

Myeloid leukemia factor is a conserved regulator of RUNX transcription factor activity involved in hematopoiesis

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Defining the function of the genes that, like *RUNX1*, are deregulated in blood cell malignancies represents an important challenge. Myeloid leukemia factors (MLFs) constitute a poorly characterized family of conserved proteins whose founding member, MLF1, has been associated with acute myeloid leukemia in humans. To gain insight into the functions of this family, we investigated the role of the *Drosophila* MLF homolog during blood cell development. Here we report that *mlf* controls the homeostasis of the *Drosophila* hematopoietic system. Notably, *mlf* participates in a positive feedback loop to fine tune the activity of the RUNX transcription factor Lozenge (LZ) during development of the crystal cells, one of the two main blood cell lineages in *Drosophila*. At the molecular level, our data in cell cultures and *in vivo* strongly suggest that MLF controls the number of crystal cells by protecting LZ from degradation. Remarkably, it appears that the human MLF1 protein can substitute for MLF in the crystal cell lineage. In addition, MLF stabilizes the human oncogenic fusion protein RUNX1-ETO and is required for RUNX1-ETO-induced blood cell disorders in a *Drosophila* model of leukemia. Finally, using the human leukemic blood cell line Kasumi-1, we show that *MLF1* depletion impairs RUNX1-ETO accumulation and reduces RUNX1-ETO-dependent proliferation. Thus, we propose that the regulation of RUNX protein levels is a conserved feature of MLF family members that could be critical for normal and pathological blood cell development.

fruit fly | cancer | proteasome

Development of the hematopoietic system relies on the activity of several signaling pathways and transcription factors that have been conserved from mammals to fly (1). *Drosophila* has emerged as a model organism to gain insight into the gene regulatory networks controlling hematopoiesis. Deregulation of these networks lies at the origin of several diseases in humans, including leukemia and lymphoma. Interestingly, these hematologic malignancies are frequently associated with recurring chromosomal abnormalities (2). Along with their clinical prognostic value, these rearrangements have led to the identification of many genes involved in the etiology of cancer; thus, characterizing the function of these genes in normal and pathological blood cell development is of particular interest.

One notorious gene identified by cloning translocation breakpoints is *RUNX1/AML1*, which is affected by the *t*(8;21)(q22;q22) translocation found in ~15% of all cases of acute myeloid leukemia (AML) (3). *RUNX1* belongs to the RUNX transcription factor family, which is characterized by the presence of a highly conserved DNA binding domain (4). RUNX proteins activate or repress transcription in a context-dependent manner, thereby regulating cell proliferation and differentiation in a variety of metazoans. In particular, *RUNX* genes have been shown to regulate hematopoiesis in both vertebrates and *Drosophila*. For instance, *RUNX1* plays a prominent role in definitive hematopoietic stem cell emergence, as well as in megakaryocyte

and lymphocyte differentiation in mammals, and mutations affecting *RUNX1* are among the most frequent genetic abnormalities associated with blood cell malignancies in humans. These alterations promote leukemia by altering RUNX1 dosage or by producing mutant proteins that act as dominant negative and/or display neomorphic activities, as for RUNX1-ETO, the product of the *t*(8;21)(q22;q22) translocation, which comprises the RUNX1 DNA-binding domain fused to the transcriptional co-repressor ETO (5). In *Drosophila*, the RUNX factor Lozenge (LZ) is specifically expressed in and required for development of one of the two main blood cell types: the crystal cells, a megakaryocyte-like lineage that participates in clotting (6). In fact, LZ interacts and cooperates with the pan-hematopoietic GATA transcription factor Serpent (SRP) to activate the crystal cell differentiation program (7–9). This cooperation is conserved in mammals, where it controls megakaryopoiesis (10–12) and hematopoietic stem cell development (13). Moreover, reminiscent of the situation in humans, RUNX1-ETO expression in the *Drosophila* LZ⁺ blood cell lineage induces a preleukemic phenotype characterized by a switch of cell fate from differentiation to self-renewal (14). Thus, *Drosophila* provides a valuable model for studying the normal and oncogenic functions of RUNX factors during hematopoiesis.

The myeloid leukemia factor (MLF) family comprises a small group of evolutionarily conserved genes whose founding member was first identified as the target of the *t*(3;5)(q25;q35) translocation associated with myelodysplastic syndrome (MDS) and AML (15). This translocation generates a fusion protein between the N-terminal region of nucleophosmin (NPM; a multifunctional nucleolar protein) and most of MLF1. Whereas NPM, which is involved in other translocations, seems to act by providing a dimerization domain and a nucleolar targeting sequence (16), little is known about MLF1 activity. Significantly, however, increased *MLF1* expression correlates with poor prognosis in AML and with malignant progression in MDS (17), and *MLF1* ectopic expression affects the myeloerythroid lineage switch and cell cycle progression in cell cultures (18–20). Nonetheless, *MLF1* function in hematopoiesis remains poorly defined. Only one *MLF* gene is present in *Drosophila*, whereas there are two *MLF* paralogs in vertebrates (21). As in mammals, *MLF* encodes for a nucleocytoplasmic shuttling protein with no recognizable

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structural domain apart from a 14-3-3 binding motif (22). However, its *in vivo* function remains unclear, given that *mlf* mutant flies are subviable and do not exhibit any obvious developmental defect (23).

Given the conservation between MLF proteins and the parallels between mammalian and fly blood cell development, we assessed whether MLF controls hematopoiesis in *Drosophila*. In particular, we found that *mlf* expression is activated in the crystal cells by SRP/LZ, and that *mlf* regulates the expansion of this lineage. At the molecular level, our data indicate that MLF controls the number of crystal cells by protecting LZ from proteasome-mediated degradation. Interestingly, human MLF1 is able to substitute for *mlf* function in the crystal cell lineage. Furthermore, *mlf* is required for the activity and stable expression of the human leukemogenic protein RUNX1-ETO in *Drosophila*, whereas human *MLF1* depletion causes a decrease in RUNX1-ETO protein and impairs RUNX1-ETO-dependent proliferation in human leukemic blood cells. Thus, we propose that the control of RUNX levels is a conserved function of MLF proteins that play an important role in the control of blood cell homeostasis.

Results

***mlf* Is Expressed in the Crystal Cell Lineage in Response to SRP/LZ.** We first explored *mlf* expression in the *Drosophila* hematopoietic system. As in vertebrates, in *Drosophila* hematopoiesis occurs in different waves (1). In the embryo, prohemocytes emerge from the head mesoderm and differentiate into plasmatocytes and crystal cells. In the larva, blood cell progenitors, plasmatocytes, and crystal cells are present in the body cavity and in a specialized organ, the lymph gland, that will release its content at pupariation. Previous work suggested that *mlf* is expressed in embryonic crystal cells (23). In line with these results, *in situ* hybridization and immunostaining showed that *mlf* is expressed from stage 11 in a bilateral cluster of cells that correspond to the crystal cells, as demonstrated by co-staining with *lz*-driven expression of GFP (LZ-GFP) (Fig. 1 A–C). Moreover, high levels of nuclear MLF were detected in larval crystal cells both in the lymph gland and in circulation (Fig. S1). In addition, low levels of MLF were consistently detected throughout the lymph gland (Fig. S1). Thus, *mlf* may control crystal cell development and larval lymph gland homeostasis.

Our analysis of *mlf cis*-regulatory regions revealed that its first intron contains closely linked GATA and RUNX consensus binding sites that are conserved in other *Drosophila* species (Fig. 1D), a hallmark of SRP/LZ direct target genes (9). To test whether this *cis* element is sufficient to recapitulate *mlf* expression in crystal cells, we generated transgenic lines expressing *lacZ* under its control. Interestingly, *mlf-lacZ* expression was detected in embryonic (Fig. 1 E–G) and larval crystal cells (Fig. S1). In addition, *lacZ* and *mlf* transcription was activated throughout the mesoderm in response to the ectopic expression of both SRP and LZ, but not in response to SRP or LZ alone (Fig. 1 H–K and Fig. S2). These results suggest that this *cis* regulatory module is a target of the SRP/LZ complex, and that SRP/LZ activates *mlf* expression in the crystal cell lineage.

***mlf* Controls Crystal Cell Development.** We then asked whether *mlf* controls hematopoiesis. *In situ* hybridizations against the plasmatocyte differentiation marker *peroxidase* (*pxn*) and the crystal cell differentiation marker *PO45* were used to monitor hemocyte differentiation in embryos carrying a homozygous null mutation in *mlf* (*mlf*^{Δ5–3/Δ5–3}) (23). Consistent with the *mlf* expression pattern, *mlf* loss did not impair plasmatocyte differentiation (Fig. S3). In contrast, *mlf* loss affected crystal cell development (Fig. 2). *PO45* levels were lower in *mlf* embryos compared with WT or heterozygous siblings (Fig. 2A–C), and the number of *PO45*-expressing cells was reduced (Fig. 2D). Importantly, reexpressing *mlf* solely in the crystal cells using the *lz-Gal4* driver restored normal crystal cell numbers in *mlf*^{Δ5–3} homozygous embryos (Fig. 2D), and similar observa-

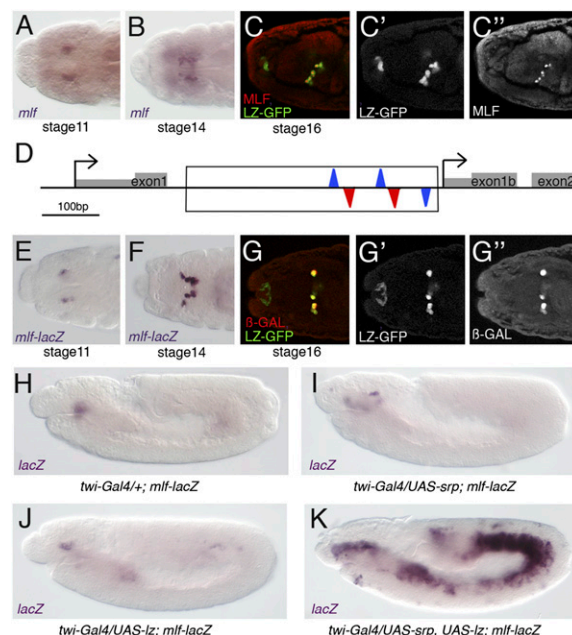


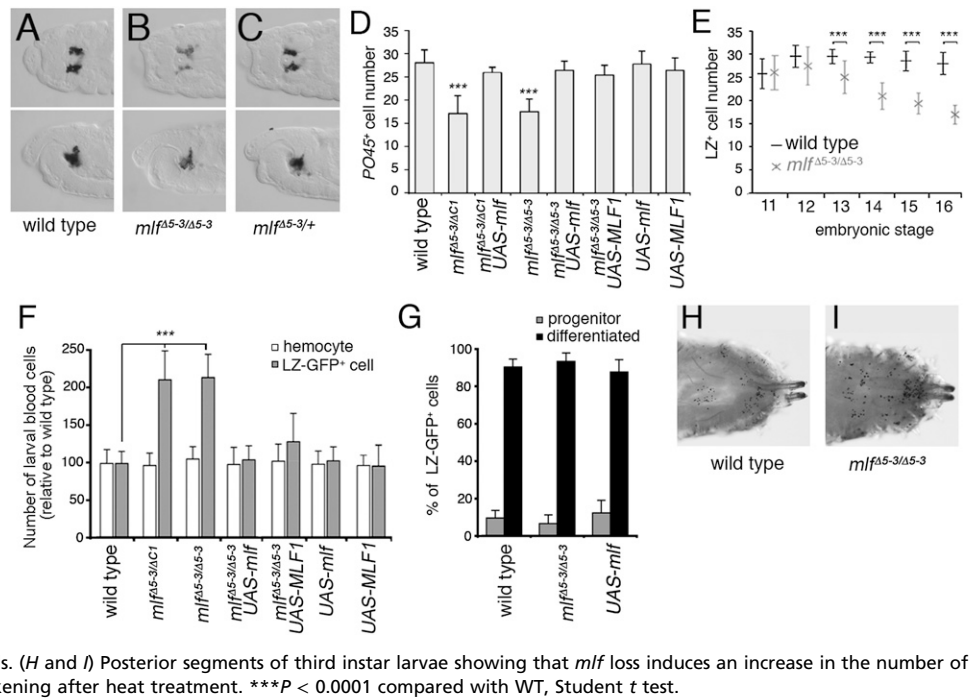
Fig. 1. *mlf* expression is activated by SRP/LZ in the crystal cell lineage. (A–C) *mlf* is expressed in crystal cells. A and B, *in situ* hybridization; C, coimmunostaining with GFP (expressed under the control of *lz-Gal4*). Embryonic stages are indicated. (D) Schematic representation of *mlf* regulatory sequences. The two alternative promoters and first exons are shown. Conserved GATA (WGATAR) and RUNX (TGYGGTY) consensus binding sites are indicated by blue and red triangles, respectively. The genomic region used to generate the transgenic lines is boxed. (E–G) *lacZ* expression driven by *mlf* enhancer is detected in crystal cells. E and F, *in situ* hybridization; G, coimmunostaining with GFP (expressed under the control of *lz-Gal4*). Embryonic stages are indicated. (H–K) *Twist*-driven coexpression of SRP and LZ activates *mlf-lacZ* expression throughout the mesoderm. *lacZ* *in situ* hybridization on stage 11 embryos of the indicated genotypes.

tions were seen using transheterozygous null mutations in *mlf* (*mlf*^{Δ5–3/ΔC1}). Furthermore, counting LZ-expressing cells from embryonic stages 11–16 showed that their number was initially WT but decreased progressively in the absence of *mlf* (Fig. 2E). Therefore, MLF is necessary for crystal cell maintenance in the embryo, and it acts cell-autonomously after the onset of *lz* expression.

We also assessed the function of *mlf* in the larval lymph gland. In third instar larvae, the lymph gland consists of a pair of primary lobes with prohemocytes in their medullary zone and differentiated cells in their cortical zone, and several smaller secondary lobes containing only prohemocytes (1). In *mlf* mutant larvae, the lymph glands were hypertrophied and displayed aberrant precocious differentiation (Fig. S4); the expression of the prohemocyte marker *tepIV* was strongly reduced, whereas both crystal cells and plasmatocytes were present in the secondary lobes. Thus, *mlf* is required to maintain lymph gland homeostasis, and it prevents premature blood cell differentiation in this organ.

Remarkably, by monitoring circulating larval blood cells, we observed that *mlf* loss induced an increase in the number of LZ-GFP⁺ cells, whereas the total hemocyte population was not significantly changed (Fig. 2F). Consequently, the proportion of circulating LZ-GFP⁺ cells rose from 4.8% in WT to 10.3% in *mlf* larvae. As in the embryo, LZ-GFP⁺ cell count was restored to WT when *mlf* was specifically reexpressed in this lineage (Fig. 2F). However, the proportion of LZ-GFP⁺ cells expressing *PO45* was similar in WT and *mlf* mutant larvae (Fig. 2G), suggesting that *mlf* loss does not prevent LZ-GFP⁺ cell differentiation in crystal cells. Consistent with these results, heat treatment, which activates the melanization cascade in mature crystal cells, re-

Fig. 2. *mlf* controls embryonic and larval crystal cell homeostasis. (A–C) Decreased expression of the crystal cell differentiation marker *PO45* is observed in the absence of *mlf*. Dorsal (Upper) and lateral (Lower) views showing *PO45* expression in stage 15 embryos of the indicated genotypes. (D) Stage 15 *mlf* embryos have fewer *PO45*-expressing cells. Expression of *mlf* or its human homolog *MLF1* under the control of *lz-Gal4* in *mlf* mutant backgrounds restores WT crystal cell number. (E) Immunostaining against LZ reveals a progressively decreasing number of LZ-expressing hemocytes in *mlf* embryos. (F and G) *lz-Gal4*, *UAS-GFP* third instar wandering larvae of the indicated genotypes were bled to determine the relative number of circulating hemocytes or LZ-GFP⁺ cells (F), as well as the proportion of differentiated (*PO45*⁺) and progenitor (*PO45*⁻) LZ-GFP⁺ cells (G). (F) Loss of *mlf* induces an increase in the number of circulating larval LZ-GFP⁺ cells that is suppressed by expression of *mlf* or human *MLF1* in this lineage. (G) *mlf* does not affect the ratio of progenitor to differentiated LZ-GFP⁺ cells. (H and I) Posterior segments of third instar larvae showing that *mlf* loss induces an increase in the number of crystal cells, as demonstrated by their blackening after heat treatment. ****P* < 0.0001 compared with WT, Student *t* test.



vealed the presence of more crystal cells in *mlf* mutants than in WT controls (Fig. 2 *H* and *I*). Thus, although *mlf* loss results in fewer crystal cells in the embryo, it promotes crystal cell expansion in the larvae. Of note, MLF overexpression did not seem to affect crystal cell number or differentiation in either the embryo or the larva (Fig. 2 *D*, *F*, and *G*), suggesting that MLF is not a limiting factor.

We then asked whether human *MLF1* could replace *mlf* function in the crystal cell lineage. Accordingly, we expressed *MLF1* under the control of the *lz-Gal4* driver, and determined the number of crystal cells present in the embryo or in circulation in the larvae. Although MLF1 expression did not alter the number of crystal cells in a WT background, it did rescue *mlf*-associated hematopoietic defects (Fig. 2 *D* and *F*). This suggests that although MLF1 exhibits only 23% of identity with MLF (21), the function of MLF proteins has been conserved from *Drosophila* to human.

***mlf* Promotes SRP/LZ-Induced Transactivation by Interfering with LZ Degradation.** As mentioned above, SRP/LZ-induced gene

transcription is pivotal for crystal cell differentiation. Interestingly, in a genome-wide RNAi screen, we recently identified *mlf* as a potential regulator of SRP/LZ in the *Drosophila* Kc blood cell line (24). We thus used this system to decipher MLF's mechanism of action. As reported previously (24), transfection of an expression plasmid for LZ (pAc-LZ) activated a reporter gene under the control of the *PO45* crystal cell-specific enhancer (*4xPO45-Fluc*), and knockdown endogenous *srp* expression by dsRNA impaired this transactivation, indicating that *4xPO45-Fluc* activity reflects SRP/LZ-induced transcription (Fig. 3*A*). Using three different dsRNAs targeting *mlf*, we showed that *mlf* depletion strongly reduced *4xPO45-Fluc* activation by SRP/LZ. Conversely, cotransfection of increasing amounts of pAc-MLF with pAc-LZ resulted in a dose-response enhancement of the reporter gene activity (Fig. 3*B*). To extend these observations, we asked whether *mlf* also controls the induction of SRP/LZ target genes within their genomic context. Real-time quantitative PCR (qPCR) experiments demonstrated that the transactivation of *PO45* and *Bc*/

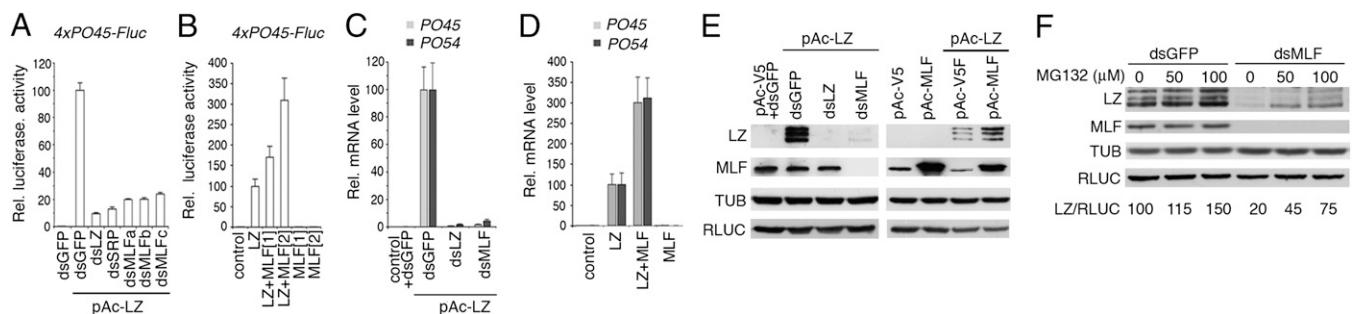


Fig. 3. MLF is required for LZ transactivation and stability in Kc cells. (A and B) Luciferase reporter assays. (A) dsRNA against *mlf* impairs LZ-induced transactivation of the p45PO45-Fluc plasmid. Three different dsRNA probes targeting *mlf* were tested. (B) Increasing amounts of pAc-MLF expression plasmid enhance pAc-LZ-induced transactivation of p45PO45-Fluc. (C and D) Real-time qPCR assays. (C) dsRNAs against *mlf* inhibit LZ-induced activation of *PO45* and *PO54* transcription. (D) Increasing levels of pAc-MLF expression plasmid promote pAc-LZ-induced *PO45* and *PO54* expression. (E) Western blot analysis showing that LZ levels decrease on depletion of MLF by dsRNA (Left) and increase on cotransfection of pAc-MLF with pAc-LZ (Right). Endogenous tubulin (TUB) and pAc-expressed *Renilla* luciferase (RLUC) were used as loading and transfection controls. (F) Inhibition of the proteasome by MG132 increases LZ levels in MLF-depleted Kc cells. The relative levels of LZ (normalized to RLUC) are shown in the lower part of the panel.

PO54 (another crystal cell-specific differentiation marker) observed on LZ expression was also impaired in the presence of dsRNA against *mlf* and was enhanced when *mlf* expression plasmid was cotransfected (Fig. 3 C and D). Therefore, MLF behaves as a coactivator for SRP/LZ.

Strikingly, Western blot analysis revealed that *mlf* dsRNA not only efficiently knocked down MLF expression, but also strongly decreased pAc-driven expression of LZ (Fig. 3E). In contrast, pAc-driven expression of *Renilla* luciferase was not affected. Conversely, overexpression of MLF increased LZ levels. Thus, it appears that MLF regulates LZ expression posttranscriptionally. Furthermore, treatment with the proteasome inhibitor MG132 resulted in increased LZ levels and partially restored LZ accumulation in MLF-depleted cells (Fig. 3F). These data raise the possibility that MLF promotes SRP/LZ-induced transactivation by protecting LZ from degradation by the proteasome.

***mlf* Controls Crystal Cell Development by Impinging on LZ Levels in Vivo.** The foregoing results suggested that MLF might regulate crystal cell development by controlling LZ levels. This hypothesis is consistent with the drop in LZ⁺ cell number in *mlf* embryos but seems at odds with its increase in *mlf* larvae. However, in line with our observation in Kc cells, immunofluorescent labeling revealed significantly reduced LZ expression in LZ-GFP⁺ circulating cells of *mlf* mutant larvae compared with control larvae (Fig. 4 A and B). Of note, GFP expression, which is driven under the control of *lz*, did not seem markedly affected by the loss of *mlf*, indicating that *mlf* is not required for *lz* transcription but is critical for LZ protein accumulation. Moreover, in the eye disk, where *mlf* is ubiquitously expressed but not up-regulated in the LZ⁺ cells, *mlf* loss also decreased LZ levels, but only modestly (Fig. S5), suggesting that LZ stability is differentially regulated in the hematopoietic and visual systems. Importantly, expressing human MLF1 in a *mlf* mutant background not only rescued crystal cell number (Fig. 2 D and F), but also restored LZ expression (Fig. 4D and Fig. S6A), providing further evidence that MLF proteins regulate LZ levels. We thus reasoned that

increasing LZ expression might rescue the crystal cell defects caused by *mlf* loss. Consequently, we generated *mlf* mutants carrying the *lz-Gal4* driver together with a *UAS-GFP* transgene and a *UAS-lz* transgene. In these conditions, we observed strong LZ expression in the crystal cells of *mlf* mutant larvae (Fig. 4C and Fig. S6A). Furthermore, the expression of the *UAS-lz* transgene suppressed not only the drop in crystal cell number in *mlf* embryos, but also the rise in crystal cell number in *mlf* larvae (Fig. 4 E and F). Therefore, these two apparently opposed phenotypes likely stem from a common cause—a decrease in LZ levels.

Human Leukemic Fusion Protein RUNX1-ETO Is Regulated by MLF Proteins. We recently showed that expression of the human RUNX oncogenic fusion protein RUNX1-ETO in the LZ⁺ blood cell lineage induces a preleukemic switch in flies (14). Indeed, it inhibits crystal cell differentiation and promotes LZ-GFP⁺ progenitor amplification in the larvae (14) (Fig. 5 A and B). In light of the foregoing results and of the link between MLF and leukemia in humans, we wondered whether *mlf* interacted genetically with RUNX1-ETO in our model. Strikingly, we found that RUNX1-ETO leukemogenic activity was almost abolished in *mlf* mutant larvae; the number of circulating LZ-GFP⁺ cells was significantly decreased, and their differentiation was restored (Fig. 5 A and B). Furthermore, in the absence of *mlf*, RUNX1-ETO expression was barely detected, whereas robust staining

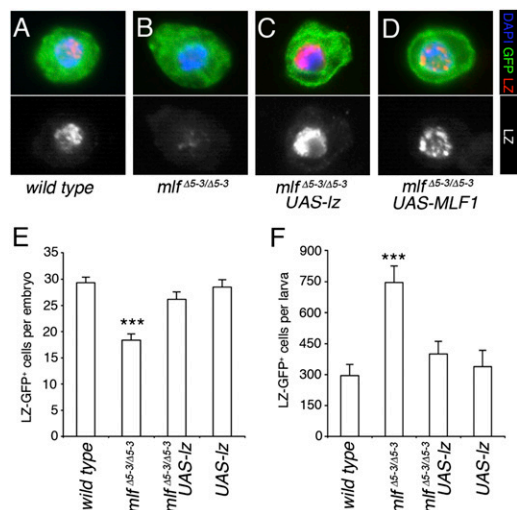


Fig. 4. Decreased LZ levels are responsible for MLF-associated crystal cell phenotypes. (A–D) MLF stabilizes LZ in vivo. Fluorescent immunostaining against LZ in circulating larval blood cells from *lz-Gal4*, *UAS-GFP* third instar larva of the indicated genotype is shown. Nuclei were stained with DAPI. The lower panels show LZ immunostaining only. (E and F) Reexpression of LZ under the control of the *lz-Gal4* restores WT LZ-GFP⁺ cell number in the embryo (E) and in third instar larvae circulation (F). GFP expression was used to score the number of LZ-GFP⁺ cells in stage 14–15 embryos (E) or in third instar wandering larvae (F) of the indicated genotypes.

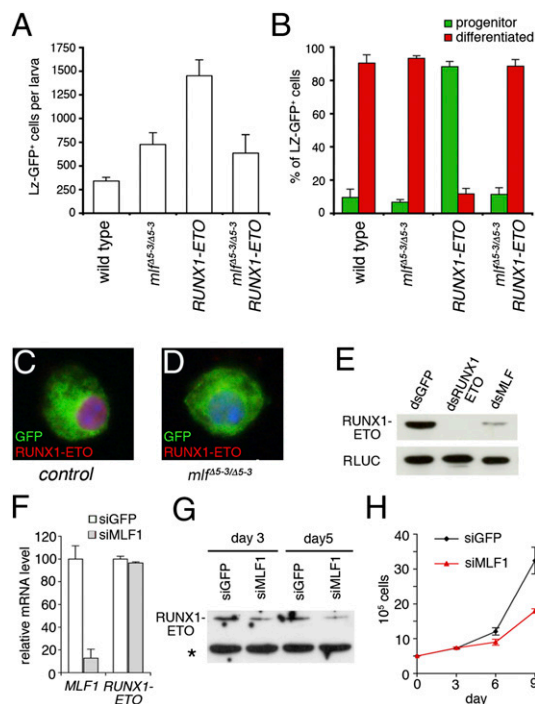


Fig. 5. MLF is required for RUNX1-ETO activity. (A and B) The phenotypes induced on expression of RUNX1-ETO in the LZ⁺ blood cell lineage are suppressed in the absence of *mlf*. (A) Absolute number of circulating LZ-GFP⁺ cells in third instar larvae. (B) Ratio of circulating progenitors (GFP⁺, *PO45*⁻) to differentiated (GFP⁺, *PO45*⁺) LZ⁺ cells in third instar larvae. (C–E) MLF is required for RUNX1-ETO expression in *Drosophila*. (C and D) Fluorescent immunostaining against RUNX1-ETO in *lz-Gal4*, *UAS-GFP*; *UAS-RUNX1-ETO* (C, control) and *lz-Gal4*, *UAS-GFP*; *mlf*^{Δ5-3/Δ5-3}; *UAS-RUNX1-ETO* (D, *mlf*^{Δ5-3/Δ5-3}) circulating larval blood cells. Nuclei were stained with DAPI. (E) Western blot analysis shows that RUNX1-ETO levels decrease after depletion of MLF by dsRNA in Kc cells. (F and G) In Kasumi-1 cells, *MLF1* depletion by siRNA does not affect *RUNX1-ETO* mRNA levels (F; real-time qPCR), but does reduce RUNX1-ETO protein levels (G; Western blot analysis). The asterisk indicates a nonspecific band that provides a loading control. (H) Knockdown of *MLF1* expression inhibits Kasumi-1 cell growth.

was observed in control larvae (Fig. 5 C and D and Fig. S6B). Thus, we propose that the suppression of RUNX1-ETO-induced blood cell phenotypes observed in the absence of *mlf* reflects the role of MLF in RUNX1-ETO stabilization. In line with this hypothesis, Western blot analysis showed that the expression of RUNX1-ETO transfected in Kc cells was strongly down-regulated on dsRNA-mediated depletion of MLF (Fig. 5E).

Finally, we asked whether MLF1 also regulates RUNX1-ETO expression in human. Kasumi-1 cells derived from a patient with AML carrying the *t*(8;21) translocation were found to constitutively express RUNX1-ETO, which is required for their proliferation (25). On transfection of an siRNA against *MLF1*, we found a significant reduction of *MLF1* transcript levels, but no effect on *RUNX1-ETO* mRNA expression (Fig. 5F). However, consistent with our observations in *Drosophila*, Western blot analysis showed strongly decreased RUNX1-ETO protein levels (Fig. 5G), paralleled by reduced cell growth (Fig. 5H). Taken together, our results reveal a conserved functional relationship between MLF family members and the leukemogenic fusion protein RUNX1-ETO.

Discussion

Although deregulation of *MLF1* has been linked to AML, the physiological role of MLF family members in hematopoiesis remains largely unknown. Focusing our analysis on *Drosophila* hematopoietic development, we have demonstrated that MLF controls blood cell homeostasis. In particular, we provide strong evidence that MLF is required to stabilize the RUNX factor LZ during crystal cell development. In addition, our findings suggest that the regulation of RUNX activity by MLF is conserved in humans, where it could play an important role in leukemogenesis.

Our findings reveal MLF's regulatory function in the control of crystal cell production. Actually *mlf*, which exhibits a rather ubiquitous expression pattern, is highly expressed in these blood cells, and *mlf* expression in this lineage is activated by SRP/LZ. We found that MLF controls crystal cell number in a cell-autonomous manner, chiefly by impinging on LZ levels. We propose that the induction of *mlf* expression by SRP/LZ contributes to crystal cell development by stabilizing LZ. As such, this gene regulatory network forms a two-component positive feedback loop that drives development forward by stabilizing the expression of lineage-specific regulators (26). Our findings also show that MLF controls lymph gland homeostasis, where it seems to promote hematopoietic progenitor maintenance. Although some of the factors controlling lymph gland development have been identified, no RUNX factor has been implicated in prohemocyte maintenance (1), suggesting that MLF has other partners in these cells. Of interest, MLF has been shown to bind Su(fu) and to possibly antagonize its function (27). Because Su(fu) negatively regulates both Hh and Wnt pathways (28, 29), which are required for prohemocyte maintenance (30), their premature differentiation in *mlf* mutants could result from increased Su(fu) activity. We anticipate that deciphering MLF's mode of action in the lymph gland will provide valuable insight into the regulation of blood cell progenitor fate.

Along with revealing the role of MLF in hematopoiesis, our findings shed light on the function and regulation of LZ. We found that decreased LZ levels in *mlf* mutant larvae resulted in an increased number of circulating LZ⁺ cells, but did not block these cells' differentiation, suggesting that low levels of LZ are sufficient to induce crystal cell differentiation. In addition, expansion of the pool of LZ⁺ cells might reflect a slowdown in the cells' rate of differentiation or a direct function of LZ in controlling blood cell proliferation or apoptosis. Along this line, LZ was shown to promote cell death in the eye, notably by regulating the expression of the *Drosophila* homolog of the Wilms' tumor gene 1 (*WT1*) (31). Alternatively, decreased LZ levels may make crystal cell progenitors more susceptible to proliferative cues

from the Notch pathway, which regulates larval crystal cell numbers (32, 33). In mammals, RUNX1 acts mostly as a brake on blood cell progenitor proliferation, and decreased *RUNX1* dosage, as well as MDS/AML-associated mutations or translocations affecting *RUNX1*, tend to promote aberrant self-renewal (34). Interestingly, RUNX1-ETO also promotes hematopoietic progenitor cell expansion in *Drosophila* (14, 35), and both WT1 overexpression and activation of the Notch signaling pathway have been linked to RUNX1-ETO-induced AML (36, 37). Given these similarities, characterizing the function of LZ in the control of crystal cell number may have broader implications.

Notwithstanding the evolutionary distance between human and fly, the human MLF1 protein rescued *mlf*-associated crystal cell defects, including LZ down-regulation, whereas *mlf* and *MLF1* were required for the stable expression of RUNX1-ETO in *Drosophila* and human leukemia cells, respectively. Thus, the regulation of RUNX turnover seems to be a conserved function of MLF family members. The proteasome was found to regulate RUNX1-ETO as well as other RUNX proteins in human cells (38, 39), and our data indicate that LZ is degraded in a proteasome-dependent manner in the absence of *mlf*. An important area of future inquiry will be to determine more precisely how RUNX stability is regulated by MLF. This is of particular interest given that altered RUNX levels are associated with several diseases in humans, including familial platelet disorders and AML for RUNX1 and leiodcranial dysplasia for RUNX2 (40). Actually, the slightly reduced LZ levels that we observed in *mlf* mutant eye discs suggests that the regulation of RUNX stability by MLF is not restricted to the hematopoietic system. Moreover, few genes required for RUNX1-ETO-induced AML have been identified so far, and our data suggest that MLF1 is a critical component for RUNX1-ETO leukemogenic activity. Indeed, along with the MDS/AML-associated *t*(3;5) translocation that generates the NPM-MLF1 fusion protein, *MLF1* overexpression was correlated with malignant progression (17). The mechanisms underlying the oncogenic activity of NPM-MLF1 or MLF1 remain largely unknown, however. Similarly, the function of *MLF1* in mammals remains poorly characterized. Exploring the relationship between MLF and RUNX factors could shed further light on MLF's role and mode of action.

Blood cell development is controlled by an intricate network of genes, the activity of which must be tightly controlled to ensure proper cell lineage choice, proliferation, and differentiation. Our present findings show that the dynamic and coordinated control of gene expression through positive feedback loops participates in the fine-tuning of hematopoiesis, and provides a framework for future investigations of the cross-regulatory interactions that control blood cell fate. Finally, our results open up new avenues of research into the mode of action of MLF family members as conserved regulators of RUNX protein stability, and we envision that *Drosophila* will provide a powerful model for deciphering MLF's function in hematopoiesis and leukemia.

Materials and Methods

Fly Strains. We used the following *Drosophila melanogaster* strains: *mlf*^{Δ5-3}, *mlf*^{ΔC1} (23), *UAS-mlfA* (23), *UAS-RUNX1-ETO* (14), *UAS-lz* (P. Gergen, Stony Brook University, NY), *lz-Gal4*, *UAS-GFP*, and *UAS-MLF1* (Bloomington Stock Center). To generate *mlf-lacZ* transgenic reporter lines, the *mlf* first intron was PCR-amplified from *D. melanogaster* genomic DNA and cloned into a *pCasper-hsp43-lacZ* vector. The resulting vector was used to produce transgenic lines by P element-mediated transformation into *w*¹¹¹⁸ flies.

In Situ Hybridization, Immunostaining, and Hemocyte Counts. Immunostaining and in situ hybridization were performed as described previously (9). For in situ hybridization, we used digoxigenin-UTP-labeled RNA probes and sheep anti-digoxigenin coupled to alkaline phosphatase (Roche). For immunostaining, we used mouse anti-LZ (Developmental Studies Hybridoma Bank), rabbit anti-MLF (27), rabbit anti-β-galactosidase (Cappel), mouse or rabbit anti-GFP (Torrey), mouse anti-P1/NimC1 (a kind gift from I. Ando, Biological

Research Center, Szeged, Hungary), rabbit anti-AML1 (Calbiochem), and secondary antibodies coupled to Alexa Fluor 488 or 555 (Molecular Probes). Circulating larval hemocyte counts and LZ⁺ cell differentiation status were assessed as described previously (14). In brief, GFP expression from *Iz-Gal4* and *UAS-GFP* was used to determine LZ⁺ cell number, and DAPI staining was used to count the whole hemocyte population on individually bled third instar female larvae. To monitor LZ⁺ cell differentiation status, female third instar larvae were bled on polylysine-coated slides, and the proportion of LZ-GFP⁺ cells expressing *PO45* was assessed after in situ hybridization coupled to immunostaining. Counts were performed on a minimum of 12 samples for each genotype.

Cell Culture, Transfection, Luciferase and Western Blot Assays, and qPCR. For transfection, dsRNA treatment, luciferase assays, Western blot analyses, and real-time qPCR, *Drosophila* Kc cells were treated essentially as described previously (24). In brief, Kc cells were grown at 25 °C in Schneider's medium supplemented with 10% (vol/vol) FBS and 50 µg of penicillin/streptomycin. For dsRNA treatment and transfections, cells were plated on in vitro-transcribed and purified dsRNA at 24 h before transfection with Effectene (Qiagen). We used the following plasmids: pAc-MLFA (23), pAc-V5 (Invitrogen), pAc-LZ-V5, and p4xPO45-Firefly luciferase (24). pAc-RUNX1-ETO was generated by subcloning RUNX1-ETO ORF from pUAST-RUNX1-ETO (14) into pAc-V5. pAc-*Renilla* luciferase was used for transfection normalization. Three independent dsRNA targeting different regions of *mlf* were produced. The sequences of the T7-containing primers used to generate the dsRNA are available on request. Firefly and *Renilla* luciferase activity was measured using the Promega Dual Luciferase Reporter Assay System. *PO45*, *BcPO54*, and *RP49* mRNA expression levels were measured

by real-time qPCR (24). For proteasome inhibition, Kc cells were incubated for 8 h with MG132 (or DMSO) before being processed for analysis. Kasumi-1 cells were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine and 10% FBS. siRNAs against *MLF1* (5'-GGUCUGGUAUAAGCAU-3') or *GFP* (5'-GCAAGCUGACCCUGAAGUUCAU-3') were transfected on day 0 and every 48 h thereafter by electroporation, as described previously (25). RNA and proteins were extracted on days 3 and 5. For real-time qPCR, *MLF1* and *RUNX1-ETO* mRNA levels were normalized to *GAPDH* expression. Kasumi-1 cell numbers were counted every 3 d using the trypan blue exclusion assay. The following primary antibodies were used for Western blot analyses: mouse anti-V5 (Invitrogen), rabbit anti-MLF (27), mouse anti-ETO (Calbiochem), mouse anti-tubulin (Sigma-Aldrich), and rabbit anti-*Renilla* luciferase (MBL International). All experiments were reproduced at least three times. Means and SDs were calculated from independent triplicates.

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