

ORIGINAL ARTICLE

Drosophila myeloid leukemia factor acts with DREF to activate the JNK signaling pathway

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Drosophila myelodysplasia/myeloid leukemia factor (dMLF), a homolog of human MLF1, oncogene was first identified by yeast two-hybrid screen using the DNA replication-related element-binding factor (DREF) as bait. DREF is a transcription factor that regulates proliferation-related genes in *Drosophila*. It is known that overexpression of dMLF in the wing imaginal discs through the *engrailed-GAL4* driver causes an atrophied wing phenotype associated with the induction of apoptosis. However, the precise mechanisms involved have yet to be clarified. Here, we found the atrophied phenotype to be suppressed by loss-of-function mutation of *Drosophila Jun N-terminal kinase (JNK)*, *basket (bsk)*. Overexpression of dMLF induced ectopic JNK activation in the wing disc monitored with the *puckered-lacZ* reporter line, resulting in induction of apoptosis. The DREF-binding consensus DRE sequence could be shown to exist in the *bsk* promoter. Chromatin immunoprecipitation assays in S2 cells with anti-dMLF IgG and quantitative real-time PCR revealed that dMLF binds specifically to the *bsk* promoter region containing the DRE sequence. Furthermore, using a transient luciferase expression assay, we provide evidence that knockdown of dMLF reduced *bsk* gene promoter activity in S2 cells. Finally, we show that dMLF interacts with DREF *in vivo*. Altogether, these data indicate that dMLF acts with DREF to stimulate the *bsk* promoter and consequently activates the JNK pathway to promote apoptosis.

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INTRODUCTION

Chromosomal translocations are frequently found in various human leukemias, and the aberrant proteins generated presumably interfere with the functions of their normal counterparts, in particular those associated with cellular growth and differentiation. Human myelodysplasia/myeloid leukemia factor 1 (hMLF1) was originally identified in the form of a fusion protein with nucleophosmin (NPM) generated by the t(3;5)(q25.1;q34) chromosomal translocation in the myelodysplastic syndrome and acute myeloid leukemia.¹ NPM is a major nucleolar phosphoprotein that is significantly more abundant in tumors and proliferating cells than in normal resting cells.^{2,3} It has also been reported that NPM shuttles between the nucleus and cytoplasm.⁴

The hMLF1 protein is mainly located in the cytoplasm, whereas its fused form with NPM is mostly located in the nucleus with highest levels in the nucleolus.¹ The hMLF1 contains no recognizable domains or motifs except for a characteristic RSXSP motif, the binding sequence for 14-3-3 protein,^{5,6} that is involved in regulating cell division, apoptosis and differentiation. It has been reported that 14-3-3 ζ interacts with hMLF1.⁷ In addition, it has been reported that hMLF1 associates with Madm, MLFIP/KLIP1, Manp and CSN3, a component of the COP9 signalosome, and regulates the cell cycle via the CSN/COP3 pathway.⁷⁻¹⁰ However, the biochemical activity of hMLF1 has yet to be fully characterized.

The MLF protein is conserved among various species in animals.^{11,12} In contrast to mammals that have two closely

related proteins, hMLF1 and hMLF2,¹¹ *Drosophila* has a single gene, *dMLF*, encoding a protein homologous to both hMLF1 and hMLF2. *Drosophila* myelodysplasia/myeloid leukemia factor (dMLF) was first identified by yeast two-hybrid screening using the DNA replication-related element-binding factor (DREF) as a bait.¹³ DREF is a transcription factor in *Drosophila* that regulates proliferation-related genes such as *PCNA*, *Cyclin A*, *DNA polymerase α* and others.¹⁴⁻²⁵ The dMLF consists of 309 amino acid residues, and especially its central region (amino acids 96 to 202) displays high homology to hMLF1 (54% identity), hMLF2 (63%) and a mouse homolog, Hemopoietic lineage switch (HLS7) (59%).¹³ Furthermore, it is reported that this highly conserved region of dMLF is necessary for binding to DREF.¹³ The dMLF protein has also been reported to interact physically and genetically with Su(fu), a negative regulator of the Gli/Ci transcription factor involved in Hedgehog (Hh) signaling.²⁶ In addition, it has also been reported that dMLF interacts with the Hh pathway component Cos2,²⁶ and suppresses the rough eye phenotype induced by overexpression of DREF.¹³ Ectopic expression of dMLF in the developing eye imaginal disc using an *eyeless-GAL4* driver resulted in a small eye phenotype rescued by coexpression of *cyclin E*, suggesting involvement of dMLF in cell-cycle regulation.²⁷ Overexpression of dMLF in eye imaginal discs using a *GMR-GAL4* driver also caused a rough eye phenotype in adults, and overexpression in wing imaginal discs induced programmed cell death and promoted transition through the S phase.²⁶ It is therefore conceivable that dMLF plays multiple roles in cell growth, survival, apoptosis and gene transcription.

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Other roles have been identified for dMLF. Thus, using a *Drosophila* model of polyglutamine disorders, it has been reported that overexpression of dMLF suppresses toxicity associated with an abnormally long polyglutamine tract expressed in the eye and central nervous system.²⁸ dMLF reduced the recruitment of the CRE binding protein and Hsp70 into polyglutamine inclusions, both of these being among essential proteins apparently trapped in the inclusions.²⁹ More recently, it has been shown that dMLF controls homeostasis of the *Drosophila* hematopoietic system by regulating the activity of the RUNX transcription factor Lozenge during development of crystal cells.³⁰

In the present study, we found that dMLF is involved in the regulation of the Jun N-terminal kinase (JNK) pathway during *Drosophila* development. The JNK cascade is an intracellular signaling pathway in which the stress-activated kinases JNK kinase and JNK play essential roles.³¹ JNK signaling is involved in processes including cell proliferation and apoptosis.^{32–34} Apoptosis induced by JNK has an important role in the morphogenesis of the wing imaginal disc.³⁵ JNK signaling controls the expression of target genes as those encoding the proapoptotic protein Reaper and the dual-specificity phosphatase Puckered (*puc*). In *Drosophila*, JNK kinase and JNK homologs are encoded by the genes *hemipterous* (*hep*) and *basket* (*bsk*), respectively.^{36–38} We have reported that the DREF-binding consensus, the DRE sequence, 5'-TATCGATA-3', exists in the *bsk* promoter region and that DREF is required for *bsk* gene expression.²⁵

In the present study, we further examined the roles of dMLF in the regulation of the JNK signaling pathway and demonstrated that dMLF acts with DREF in the *bsk* promoter to stimulate *bsk* expression and consequently activate the JNK pathway and apoptosis.

RESULTS

dMLF genetically interacts with *bsk*

Transgenic fly lines overexpressing dMLF in the wing imaginal discs exhibit an atrophied phenotype of the posterior compartment of adult wings associated with caspase-3 activation and apoptosis in the wing imaginal discs.²⁶ In the wing disc, JNK activation is usually linked to the activation of caspase-3.³⁹ Therefore, we tested whether dMLF apoptotic effects in the wing discs could be due to the activation of the JNK pathway. Overexpression of dMLF in the wing discs using the posterior *engrailed* (*en*)-*GAL4* driver (*en-GAL4, UAS-dMLF* flies) exhibited a

severe reduction of the posterior part of the adult wing but not of the anterior compartment used here as a control (Figure 1b). Strikingly, half-dose reduction of *bsk* (*en-GAL4, UAS-dMLF/bsk¹*) resulted in a suppression of this atrophied phenotype (Figure 1c). The same result was also seen with another allele of *bsk*, *bsk²* (Figure 1d). These data suggest that dMLF apoptotic effect requires Bsk activity.

Overexpression of dMLF induces Bsk (JNK) activation and apoptosis

The dMLF overexpression may act through Bsk to positively regulate the JNK pathway in the wing disc. Therefore, we monitored the effect of dMLF overexpression on the expression of *puc*, a gene highly expressed in Bsk-activated cells.^{39,40} For that purpose, we used an enhancer trap line, *puc^{E69}*, in which *LacZ* is inserted into the *puc* gene intron^{39,40} so that *puc* enhancer activity can be monitored with reference to *LacZ* expression. It is well known that the *puc* gene is highly expressed in Bsk-activated cells^{39,40} and the *puc* enhancer trap line has been widely employed to monitor Bsk activity *in vivo*.^{35,41,42} As shown in Figure 2Ab, *puc-LacZ* enhancer trap line is normally expressed in the stalk region of wing imaginal discs (Figure 2Aa).^{25,39,43} The dMLF overexpression driven by *en-GAL4* in the posterior region of the wing discs (Figure 2Ba) induced a strong ectopic expression of *puc-lacZ* in the posterior region of the wing discs (Figure 2Bb). In contrast, ectopic expression of *puc-lacZ* was not seen in the control anterior region. These results indicate that overexpression of dMLF can promote ectopic activation of the JNK pathway.

As noted previously,²⁶ *en-GAL4*-driven overexpression of dMLF in the wing imaginal discs induced programmed cell death in the posterior region, although restricted in some areas (Figure 3). Altogether, these data indicate that overexpression of dMLF induces apoptosis through JNK activation during wing development, and consequently an atrophied phenotype of the wing posterior is exhibited in adults.

dMLF binds to genomic regions containing the DRE sequence of the *bsk* promoter in S2 cells

A direct interaction between dMLF and DREF has been demonstrated by both yeast two-hybrid assay using DREF as a bait and glutathione *S*-transferase pull-down assays.¹³ We reported that the DREF-binding consensus, the DRE sequence, 5'-TATCGATA-3' exists in the *bsk* promoter region and DREF activates *bsk* gene transcription.²⁵ Therefore, an attractive hypothesis is that dMLF

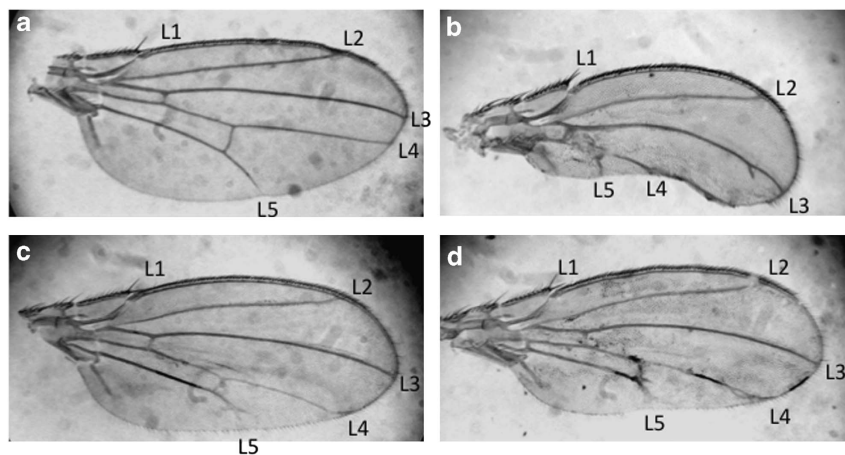


Figure 1. The *bsk* mutant suppresses the wing phenotype induced by overexpression of dMLF. (a) *en-GAL4/+*. Normal wings have five wing veins (L1, L2, L3, L4 and L5). The wing anterior includes L1, L2 and L3, whereas the wing posterior includes L4 and L5. (b) *en-GAL4, UAS-dMLF/+*. Overexpression of dMLF in wing imaginal discs results in an atrophied phenotype of the wing posterior. (c) *en-GAL4, UAS-dMLF/bsk¹*. (d) *en-GAL4, UAS-dMLF/bsk²*. The aberrant wing phenotype was suppressed by crossing with loss-of-function alleles of *bsk*. The results suggest that dMLF positively regulates the JNK pathway.

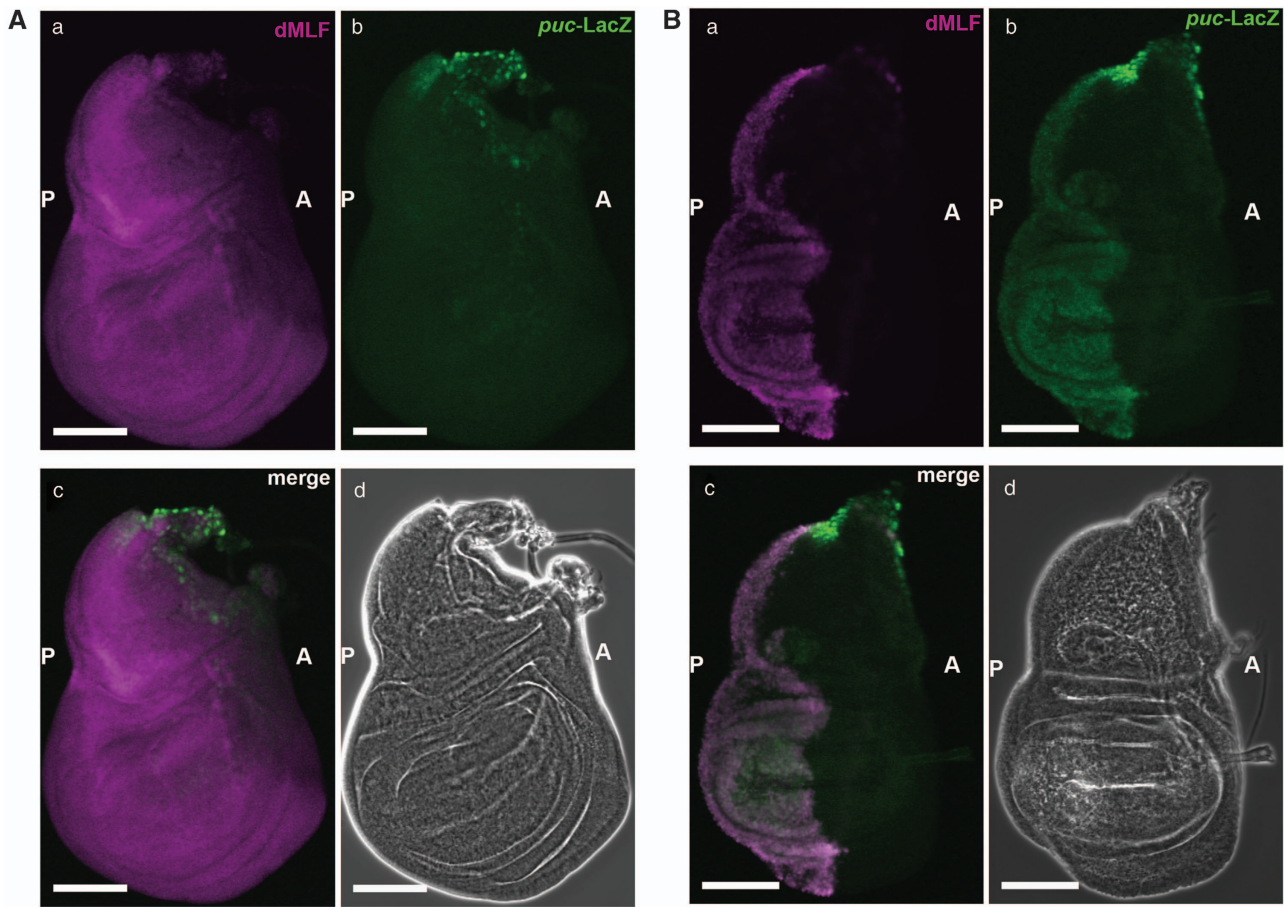


Figure 2. Expression of dMLF and *puc-LacZ* driven by *en-GAL4* in wing imaginal discs. **(A)** Immunostaining of a wing disc from an *en-GAL4/+; puc^{E69}/+* fly with anti-dMLF IgG and anti-LacZ IgG. The settings were different from those in **(B)** in order to visualize the weak signals of endogenous dMLF in the control strain. (a) Immunostaining with anti-dMLF IgG (magenta). (b) Immunostaining with anti-LacZ IgG (green). (c) Merged image of dMLF and LacZ signals. (d) Phase contrast image of the wing disc. Scale bars, 100 μ m. A, anterior; P, posterior. **(B)** Immunostaining of a wing disc from an *en-GAL4, UAS-dMLF/+; puc^{E69}/+* fly with anti-dMLF IgG and anti-LacZ IgG. (a) Expression of dMLF (magenta) driven by *en-GAL4*. (b) Ectopic expression of *puc* (green) detected in the posterior of the wing disc on overexpression of dMLF. (c) Merged image of anti-dMLF and anti-LacZ signals. These results indicate that overexpression of dMLF induces ectopic JNK activation. (d) Phase contrast image of the wing disc. Scale bars, 100 μ m.

and DREF could act together to upregulate *bsk* transcription. We therefore performed ChIP assays of S2 cell extracts immunoprecipitated with anti-dMLF immunoglobulin G (IgG) followed by quantitative real-time PCR using primers that amplify the *bsk* gene promoter region containing the DRE sequence (Figure 4a, region 1). The 2 kb upstream region from the transcription initiation site of the *bsk* gene was chosen as a negative control because it does not contain a DRE sequence (Figure 4a, region 2).

Amplification of the region 1 in the immunoprecipitates with an anti-dMLF IgG was 5.81-fold higher than that with the control IgG (Figure 4b). In contrast, no amplification was observed for the far upstream region 2. These results indicate that dMLF binds to the *bsk* gene promoter region containing the DRE sequence *in vivo*.

dMLF is required for *bsk* gene promoter activity in S2 cells

To further examine the requirement of dMLF for *bsk* gene promoter activity, we carried out dMLF RNA interference (RNAi) experiments in cultured *Drosophila* S2 cells (Figure 5). Measuring levels of dMLF proteins in S2 cells by western immunoblot analysis confirmed efficient knockdown of the *dMLF* gene after treatment with *dMLF*dsRNA (Figure 5b). We conducted transient luciferase expression assays with the wild-type *bsk* gene promoter-luciferase reporter gene after treating S2 cells with *dMLF*dsRNA, *DREF*dsRNA,

control *YFP*dsRNA or no dsRNA (Mock). Treatment of S2 cells with *dMLF*dsRNA and *DREF*dsRNA reduced *bsk* gene promoter activity by 80% and 90%, respectively, whereas control *YFP*dsRNA treatment exerted no effect (Figure 5c). These results indicate that both DREF and dMLF are required for *bsk* promoter activity.

dMLF interacts with DREF *in vivo*

As reported previously, dMLF genetically interacts with DREF and suppresses the rough-eye phenotype induced by overexpression of DREF.¹³ Therefore, to further examine the relationship between dMLF and DREF in the wing development, we tested whether changing DREF levels could affect the consequences of dMLF overexpression. Thus, transgenic fly lines carrying *UAS-DREF*, *UAS-dref-IR* or *UAS-GFP* (as a control) were crossed with dMLF overexpression lines. Flies with *en-GAL4, UAS-dMLF/ UAS-DREF* and *UAS-dref-IR/+*; *en-GAL4, UAS-dMLF/+* showed lethality (Table 1). However, when the *UAS-GFP* transgenic line was used for crossing as a control, the flies were viable (Table 1). In addition, although it is reported that *UAS-dref-IR* strain 15 was lethal when driven by *en-GAL4*,⁴⁴ the *en-GAL4* derived expression of *UAS-DREF* or *UAS-dref-IR* alone under these conditions exerted no effect on viability (Table 1).⁴⁵ The *en-GAL4* driver is expressed very early and throughout development, and thus its use could lead to early development defects before any adult phenotype could be

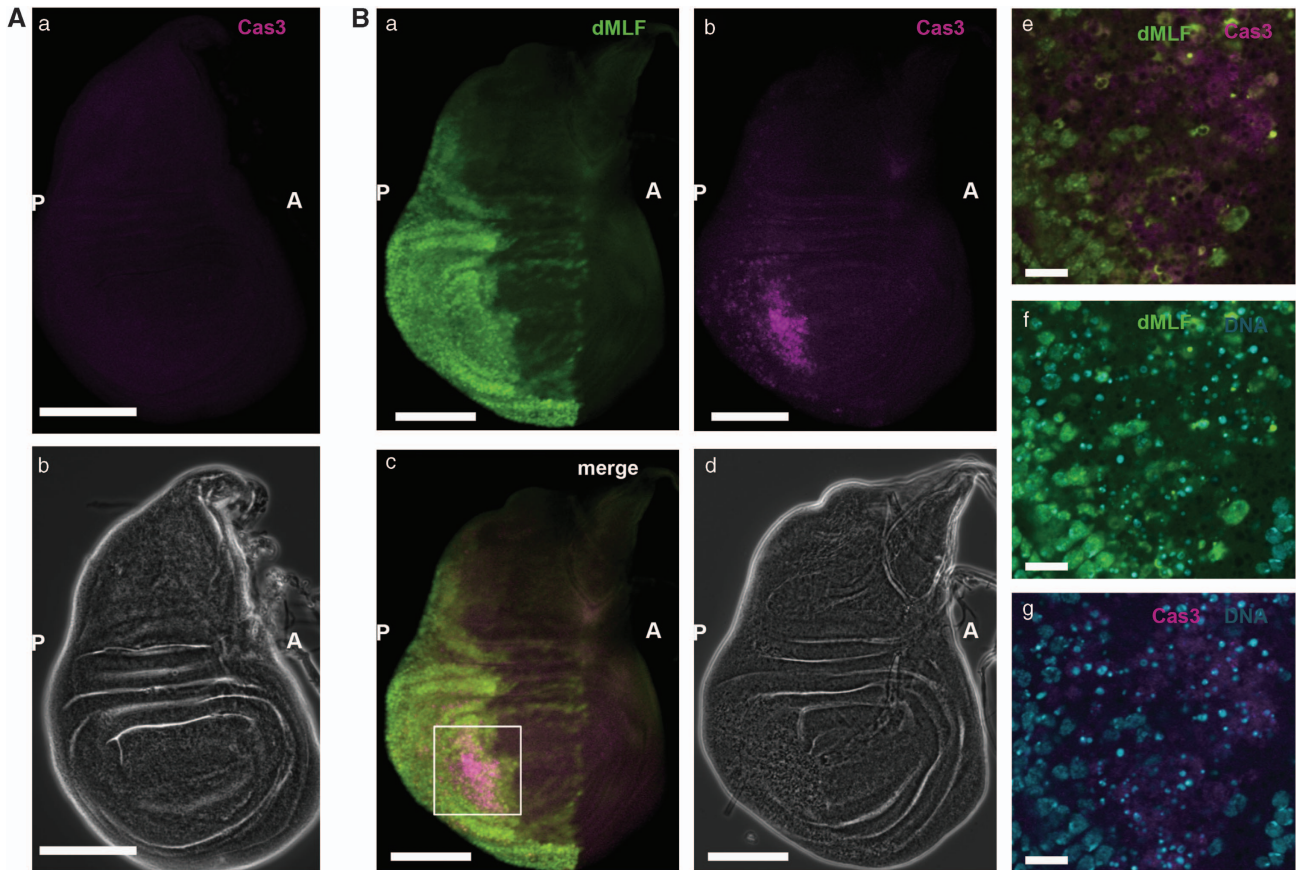


Figure 3. Overexpression of dMLF in the wing imaginal discs induces programmed cell death. **(A)** Immunostaining of a wing disc from an *en-GAL4/+* fly with anti-activated caspase-3 IgG. (a) Activated Caspase-3 is not apparent without overexpression of dMLF. (b) Phase contrast image. Scale bars, 100 μ m. A, anterior; P, posterior. **(B)** Immunostaining of a wing disc from an *en-GAL4, UAS-dMLF/+* fly with anti-dMLF IgG, anti-activated caspase-3 IgG. DNA was labeled with DAPI. (a) Expression of dMLF (green) driven by *en-GAL4* in wing discs. (b) Activated Caspase-3 (magenta) is present in posterior of wing discs on overexpression of dMLF. (c) Merged image of anti-dMLF and anti-active Caspase-3 signals. (d) Phase contrast image. Scale bars are for 100 μ m. (e–g) A higher magnification of the region marked with the square in **(Bc)**. Fragmentation of DNA (blue) by apoptosis via caspase-3. Scale bars, 20 μ m.

visualized. The synthetic lethality resulting from DREF and dMLF overexpression could simply be because of the fact that overexpression of each protein leads to cell death. The lethal effect of dMLF overexpression when DREF levels are reduced could be due to the trapping by DNA-unbound dMLF of the remaining low amounts of DREF that would thus become unavailable to control the expression of essential genes. In any of the events, these results suggest that dMLF and DREF act together to play a critical role during *Drosophila* development.

In addition, dMLF and DREF have been shown to interact directly by both yeast two-hybrid assay using DREF and glutathione *S*-transferase pull-down assays *in vitro*.¹³ Therefore, to investigate this interaction between dMLF and DREF *in vivo*, immunoprecipitation assays were performed using an anti-dMLF IgG with crude lysates of S2 cells. In immunoblot analyses of the immunoprecipitates with anti-dMLF IgG, bands for DREF were detected (Figure 5d, lane 2), whereas none were found in the immunoprecipitates with control IgG or no antibody sample (Figure 5d, lanes 3 and 4). These data support the idea that dMLF and DREF truly interact *in vivo* to regulate target gene expression.

DISCUSSION

Since the identification of dMLF as a partner of DREF by yeast two-hybrid screen, biological significance of interactions of these two proteins has been a long-term puzzle. In the present study, we demonstrated that dMLF acts with DREF to stimulate *bsk* promoter

activity and consequently activates the JNK pathway. Our present data thus shed light on a dMLF function as a co-activator of the transcription factor DREF. DREF is a major regulator of transcription that has been under intensive studies for more than a decade.^{22,46} So far, only two negative regulators of DREF have been identified that inhibit the DNA-binding activity of DREF: dMi-2, a component of chromatin remodeling complex,⁴⁷ and the transcription factor Distal-less.⁴⁸ The present work thus also constitutes the first example of a possible co-activator of DREF.

Interestingly, dMLF is known to also interact physically and genetically with Su(fu), a negative regulator of the Gli/Ci transcription factor involving Hh signaling pathway.²⁶ Notably, both DREF and Su(fu) share common interactors. Indeed, Yeast two-hybrid screen using DREF and Su(fu) as bait identified both dMLF and dMi-2.^{26,47} It would be interesting to compare chromatin immunoprecipitation (ChIP) sequence data with DREF, Su(fu), dMi-2 and dMLF. In addition, dMLF controls the activity of the transcription factor Lozenge by regulating its stability during development of crystal cells.³⁰ Therefore, dMLF appears to contribute to regulation of transcription factor activity by several distinct mechanisms.

In the present study, we found that dMLF overexpression results in an atrophied wing phenotype associated with apoptosis and activation of the JNK signaling pathway. It has been reported that overexpression of dMLF in the wing imaginal disc induces apoptosis and DNA replication,²⁶ whereas the JNK pathway is known to be involved in apoptosis and cell proliferation.^{32–34}

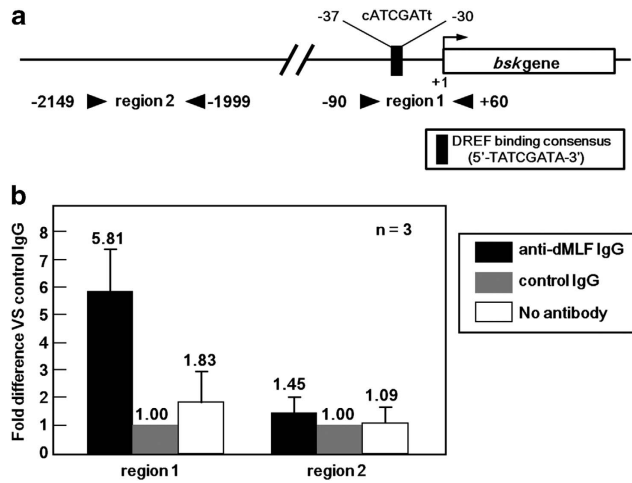


Figure 4. (a) Schematic illustration of the DREF-binding consensus sequence in the 5'-flanking region of the *bsk* gene. Arrowheads show the positions of primers used for the ChIP assays for two genomic regions (region 1, proximal, and region 2, distal). (b) Chromatin immunoprecipitation results. The data shown are derived from quantitative real-time PCR analysis of two genomic regions 1 and 2. Chromatin from S2 cells was immunoprecipitated with either anti-dMLF IgG or control rabbit IgG. The fold different values are for anti-dMLF IgG immunoprecipitated samples compared with the corresponding control rabbit IgG immunoprecipitated sample defined as 1.00. A sample without antibody treatment was also included as a negative control (no antibody column).

Therefore, it is suggested that dMLF induces cell proliferation as well as apoptosis via activation of the JNK pathway. There is recent evidence that the JNK pathway activates the transcriptional co-activator Yorkie (Yki) in the Hippo pathway.⁴⁹ The latter is well known as a tumor suppressor pathway and it represses expression of the apoptosis inhibitor DIAP1 and the cell cycle regulator Cyclin E via inactivation of the Yki by Warts, resulting in cell cycle arrest and induction of apoptosis.^{50,51} Therefore, the JNK pathway activated by dMLF may promote cell proliferation through the Hippo pathway. It should also be noted that DREF activates transcription of both the *bsk* gene²⁵ and the *warts* gene.²⁴

In the present study, we found that dMLF binds to the *bsk* gene promoter region containing the DRE sequence *in vivo* and is required for activation of the *bsk* promoter. In addition, we confirmed that the dMLF protein interacts with DREF *in vivo* using immunoprecipitation assays. Although we could not exclude other possibilities, our data support the notion that dMLF-induced JNK activation is, at least in part, mediated by DREF. Indeed, these results suggest that dMLF forms complexes with DREF and both factors act together to enhance *bsk* promoter activity and consequently activate the JNK pathway. In addition, we cannot exclude the possibility that dMLF may directly binds to the *bsk* gene promoter region. However, as dMLF is known to physically interact with DREF,¹³ it is more likely recruited on the *bsk* promoter via DREF. Although our findings were obtained with the wing system, JNK pathway regulation by dMLF may also be important in hemocyte differentiation. In any event, dMLF and DREF might control complex signaling networks in a well-balanced way. Identification of additional genetic interactants with dMLF by genetic screening may provide clues for deeper understanding of dMLF functions *in vivo*.

MATERIALS AND METHODS

Oligonucleotides

To carry out ChIP assays, the following PCR primers were chemically synthesized. These primer sets were designed to amplify 150 base pair (bp) amplicons: *bsk* + 60, F: 5'-GCGGCACCTTCGCATGAGAATAATTG-3' *bsk*-90,

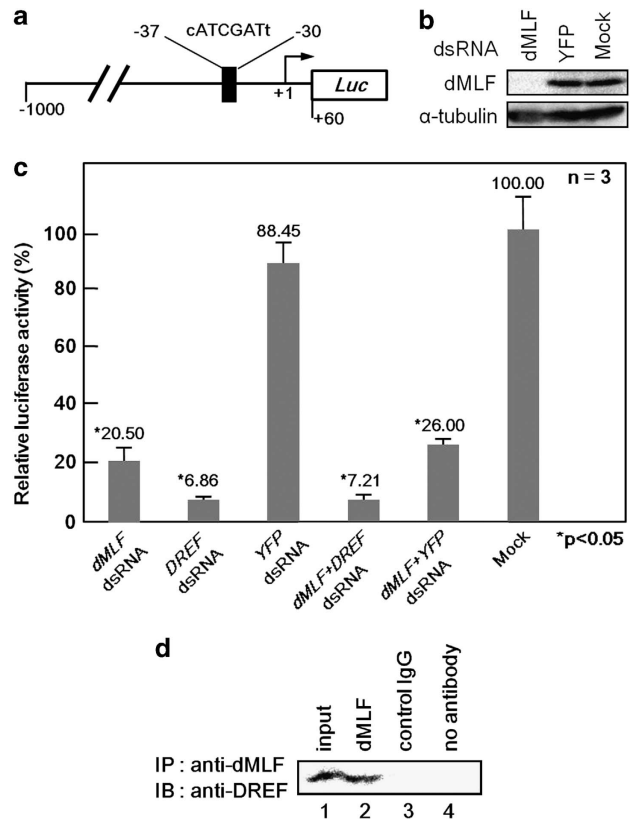


Figure 5. (a) Schematic features of the *bsk* promoter-luciferase fusion plasmid. The DRE-like sequence is indicated. (b) Western immunoblot analysis of S2 cells treated with *dMLF*dsRNA, *YFP*dsRNA or no dsRNA (Mock). Proteins were probed with anti-dMLF IgG and anti- α -tubulin IgG. (c) Effects of *dMLF*dsRNA treatment on *bsk* gene promoter activity in S2 cells. Mean activities with s.d. from three independent transfections are shown, with the *P*-value by Welch's *t*-test. (d) dMLF and DREF interact *in vivo*. Extracts of S2 cells were first immunoprecipitated with anti-dMLF IgG followed by immunoblotting with anti-DREF IgG.

R: 5'-TCGATTGGCTGACTTTAGCCGTTTC-3'; *bsk*-1999, F: 5'-TTCAGGGATATGAACGCAAATTGCCG-3'; *bsk*-2149, R: 5'-AATGCTGACGTTCTTCAGTTGCTGG-3'.

Plasmids

The plasmids p5'-1000bskwt-Luc and p5'-1000bskmutDRE-Luc used in the luciferase transient expression assays were as described previously.^{25,43}

Fly stocks

Flies were cultured at 18 °C or 25 °C on standard food. Canton S flies were used as the wild-type strain. The *UAS-dMLF*(II) line has been described previously²⁶ and the *en-GAL4* driver line was kindly provided by Dr N Dyson (Harvard Medical School, Charlestown, MA, USA). The *puc*^{E69}/*TM3* line was kindly provided by Dr T Adachi-Yamada (Gakushuin University, Tokyo, Japan). The mutant stocks *bsk*¹/*CyO* and *bsk*²/*CyO* used in this study were obtained from the Bloomington Drosophila stock center (Bloomington, IN, USA). The *UAS-DREF* (II) and *UAS-dref-IR* (X) lines were as described previously.⁴⁴

Inspection of wing phenotype

Ripped wings of adult flies were mounted on slides with Hoyer's medium and inspected with an Olympus SZX12 microscope equipped with an Olympus CAMEDIA C-3030 ZOOM (Olympus, Tokyo, Japan).

Immunohistochemistry

Third instar larvae were dissected in *Drosophila* Ringer's solution and imaginal discs were collected and fixed in 4% paraformaldehyde in

Table 1. Summary of effects on viability by the genetic interaction between dMLF and DREF

Genotype		Percentage
<i>en-GAL4, UAS-dMLF/CyO</i>	<i>en-GAL4, UAS-dMLF/UAS-GFP</i>	39% (<i>n</i> = 57)
<i>UAS-GFP</i> (II)	<i>UAS-GFP/CyO</i>	61%
<i>en-GAL4, UAS-dMLF/CyO</i>	<i>en-GAL4, UAS-dMLF/UAS-DREF</i>	0% (<i>n</i> = 40)
<i>UAS-DREF</i> (II)	<i>UAS-DREF/CyO</i>	100%
<i>en-GAL4, UAS-dMLF/CyO</i>	<i>UAS-dref-IR/+ or y;</i> <i>en-GAL4, UAS-dMLF/+</i>	0% (<i>n</i> = 81)
<i>UAS-dref-IR</i> (X) #10	<i>UAS-dref-IR/+ or y; +/CyO</i>	100%
<i>en-GAL4/CyO</i>	<i>UAS-dref-IR/+ or y; en-GAL4</i>	49% (<i>n</i> = 199)
<i>UAS-dref-IR</i> (X) #10	<i>UAS-dref-IR/+ or y; +/CyO</i>	51%
<i>en-GAL4, UAS-dMLF/CyO</i>	<i>UAS-dref-IR/+ or y;</i> <i>en-GAL4, UAS-dMLF/+</i>	0% (<i>n</i> = 38)
<i>UAS-dref-IR</i> (X) #15	<i>UAS-dref-IR/+ or y; +/CyO</i>	100%
<i>en-GAL4/CyO</i>	<i>UAS-dref-IR/+ or y; en-GAL4</i>	59% (<i>n</i> = 228)
<i>UAS-dref-IR</i> (X) #15	<i>UAS-dref-IR/+ or y; +/CyO</i>	41%

Abbreviations: dMLF, *Drosophila* myelodysplasia/myeloid leukemia factor; DREF, DNA replication-related element-binding factor.

phosphate-buffered saline (PBS) for 15 min at 25 °C. After washing with PBS containing 0.3% Triton X-100 (PBS-T), the samples were blocked with PBS-T containing 10% normal goat serum for 30 min at 25 °C and incubated with rabbit anti-dMLF antibody (1:500), rat anti-dMLF polyclonal antibody (1:500), mouse anti- β -galactosidase monoclonal antibody (1:500) or rabbit anti-active caspase-3 antibody (1:500; BD Pharmingen, Tokyo, Japan) at 4 °C for 16 h. After extensive washing with PBS-T, the imaginal discs were incubated with anti-rabbit IgG conjugated with Alexa 488 (1:400; Invitrogen, Life Technologies, San Diego, CA, USA) or anti-mouse IgG conjugated with Alexa 546 (1:400; Invitrogen) for 3 h at 25 °C. After extensive washing with PBS-T and PBS, samples were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and inspected with an Olympus FLUOVIEW FV10i.

Preparation of double-stranded RNA (dsRNA) for RNAi experiments

The dsRNA was prepared using a RiboMax T7 kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNAi analysis was carried out as described earlier.^{21,52}

Chromatin immunoprecipitation

ChIP was performed using a ChIP Assay kit as recommended by the manufacturer (Merck Millipore, Billerica, MA, USA) with minor modifications.⁵³ Approximately 2×10^7 S2 cells were fixed in 1% formaldehyde at 37 °C for 10 min, quenched in 125 mM glycine for 5 min at 25 °C, collected and washed twice in PBS containing protease inhibitors (1 mM PMSF, 1 μ g/ml aprotinin and 1 μ g/ml pepstatin A) and then lysed in 2 ml of SDS lysis buffer (Merck Millipore). Lysates were sonicated to break DNA into fragments of <1 kb and centrifuged at 15 300 *g* for 10 min at 4 °C. The sonicated cell supernatants were diluted 10-fold in ChIP Dilution Buffer (Merck Millipore) and precleared with 80 μ l of salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4 °C. After brief centrifugation, supernatants were incubated with 1 μ g of normal rabbit IgG (Sigma-Aldrich, St Louis, MO, USA) or anti-dMLF rabbit IgG for 16 h at 4 °C. Salmon sperm DNA/protein A agarose-50% slurry was added, followed by incubation for 1 h at 4 °C. After washing, immunoprecipitated DNA was eluted with elution buffer containing 1% SDS and 0.1 M NaHCO₃. Then, protein-DNA crosslinks were reversed by heating at 65 °C for 4 h. After deproteinization with proteinase K, DNA was recovered. Then, the immunoprecipitated DNA fragments were detected by quantitative real-time PCR using SYBR Green I (Takara Bio Inc., Shiga, Japan) and the Applied Biosystems 7500 Real Time PCR system (Life Technologies, Foster City, CA, USA). The $\Delta\Delta$ CT value of each sample was calculated by subtracting the CT value for the input sample from the CT value obtained for the

immunoprecipitated sample. Fold change was calculated by raising 2 to the $\Delta\Delta$ CT power. The $\Delta\Delta$ CT was calculated by subtracting the Δ CT value for the sample immunoprecipitated with control IgG.⁵⁴

Luciferase transient expression assays

For luciferase transient expression assays, 1×10^5 S2 cells were plated in 24-well dishes. Transfection of various DNA mixtures was performed using Cell-Fectin reagent (Invitrogen) and cells were harvested 48 h thereafter. Luciferase activity was measured as described earlier^{21,52,55} and normalized to *Renilla* luciferase activity using pAct5C-seapansy as an internal control.¹⁸ All plasmids for transfection were prepared using a QIAGEN plasmid Kit (Qiagen, Venlo, Netherlands).

For dsRNA interference experiments, 30 μ g of *dMLF*dsRNA, *DREF*dsRNA or *YFP*dsRNA were added to 1×10^6 S2 cells plated in each of six-well dishes. At 72 h after RNAi treatment, the cells were transfected with various DNA mixtures and harvested 48 h later for processing for the luciferase assay as described above.

All transient expression data reported in this study are means from three independent experiments, each performed in triplicate. Average relative luciferase activity was graphed and statistically analyzed with the Welch's *t*-test.

Western immunoblot analysis

Whole-cell extracts from S2 cells, prepared as described earlier,⁴³ were applied to 10% polyacrylamide gels containing 0.1% SDS and transferred to polyvinylidene difluoride membranes. Blotted membranes were blocked with Tris-buffered saline (50 mM Tris-HCl, pH 8.3 and 150 mM NaCl) containing 10% skim milk for 1 h at 25 °C and incubated with an anti-dMLF IgG in a 1:1000 dilution, or an anti- α tubulin monoclonal antibody (Sigma-Aldrich) in a 1:5000 dilution at 4 °C for 16 h. After washing with Tris-buffered saline, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG (Life Technologies, Waltham, MA, USA) in a 1:5000 dilution for 1 h at 25 °C. Detection was performed with ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK), and images were analyzed with a Lumivision Pro HSII image analyzer (Aisin Seiki, Aichi, Japan).

Immunoprecipitation

Whole-cell extracts were prepared in lysis buffer containing a proteinase inhibitor mix. Immunoprecipitation was performed with 800 μ g of lysates with anti-dMLF rabbit IgG or the normal rabbit IgG (Sigma-Aldrich) and protein A-Sepharose (GE Healthcare). The protein A-antibody complexes were washed with the same buffer. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with mouse anti-DREF IgG.¹⁵

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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