the eye imaginal disc the morphogenetic furrow (MF) sweeps from the posterior to the anterior, orchestrating ommatidial development as it progresses. Posterior to the MF the first cell to differentiate is the R8 photoreceptor, which recruits additional cells to the forming ommatidium from a pool of undifferentiated cells. The ommatidia form in a step-wise manner: first, the photoreceptors join the ommatidium, followed by the cone cells and, lastly, the pigment cells and interommatidial bristles. *lz* is expressed in the undifferentiated precursors, photoreceptors R1, R6, R7, cone cells and pigment cells and is necessary for the differentiation of these cell types (Fig. 1C) (Daga et al., 1996; Batterham et al., 1996; Crew et al., 1997; Flores et al., 1998).

As a first step towards testing the dominant-negative model of AML1-ETO function, we analyzed the adult phenotypes resulting from the expression of AML1-ETO and other RD proteins (such as AML1 and Run) in the eye. We used two different Gal4 driver lines: *lz-Gal4* and *Glass Multimer Reporter* (*GMR*)-*Gal4*. *lz-Gal4* drives expression in cells that normally express *lz*, namely, the undifferentiated cells, R1, R6, R7 and cone and pigment cells, but is also expressed outside the eye. *GMR-Gal4* is more eye specific and expressed in all cells that are within and posterior to the morphogenetic furrow.

lz-Gal4 UAS-lz eyes appear wild type, demonstrating that elevating Lz levels in *lz*-expressing cells has no effect on eye development (Fig. 2B). By contrast, the expression of either *run* or *AML1* results in eyes that appear 'glazed', meaning that individual ommatidium are virtually impossible to discern (Fig. 2C,D). This suggests that *run* and *AML1* interfere with normal eye development and are not functionally equivalent to *lz*. However, *lz-Gal4 UAS-run* eyes are slightly more pigmented than the *lz-Gal4 UAS-AML1* eyes, suggesting that *run* and *AML1* may be functionally distinct from each other. Flies that express AML1-ETO via *lz-Gal4* die during pupal stages, probably owing to expression outside the eye (for example, *lz-Gal4* also drives expression in the hematopoietic system). However, in the eyes of *lz-Gal4 UAS-AML1-ETO* animals that survive to late pupal stages individual ommatidia cannot be observed and part of the eye is frequently covered by scar tissue (Fig. 2E). This phenotype is more severe than both the *lz-Gal4 UAS-AML1* and lz^{null} phenotypes. Owing to the pupal lethality of *lz-Gal4*; *UAS-AML1-ETO* flies, we also used *GMR-Gal4* to express these proteins in the fly eye. *GMR-Gal4 UAS-AML1-ETO* eyes are greatly reduced in size and no ommatidia are visible (Fig. 2K). This phenotype is distinct from that produced by the expression of the other *RD* genes using this driver. The difference in phenotype resulting from

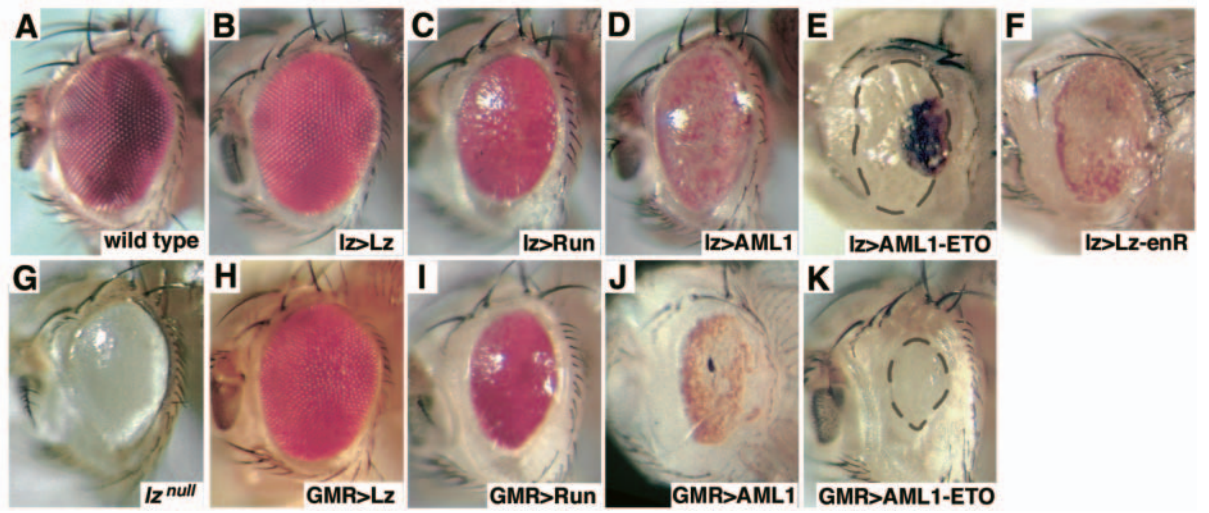

Fig. 2. Adult eye phenotypes resulting from altering *RD* gene expression. Photographs of adult eyes, except for E, F and J, which were dissected from unhatched pupae. For clarity, the boundary of eye tissue is outlined in the *GMR-Gal4 UAS-AML1-ETO* and *lz-Gal4 UAS-AML1-ETO* flies. Two different driver lines (*lz-Gal4*, B-F; *GMR-Gal4*, H-K) were used to express ectopically different fly and mammalian RD proteins. The eye color differences are due to the presence or absence of *white* (or a *mini-white* transgene), which is necessary for eye pigmentation. The relevant genotypes of the flies are as follows: (A) wild type; (B) *lz-Gal4*; *UAS-lz*; (C) *lz-Gal4*; *UAS-run*; (D) *lz-Gal4*; *UAS-AML1*; (E) *lz-Gal4; UAS-AML1-ETO*; (F) *lz-Gal4*; *UAS-lz-enR*; (G) lz^{null}; (H) *GMR-Gal4*; *UAS-lz*; (I) *GMR-Gal4*; *UAS-run*; (J) *GMR-Gal4*; *UAS-AML1*; (K) *GMR-Gal4 UAS-AML1-ETO*.

AML1 versus AML1-ETO is not due to variations in expression levels, as antibody stains show that these two factors are expressed at similar levels (data not shown). *GMR-Gal4 UAS-lz* eyes are mildly rough and *GMR-Gal4 UAS-run* eyes have the same 'glazed' appearance as *lz-Gal4 UAS-run* eyes (Fig. 2H,I). *GMR-Gal4 UAS-AML1* eyes are reduced in size, although more eye tissue is present than in *GMR-Gal4 UAS-AML1-ETO* eyes (Fig. 2J). Thus, the ectopic expression of AML1-ETO in the eye via *GMR-Gal4* results in a phenotype that is distinct from the phenotypes produced by other RD factors.

We also compared the affects of expressing AML1-ETO to lz^{null} eyes. As described above, *GMR-Gal4 UAS-AML1-ETO* eyes are very small (Fig. 2K). By contrast, lz^{null} eyes are wild-type in size, although the eyes have a 'glazed' appearance (Fig. 2G). In addition to comparing adult phenotypes, we also analyzed the expression of two markers of eye development in *GMR-Gal4 UAS-AML1-ETO* and lz^{null} eye discs. In lz^{null} eye discs, cone cells are transformed into photoreceptors (Daga et al., 1996). The transformed cone cells express *elav*, a pan-neural marker, and *seven-up* (*svp*)-*lacZ*, which is normally expressed in only a subset of photoreceptors (R3, R4, R1 and R6; Fig. 3B,D) (Daga et al., 1996). Using these markers, we did not find any evidence of a cone cell-to-photoreceptor transformation in *GMR-Gal4 UAS-AML1-ETO* eye discs (Fig. 3F). Instead, we found that *svp-lacZ* expression is decreased: in most of the ommatidia of *GMR-Gal4 UAS-AML1-ETO* eye discs, *svp-lacZ* is limited to R3 and R4. This is very different

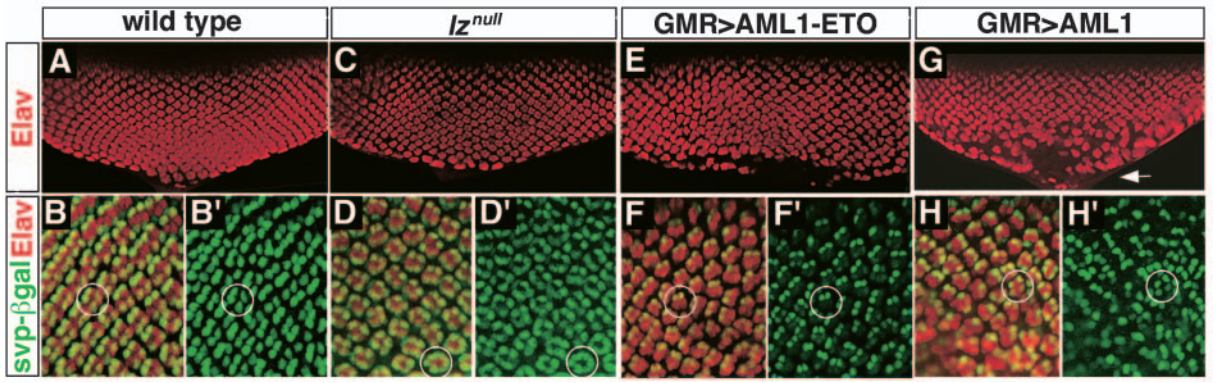

Fig. 3. *elav* and *svp-lacZ* expression in larval eye discs expressing RD proteins. Discs were stained for Elav (red) and β-gal (green). The ommatidia in the posterior of the *GMR-Gal4 UAS-AML1* eye disc (arrow in G) are more disorganized and degenerated than in *GMR-Gal4 UAS-AML1-ETO* discs (E). In the higher magnification photographs (B,D,F,H), a single ommatidium is circled. The eye discs were taken from larvae of the following genotypes: (A,B) wild type, four cells express *svp-lacZ* per ommatidium; (C,D) lz^{null}, more than four cells express *svp-lacZ* per ommatidium; (E,F) *GMR-Gal4 UAS-AML1-ETO*, the majority of ommatidia have only two *svp-lacZ*-expressing cells; (G,H) *GMR-Gal4 UAS-AML1*, many ommatidia have four cells that express *svp-lacZ*.

from the lz^{null} eye discs, where more cells express *svp-lacZ* than in wild type (compare Fig. 3D,F). We also examined *elav* and *svp-lacZ* expression in *GMR-Gal4 UAS-AML1* eye discs to determine if *GMR-Gal4 UAS-AML1-ETO* phenotypes are similar to *GMR-Gal4 UAS-AML1* phenotypes. In the posterior of the *GMR-Gal4 UAS-AML1* eye discs, the ommatidial clusters appear to degenerate (Fig. 3G). The amount of ommatidial degeneration is much greater in *GMR-Gal4 UAS-AML1* eye discs than in *GMR-Gal4 UAS-AML1-ETO* eye discs (Fig. 3E). This difference in the larval eye disc is striking, given that the adult *GMR-Gal4 UAS-AML1-ETO* eyes are smaller and have less eye tissue than *GMR-Gal4 UAS-AML1* eyes. In addition, despite the dramatic effect on photoreceptor differentiation, *svp-lacZ* expression is affected only in a subset of the ommatidia in *GMR-Gal4 UAS-AML1* eye discs (Fig. 3H). These results suggest that expressing AML1-ETO produces a phenotype that is different from both lz^{null} eyes and *GMR-Gal4 UAS-AML1* eyes, and therefore do not support the dominant-negative model of AML1-ETO function.

Additional *bro* or *bgb* does not suppress the ectopic *AML1-ETO* phenotype

The dominant-negative model posits that AML1-ETO interferes with the function of endogenous RD proteins such as AML1 by competing for a common co-factor. The best characterized co-factor for the RD family of transcription factors is CBFβ, which has two *Drosophila* homologs, Bro and Bgb. Consistent with the idea that these co-factors interact with Lz in vivo, removing one copy of *bgb* enhances a partial *lz* loss-of-function eye phenotype while additional Bgb (or Bro) suppresses this phenotype (Kaminker et al., 2001; Li and Gergen, 1999). For AML1-ETO, the dominant-negative model predicts that increasing Bro or Bgb levels should also suppress the *GMR-Gal4 UAS-AML1-ETO* phenotype. To test this, we took advantage of the fact that the *GMR-Gal4 UAS-AML1-ETO* phenotype is temperature-sensitive (Fig. 4D,E). However, contrary to the dominant-negative model, expressing Bro or Bgb increased the severity of the *GMR-Gal4 UAS-AML1-ETO* phenotype at 22°C (Fig. 4E,F; data not shown). The *GMR-Gal4 UAS-AML1-ETO UAS-bro* eyes were greatly reduced in size and resembled *GMR-Gal4 UAS-AML1-ETO* flies raised at a higher temperature (Fig. 4F,D). Expressing Bro or Bgb on their own had no discernable phenotypic consequences (Fig. 4B; data not shown). Consistent with these observations, we found that removing one copy of *bgb* partially suppressed the *GMR-Gal4 UAS-AML1-ETO* phenotype (Fig. 4G). Thus, the *GMR-Gal4 UAS-AML1-ETO* phenotype is sensitive to Bro and Bgb levels, but in the opposite direction predicted by the dominant-negative model.

We also found that the *GMR-Gal4 UAS-AML1-ETO* phenotype is sensitive to the dose of *lz*. Increasing Lz levels suppressed the *GMR-Gal4 UAS-AML-ETO* phenotype whereas decreasing Lz levels enhanced the phenotype (Fig. 4H; data not shown). These findings suggest that AML1-ETO and Lz may be competing for a common factor or binding site, a result that is consistent with both the dominant-negative and constitutive repressor models.

The ETO region of AML1-ETO is required for its activity

Another test of the dominant-negative model is to determine if

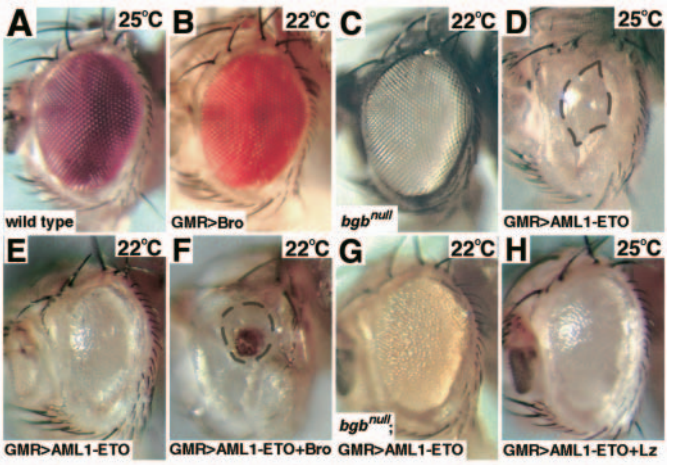

Fig. 4. The AML1-ETO-induced phenotype is modified by changes in *bro*, *bgb* and *lz* levels. Photographs of adult eyes raised at 22°C (B,C,E-G) and 25°C (A,D,H). The eye tissue of the *GMR-Gal4 UAS-AML1-ETO* (D) and *GMR-Gal4 UAS-AML1-ETO UAS-bro* (F) flies is outlined. The eye color differences are due to the presence or absence of *white* (or a *mini-white* transgene), which is necessary for eye pigmentation. The genotypes of the flies are as follows: (A) wild type; (B) *GMR-Gal4*; *UAS-bro*; (C) bgb^{null}/+; (D) *GMR-Gal4 UAS-AML1-ETO*; (E) *GMR-Gal4 UAS-AML1-ETO*; (F) *GMR-Gal4 UAS-AML1-ETO*; *UAS-bro*; (G) *GMR-Gal4 UAS-AML1-ETO*; bgb^{null}/+; (H) *GMR-Gal4 UAS-AML1-ETO*; *UAS-lz*.

an AML1-ETO truncation that still has the RD, and therefore retains the potential to interact with Bro and Bgb, produces a similar phenotype as the full-length protein. We truncated AML1-ETO C terminal to the RD, removing ETO completely (NLS-AML1ΔETO) (Fig. 1A). Similarly truncated AML1 proteins have been previously shown to maintain their ability to bind CBFβ, which interacts with residues present in the RD (Tahirov et al., 2001; Warren et al., 2000; Kim et al., 1999; Kanno et al., 1998). Because AML1-ETO is a constitutively nuclear protein, we also added an exogenous NLS to ensure that the truncated protein enters the nucleus (inset in Fig. 5D). We expressed *NLS-AML1ΔETO* using *lz-Gal4* so that its expression would be restricted to *lz*-expressing cells in the eye. *lz-Gal4 UAS-NLS-AML1ΔETO* animals hatch and their eyes appear wild type (Fig. 5C). This is in contrast to *lz-Gal4 UAS-AML1-ETO* animals, which die during pupation, and *GMR-Gal4 UAS-AML1-ETO* flies, which have very reduced eyes (Fig. 2K). The expression of two different markers of cell differentiation, *elav* and the cone cell marker *SME-lacZ* (a direct target of Lz, see below), is also wild type in *lz-Gal4 UAS-NLS-AML1ΔETO* eye discs (Fig. 5D). These results are consistent with the idea that the RD domain in AML1-ETO is not titrating away factor(s) that Lz requires to function and thus provides further evidence against the dominant-negative model. These results also demonstrate that the ETO region of the AML1-ETO chimera is necessary for the AML1-ETO-induced phenotypes.

AML1-ETO blocks the expression of an activated Lz target in cone cells

Because the results described above do not support the dominant-negative model of AML1-ETO function, we designed experiments to test the constitutive repressor model.

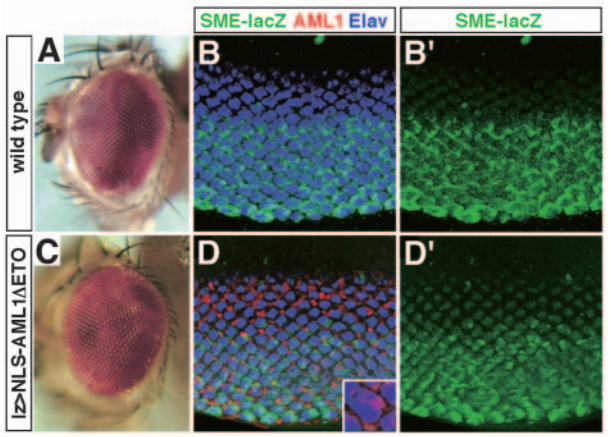

Fig. 5. The ETO region of AML1-ETO is required for activity. Larval eye discs are stained for AML1 (red), β-gal (green) and Elav (blue). (A,B) Wild-type eye (A) and larval eye disc (B), showing the normal expression pattern of *SME-lacZ*. anti-AML1 does not recognize any of the endogenous fly RD proteins. (C,D) *lz-Gal4 UAS-NLS-AML1ΔETO* eyes appear wild type (C) and expression of NLS-AML1ΔETO does not affect *SME-lacZ* expression or eye disc development (D). (D, inset) An ommatidium is enlarged to show that NLS-AML1ΔETO colocalizes with Elav in the nucleus.

This model proposes that AML1-ETO represses the expression of AML1 targets, regardless of whether AML1 normally activates or represses these genes. In the *Drosophila* eye, several *lz* targets have been identified. One of these is *Drosophila Pax2*, which is expressed in cone cells and is directly activated by Lz via the *SME* enhancer (Fu and Noll, 1997; Fu et al., 1998; Flores et al., 2000). We used the *SME-lacZ* reporter gene to monitor the effect of AML1-ETO on this Lz target in vivo (Flores et al., 2000). In addition, we followed the expression of *cut*, which is a Lz-independent marker for cone cells, to determine if cone cells can still differentiate when expressing AML1-ETO. There was a small reduction in the number of *cut*-expressing cone cells, suggesting that AML1-ETO has some potential to interfere with the differentiation of this cell type. However, in the many *cut*-expressing cone cells that remain, AML1-ETO repressed *SME-lacZ* expression (Fig. 6I,J). Although there was some residual *SME-lacZ* expression in larval eye discs, expression of this reporter gene is nearly abolished by pupal stages (Fig. 6K,L).

If AML1-ETO is a constitutive repressor, we would expect it to have similar properties to other RD proteins that are fused to a potent and constitutive repressor domain. To test this idea, we expressed a Lz protein that is fused to the *engrailed* repressor domain (Lz-enR, Fig. 1A). As with AML1-ETO, flies expressing *lz-enR* via *lz-Gal4* die during pupal stages. In pupae where eyes form, they have an eye phenotype that is similar to that produced by AML1-ETO (Fig. 2F). Moreover, like AML1-ETO, expression of Lz-enR inhibits *SME-lacZ* expression in cone cells (Fig. 6M-P). Thus, expressing Lz-enR results in a similar phenotype to expressing AML1-ETO that is distinct from the lz^{null} phenotype. Unlike lz^{null} eye discs, cone cells are present in both the *lz-Gal4 UAS-AML1-ETO* and *lz-Gal4 UAS-lz-enR* eye discs. Therefore, the loss of *SME-lacZ* expression is not due to the absence of this cell type.

AML1-ETO represses *deadpan*, a gene normally repressed by Lz

The results described above indicate that AML1-ETO is capable of inhibiting the expression of *Drosophila Pax2*, a gene that is directly activated by Lz. Our next question was whether expression of AML1-ETO also blocks the expression of targets that Lz negatively regulates. The dominant-negative and constitutive repressor models predict different outcomes for this experiment (Fig. 1B). If AML1-ETO is acting as a dominant-negative factor, a gene that Lz negatively regulates should be de-repressed. In contrast, if AML1-ETO is acting as a constitutive repressor this gene will remain repressed. *dpn* is directly and negatively regulated by Lz in cone cells (Canon and Banerjee, 2003). *dpn* is normally expressed in the R3/R4 photoreceptors just posterior to the furrow and is then transiently expressed in differentiating R7 photoreceptors (Fig. 7A; see Fig. 1C for a summary of its

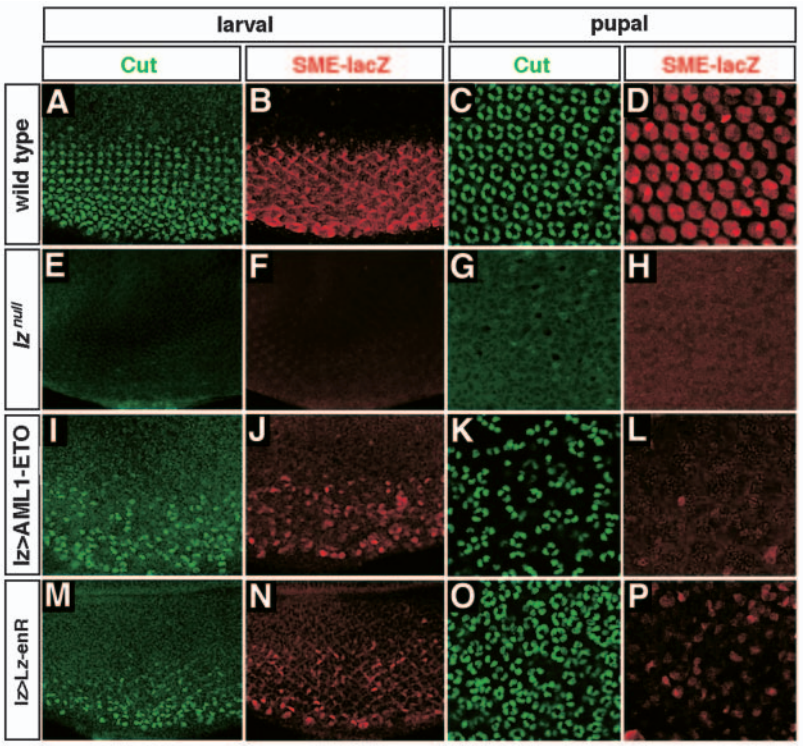

Fig. 6. Expression of AML1-ETO and Lz-enR inhibits *SME-lacZ* expression in larval and pupal eye discs. The eye discs are stained for Cut (green) and β-gal (red). Larval eye discs are shown in A,B,E,F,I,J,M,N and pupal eye discs are shown in C,D,G,H,K,L,O,P. (A-D) In wild type, *SME-lacZ* is expressed in cone cells, which also express *cut*, during larval and pupal stages (the green and red channels are separated). (E-H) *SME-lacZ* is directly activated by Lz, and is therefore not expressed in lz^{null} eye discs, which also do not have cone cells. (I-L) *SME-lacZ* levels are significantly reduced in *lz-Gal4 UAS-AML1-ETO* eye discs. This effect is more obvious at pupal stages (K,L). Cut expression remains. (M-P) Ectopic expression of Lz-enR also inhibits *SME-lacZ* expression (*lz-Gal4 UAS-lz-enR*). Cut expression remains.

expression pattern) (Canon and Banerjee, 2003). In lz^{null} eye discs *dpn* is expressed normally in R3, R4 and R7 but is also expressed in the transformed cone cells (Fig. 7B) (Canon and Banerjee, 2003). In *GMR-Gal4 UAS-AML1-ETO* eye discs, *dpn* expression in R7 is virtually abolished and there is no ectopic expression in other cells (Fig. 7C). *dpn* expression in R7 is also repressed in *GMR-Gal4 UAS-lz-enR* eye discs, similar to *GMR-Gal4 UAS-AML1-ETO* eye discs (Fig. 7D). The expression of *dpn* in R3/R4 is not affected, despite the fact that *GMR-Gal4* is active in these cells. One explanation for this observation is that *bgb* is not expressed in R3/R4 (although it is not known if Bro is expressed in these cells) (Kaminker et al., 2001). The repression of *dpn* by AML1-ETO is inconsistent with a model in which AML1-ETO inhibits Lz activity. Instead, these results provide further support for the idea that AML1-ETO is a constitutive transcriptional repressor.

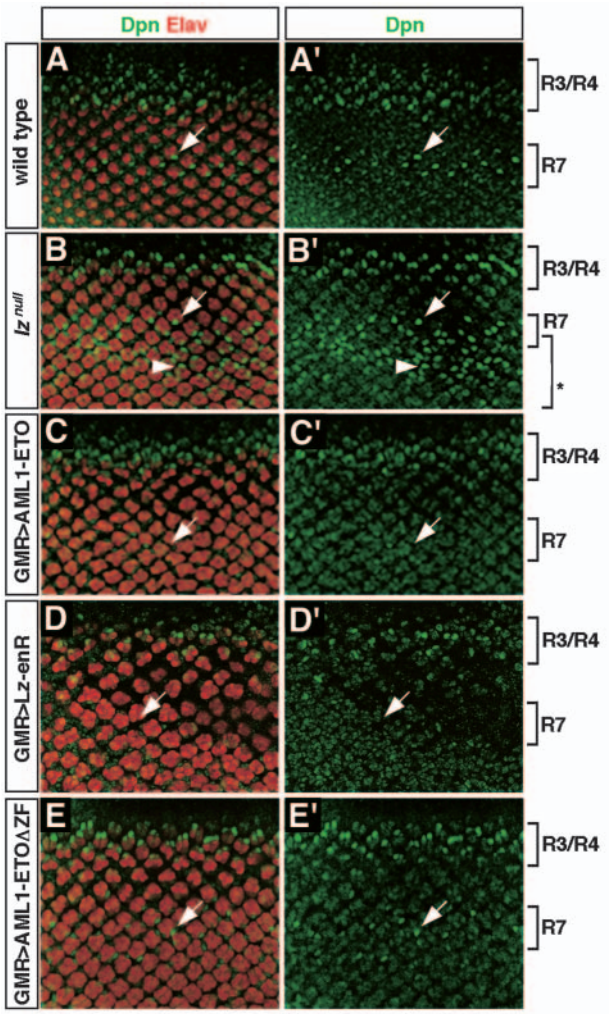

Fig. 7. Effects of AML1-ETO and AML1-ETOΔZF expression on *dpn*. Discs were stained for Elav (red) and Dpn (green). The identity of the *dpn*-expressing photoreceptors is indicated to the right of the panels and an arrow indicates an individual R7 cell in each photograph. The asterisk next to B indicates cone cells differentiating as photoreceptors in lz^{null} eye discs. (A) *dpn* is normally expressed in differentiating R3, R4 and R7 photoreceptors (see also Fig. 1C). (B) In lz^{null} eye discs, *dpn* is expressed in the transformed cone cells (arrowhead). (C) In *GMR-Gal4 UAS-AML1-ETO* eye discs, *dpn* is repressed in R7 (although an occasional R7 cell weakly expresses *dpn*). No ectopic Dpn expression is observed. (D) *dpn* is not expressed in R7 in *GMR-Gal4 UAS-lz-enR* eye discs. (E) In *GMR-Gal4 UAS-AML1-ETOΔZF* eye discs there are more *dpn*-expressing R7 cells than in *GMR-Gal4 UAS-AML1-ETO* eye discs, suggesting that the zinc-finger domain is required for complete *dpn* repression in this assay.

In the constitutive repressor model, the ETO region of AML1-ETO recruits transcriptional co-repressors (Hug and Lazar, 2004). Although multiple regions within the ETO C terminus can interact with transcriptional repressors, the zinc-finger domain is necessary to mediate repression in cell culture assays (Lutterbach et al., 1998a; Wang et al., 2004). Based on these results, we tested whether the zinc-finger domain is also crucial for AML1-ETO function in vivo in *Drosophila* by expressing an AML1-ETO protein that lacks the zinc-finger domain (AML1-ETOΔZF). Expression of AML1-ETOΔZF using *GMR-Gal4* produced an adult eye phenotype that was intermediate between the AML1 and AML1-ETO phenotypes (Fig. 2J,K; data not shown). Furthermore, expression of this protein had an inconsistent effect on *dpn* expression in R7 (Fig. 7E). There were some R7 cells that expressed *dpn* and some that did not. No ectopic *dpn* expression was observed in these eye discs. However, expression of AML1-ETOΔZF repressed *SME-lacZ*, similar to full-length AML1-ETO, although AML1-ETOΔZF showed less inhibition of cone cell differentiation (monitored by *cut* staining) than did AML1-ETO (data not shown). These results suggest that the ETO zinc-finger domain plays a role in AML1-ETO function in vivo, but for the repression of some target genes this domain appears not to be required and may be redundant with other ETO domains.

Discussion

Drosophila as a model system to study AML1-ETO function

AML1-ETO has been widely studied using both cell culture and animal models. Several *AML1-ETO* knock-in murine models now exist, including an inducible knock-in that bypasses the embryonic lethality observed in other *AML1-ETO* knock-in mice (Yergeau et al., 1997; Okuda et al., 1998; Rhoades et al., 2000; Yuan et al., 2001; Higuchi et al., 2002). Surprisingly, these knock-in murine models indicate that *AML1-ETO* alone is not sufficient to cause leukemia. The *AML1-ETO*-expressing mice develop leukemia only after additional mutations are induced (Yuan et al., 2001; Higuchi et al., 2002). The need to induce secondary mutations to trigger the disease state could complicate the use of mice as a model organism to study how *AML1-ETO* acts at a mechanistic level. Complementary animal models to investigate *AML1-ETO* function in vivo may provide additional insights into the activity of this oncogene. For example, *AML1-ETO* has recently been expressed in zebrafish embryos, resulting in abnormal hematopoiesis (Kalev-Zylinska et al., 2002). Here, we describe the phenotypes resulting from the expression of *AML1-ETO* in flies, establishing another animal model in which to study *AML1-ETO* function. *Drosophila* is a particularly attractive model organism, given the relative ease of performing large-scale genetic screens in flies. In support of this idea, we show here that the *AML1-ETO* eye phenotype is sensitive to changes in the levels of *bro* and *bgb*. Thus, it should be possible to perform a modifier screen to identify additional genes that interact with *AML1-ETO*. In

fact, we have already performed an initial screen and identified several genomic regions that show a genetic interaction with *AML1-ETO* (J.W. and R.S.M., unpublished).

AML1-ETO as a constitutive transcriptional repressor

We used the *Drosophila* eye to investigate two different models of *AML1-ETO* function. Although previous studies showed that AML1-ETO interferes with endogenous AML1 activity, it was unclear how AML1-ETO might act in vivo. AML1-ETO contains the AML1 RD, which interacts with DNA and co-factors such as CBFβ. Thus, one plausible model is that AML1-ETO titrates CBFβ away from AML1, inhibiting AML1 from acting effectively. CBFβ is crucial for AML1 activity, as demonstrated by the fact that *Cbfb*-null mice phenocopy *AML1* mutants (Wang et al., 1996b; Sasaki et al., 1996; Niki et al., 1997). In addition, AML1-ETO has been shown to compete with AML1 for CBFβ (Meyers et al., 1995; Tanaka et al., 1998). In flies, the CBFβ homologs *bro* and *bgb* are required for RD function (Li and Gergen, 1999; Kaminker et al., 2001). However, in contrast to the prediction of a dominant-negative model, we found that supplying higher levels of Bro (or Bgb) increased the severity of the AML1-ETO phenotype instead of suppressing it. This result suggests that AML1-ETO uses these co-factors to generate the observed phenotypes and that supplying additional Bro or Bgb results in a higher concentration of functional AML1-ETO/co-factor complexes. In an analogous manner, reducing the dose of Bgb enhances a *lz* hypomorphic phenotype (Kaminker et al., 2001), consistent with the idea that Lz uses this co-factor and that reducing its concentration results in lower amounts of functional Lz/co-factor complexes. In both cases, changes in Bgb or Bro levels only show an effect when AML1-ETO or Lz are present in limiting amounts (lz^{ts} and *GMR-Gal4; UAS-AML1-ETO* at 22°C); changing the levels of these co-factors does not produce a visible phenotype in an otherwise wild-type background. Similarly, we found that expression of an AML1-ETO truncation that still contains the Bro- and Bgb-interaction domain had no effect on eye development in an otherwise wild-type background. Thus, it appears that these co-factors are not normally present in limiting amounts, but become limiting when their partners (e.g. AML1-ETO or Lz) are present at low levels. Taken together, these results suggest that AML1-ETO does not compete with endogenous RD factors for these co-factors and provide evidence against a dominant-negative model of AML1-ETO function.

By contrast, our results, in particular showing that AML1-ETO represses genes that are directly activated (*Drosophila Pax2*) or directly repressed (*dpn*) by Lz, support the idea that AML1-ETO behaves as a constitutive repressor. These results are also consistent with previous findings showing that AML1-ETO represses gene expression (Meyers et al., 1995; Lutterbach et al., 1998a). Although AML1 functions as a transcriptional activator and repressor, neither the AML1 transactivation domain nor repressor domain are present in AML1-ETO. Instead, the RD is fused to nearly the entire ETO protein, which is capable of recruiting several co-repressors through multiple domains (Peterson and Zhang, 2004; Hug and Lazar, 2004). In our experiments, we propose that AML1-ETO binds to Lz-binding sites via its RD and represses the expression of Lz target genes, regardless of whether these genes are normally activated or repressed by Lz. By extension, we suggest that AML1-ETO acts similarly to repress AML1 target genes when expressed in humans. Although there are a few reports suggesting that AML1-ETO activates transcription, it is unclear if this regulation is direct. Furthermore, for at least one of these activated targets (*bcl2*), there is conflicting evidence whether AML1-ETO causes an in increase in gene expression (Klampfer et al., 1996; Banker et al., 1998; Shikami et al., 1999; Burel et al., 2001). In sum, our results support the idea that AML1-ETO is a constitutive transcriptional repressor of AML1 targets and fit with a large body of evidence showing that AML1-ETO represses transcription in a RD binding site-dependent manner (Peterson and Zhang, 2004).

Recently, AML1-ETO was also shown to affect transcription by interacting with the basic helix-loop-helix factor called HeLa E-box binding factor (HEB) (Zhang et al., 2004). In these experiments, AML1-ETO and ETO were shown to block the transactivation activity of HEB in cell culture assays by interfering with the ability of HEB to recruit CBP/p300. For AML1 target genes that are activated by E proteins, the mechanism defined by these experiments may be one way in which AML1-ETO causes transcriptional repression. In addition, inhibition of E protein activity may represent another mechanism by which AML1-ETO carries out its leukemogenic functions. As our experiments specifically examined the regulation of previously characterized *lz* target genes, for which it is not known if there is an E protein input, we cannot at present distinguish between these two possibilities. However, we emphasize that these mechanisms are not mutually exclusive and that both may be operating in vivo.

Our results also tested if the zinc fingers are necessary for AML1-ETO to inhibit gene expression. When compared with AML1-ETO, we found that AML1-ETOΔZF is slightly less potent at repressing *dpn* expression but is able to repress *SME-lacZ* equally well. These results suggest that the zinc fingers may be more important for repressing some target genes than others and that this domain might be functionally redundant with other parts of the protein. This is not surprising, as multiple transcriptional repressor complexes can interact with different regions of ETO (Davis et al., 2003). Although the zinc fingers mediate an interaction with N-CoR/SMRT, the amino acids surrounding NHR2, for example, are capable of recruiting HDAC-1, HDAC-3 and Sin3a (Lutterbach et al., 1998a; Lutterbach et al., 1998b; Gelmetti et al., 1998; Wang et al., 1998; Amann et al., 2001; Hildebrand et al., 2001). Furthermore, an attempt to define a single region of ETO that disrupts its function in vivo was unsuccessful (Cao et al., 2002). Thus, although the zinc-finger domain is highly conserved, blocking its function may only interfere with the repression of a small subset of AML1-ETO target genes.

In conclusion, these data provide strong support for a model in which AML1-ETO is a constitutive transcriptional repressor rather than a factor that dominantly interferes with the activity of endogenous RD protein function. One implication from these findings is that AML might be caused by the repression of genes that AML1 normally activates, rather than a reduction of normal AML1 activity. Accordingly, we suggest that a deeper understanding of how AML1-ETO contributes to AML will require the identification of genes that are normally activated by AML1.

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