# Lozenge directly activates *argos* and *klumpfuss* to regulate programmed cell death

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**We show that reducing the activity of the *Drosophila* Runx protein Lozenge (Lz) during pupal development causes a decrease in cell death in the eye. We identified Lz-binding sites in introns of *argos* (*aos*) and *klumpfuss* (*klu*) and demonstrate that these genes are directly activated targets of Lz. Loss of either *aos* or *klu* reduces cell death, suggesting that Lz promotes apoptosis at least in part by regulating *aos* and *klu*. These results provide novel insights into the control of programmed cell death (PCD) by Lz during *Drosophila* eye development.**

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Members of the Runx family of transcription factors are critical for multiple aspects of development, including cell differentiation, proliferation, neurogenesis, and hematopoiesis, and mutations in *Runx1* in humans are frequently associated with leukemia (Coffman 2003). The Runx DNA-binding domain (the Runt Domain, RD) is highly conserved throughout the animal kingdom, and the *Drosophila* RD factor Runt (Run) binds similar sequences as its mammalian homologs (Pepling and Gergen 1995). Moreover, the *Drosophila Runx* homologs *run* and *lozenge* (*lz*) play analogous roles in development as their mammalian counterparts, indicating that Runx function is also conserved (Canon and Banerjee 2000). There also is evidence that *Runx3* may regulate apoptosis in the murine gastric epithelium, suggesting that *Runx3* might function as a tumor suppressor by regulating programmed cell death (PCD) (Li et al. 2002).

The *Drosophila Runx* gene *lz* is expressed in the eye, an accessible model tissue that is frequently used to study gene function. The fly eye is comprised of ~800 ommatidia, each of which is a light-sensing unit composed of eight photoreceptors, four lens-secreting cone cells, 11 pigment cells, and three bristle cells (Wolff and Ready 1993). Lz controls the differentiation of a subset of ommatidial cell types, namely photoreceptors R1, R6, R7, and the cone and pigment cells (Daga et al. 1996; Crew et al. 1997). Although the role of *lz* in promoting cell differentiation during larval stages has been explored in detail, the expression of *lz* during pupal stages suggests that it may have additional functions in eye development.

To test for a novel function of *lz*, we used a temperature-sensitive allele of *lz* ($lz^{ts}$) to reduce Lz activity during pupal stages of eye development. Our results suggest that *lz* is required for PCD that normally occurs at this stage. In addition, we used an in silico approach to discover novel Lz-regulated enhancers that control the expression of two genes, *aos* and *klu*, which have been implicated in PCD in the eye. DNA-binding experiments and mutagenesis of these enhancers suggest that *aos* and *klu* are direct Lz targets in cone and pigment cells. Based on these results, we propose a model for Lz-mediated regulation of cell death in the eye.

## Results and Discussion

### lz *is required for cell death in the pupal retina*

*lz* is expressed in the *Drosophila* pupal eye during PCD, suggesting that *lz* might have a role in this process (Flores et al. 1998). To determine if *lz* has any effect on apoptosis, we used a $lz^{ts}$ allele to reduce Lz activity during PCD, which is later and distinct from the time when Lz promotes cell differentiation and survival in the larval eye disc (Fig. 1A; Daga et al. 1996; Crew et al. 1997; Siddall et al. 2003). Photoreceptor, cone, and 1° pigment cell (1° cell) differentiation is complete by ~20 h after pupal formation (APF). Excess presumptive 2° and 3° pigment cells (2° and 3° cells, respectively) die between 25 and 35 h APF, and the ommatidia have their full complement of differentiated cells at 42 h APF. We shifted $lz^{ts}$ flies to the nonpermissive temperature at 20 h APF and then examined retinas 42 h APF. In temperature-shifted $lz^{ts}$ pupal retinas, there was an increased number of 2° and 3° cells, indicating a decrease in cell death (Fig. 1F,G; 2° and 3° cell number is normal in $lz^{ts}$ flies raised at 25°C; data not shown). On average, there were an additional two cells/ommatidium ($n = 53$), which is similar to the number of cells that normally die (two to three cells/ommatidium) (Wolff and Ready 1991). Fifty-five-hour-APF retinas also have additional 2° and 3° cells, indicating that PCD is not delayed (data not shown). Only 2° and 3° cell death is affected; perimeter cell death, which occurs 42 h APF (Lin et al. 2004), is normal in temperature-shifted $lz^{ts}$ animals (data not shown). dUTP nick-end labeling (TUNEL) and acridine orange staining, two methods to label dying cells, demonstrated that there is a reduction in apoptosis in temperature-shifted $lz^{ts}$ retinas (Fig. 1I; data not shown). Thus, the extra cells in $lz^{ts}$ retinas probably result from a decrease in cell death. Moreover, these cells appear to differentiate as 2° and 3° cells because they express *homothorax* (*hth*), a marker for these cell types (Fig. 1K; Wernet et al. 2003). In addition, the eyes of temperature-shifted $lz^{ts}$ flies have wild-type pigmentation, indicating that the 2° and 3° cells have differentiated normally (Fig. 1H).

*hid*, *grim*, and *rpr* encode the principal pro-apoptotic factors in *Drosophila* (Bergmann et al. 2003). In the eye,

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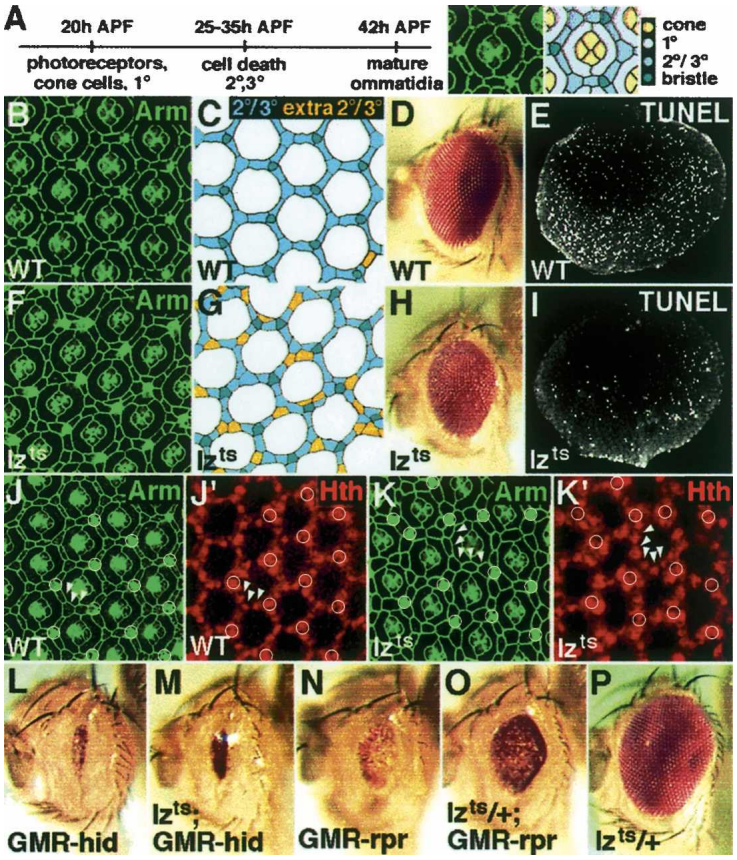


**Figure 1.** Lz promotes pupal cell death. Anti-armadillo (Arm; green) marks the cell boundaries in 42-h-APF pupal retinas. The 2°, 3°, and bristle cells in *B* and *F* were traced and are shown to the *right* of the corresponding photograph (*C* and *G*, respectively). The cells in the trace are color-coded: bristle cells are dark green, 2° and 3° cells are blue, and extra 2° and 3° cells are orange; posterior is to the *left*. (*L–P*) Adult eyes of the indicated genotypes. (*L*,*M*) Male. (*N–P*) Female. For each genotype, the phenotype is consistent between individuals. (*A*, *left*) Timeline of pupal eye development. By 20 h APF the photoreceptors, cone, and 1° cells have differentiated. 2° and 3° cells differentiate shortly after PCD. (*Right*) One ommatidium from a 42-h-APF retina and corresponding trace with color-coded cells to show the relative location of each cell type. The ommatidia share three 3° cells at the apices of the ommatidial hexagon and six 2° cells between the bristle and 3° cells. (*B*,*C*) Wild-type (WT) pupal retina, in which a rare extra 2°/3° cell can be seen in the *lower right* corner. (*D*) Wild-type (WT) eye. (*E*) TUNEL (white) identified dying cells in a whole-mounted 32-h-APF wild-type (WT) retina. (*F*,*G*) Temperature-shifted $lz^{ts}$ retina. (*H*) Temperature-shifted $lz^{ts}$ adult eyes are only mildly rough and have wild-type pigmentation. (*I*) TUNEL (white) stain of a whole-mounted 32-h-APF temperature-shifted $lz^{ts}$ pupal retina. (*J*,*K*) Separated channels of wild-type (WT) (*J*) and temperature-shifted $lz^{ts}$ (*K*) retinas showing the expression of Hth (red) in the 2° and 3° (arrowheads) and bristle sensory organ, which is comprised of four cells (circle). Apically, Arm marks the cell boundaries, and basally, Hth marks nuclei. (*J*) There are no bristle cells in the *upper left* corner because this is a part of the rim of the eye. (*L*) *GMR–hid*. (*M*) Temperature-shifted $lz^{ts}$; *GMR–hid*. (*N*) *GMR–rpr*. (*O*) Temperature-shifted $lz^{ts}$/+; *GMR–rpr*. (*P*) Temperature-shifted $lz^{ts}$/+.

ectopic expression of *hid* or *rpr* under control of the *glass multimer reporter* (*GMR*) reduced the overall size and pigmentation of the eye (Fig. 1L,N). *GMR–hid*- and *GMR–rpr*-induced eye phenotypes are sensitive to loss- and gain-of-function mutations in genes that affect PCD (Bergmann et al. 1998; Kurada and White 1998). We found that reducing Lz activity using the same temperature-shift protocol described above partially suppresses both the *GMR–hid* and *GMR–rpr* adult phenotypes (Fig. 1M,O). The level of suppression is similar to the effect of mutations that stimulate Epidermal Growth Factor (EGF) signaling, which antagonizes cell death by suppressing *hid* function (Bergmann et al. 1998; Kurada and White 1998). These results suggest that a reduction of Lz activity causes a decrease in cell death and that Lz modulates *hid*- and *rpr*-mediated cell death.

### *Computer-based search for Lz-regulated genes controlling apoptosis*

As a transcription factor Lz most likely affects cell death by regulating the expression of gene(s) that have pro- or anti-apoptotic functions. We performed a search for Lz-binding sites in the *Drosophila* genome to identify genes that Lz might regulate to control cell death. Previous work has shown that Lz regulates gene expression in combination with cofactors (Flores et al. 2000; Xu et al. 2000; Canon and Banerjee 2003). That Lz works with the Ets transcription factor Pointed (Pnt) to regulate both *prospero* (*pros*) and *D-Pax2* is especially striking given that Runx1 also interacts with Ets factors to regulate transcription (Flores et al. 2000; Wheeler et al. 2000; Xu et al. 2000). Moreover, Pnt is an effector of EGFR signaling, which influences cell death in the *Drosophila* eye (Bergmann et al. 1998; Kurada and White 1998). Based on these observations, we used the binding site search program Target Explorer to search for clusters of RD- and Ets-binding sites in the *Drosophila* genome (Sosinsky et al. 2003; see Materials and Methods). In brief, we created positional weight matrices representing RD- and Ets-binding sites (Supplementary Table 1) and searched for clusters of these sites near genes annotated by FlyBase to be expressed or have activity in the eye (so-called eye genes). Using these criteria, we found >500 RD/Ets clusters. Next, we used the cluster score (Sosinsky et al. 2003) to rank the putative enhancers and selected a cut-off score (see Materials and Methods; Supplementary Fig. 1). We found 135 clusters in 112 eye genes that were above this score, including clusters that correspond to the previously identified *D-Pax2* and *pros* enhancers (Supplementary Table 2).

From this list of high-scoring clusters, we selected 16 putative enhancers located in 11 eye genes that have pro- or anti-apoptotic functions (Table 1). When available, we used antibodies and *lacZ* reporter lines to determine if the expression of these genes changed in temperature-shifted $lz^{ts}$ pupal retinas. We cloned the remaining putative enhancers into a *lacZ* reporter gene vector and assayed for *lacZ* expression in vivo. We found that two enhancers located in two genes, *aos* and *klu*, drove expression of *lacZ* in the pupal retina, suggesting that these genes are Lz targets (Table 1).

### *The* aos *and* klu *enhancer expression patterns*

We cloned a 923-bp fragment of the first *aos* intron containing a cluster of 8 RD sites and two Ets sites to generate $aos^{923}$*–lacZ* (Fig. 2A). In larval eye discs $aos^{923}$*–lacZ* is expressed in a subset of the cell types that express

**Table 1.** *High-scoring clusters located in eye genes implicated in cell death*

| Putative target | Cluster score | Change in $lz^{ts}$ [a] | DNA size (kb) | Reporter expression (pupal retina) |
|---|---|---|---|---|
| *reaper* | 3.74 | N.D.[b] | 1.476 | None detected |
| | 3.57 | | 1.088 | None detected[c] |
| *roughest* | 3.73 | No | — | |
| | 3.31 | | — | |
| *hid* | 3.71 | No | — | |
| *Notch* | 3.71 | No | — | |
| *klu* | 3.71 | Yes | 0.560 | R1 R6 R7, cone, 1°, 2°, 3° |
| | 3.50 | | 1.145 | None detected |
| *Damm* | 3.52 | N.D. | 0.912 | None detected |
| *peanut* | 3.47 | N.D.[b] | — | |
| | 3.40 | | — | |
| *sickle* | 3.40 | No | — | |
| *aos* | 3.38 | N.D. | 1.036 | None detected |
| | 3.35 | | 0.923 | Cone, 2°, 3° |
| *pointed* | 3.38 | N.D. | 0.424 | None detected |
| *Delta* | 3.30 | No | — | |

(N.D.) not determined; (—) not cloned.
[a]Monitored by antibody stain or *lac-Z* reporter.
[b]Neither *rpr-11-lacZ* (Nordstrom et al. 1996) nor Peanut is expressed in wild-type retinas (data not shown).
[c]$rpr^{1088}$*-lacZ* is expressed in a subset of glia in the CNS (data not shown).

an *aos lacZ* enhancer trap, $aos^{w11}$*–lacZ*, indicating that this enhancer is responsible for a subset of the *aos* expression pattern (Fig. 2C). The expression of $aos^{w11}$*–lacZ* in photoreceptors is unaffected in $lz^{null}$ larval eye discs, indicating that Lz does not regulate *aos* during early stages of eye development (data not shown). During pupal stages, $aos^{923}$*–lacZ* is expressed in cone, 2°, 3°, and bristle cells (Fig. 2D). The coexpression of $aos^{923}$*–lacZ* and *lz* suggests that *aos* may be directly activated by Lz in cone and pigment cells. However, $aos^{923}$*–lacZ* is also expressed outside the eye, where Lz is not present (data not shown). To focus on the Lz-dependent portion of the enhancer we made a shorter construct ($aos^{292}$*–lacZ*) that spans the first and last RD-binding sites (Fig. 2A). $aos^{292}$*–lacZ* expression is restricted to cone and pigment cells in the eye and *lz*-positive cells in the antenna and leg disc (Fig. 2E; data not shown). Thus, $aos^{292}$ is an *aos* enhancer whose activity is largely limited to *lz*-expressing cells.

A 560-bp piece of the first *klu* intron contains a cluster of three RD sites and two Ets sites ($klu^{560}$*–lacZ*) (Fig. 2B). During pupal stages, $klu^{560}$*–lacZ* is expressed in cone, 1°, 2°, and 3° cells (Fig. 2G). The overlapping expression patterns of $klu^{560}$*–lacZ* and *lz* suggest that *klu* is also likely to be activated by Lz. A truncated $klu^{560}$*–lacZ* construct ($klu^{363}$*–lacZ*) that contains only the sequence flanked by the first and last RD sites is expressed in a same pattern as $klu^{560}$*–lacZ* (Fig. 2H).

### $aos^{292}$–lacZ *and* $klu^{363}$–lacZ *are directly activated by Lz*

We tested the idea that Lz directly regulates *aos* and *klu* by examining the expression of $aos^{292}$*–lacZ* and $klu^{363}$*–lacZ* in three different genetic backgrounds. First, neither $aos^{292}$*–lacZ* nor $klu^{363}$*–lacZ* was expressed in $lz^{null}$ pupal retinas (Fig. 3B,F). Second, we expressed a protein that has the Engrailed repressor domain fused to Lz (Lz–enR), which is predicted to repress Lz target genes. Using *lz-Gal4*, we expressed Lz–enR in the eye and found that $aos^{292}$*–lacZ* and $klu^{363}$*–lacZ* expression was abolished (Fig. 3C,G). However, cell differentiation was also affected in both the $lz^{null}$ and *lz-G4 UAS-lz-enR* eyes, raising the possibility that the lack of expression could be due to a change in cell fate. Therefore, we used the $lz^{ts}$ allele to reduce *lz* activity after ommatidial cells have differentiated. Neither $aos^{292}$*–lacZ* nor $klu^{363}$*–lacZ* was expressed in $lz^{ts}$ retinas shifted to the nonpermissive temperature at 20 h APF (Fig. 3D,H). These results suggest that *aos* and *klu* are positive targets of *lz* regulation in cone and pigment cells.

To test if Lz directly regulates $aos^{292}$*–lacZ* and $klu^{363}$*–lacZ*, we performed electrophoretic mobility shift assays (EMSAs) using oligos containing RD-binding sites found in each enhancer. We tested five of the eight RD-binding sites in $aos^{292}$ and found that Lz bound to each oligo probe (Fig. 3I,J; data not shown). Lz did not, however, bind to probes that have mutated RD sites, demonstrating that Lz binding is specific (Fig. 3I). Similar experiments showed that Lz binds each of the three RD sites present in $klu^{363}$ (Fig. 3J; data not shown).

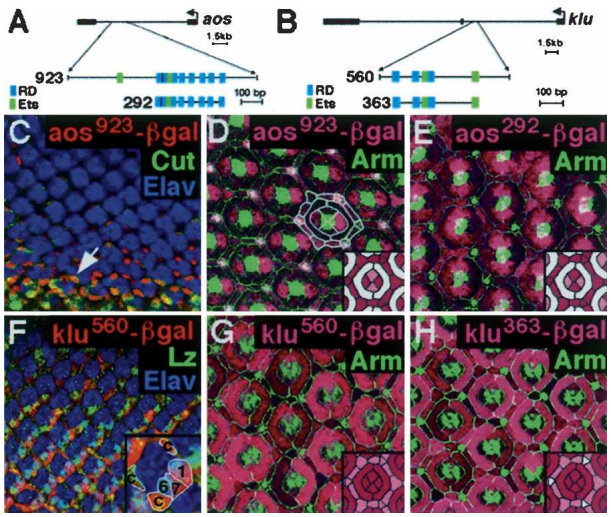


**Figure 2.** Activity of *aos* and *klu* enhancers in the eye. Eye tissue stained for anti-β-gal (red, pink) and Elav (blue). (*C*,*F*) The morphogenetic furrow is located at the *top* of the panel. (*D*,*E*,*G*,*H*) The cartoons at the *bottom right* schematize the β-gal expression patterns. (Dark pink) Strong expression; (light pink) weaker expression (*D*,*E*), or cells with variable expression (*G*,*H*); (white) no expression. (*A*) The $aos^{923}$ enhancer. (*B*) The $klu^{560}$ enhancer. (*C*) During larval stages, $aos^{923}$*–lacZ* is expressed in Cut-positive (green) cone cells (arrow). (*D*,*E*) Forty-two-hour-APF retinas stained with anti-Arm (green). (*D*) $aos^{923}$*–lacZ* is expressed in the cone, 2°, 3°, and bristle cells (the narrow gap in staining between the cone cells and the 2°/3° cells is the 1° cell). The cell boundaries of a single ommatidium are outlined. (*E*) $aos^{292}$*–lacZ* is expressed in cone, 2°, 3°, and bristle cells. $aos^{923}$*–lacZ* and $aos^{292}$*–lacZ* are expressed in the same cell types, although at different expression levels. (*F*) $klu^{560}$*–lacZ* is initially expressed in a subset of undifferentiated cells (not visible in this confocal plane). $klu^{560}$*–lacZ* is coexpressed with *lz* (green) in photoreceptors R1, R6, and R7 and cone cells (a single ommatidium is enlarged to better show the expression of $klu^{560}$*–lacZ*). (*G*,*H*) Forty-two-hour-APF retinas stained with anti-Arm (green). (*G*) $klu^{560}$*–lacZ* is expressed in cone, 1°, 2°, 3°, and bristle cells. (*H*) $klu^{363}$*–lacZ* is expressed in cone, 1°, 2°, and 3° cells.

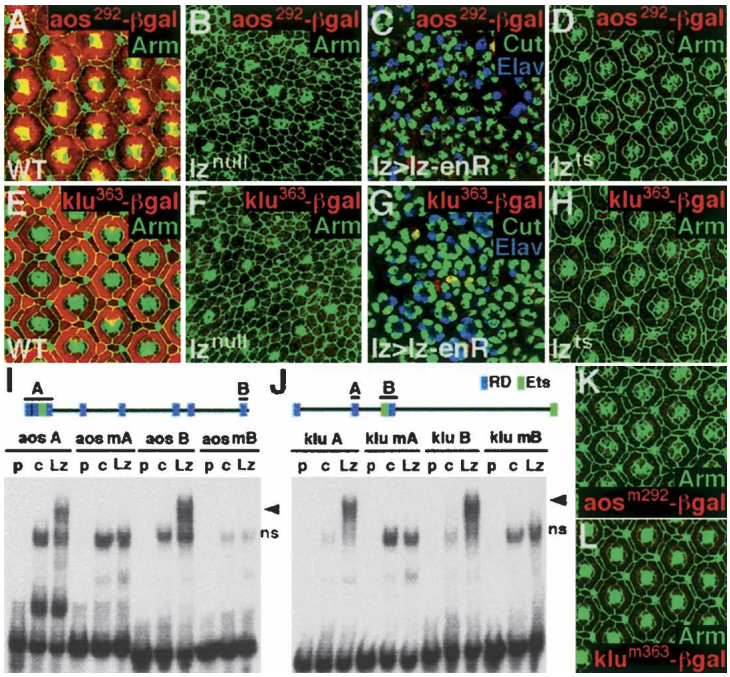


**Figure 3.** Lz directly activates *aos*$^{292}$–*lacZ* and *klu*$^{363}$–*lacZ*. Arm (green) delineates cell boundaries (*A*,*B*,*D*,*E*,*F*,*H*,*K*,*L*) and Cut (green) marks cone cell nuclei (*C*,*G*). The retinas were stained with anti-β-gal (red) and are 42 h APF unless otherwise noted. (*A*,*E*) Wild-type expression patterns of *aos*$^{292}$–*lacZ* (*A*) and *klu*$^{363}$–*lacZ* (*E*). (*B*,*F*) The expression of *aos*$^{292}$–*lacZ* (*B*) and *klu*$^{363}$–*lacZ* (*F*) is abolished in *lz*$^{null}$ pupal retinas. (*C*,*G*) In *lz-Gal4 UAS-lz-enR* retinas the expression of *aos*$^{292}$–*lacZ* (*C*) and *klu*$^{363}$–*lacZ* (*G*) is absent (Elav in blue marks the photoreceptors; retinas are <42 h APF). (*D*,*H*) Temperature-shifted *lz*$^{ts}$ retinas do not express *aos*$^{292}$–*lacZ* (*D*) and *klu*$^{363}$–*lacZ* (*H*). (*I*,*J*) EMSAs with radiolabeled oligonucleotide probes containing wild-type or mutant RD sites from the *aos*$^{292}$ and the *klu*$^{363}$ enhancers. (p) Probe alone, (c) control lysate, (Lz) Lz lysate; (ns) nonspecific band. The arrowhead points to the Lz–DNA complex. *Above* each EMSA is a cartoon of the *aos*$^{292}$ or *klu*$^{363}$ enhancers with lines *above* the binding sites used as probes; (blue boxes) RD sites, (green boxes) Ets sites. (*I*) Lz forms a complex with two different *aos*$^{292}$ probes containing RD sites 1, 2, 3 (aos A) and RD site 8 (aos B). Although aos A contains three putative RD sites, there is no evidence that multiple Lz molecules are bound because the shift is similar to aos B, which has only one RD site. Lz also binds to RD site 4 (data not shown). Lz does not bind to probes with mutant RD sites (mA, mB). (*J*) Lz binds *klu*$^{363}$ probes containing RD sites 2 and 3 (klu A and klu B, respectively). Lz also binds to a probe containing RD site 1 (data not shown). Lz does not bind to probes with mutant RD sites (mA, mB). (*K*,*L*) Mutating all the RD sites in each enhancer abolished expression of both reporters: *aos*$^{292mRD}$–*lacZ* (*K*) and *klu*$^{363mRD}$–*lacZ* (*L*).

To determine if the RD sites are necessary for *aos*$^{292}$–*lacZ* and *klu*$^{363}$–*lacZ* expression in vivo, we mutated all of the RD sites present in the *aos*$^{292}$ and *klu*$^{363}$ enhancers (Fig. 3K,L). These reporter genes (*aos*$^{292mRD}$–*lacZ* and *klu*$^{363mRD}$–*lacZ*) were inactive, demonstrating that the RD sites are necessary for the activity of these enhancers in vivo.

### *EGFR loss of function is epistatic to Lz loss of function*

Decreasing EGFR activity results in an increase in cell death in the pupal eye (Brachmann and Cagan 2003). Both *aos* and *klu* antagonize EGFR signaling and promote cell death in the pupal retina (Freeman 1994; Sawamoto et al. 1998; Rusconi et al. 2004). If, as we propose, Lz activates these factors, a decrease in EGFR activity should be epistatic to *lz* loss of function. We tested this by decreasing EGFR activity in two ways. First, we used a *heat shock* (*hs*) *Gal4* driver to express *aos* at the same time we reduced Lz activity. As previously reported, ectopic *aos* caused a decrease in cone, 2°, and 3° cell number (Fig. 4E,F; Freeman 1994; Sawamoto et al. 1998). Ectopic *aos* produced a similar phenotype in temperature-shifted *lz*$^{ts}$ retinas, indicating that *aos* normally functions downstream of *lz* during PCD, albeit we cannot rule out the possibility that *aos* affects differentiation in these experiments (Fig. 4G,H). Although the overall number of 2° and 3° cells was reduced, a few small clusters of 2°/3° cells remained in the temperature-shifted *lz*$^{ts}$*; hs-Gal4 UAS-aos* retinas that were not seen in the *hs-Gal4 UAS-aos* retinas. These residual pigment cells could be due to a reduction of another Lz-dependent regulator of cell death (such as *klu*). Nevertheless, ectopic *aos* is able to at least partially suppress the *lz* loss-of-function phenotype, supporting the idea that *lz* promotes cell death by activating *aos*. In a second test, reducing EGFR signaling (using a temperature-sensitive allele of *EGFR*) in *lz*$^{ts}$ retinas resulted in an increase in cell death (Fig. 4I,J), also suggesting that EGFR activity is epistatic to Lz.

These findings, together with what is known about *aos* and *klu*, support the following model: Lz induces *aos* expression in cone cells, wherefrom Aos diffuses to antagonize EGFR activity in the surrounding 2° and 3° cells (Fig. 4K). The expression pattern of *aos*$^{923}$–*lacZ* indicates that Lz also regulates *aos* expression in 2° and 3° cells, suggesting that these cells may also send antisurvival signals. Our data further suggest that within the 2° and 3° cells, Lz activates *klu*, which antagonizes EGFR signaling downstream of the receptor. Lz also activates *klu* expression in cone and 1° cells, but it is unclear what function *klu* has in these cells. Although two phases of PCD during retinal development have been proposed (Cordero et al. 2004), our experiments support a role for Lz in promoting only the EGFR-dependent phase. An alternative possibility is that the decrease in cell death in *lz* mutant retinas is due to an increase in 2° and 3° cell differentiation stimulated by an increase in EGFR signaling. However, given the large body of evidence demonstrating that *lz* normally functions to promote differentiation, we do not favor a model in which *lz* acts to suppress differentiation.

The mammalian homolog of Lz, Runx1 (also known as AML1), is also a transcriptional regulator. In humans, translocations that affect *Runx1* are associated with acute myelogenous leukemia (AML), which is characterized by the proliferation of undifferentiated hematopoietic cells. Effects on cell cycle regulators have been implicated in contributing to this overproliferation, but it is likely that PCD also plays a role (Alcalay et al. 2001; Bernardin and Friedman 2002). Changes in the amount of the apoptotic regulator Wilms Tumor 1 (WT1) are often found in AML patients (Ostergaard et al. 2004). We have shown here that *lz* promotes cell death in the *Drosophila* eye in part by activating the expression of *klu*, the *Drosophila* homolog of *WT1*. We suggest that our findings may be relevant to how Runx1 chimeras lead to the de-

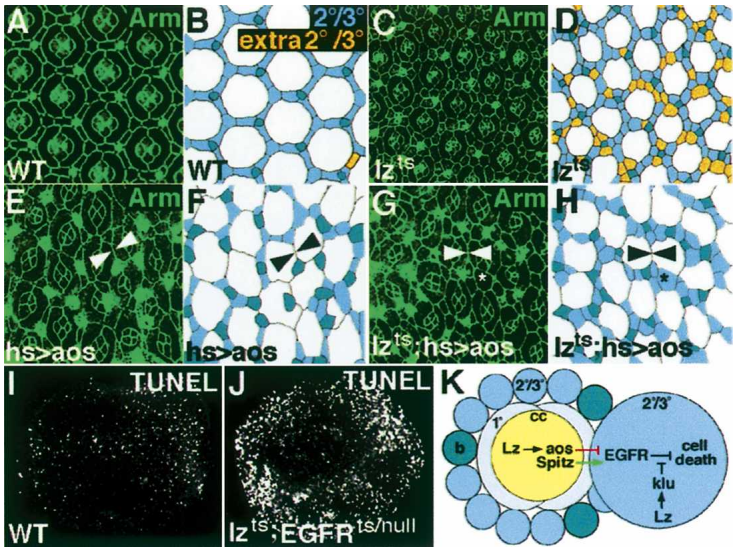


**Figure 4.** Reduction of EGFR activity is epistatic to *lz* loss of function. Arm (green) marks cell boundaries. To the *right* of each photograph is a trace of the 2°, 3°, and bristle cells (see Fig. 1). All retinas are 42 h APF. (*A*,*B*) Wild-type (WT) retina and corresponding trace (*B*). (*C*,*D*) Temperature-shifted $lz^{ts}$ retina. (*E*,*F*) *hs-Gal4 UAS-aos* retina. Because 2° and 3°s are lost, 1°s of neighboring ommatidia contact each other (arrowheads; some cone cells are missing). (*G*,*H*) Ectopic expression of *aos* in temperature-shifted $lz^{ts}$ retinas reduced 2° and 3° cell number (some ommatidia also lack cone cells). 1° cells from neighboring ommatidia touch (arrowheads), and there are also small clusters of 2°/3° cells (asterisk). (*I*,*J*) TUNEL stain (white) of wild-type (WT) (*I*) and $lz^{ts}$; $EGFR^{ts}/EGFR^{null}$ (*J*) pupal retinas. (*K*) Model of Lz's control of PCD in the pupal retina.

velopment of AML in humans. Furthermore, they suggest that *WT1* may be a direct target of *Runx1*.

## Materials and methods

*Search for putative Lz targets*

We searched for clusters of RD and Ets sites in the *Drosophila* genome using Target Explorer (Sosinsky et al. 2003; http://trantor.bioc.columbia.edu/Target_Explorer). Positional weight matrices representing binding sites for each transcription factor family were constructed from training sets of experimentally identified binding sites retrieved from the literature (Supplementary Table 1). We selected the lowest score in each training set as the cut-off score for that matrix. The minimal requirements for defining a putative enhancer were based on the previously identified *D-Pax2* and *pros* enhancers. We searched the noncoding euchromatin fraction of the whole genome with each RD and Ets matrix for clusters of at least two nonoverlapping sites within 500 bp. Additional criteria were that the RD/Ets clusters should: (1) have at least one pair of RD/Ets sites separated by 10 bp or less (based on constraints that limit the cooperative interaction between AML1 and Ets) (Kim et al. 1999); (2) have a cluster score above 3.30 (for a definition of the cluster score, see Supplementary Fig. 1 and Sosinsky et al. 2003); and (3) be located within 15 kb of the transcriptional start site of an "eye" gene. Eye genes were defined as genes annotated by FlyBase as having a loss- or gain-of-function phenotype and/or a known pattern of expression in the eye (511 genes qualified as eye genes). These criteria identified 135 clusters in 112 eye genes. This list was annotated to include information about the expression pattern and mutant phenotypes described in FlyBase and the literature. We selected 16 putative enhancers in genes that were previously shown to have pro- or anti-apoptotic functions (Table 1).

*Fly strains*

$lz^{r15}$, a null allele of *lz*; $lz^{ts}$, and *lz-Gal4* were kindly provided by the Banerjee lab. We thank N. Baker (Albert Einstein College of Medicine, New York) for *UAS-aos* flies. $klu^{P819}$*–lacZ* was a gift of T. Klein (Universitat zu Koln, Koln, Germany). H.D. Ryoo (The Rockefeller University, New York) provided *GMR–hid* and *GMR–rpr* flies. $EGFR^{f24}$, a null allele, and $EGFR^{ts}$ were obtained from the Bloomington stock center.

*Temperature-shift protocols*

Oregon-R wild-type and $lz^{ts}$ flies were cultured at 25°C. Brown pupae just prior to head eversion that displayed a prominent bubble and were buoyant were considered to be 15 h APF. At 20 h APF, the wild-type and $lz^{ts}$ pupae were transferred to 33°C and kept at this temperature until 42 h APF, when the pupae were dissected and fixed. Cone and 1° cell differentiation was not affected in temperature-shifted $lz^{ts}$ retinas, and a reduction in Lz immunostaining was observed. Temperature-shifted $lz^{ts}/lz^{null}$ retinas also had an increased number of 2° and 3° cells. A similar increase in cell number was also observed in retinas shifted to 31°C at 15 h APF. For the TUNEL assay, temperature-shifted pupae were dissected at 32 h APF. For the *aos* and *EGFR* epistasis experiments, flies were cultured at 18°C. For the ectopic *aos* experiment, staged 15-h-APF pupae were kept at 18°C for 11 h (when they resembled 20-h-APF pupae raised at 25°C), transferred to 35°C, and fixed at 42 h APF. For the *EGFR* epistasis experiment, staged 15-h-APF pupae were kept at 18°C for 26 h, transferred to 33°C for 5 h, and then fixed.

*Transgenic flies*

Genomic DNA isolated from wild-type flies was used to amplify putative enhancers by the PCR. Fragments were cloned into pCaSperR-hs43. Mutant versions of $aos^{292}$ and $klu^{363}$ were created using oligos with mutations in the RD-binding sites. *UAS-lz-enR* was created by substituting the Engrailed Repressor domain (2–297 amino acids of Engrailed) in frame for 542–750 amino acids of Lz (GenBank accession no. AAC47196) and cloning into pUAST.

*Immunohistochemistry and cell death assays*

Dissected tissue was prepared by standard procedures. The primary antibodies used were rat anti-Elav (1/50; Hybridoma Bank), mouse anti-Arm (1/10; Hybridoma Bank), mouse anti-Cut (1/10; Hybridoma Bank), mouse anti-Lz (1/10; gift of U. Banerjee, University of California at Los Angeles, Los Angeles, CA), rabbit anti-β-gal (1/1000; Cappell), guinea pig anti-Hth (1/1000) (Ryoo and Mann 1999), mouse anti-Dl (1/1000; Hybridoma Bank), mouse anti-N C17.9C6 (1/1000; Hybridoma Bank), mouse anti-Rst (1/10; gift of K. Fischbach, Albert-Ludwigs-Universitat Freiburg, Freiburg, Germany), rabbit anti-Hid (1/1000) (Yoo et al. 2002), anti-Pnut (1/10; Hybridoma Bank), anti-Skl (1/300) (Srinivasula et al. 2002). TUNEL assays were carried out with the ApopTag Fluorescein In Situ Apoptosis Detection kit (Serologicals).

*EMSAs*

Lz and control lysates were produced using the TNT coupled reticulocyte system (Promega). Lz was transcribed from a full-length *lz* cDNA in pET3c template (kindly provided by R. Carthew, Northwestern University, Evanston, IL). $^{32}$P-end-labeled oligo probes were mixed with 5 µL of either control or Lz lysates in 10 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 67 µg/mL BSA, 13.4 µg/mL poly(dI/dC), and incubated for 20 min at room temperature. The entire 20-µL reaction was run on a 4% polyacrylamide gel.

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