Ph.D.Thesis

Cell biological and genetical studies on *Drosophila* myeloid leukemia factor (dMLF) and DRE/DREF transcriptional regulatory pathway

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Chapter 1

General introduction

General introduction

The myelodysplasia / myeloid leukemia factor

The myelodysplasia / myeloid leukemia factor (MLF) family is a group of proteins conserved in human, mouse and fly (Kuefer *et al*. 1996; Ohno *et al*. 2000; Williams *et al*. 1999; Yoneda-Kato *et al*. 1996). The human MLF1 was originally found in the form of a fusion protein with nucleophosmin (NPM) generated by the t(3;5)(q25.1;q34) chromosomal translocation, which is associated with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Yoneda-Kato *et al*. 1996). 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 hematopoietic cellular growth and differentiation (Yoneda-Kato *et al*. 1996). NPM is a major nucleolar phosphoprotein, which is significantly more abundant in tumors and proliferating cells than in normal resting cells (Chan 1989; Feuerstein *et al*. 1988). It has also been reported that NPM shuttles between the nucleus and cytoplasm, although its precise function is yet unknown (Borer *et al*. 1989).

The hMLF1 protein is normally located in the cytoplasm, whereas its fused form with NPM is mostly located in the nucleus with highest levels in the nucleolus (Yoneda-Kato *et al*. 1996). hMLF1 contains no recognizable domains or motifs except for a characteristic RSXSXP motif, the binding sequence for 14-3-3 protein (Fig. 1A) (Muslin *et al*. 1996; Yaffe *et al*. 1997), which is involved in regulating cell division, apoptosis, and differentiation. In addition, it has been reported that mammalian MLF1 is associated with 14-3-3ζ, Madm (MLF1-adaptor molecule), MLF1IP/KLIP1, Manp (MLF1-associated nuclear protein) and CSN3 (COP9 signalosome complex subunit 3), and hMLF1 regulates the cell cycle via the CSN3/COP1 pathway that influences tumor suppressor p53 levels (Hanissian *et al*. 2004; Lim *et al*. 2002; Winteringham *et al*. 2006; Yoneda-Kato *et al*. 2005), although the biochemical activity of hMLF1 has yet to be fully characterized.

In contrast to mammals, which contains two closely related protein, hMLF1 and hMLF2 (Kuefer *et al*. 1996), *Drosophila melanogaster* has a single gene (*dMLF*) encoding a protein homologous to hMLF1 and hMLF2. dMLF was first identified by yeast two-hybrid screening using DNA replication-related element-binding factor (DREF) as the bait (Ohno *et al*. 2000). DREF is a transcription factor in *Drosophila* that regulates proliferation-related genes such as *PCNA*, *Cyclin A, DNA polymerase α* and others (Hirose *et al*. 1993; Hirose *et al*. 1996; Ohno *et al*. 1996; Takahashi *et al*. 1996; Sawado *et al*. 1998; Okudaira *et al*. 2005; Tsuchiya *et al*. 2007;

Ida *et al*. 2007; Matsukage *et al*. 2008; Nakamura *et al*. 2008; Fujiwara *et al*. 2012; Yoshioka *et al*. 2012). dMLF consists of 309 amino acid residues and especially, the central region of the dMLF protein (amino acids 96 to 202) displays the highest homology to those of hMLF1 (54% identity), hMLF2 (63%) (Fig. 1) and mouse homologue, Hemopoietic lineage switch (HLS7) (59%) (Ohno *et al*. 2000). And, it is reported that this highly conserved region of dMLF is necessary for binding to DREF (Ohno *et al*. 2000). dMLF localizes mainly in the nucleus, whereas dMLF that appears to be produced by the alternative splicing displays both nuclear and cytoplasmic localization (Sugano *et al*. 2007). It has been reported that the C-terminal 40 amino acid region of dMLF is necessary and sufficient for nuclear localization (Fig. 1A) (Sugano *et al*. 2007). Although dMLF also contains RSXSXP motif like hMLF1, this motif appears to be dispensable for subcellular localization of dMLF and its function is yet to be clarified (Fig. 1A) (Ohno *et al*. 2000). dMLF protein has been reported to interact physically and genetically with Suppressor of Fused (Su(fu)), a negative regulator of the Gli/Ci transcription factor involved in Hedgehog (Hh) signaling (Fouix *et al*. 2003). In addition, it has also been reported that dMLF interacts with the Hh pathway component Cos2 (Fouix *et al*. 2003) and suppresses the rough eye phenotype induced by overexpression of DREF (Ohno *et al*. 2000). Furthermore, 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 (Kazemi-Esfarjani *et al*. 2002). dMLF reduced the recruitment of the CRE Binding Protein (CBP) and Hsp70 into polyglutamine inclusions, both of these being among essential proteins apparently trapped in the inclusions (Kim *et al*. 2005). 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 (Bras *et al*. 2012). Lozenge is essential for differentiation of crystal cells and dMLF appears to play a role in stabilization of Lozenge protein (Bras *et al*. 2012).

It is also reported that overexpression of dMLF protein in various tissues induces abnormal development. For instance, ectopic expression of dMLF in the developing eye imaginal disc using an *eyeless-GAL4* driver resulted in a small-eye phenotype rescued by co-expression of *cyclin E*, suggesting involvement of dMLF in cell-cycle regulation (Sugano *et al*. 2008). In addition, 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 (Fouix *et al*. 2003). These observations suggest that dMLF plays multiple roles *in vivo*. However, the precise molecular mechanisms in which dMLF

induces abnormal development have yet to be understood. In this thesis study, based on the results and findings from genetic and cytological studies, possible roles of dMLF in regulating the signal transduction pathway via the DRE/DREF transcriptional regulatory system during *Drosophila* development are discussed.

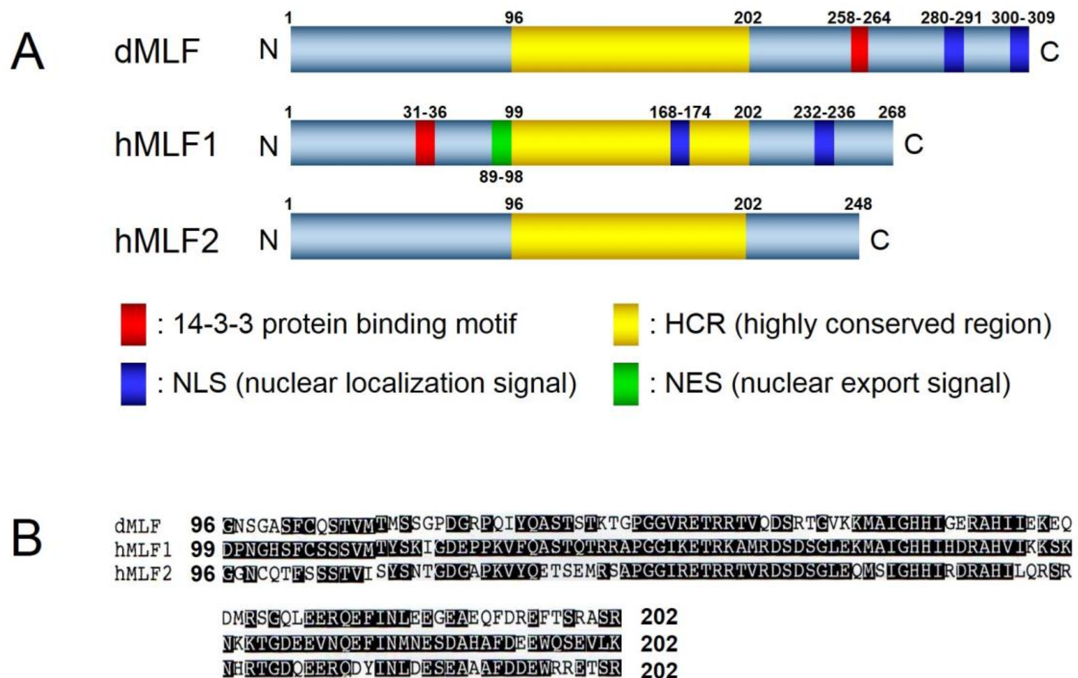

Fig. 1. Schematic representation of dMLF, hMLF1 and hMLF2 proteins.

(A) Structure of dMLF, hMLF1 and hMLF2 proteins.

dMLF contains RSXSXP motif (14-3-3 protein binding motif) like hMLF1, although its function is yet to be clarified. The highly conserved region (HCR) of dMLF is necessary for binding to DREF. The C-terminal 40 amino acid region of dMLF is necessary for nuclear localization.

(B) Amino acid sequences of the HCR of the dMLF, hMLF1 and hMLF2 proteins.

The HCR of dMLF displays the highest homology to those of hMLF1 (54% identity), hMLF2 (63%).

The DRE/DREF transcriptional regulatory system

As described previously, DREF is a transcription factor in *Drosophila* that regulates a wide variety of proliferation-related genes. *Drosophila* DREF is a homo-dimer of a polypeptides of 709 amino acid residues, and shares 22% identity in its amino acid sequence with the human homolog of 694 amino acid residues (Hirose *et al*. 1996; Ohshima *et al*. 2003). DREF specifically binds to the DNA replication-related element (DRE) sequences (5' TATCGATA), a common palindromic sequence in the promoter region of genes requiring for DNA replication/cell proliferation in *Drosophila*, and activates their gene transcription (Hirose et al., 1993; Ohno *et al*. 1996; Ryu *et al*. 1997).

Ectopic expression of a dominant negative form of DREF using GAL4-UAS targeted expression system results in inhibition of both DNA replication in eye imaginal disc and endo-replication in salivary gland (Hirose *et al*. 1999). In addition, overexpression of full length DREF in eye imaginal discs induced ectopic DNA synthesis and apoptosis in otherwise post-mitotic cells, and inhibits photoreceptor cell differentiation so that a severe rough eye phenotype develops in adults (Hirose *et al*. 2001). Knockdown of the *DREF* gene in imaginal discs causes defects in cell growth and cell cycle progression (Hyun *et al*. 2005), whereas more efficient knockdown in wing imaginal discs induces loss-of-vein phenotype (Otsuki *et al*. 2004). These results indicate that DREF is involved in regulation of cell proliferation during development of tissues.

Additionally, computational prediction indicated that 277 genes carry DRE motifs within their promoter regions (from -60 to +40) in *Drosophila* genome (Ohler *et al*. 2002). Moreover, it was reported that the DRE/DREF system involves in regulation of genes that are not only positive but also negative regulators of cell proliferation such as *PCNA*, the *DNA polymerase α, Cyclin A, D-raf, E2F, caudal, TBP, Orc2, skpA, eIF4a*, *rfc140*, *moira*, *osa*, *p53*, *wts*, *bsk* genes (Takahashi *et al*. 1996; Ohno *et al*. 1996; Ryu *et al*. 1997; Sawado *et al*. 1998; Choi *et al*. 2004; Okudaira *et al*. 2005; Thao *et al*. 2006; Ida *et al*. 2007; Tsuchiya *et al*. 2007; Nakamura *et al*. 2008; Trong-Tue *et al*. 2010; Fujiwara *et al*. 2012; Fujiwara *et al*. 2013). In addition, DREF is a component of the transcription complex containing TATA-box-binding protein-related factor 2 (TRF2) (Hochheimer *et al*. 2002). The transcription factor *Distal-less* (*Dll*) and the chromatin remodeling factor *Mi-2* genes were identified as inhibitors of DNA binding activity of DREF (Hayashi *et al*. 2006; Hirose *et al*. 2002). Therefore, these observations suggest that the DRE/DREF system is required for the expression of many genes *in vivo*. In this thesis study, I found that dMLF cooperates with DREF on the *bsk* promoter to activate its transcription.

Taken together with these reports, DREF appears to play an important role in transcription of the positive or negative regulators for cell proliferation via the signal transduction systems. Therefore, additional searching for DREF-target genes and factors interacting with DREF will provide clues of the regulatory mechanism for coordinated expression of many cell proliferation-related genes. In this thesis study, I examined the regulatory mechanism of DREF for cell proliferation through the JNK and the Hippo pathway, regulating cell proliferation by inducing apoptosis.

Outline of this thesis

Chapter 2: *Drosophila* myeloid leukemia factor acts with DREF to activate the JNK signaling pathway

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, I 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, I provide evidence that knockdown of dMLF reduced *bsk* gene promoter activity in S2 cells. Finally, I 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.

Chapter 3: The Hippo pathway as a target of the *Drosophila* DRE/DREF transcriptional regulatory pathway

The DRE/DREF transcriptional regulatory system has been demonstrated to activate a wide variety of genes with various functions. In *Drosophila*, the Hippo pathway is known to suppress cell proliferation by inducing apoptosis and cell cycle arrest through inactivation of Yorkie, a transcription co-activator. In the present study, it is found that half dose reduction of the *hippo* (*hpo*) gene induces ectopic DNA synthesis in eye discs that is suppressed by overexpression of DREF. Half reduction of the *hpo* gene dose reduced apoptosis in DREF-overexpressing flies. Consistent with these observations, overexpression of DREF increased the levels of hpo and phosphorylated Yorkie in eye discs. Interestingly, the *diap1-lacZ* reporter was seen to be significantly decreased by overexpression of DREF. Luciferase reporter assays in cultured S2 cells revealed that one of two DREs identified in the *hpo* gene promoter region was responsible for promoter activity in S2 cells. Furthermore, endogenous *hpo* mRNA was reduced in DREF knockdown S2 cells, and chromatin immnunoprecipitation assays with anti-DREF antibodies proved that DREF binds specifically to the *hpo* gene promoter region containing DREs *in vivo*. Together, these results indicate that the DRE/DREF pathway is required for transcriptional activation of the *hpo* gene to positively control Hippo pathways.

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Chapter 2

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

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 (Yoneda-Kato *et al*. 1996). NPM is a major nucleolar phosphoprotein that is significantly more abundant in tumors and proliferating cells than in normal resting cells (Feuerstein *et al*. 1988; Chan 1989). It has also been reported that NPM shuttles between the nucleus and cytoplasm (Borer *et al*. 1989).

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The MLF protein is conserved among various species in animals (Kuefer *et al*. 1996; Williams *et al*. 1999). In contrast to mammals that have two closely related proteins, hMLF1 and hMLF2 (Kuefer *et al*. 1996), *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 (Ohno *et al*. 2000). DREF is a transcription factor in *Drosophila* that regulates proliferation-related genes such as *PCNA*, *Cyclin A, DNA polymerase α* and others (Hirose *et al*. 1993; Hirose *et al*. 1996; Ohno *et al*. 1996; Takahashi *et al*. 1996; Sawado *et al*. 1998; Okudaira *et al*. 2005; Tsuchiya *et al*. 2007; Ida *et al*. 2007; Matsukage *et al*. 2008; Nakamura *et al*. 2008; Fujiwara *et al*. 2012; Yoshioka *et al*. 2012). 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%) (Ohno

et al. 2000). Furthermore, it is reported that this highly conserved region of dMLF is necessary for binding to DREF (Ohno *et al*. 2000). 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 (Fouix *et al*. 2003). In addition, it has also been reported that dMLF interacts with the Hh pathway component Cos2 (Fouix *et al*. 2003), and suppresses the rough eye phenotype induced by overexpression of DREF (Ohno *et al*. 2000). 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 (Sugano *et al*. 2008). 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 (Fouix *et al*. 2003). It is therefore conceivable that dMLF plays multiple roles in cell growth, survival, apoptosis and gene transcription.

In addition, other roles have been identified for dMLF. 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 (Kazemi-Esfarjani *et al*. 2002). 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 (Kim *et al*. 2005). 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 (Bras *et al*. 2012).

In this thesis study, I 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 (Martin-Blanco *et al*. 2000). JNK signaling is involved in processes including cell proliferation and apoptosis (Noselli *et al*. 1999; Davis 2000; Igaki *et al*. 2006). Apoptosis induced by JNK has an important role in the morphogenesis of the wing imaginal disc (Adachi-Yamada *et al*. 1999). 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 (Riesgo-Escovar *et al*. 1996; Sluss *et al*. 1996; Agnes *et al*. 1999). It is 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 (Yoshioka *et al*. 2012).

In this thesis study, I 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.

Materials and methods

Oligonucleotides

To carry out chromatin immunoprecipitation assays, the following PCR primers were chemically synthesized. These primer sets were designed to amplify 150 base pair (bp) amplicons:

bsk+60, F: 5’-GCGGCACTTTCGCATGAGAATAATTG-3’

bsk–90, R: 5’-TCGATTGGCTGACTTTAGCCGTTTCT-3’

bsk–1999, F: 5’-TTCAGGGATATGAACGCAAATTGCCG-3’

bsk–2149, R: 5’-AATGCTGACGTTCTTCAGTTGCTTGG-3’

Plasmids

The plasmids p5’-1000bskwt-Luc and p5’-1000bskmutDRE-Luc used in the luciferase transient expression assays were as described previously (Yoshioka *et al*. 2012; Yoshioka *et al*. 2008).

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 previously (Fouix *et al*. 2003) 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 (Indiana). The *UAS-DREF* (II) and *UAS-dref-IR* (X) lines were as described previously (Yoshida *et al*. 2004).

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).

Immunohistochemistry

Third instar larvae were dissected in *Drosophila* Ringer’s solution and imaginal discs were collected and fixed in 4% paraformaldehyde in 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 pharmigen, 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) 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) 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) according to the manufacturer's instructions. RNAi analysis was carried out as described earlier (Ida *et al.* 2007; Seto *et al.* 2006).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using a ChIP Assay kit as recommended by the manufacturer (Millipore) with minor modifications (Thao et al. 2006). 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 μg/ml aprotinin and 1 μg/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 (Millipore) and precleared with 80 μl of salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4 °C. After brief centrifugation, supernatants were incubated with 1 μg of normal rabbit IgG (Sigma-Aldrich) 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 0.1M $NaHCO_3$. 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) and the Applied Biosystems 7500 Real Time PCR system (Life Technologies). 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 $^{\Delta\Delta}C_T$ power. The $^{\Delta\Delta}C_T$ was calculated by subtracting the $^{\Delta}C_T$ value for the sample immunoprecipitated

with control IgG (Morrison *et al*. 1998).

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 (Ida *et al*. 2007; Seto *et al*. 2006; Hayashi *et al*. 1999) and normalized to *Renilla* luciferase activity using pAct5C-seapansy as an internal control (Sawado *et al*. 1998). All plasmids for transfection were prepared using a QIAGEN plasmid Kit (Qiagen).

For dsRNA interference experiments, 30 μg 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 (Yoshioka *et al*. 2008), 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 1:1,000 dilution, or an anti-α tubulin monoclonal antibody (Sigma-Aldrich) in a 1:5,000 dilution at 4 °C for 16 h. After washing with Tris-bufferd saline, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG (Life Technologies) in a 1:5,000 dilution for 1 h at 25 °C. Detection was performed with ECL Western blotting detection reagents (GE healthcare), and images were analyzed with a Lumivision Pro HSII image analyzer (Aisin Seiki).

Immunoprecipitation

Whole-cell extracts were prepared in lysis buffer containing a proteinase inhibitor mix. Immunoprecipitation was performed with 800 μg 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 (Hirose *et al*. 1996).

Results

dMLF* genetically interacts with *bsk

Transgenic fly lines overexpressing dMLF in the wing imaginal disc exhibit an atrophied phenotype of the posterior compartment of adult wings associated with caspase-3 activation and apoptosis in the wing imaginal disc (Fouix *et al*. 2003). In the wing disc, JNK activation is usually linked to the activation of caspase-3 (Adachi-Yamada *et al*. 2002). Therefore, I tested whether dMLF apoptotic effects in the wing dics 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 (Fig. 1B). Strikingly, half-dose reduction of *bsk* (*en-GAL4, UAS-dMLF/*bsk^1) resulted in a suppression of this atrophied phenotype (Fig. 1C). The same result was also seen with another allele of *bsk*, bsk^2 (Fig. 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 though Bsk to positively regulate the JNK pathway in the wing disc. Therefore, I monitored the effect of dMLF overexpression on the expression of *puc*, a gene highly expressed in Bsk-activated cells (Adachi-Yamada *et al*. 2002; Martin-Blanco *et al*. 1998). For that purpose, I used an enhancer trap line, puc^{E69}, in which *LacZ* is inserted into the *puc* gene intron (Adachi-Yamada *et al*. 2002; Martin-Blanco *et al*. 1998) 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 (Adachi-Yamada *et al*. 2002; Martin-Blanco *et al*. 1998) and the *puc* enhancer trap line has been widely employed to monitor Bsk activity *in vivo* (Adachi-Yamada *et al*. 1999; Tateno *et al*. 2000; Igaki *et al*. 2002). As shown in Fig. 2A, panel b, *puc-LacZ* enhancer trap line is normally expressed in the stalk region of wing imaginal discs (Fig. 2A, panel a) (Yoshioka *et al*. 2012; Yoshioka *et al*. 2008; Adachi-Yamada *et al*. 2002). The dMLF overexpression driven by *en-GAL4* in the posterior region of the wing disc (Fig. 2B, panel a) induced a strong ectopic expression of *puc-lacZ* in the posterior region of the wing disc (Fig. 2B, panel b). 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 (Fouix *et al*. 2003), *en-GAL4*-driven overexpression of dMLF in the wing

imaginal discs induced programmed cell death in the posterior region, although restricted in some areas (Fig. 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 (Ohno *et al*. 2000). It is reported that the DREF-binding consensus, the DRE sequence, 5'-TATCGATA-3' exists in the *bsk* promoter region and DREF activates *bsk* gene transcription (Yoshioka *et al*. 2012). Therefore, an attractive hypothesis is that dMLF and DREF could act together to upregulate *bsk* transcription. I therefore performed chromatin immunoprecipitation assays of S2 cell extracts immunoprecipitated with anti-dMLF IgG followed by quantitative real-time PCR using primers that amplify the *bsk* gene promoter region containing the DRE sequence (Fig. 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 (Fig. 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 (Fig. 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, I carried out dMLF RNA interference (RNAi) experiments in cultured *Drosophila* S2 cells (Fig. 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 (Fig. 5B). I 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 (Fig. 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 (Ohno *et al*. 2000). Therefore, to further examine the relationship between *dMLF* and *DREF* in the wing development, I 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* (Yoshida *et al*. 2004), the *en-GAL4* derived expression of *UAS-DREF* or *UAS-dref-IR* alone under these conditions exerted no effect on viability (Table. 1) (Yoshida *et al*. 2001). 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 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 a yeast two-hybrid assay using DREF and glutathione S-transferase pull-down assays *in vitro* (Ohno *et al*. 2000). 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 imunoprecipitates with anti-dMLF IgG, bands for DREF were detected (Fig. 6, lane 2), whereas none were found in the immunoprecipitates with control IgG or no antibody sample (Fig. 6, 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, I demonstrated that dMLF acts with DREF to stimulate *bsk* promoter activity and consequently activates the JNK pathway. My 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 (Matsukage *et al*. 2008; Fujiwara *et al*. 2013). So far, only three negative regulators of DREF have been identified that inhibit the DNA-binding activity of DREF: dMi-2, a component of chromatin remodeling complex (Hirose *et al*. 2002), the chromatin regulator XNP/dATRX (Valadez-Graham *et al*. 2012) and the transcription factor Distal-less (Hayashi *et al*. 2006). 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 (Fouix *et al*. 2003). 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 (Fouix *et al*. 2003; Hirose *et al*. 2002). It would be interesting to compare chromatin immunoprecipitation 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 (Bras *et al*. 2012). Therefore, dMLF appears to contribute to regulation of transcription factor activity by several distinct mechanisms.

In the present study, I 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 (Fouix *et al*. 2003), whereas the JNK pathway is known to be involved in apoptosis and cell proliferation (Noselli *et al*. 1999; Davis 2000; Igaki *et al*. 2006). 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 (Sun *et al*. 2011). 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 (Xu *et al*. 1995; Huang *et al*. 2005). 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* gene

(Yoshioka *et al*. 2012) and the *warts* gene (Fujiwara *et al*. 2012) and the *hippo* gene (Vo *et al*. 2014; See Chapter 3).

In this thesis study, I 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, I confirmed that the dMLF protein interacts with DREF *in vivo* using immunoprecipitation assays. Although other possibilities can not be excluded, the present 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, I 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 (Ohno *et al*. 2000), it is more likely recruited on the *bsk* promoter via DREF. Although my 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*.

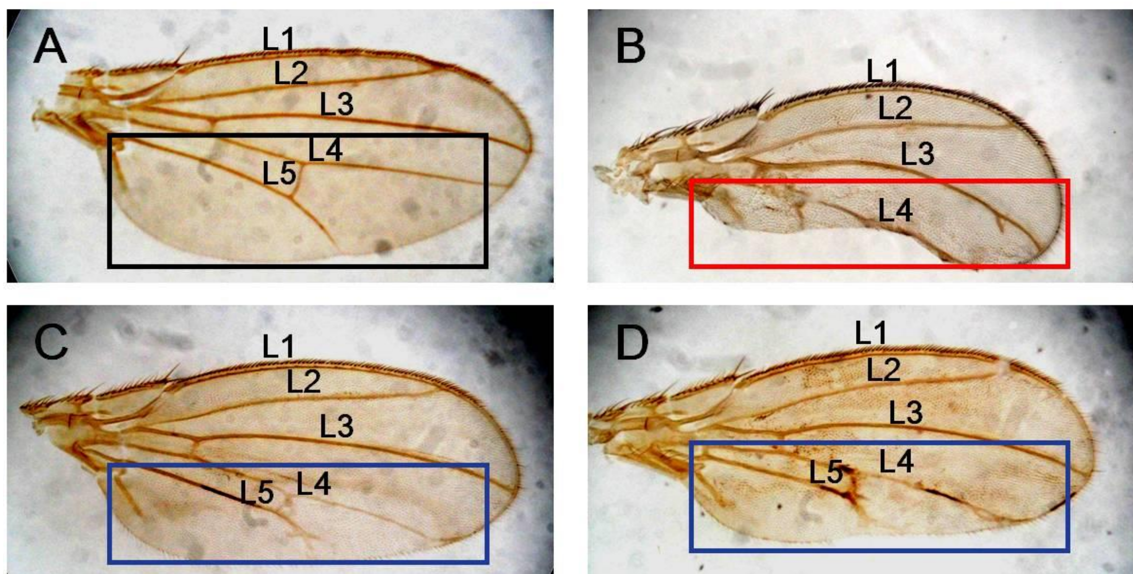

Fig. 1. The *bsk* mutant suppresses the wing phenotype induced by overexpression of dMLF.

(A) *en-GAL4/+*

Nomal 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 (black square).

(B) *en-GAL4, UAS-dMLF/+*

Overexpression of dMLF in wing imaginal discs results in an atrophied phenotype of wing posterior (red square).

(C) *en-GAL4, UAS-dMLF/bsk*^{1} (D) *en-GAL4, UAS-dMLF/bsk*^{2}

The aberrant wing phenotype was suppressed by crossing with loss of function alleles of *bsk* (blue square). The results suggest that dMLF positively regulates the JNK pathway.

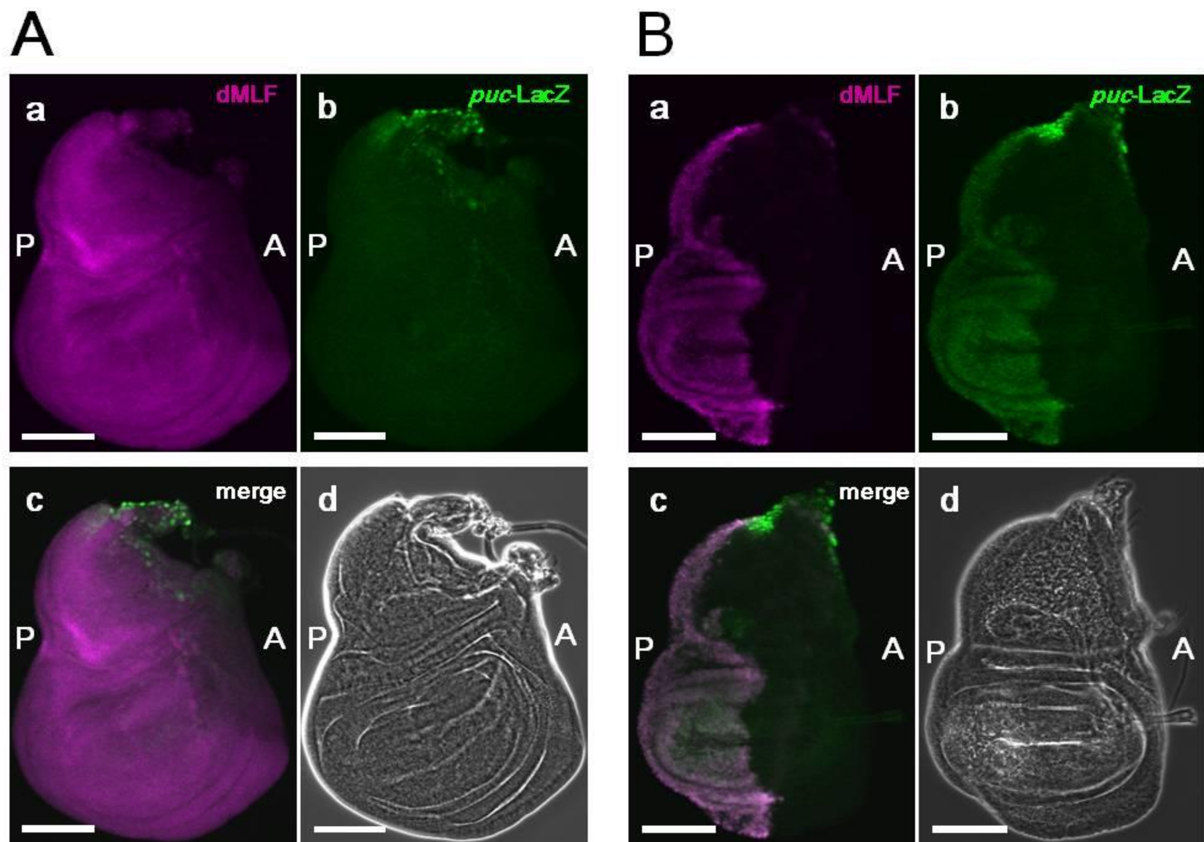

Fig. 2. Expression of dMLF and *puc-LacZ* driven by *en-GAL4* in wing imaginal discs.

(A) Immunostaining of a wing discs from *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 anti-dMLF and anti-LacZ signals. (d) Phase contrast image of the wing disc. Scale bars are for 100 µm. A: anterior, P: posterior.

(B) Immunostaining of a wing disc from *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 are for 100 µm. A: anterior, P: posterior.

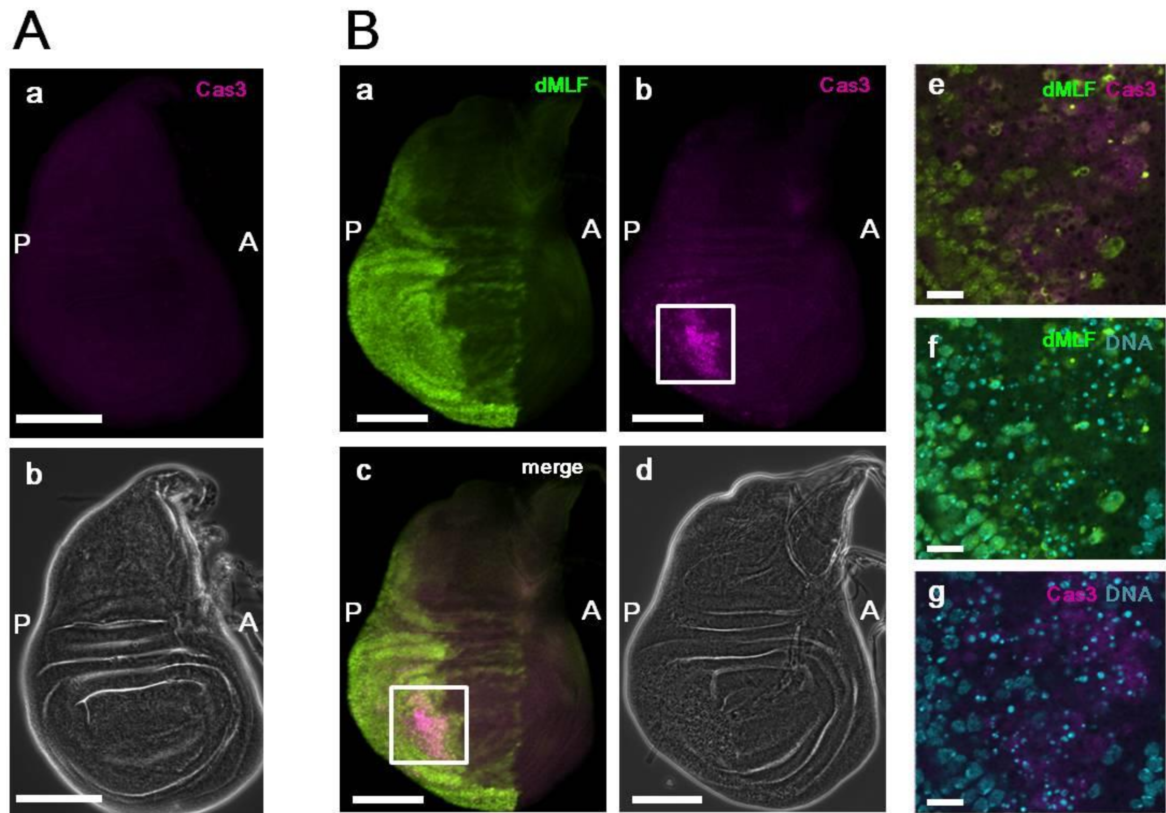

Fig. 3. Overexpression of dMLF in the wing imaginal discs induced programmed cell death.

(A*)* Immunostaing of a wing disc from *en-GAL4*/+ fly with anti-activated Caspase-3 IgG. (a) Activated Caspase-3 is not apparent without overexpression of dMLF. (b) Phase contrast image of the wing disc. Scale bars are for 100 µm. 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 disc. (b) Activated Caspase-3 (magenta) is present in posterior of wing discs on overexpression of dMLF. (c) Merged image of anti-dMLF and anti-activated Caspase-3 signals. (d) Phase contrast image of the wing disc. Scale bars are for 100 µm. A: anterior, P: posterior. (e), (f), (g) A higher magnification of the region marked with the square in panel B-b, c. Fragmentation of DNA (blue) by apoptosis via Caspase-3. Scale bars are for 20µm.

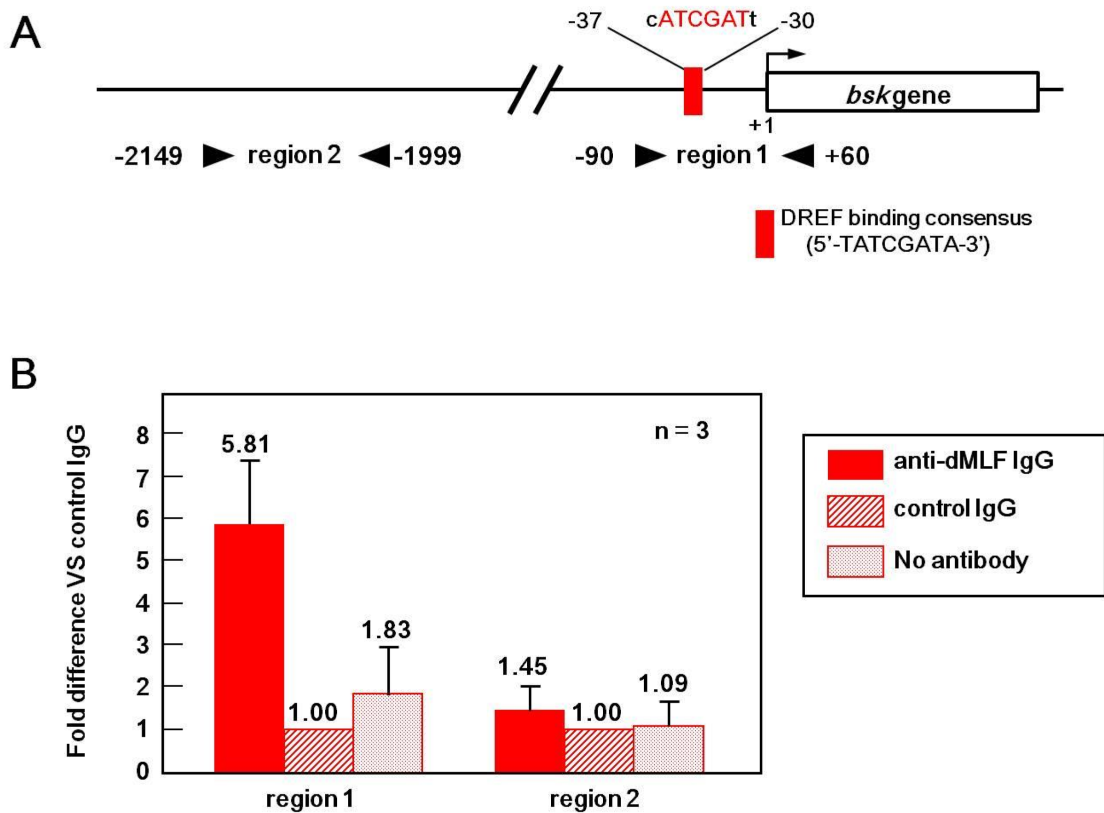

Fig. 4. (A) Schematic illustration of the DREF-binding consensus sequence in the 5'-flanking region of the *bsk* gene. Arrowheads show the positions of primers used for the chromatin immunoprecipitation assays for two genomic regions (region 1, proximal and region 2, distal).
(B) Chromatin immunoprecipitation 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).

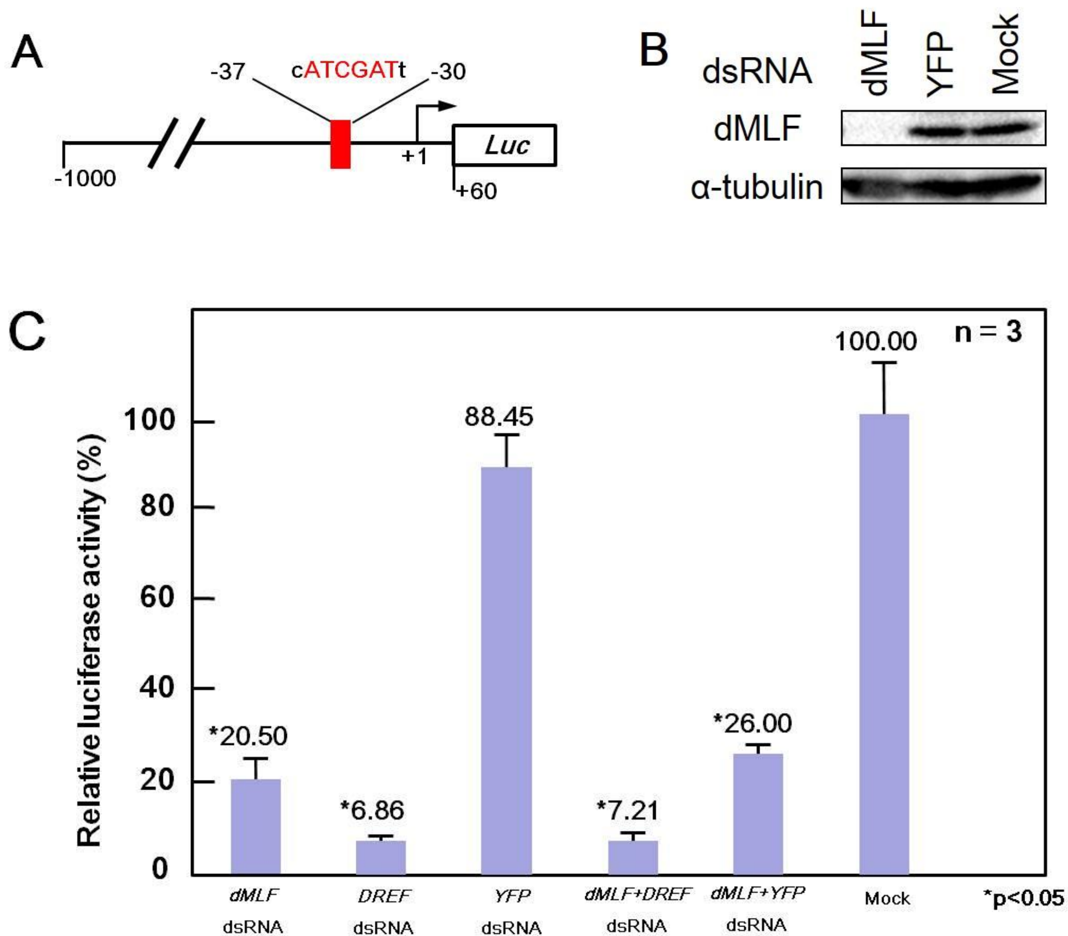

Fig. 5. (A) Schematic features of the *bsk* promoter-*luciferase* fusion plasmid. The DRE-like sequence is indicated.

(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 standard deviations from three independent transfections are shown, with the *P*-value by Welch's *t*-test.

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

	Genotype	Percentage	
en-GAL4, UAS-dMLF/ CyO × *UAS-GFP* (Ⅱ)	*en-GAL4, UAS-dMLF/ UAS-GFP*	39%	(n = 57)
	UAS-GFP/ CyO	61%	
en-GAL4, UAS-dMLF/ CyO × *UAS-DREF* (Ⅱ)	*en-GAL4, UAS-dMLF/ UAS-DREF*	0%	(n = 40)
	UAS-DREF/ CyO	100%	
en-GAL4, UAS-dMLF/ CyO × *UAS-dref-IR* (X) #10	*UAS-dref-IR/* + or *y*; *en-GAL4, UAS-dMLF/* +	0%	(n = 81)
	UAS-dsRNADREF/ + or *y*; *+/ CyO*	100%	
en-GAL4, UAS-dMLF/ CyO × *UAS-dref-IR* (X) #15	*UAS-dref-IR/* + or *y*; *en-GAL4, UAS-dMLF/* +	0%	(n = 38)
	UAS-dref-IR/ + or *y*; *+/ CyO*	100%	

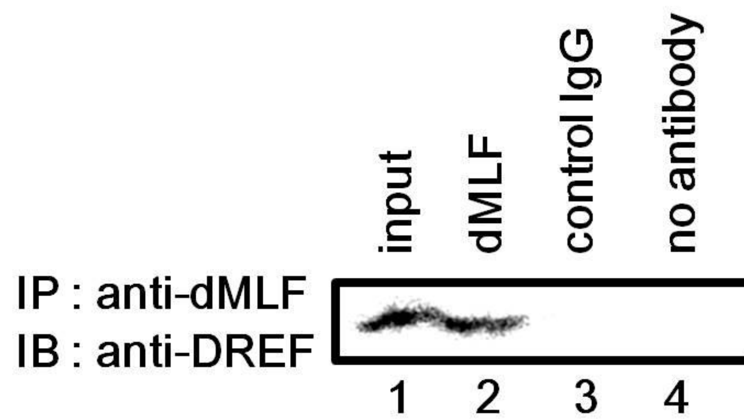

Fig. 6. dMLF and DREF interact *in vivo*. Extracts of S2 cells were first immunoprecipitated with anti-dMLF IgG followed by immunoblotting with anti-DREF IgG.

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Chapter 3

The Hippo pathway as a target of the *Drosophila* DRE/DREF transcriptional regulatory pathway

Introduction

The BED finger-type transcription factor DREF (DNA replication-related element-binding factor) (Hirose *et al*. 1993) binds to the DRE sequence (5'-TATCGATA), a highly conserved sequence in the core promoters of many *Drosophila* genes (Ohler *et al*. 2002). Target genes of DREF are involved in a variety of diverse processes (Matsukage *et al*. 2008), such as DNA replication (Hirose *et al*. 1993; Yamaguchi *et al*. 1995; Okudaira *et al*. 2005; Tsuchiya *et al*. 2007), cell cycle regulation (Ohno *et al*. 1996; Sawado *et al*. 1998), apoptosis (Trong-Tue *et al*. 2010), protein synthesis (Ida *et al*. 2007) and degradation (Dang *et al*. 2006), and maintenance of chromatin structure (Nakamura *et al*. 2008; Ida *et al*. 2009).

Analyses of the cellular function of DREF suggest that it is a multifunctional protein. As a transcription factor DREF has been shown to be important for *Drosophila* development. It is important for bristle development through its regulation of endoreplication in shaft cells (Kawamori *et al*. 2011). It co-operates with *Drosophila* myeloid leukemia factor (Yoshioka *et al*. 2012; Yanai *et al*. 2014) and the chromatin regulator XNP/dATRX (Valadez-Graham *et al*. 2012) in thorax development to regulate the JNK pathway. It also functions downstream of the Target-of-Rapamycin (TOR) pathway (Killip *et al*. 2012) to modulate cell and organ growth in *Drosophila*. Genome-wide ChIP-sequence analyses also suggest that as well as functioning as a transcription factor DREF, in collaboration with insulator proteins may contribute to maintaining chromosome organization during the cell cycle, marking a subset of genomic sites for the assembly of pre-replication complexes, and gene bookmarking during the M/G1 transition (Gurudatta *et al*. 2013). Furthermore, a DREF/TRF2 complex (Hochheimer *et al*. 2003) appears to be important for maintenance of telomere length in *Drosophila* (Takacs *et al*. 2012).

The DRE/DREF pathway appears to be required for transcriptional regulation of the *warts* (*wts*) gene, an essential component of the Hippo pathway (Fujiwara *et al*. 2012). The Hippo pathway itself was firstly identified in *Drosophila* as a tumor-suppressive signal cascade which plays a crucial role in controlling organ size (Justice *et al*. 1995; Xu *et al*. 1995; Tapon *et al*. 2002; Harvey *et al*. 2003; Pantalacci *et al*. 2003; Udan *et al*. 2003).This pathway is defined by a kinase cascade whereby the Ste-20-like kinase Hippo (Hpo), facilitated by the WW-domain-containing adaptor protein Salvador (Sav), phosphorylates Wts (Tapon *et al*. 2002; Pantalacci *et al*. 2003; Udan *et al*. 2003; Wu *et al*. 2003). Activated Wts then phosphorylates and inactivates the transcriptional co-activator Yorkie (Yki) (Huang *et al*. 2005; Oh *et al*. 2008; Dong *et al*. 2007), leading to transcriptional down-regulation of target genes such as the cell-cycle regulator *cyclin E*,

the cell death inhibitor *diap1*, the Hippo pathway regulator *expanded* (*ex*) and the microRNA bantam (Edgar 2006; Bandura *et al*. 2008; Goulev *et al*. 2008; Wu *et al*. 2008; Zhang *et al*. 2008; Pan 2007; Reddy *et al*. 2008; Saucedo *et al*. 2007; Zeng *et al*. 2008; Badouel *et al*. 2009; Peng *et al*. 2009). Inactivation of the Hpo, Sav, or Wts tumor suppressors, or overexpression of Yki, results in massive tissue over-growth, characterised by excessive cell proliferation and diminished apoptosis. A recent study showed that the Hippo pathway also regulates normal proliferation of intestinal stem cells in *Drosophila* midgut, playing an essential role in maintaining homeostasis and regeneration in response to tissue damage (Ren *et al*. 2010). Although, a variety of factors have been identified which interact with the Hippo pathway, indicating wide-ranging functions, the mechanisms of, transcriptional regulation of the genes encoding these factors is largely unknown and poorly studied.

By extensive genetic screening with DREF overexpressing flies, several genes related to the Hippo pathway have been identified (Ida *et al*. 2009). However, although some preliminary indications were obtained regarding the *wts* gene (Fujiwara *et al*. 2012) the biological significance of these interactions, and the involvement of DREF in regulation of the Hippo pathway are poorly understood. In this thesis study, it was observed that overexpression of DREF induced increased hpo signals in eye imaginal discs coupled with apoptosis. Significant increase of phospho-Yki in DREF-overexpressing flies, and a significant reduction of the *diap1-lacZ* reporter in DREF-overexpressing eye discs were also observed. In S2 cells, knockdown of DREF reduced the level of endogenous *hpo* mRNA. These results suggest that DREF positively regulates the Hippo pathway to restrict apoptosis. Consistent with this, at least one of the two DREs identified in the *hpo* gene promoter region was found to be responsible for promoter activity determined by a luciferase transient expression assay in *Drosophila* S2 cells. In addition, chromatin immunoprecipitation assays with anti-DREF antibodies revealed that DREF binds specifically to the *hpo* gene promoter region containing DREs *in vivo*. These results indicate that the DRE/DREF pathway is required for transcriptional activation of the *hpo* gene to positively control Hippo pathways.

Materials and methods

Fly stocks

Fly strains were maintained at 25 ºC on standard food. The *UAS-DREF* line was as described earlier as well as the transgenic fly line carrying *GMR-GAL4* on the X chromosome (Ida *et al*. 2009; Hirose *et al*. 1999). The *UAS-DREFIR* fly line was obtained from the Vienna Drosophila Resource Center. The fly line *hpo*^{KC202}, *th*^{j5c8}*P[lacZ]*, *UAS-GFP*, *UAS-GFPIR* and all other stocks used in this study were obtained from the Bloomington stock center (Indiana) and Drosophila Genetic Resource Center (Kyoto).

Flip-out experiments

RNAi clones in eye discs were generated with a flip-out system (Sun *et al*. 1999). Female flies with *hs-flp*; *Act5C>FRT y FRT>GAL4*, *UAS-GFP* were crossed with *UAS-DREFIR* and clones were identified by the presence of green fluorescent protein (GFP) expressed under control of the *Act5C* promoter. In the control, the female flies with *hs-flp; Act5C>FRT y FRT>GAL4, UAS-GFP* were crossed with Canton S. Flip-out was induced by heat shock (60 min at 37 ºC) at 24-48 h after the eggs were laid.

Plasmid construction

To construct the plasmid p5'-*hpo*wt-luc, PCR was performed using *Drosophila* genomic DNA as a template and the following primer combination:

hpo5': 5'-ACTACGCGTGCATGTAGATTTCCCAAGCAC

hpo3': 5'-TCACTCGAGGTGCCCTCGGCACTTTGCAA

PCR products were digested with Mlu1 and Xho1 and inserted between the f1 origin and luciferase (luc) coding region of the pGV-B plasmid (Toyo Ink). Base-substituted derivatives of p5'-hpowt-luc were constructed using a QuickChange Site-Directed Mutagenesis Kit (STRATAGENE) according to the manufacturer's protocol. The following oligonucleotide pairs carrying base substitutions in DRE were used as primers:

hpoDRE1mutF: 5'-GCCAAGAGAACTTCGCGGCAAATGGCTAATTCTGC

hpoDRE1mutR: 5'-GCAGAATTAGCCATTTGCCGCGAAGTTCTCTTGGC

hpoDRE2mutF: 5'-GATGAACAACAACTCGCGGCCCATAAAAGCAATTT

hpoDRE2mutR: 5'-AAATTGCTTTTATGGGCCGCGAGTTGTTGTTCATC

DNA transfection into S2 cells and luciferase assays

Approximately $2x10^5$ cells were plated 24 h before plasmid transfection, then 500 ng of reporter plasmid and 1 ng of pAct5C-seapanzy as an internal control were co-transfected into cells using CellFectin II reagent (Invitrogen). After 48 h from transfection, S2 cells were harvested and luciferase activities were normalized to Renilla luciferase activity. Transfections were performed several times with at least two independent plasmid preparations.

For dsRNA interference (RNAi) experiments, $1x10^5$ cells were plated in 24-well dishes in the presence of 3 μg/well of *DREF*-dsRNA, *YFP*-dsRNA and mock for control (dsRNA free incubation) in fetal bovine serum (FBS) free M3 medium for 1 h. After the incubation, three volumes of M3 containing 10 % FBS were added to each well. Four days after RNAi treatment, cells were co-transfected with the luciferase reporter plasmid (500 ng) and pAct5C-seapanzy plasmid (1 ng) as an internal control, then harvested after 48 h for measurement of luciferase activities according to the standard instructions for the kit (Promega).

Quantitative RT-PCR

$1x10^6$ S2 cells were plated in 6-well dishes in 2 ml M3 medium containing 30 μg /well of double-strand RNAs for *DREF* (*DREF*dsRNA) or *β-tubulin* (*β-tubulin*dsRNA) for 1 h. After incubation, 3 ml of M3 medium containing 10 % FBS was added to each well. At 5 days after the RNAi treatment, total RNA was isolated from cells using Trizol Reagent (Invitrogen) and 1 μg aliquots were reverse transcribed with an oligo (dT) primer using a Takara high fidelity RNA PCR kit (Takara). Real-time PCR was performed with SYBR Green I kit (Takara) and the Applied Biosystems 7500 Real-time PCR system using 1 μl of reverse transcribed sample per reaction. *β-tubulin* mRNA was chosen as a negative control and *Ribosomal protein L32* (*RpL32*/*Rp49*) as an endogenous reference gene. To carry out quantitative real-time PCR, the following PCR primers were chemically synthesized:

Hpo2nd Primer: 5'-CCTCTTCGGCAGCATCTC

Hpo3rd Primer: 5'-CCGAATCGGAGTTGATTACCATA

DREF-F: 5'-GGCAATCTCCGTTGAATGACG

DREF-R: 5'-TTCACCTCCGAGAAGCCCTT

β-tubulin-F: 5'-AGTTCACCGCTATGTTCA

β-tubulin-R: 5'-CGCAAAACATTGATCGAG

Wts-F: 5'-GCGAGAACAAAGAGGCGAAA

Wts-R: 5'-GGGAATTCTTCCACTCGCATTC

RP49-F: 5’-GCTTCTGGTTTCCGGCAAGCTTCAAG
RP49-R: 5’-GACCTCCAGCTCGCGCACGTTGTGCACCAGGAAC

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using a ChIP assay kit according to the protocol recommended by the manufacturer (Millipore). Approximately $1x10^7$ S2 cells were fixed in 1 % formaldehyde at 37 ºC for 10 min and then quenched in 125 mM glycine for 5 min at 25 ºC. Cells were washed twice in PBS containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstain A), lysed in 200 μl of SDS lysis buffer, sonicated to break DNA into fragments of less than 1 kb and centrifuged at 15,300 x g for 10 min at 4 ºC. The sonicated cell supernatants were diluted 10-fold in ChIP Dilution Buffer and pre-cleared with 80 μl salmon sperm DNA/protein A agarose-50 % slurry for 30 min at 4 ºC. After brief centrifugation, each supernatant was incubated with 1 μg of normal rabbit IgG or rabbit anti-DREF IgG (Ida *et al*. 2009) for 16 h at 4 ºC. Salmon sperm DNA/protein A agarose-50 % slurry was added with incubation for 1 h at 4 ºC. After washing, immunoprecipitated DNA was eluted with elution buffer containing 1 % SDS and 0.1 M $NaHCO_3$. The protein-DNA crosslinks were reversed by heating at 65 ºC for 4 h. After deproteinization with proteinase K, DNA was recovered by phenol-chloroform extraction and ethanol precipitation. Quantitative real-time PCR was performed with the following PCR primers:
ChIPhpoDRE15’Primer: 5’-AGGTAAGCATGCGTGAGTTG
ChIPhpoDRE13’Primer: 5’-GAACACCGCAGAATGTAAACAAAG
ChIPhpoDRE25’Primer: 5’-ACAACGACAAATTGGCACAG
ChIPhpoDRE23’Primer: 5’-TGGATCGATTGTTGTTGTTCATC
ChIPhpoupstreamregion5’Primer: 5’-CACCACCCAAATGACATACG
ChIPhpoupstreamregion3’Primer: 5’-CGCTGCAATCGGAAAGTTA
ChIPwtsDRE1,2 5’Primer: 5’-CCGATAACATTTACTTGCTCTC
ChIPwtsDRE1,2 3’Primer: 5’-GACCGATATCGATAGACAGAG

Immunohistochemistry

Third instar larval eye imaginal discs were dissected and fixed in 4 % paraformaldehyde in PBS for 15 min at 25 °C. After washing with PBS containing 0.3 % Triton X-100 (PBST), samples were blocked with PBS containing 0.15 % Triton X-100 and 10 % normal goat serum for 30 min at 25 °C and incubated with diluted primary antibodies in PBS or in PBS containing 0.15% Triton X-100 and 10% normal goat serum for 16 h at 4 °C. The following antibodies were used as

primary antibodies: mouse anti-DREF IgG (diluted at 1:200) (Hirose *et al*. 1996), rabbit anti-hpo IgG (1:200; a kind gift from Dr. Helen McNeil) (Badouel *et al*. 2009), rabbit anti-phosphoYki S168 IgG (1:200; a kind gift from Dr. Duojia Pan) (Dong *et al*. 2007), mouse anti-GFP IgG (1:400; Nacalai tesque), mouse anti-β-galactosidase (1:400, DSHB). After extensive washing with PBST, samples were incubated with secondary antibodies labeled with Alexa 647, Alexa594 and Alexa488 (1:400; Invitrogen) for 3 h at 25 °C. After further washing with PBST and PBS, samples were mounted in Vectashield Mounting Medium (Vector laboratories) and inspected with a confocal laser scanning microscope (Olympus FLUOVIEW FV10i).

5-ethynyl-2´-deoxyuridine (EdU) labeling

Detection of cells in S phase was performed using an EdU-labeling kit from Invitrogen (Click-iT EdU Alexa Fluor 594 Imaging Kit). Third instar larvae were dissected in PBS and the imaginal discs were suspended in Grace's insect medium in the presence of 10 μM EdU for 60 min at 25 °C. The samples then were fixed with 3.7 % formaldehyde in PBS for 15 min at 25 °C. After fixing, samples were washed with 3 % BSA in PBS and permeabilized in 0.5 % Triton X-100 in PBS for 20 min at 25 °C. Samples were washed with 3 % BSA in PBS and incubated with Click-iT reaction cocktails for 30 min at 25 °C according to the manufacturer's instructions. After further washing with 3 % BSA in PBS and PBS, samples were stained with Hoechst 33342 (Invitrogen) for labeling DNA, and finally samples were mounted and observed as described in immunostaining section.

Apoptosis assay

Detection of apoptotic cells was performed using a Cell Event Caspase-3/7 Green Detection Reagent (Molecular Probes, Invitrogen). Third instar larvae were dissected in PBS and their imaginal discs were incubated in Grace's insect medium containing 5 μM Cell Event Caspase3/7 Green Detection Reagent for 30 min at 37 °C. Then, the eye discs were fixed with 3.7% formaldehyde for 15 min at 25 °C. After fixing, samples were washed with PBS and permeabilized in 0.5 % Triton X-100 in PBS for 20 min at 25 °C. After several washings with PBS, samples were mounted and observed as described in the immunostaining section.

Quantification and statistics analysis

EdU signals, apoptosis signals and immunostaining signals in the region posterior to the MF were counted and measured from six independent eye imaginal discs using MetaMorph software

(Molecular Devices). The experiments were repeated at least three times. Then, statistical analysis was conducted, as indicated in the figure legends, using GraphPad Prism 6. Every single set of data was assessed using Welch's t-test. Significance is described in the figure legends as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$.

Results

Half dose reduction of the *hpo* gene induces ectopic DNA synthesis in eye discs, and this can be suppressed by overexpression of DREF

A number of previous studies have implicated both Hippo and DREF in the control of cell proliferation and apoptosis and both factors have been suggested to affect the balance of cell proliferation and apoptosis via p53. In addition, it is known that overexpression of DREF in eye discs induces a severe rough eye phenotype in adults without impairing viability or fertility (Hirose *et al*. 2001). The rough eye phenotype has been used to identify proteins that interact with DREF genetically, by screening for mutations that modify the rough eye phenotype. This analysis revealed several modifier genes related to Hippo pathway, such as *ex*, and *fat* (Ida *et al*. 2009). It is also reported that DRE/DREF pathway may be required for transcriptional regulation of the *wts* gene, an essential component of the Hippo pathway (Fujiwara *et al*. 2012).

To further investigate the relationship between DREF and the Hippo pathway, DNA synthesis in the posterior region of the eye imaginal disc was visualized using an EdU incorporation assay, and how this was affected by altered expression of DREF and Hippo was determined. In eye discs of wild type flies, DNA synthesis was detected in the region anterior to the morphogenetic furrow (MF), where cells undergo non synchronous proliferation, and also in the synchronized S phase zone behind the MF. In the eye discs of flies heterozygous for *hpo* mutation, ectopic DNA synthesis was detected in the region posterior to MF (Fig. 1E, G). Slight induction of DNA synthesis in the posterior region of the eye disc was observed in DREF-overexpressing flies, as reported previously (Hirose *et al*. 2001) (Fig. 1D, G). However, a similar extent of ectopic DNA synthesis was observed with GFP-overexpressing flies (Fig. 1C, G), suggesting that overexpression of DREF only marginally induced ectopic DNA synthesis. Overexpression of DREF and GFP was confirmed by immunostaining with specific antibodies (Fig. 1A, B). Immunostaining of eye discs with anti-DREF antibody further confirmed ubiquitous expression of endogenous DREF together with overexpression and knockdown of DREF by *GMR-GAL4* driver (data not shown). Interestingly, extensive induction of DNA synthesis in flies heterozygous for the *hpo* mutation was extensively suppressed by overexpression of DREF (Fig. 1F, G).

Half reduction of the *hpo* gene dose reduces the cell death program in DREF-overexpressing flies

Effects of alterations in these proteins on apoptosis was also examined, using the Cell Event Caspase-3/7 Green Detection Reagent assay which uses a fluorogenic substrate to detect activated Caspases 3 and 7. This cell-permeant reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. It is intrinsically non-fluorescent, because the DEVD peptide inhibits the ability of the dye to bind to DNA, however activation of caspase-3 or caspase-7 in apoptotic cells causes cleavage of the DEVD peptide, enabling the dye to bind to DNA and produce a bright, fluorogenic response. In eye imaginal discs of flies expressing either *UAS-GFP* (control) (Fig. 2A) or heterozygous for the *hpo* mutation (Fig. 2C), no apoptotic cells were detected. However, eye imaginal discs of DREF overexpression flies showed extensive cell death signals in the region posterior to the morphogenetic furrow (Fig. 2B) as compared to the control (Fig. 2A) and the *hpo* heterozygous mutant (Fig. 2C). Furthermore, these signals were dramatically reduced when heterozygous *hpo* mutation was combined with overexpression of DREF in the posterior region (Fig. 2D, E). These observations are consistent with other studies reporting that *hpo* mutation in *Drosophila* imaginal discs is associated with severe tumour-like phenotypes (Udan *et al*. 2003) and resistance to apoptotic signals (Tapon *et al*. 2002; Harvey *et al*. 2003; Pantalacci *et al*. 2003; Wu *et al*. 2003; Kango-Singh *et al*. 2002; Jia *et al*. 2003; Lai *et al*. 2005).

Overexpression of DREF increases the hpo level in eye imaginal discs.

The observations described above might be explained by activation of the Hippo pathway by overexpression of DREF. Therefore immunostaining of eye imaginal discs with anti-hpo antibodies was performed. In eye discs of DREF overexpressing flies, hpo signals increased extensively in the posterior region to MF (Fig. 3B) as compared to control GFP-overexpressing flies (Fig. 3A). The *hpo* heterozygous mutant showed no detectable anti-hpo signal (Fig. 3C). With the combination of overexpression of DREF and *hpo* mutation, the anti-hpo signal was almost comparable to that in the controls (Fig. 3D, E). These results indicate that overexpression of DREF indeed increased the hpo level in eye discs.

To further confirm the effects of overexpression of DREF on the Hippo signal in eye discs, flip-out experiments with DREF knockdown flies was performed. The knockdown of DREF was confirmed by immunostaining of eye discs with the anti-DREF antibody in the flip-out experiments (Fig. 4A-C, 4A’-C’). The flip-out clones expressing GAL4 alone were used as a control (Fig. 4D-F). Immunostaining of eye imaginal discs of control flies overexpressing GFP

with anti-hpo antibody revealed that Hippo is expressed as punctate pattern throughout eye discs (Fig. 5A, B). In the flip-out experiment, cells marked with GFP (Green) express DREF dsRNA (Fig. 5D, D'), and in these cells the hpo signal was significantly reduced (Fig. 5C, E). However, in the clone area without GFP, hpo signals (Red) were still detectable (Fig. 5C, E). These data further confirmed that DREF is required for expression of Hippo in the eye discs.

Overexpression of DREF increases the phospho-Yki level in eye imaginal discs.

Yorkie (Yki) transcriptionally activates *cycE* and *diap1*, which are associated with cell proliferation and anti-apoptosis, respectively. Therefore, to investigate the effect of DREF downstream of the Hippo pathway, immunostaining was performed to determine how DREF affects the levels of anti-phospho-Yki, an inactive form of Yki, in eye discs. It was found that overexpression of DREF increased phospho-Yki signals in the region posterior to the MF (Fig. 6B, E). The signals were reduced if DREF overexpression was combined with *hpo* mutation (Fig. 6D, E). In heterozygous *hpo* mutant flies, phospho-Yki signals were found to be lower than in control flies (Fig. 6C, E).

To further investigate the effects of DREF on phospho-Yki level, the flip-out experiment with DREF knockdown flies was carried out. The phospho-Yki positive cells were detected mostly in the GFP-negative area where DREF was not knocked down (Fig. 7B-B', E-E', F-F'). In contrast, in the GFP-positive area, only a few phospho-Yki signals were detected (Fig. 7, white arrows). It was found that these phospho-Yki-positive cells are also DREF-positive even in the GFP clone area (Fig. 7, white arrows), suggesting some variation in DREF-knockdown efficiency in individual cells within the same clone. It should be noted that these cells are still red even in merged image with GFP (Fig. 7E). The flip-out experiments were also carried out with the flies expressing GAL4 alone as a control to confirm these observations. The phospho-Yki signals were scattered throughout the whole eye discs including both GFP and non-GFP clones (Fig. 8B, C, D, F). Whereas, there was no change in the expression of DREF in the eye discs of these flies (Fig. 8A, D, E, F). In contrast to the data with the DREF-knockdown clone analysis, phospho-Yki signals become yellow in merged image with GFP in this control flip-out experiments (Fig. 8C). These data taken together suggest that DREF positively regulates the Hpo level and consequently increases the level of phospho-Yki in the eye disc.

An increase in phospho-Yki should cause a decrease of the DIAP1 level. Therefore expression of DIAP1 was examined by monitoring lacZ expression of the *diap1-lacZ* enhancer trap line, *th*^{j5c8} *P[lacZ]* that carries a *P[lacZ]* insertion in the 5' untranslated region of *diap1 (th)* (Hay *et al*. 1995).

It is reported that the expression pattern of the lacZ mimics that of endogenous diap1 (Ryoo *et al.* 2002). Expression of the *diap1-lacZ* reporter was significantly decreased in DREF-overexpressing flies (Fig. 6F, G). Quantification revealed that in eye discs overexpressing DREF, *diap1-lacZ* signals decreased by 73% of that of flies expressing GAL4 alone (Fig. 6H).

The 5'-flanking region of the *hpo* gene contains DRE and DRE-like sequences

The DRE sequence is an 8bp sequence of which the central 6bp (5'-ATCGAT) is essential for DREF binding and activation of promoters (Hirose *et al.* 1999), and previous studies have demonstrated that DRE sequences in the genomic region 5' of the transcription initiation site can up-regulate transcription of many target genes.

To determine whether transcription of the *hpo* gene is directly regulated by DREF, the DRE sequences within the 1.4kb genome region to 5' of the transcription initiation site of the *hpo* gene wsa searched as documented on the *Drosophila* genome database, Flybase. A perfect match to the DRE sequence, named DRE1 (5'-TATCGATA) was found to be located at nucleotide position from -136 to -129 with respect to the transcription initiation site and a DRE-like sequence with a 7 out of 8 bp match DRE2 (5'-TATCGATc) at -450 to -443 (Fig. 9A).

DREF binds to the genomic region containing DRE and DRE-like sequences of the *hpo* gene *in vivo*

To test whether DREF binds to the DRE-containing 5'-flanking region of the *hpo* gene in S2 cells, chromatin immunoprecipitation assays with anti-DREF polyclonal antibodies were carried out (Fig. 9B). The *wts* locus, previously shown to bind DREF (Fujiwara *et al.* 2012), was used as a positive control, and the upstream genomic devoid of DREs was used as a negative control. Amplification of the *wts* gene promoter region containing DRE in immunoprecipitates with anti-DREF IgG was 77-fold higher than that with control IgG (Fig. 9B). In contrast, no significant amplification of the upstream region was observed. Amplification of the *hpo* gene region containing DRE1 from immunoprecipitates with the anti-DREF IgG was about 9.9-fold higher than that with control IgG (Fig. 9B). Binding of DREF to the DRE2 region of the *hpo* promoter, was also detected, but this binding was weaker than that for the DRE1 containing region. These results indicate that DREF mainly binds to the genomic region containing DRE1 and less effectively to DRE2 under cellular conditions.

DRE1 and DRE2 both influence *hpo* promoter activity

To investigate the role of the DREs in *hpo* promoter activity, transient luciferase expression assays were carried out in cultured *Drosophila* S2 cells. A *hpo* promoter and *luciferase* fusion plasmid and a set of derivatives carrying mutations in DRE1 and/or DRE2 were constructed. These plasmids were transfected into S2 cells and luciferase activity was measured. A mutation in DRE1 (*hpo*-DRE1mut) and mutations in both DREs (*hpo*-DREallmut) reduced the *hpo* promoter activity by 85% and 70%, respectively (Fig. 10A). However, the mutation in DRE2 (hpo-DRE2mut) slightly increased the *hpo* promoter activity (Fig. 10A). These results indicate that DRE1 is mainly responsible for promoter activity and DRE2 may have some negative role in promoter activity.

DREF is required for *hpo* promoter activity in S2 cells

To further examine the requirement of DREF for *hpo* promoter activity, DREF RNA interference (RNAi) assays with cultured *Drosophila* S2 cells were carried out. Transient luciferase expression assays with the wild-type *hpo* gene promoter-luciferase reporter gene were conducted, after treating S2 cells with *DREF*dsRNA, control *YFP*dsRNA or no dsRNA (Mock). Treatment of S2 cells with *DREF*dsRNA reduced *hpo* gene promoter activity by 50%, while control *YFP*dsRNA treatment exerted only a marginal effect (Fig. 10B). When DRE1 and 2 were mutated, no further decrease of *hpo* promoter activity was observed with knockdown of DREF (Fig. 10B). These results indicate that DREF is required for *hpo* promoter activity.

Knockdown of the *DREF* gene reduces endogenous *hpo* gene expression in cultured S2 cells

The DREF knockdown S2 cells were also used to examine whether DREF affects expression of the endogenous *hpo* gene. Total RNAs from dsRNA treated S2 cells were isolated at 5 days after addition of dsRNA for assessment by quantitative RT-PCR. The *β-tubulin* gene was used as a negative control and the *wts* gene as a positive control. The *DREF* mRNA level was reduced by 97% with *DREF*dsRNA treatment as compared with no dsRNA (Mock) (Fig. 10C). The *hpo* mRNA level in *DREF*dsRNA treated cells was reduced to 58% relative to no dsRNA treated cells, while no significant change was observed with *YFP*dsRNA treated cells (Fig. 10C). Similar effects on *hpo* mRNA level were obtained for the positive control *wts*. These results indicate that DREF is truly required for *hpo* gene expression.

Discussion

In this thesis study, it is demonstrated that the *hpo* gene is a major DREF-target, featuring one DRE and one DRE-like sequence in its 5'-flanking region. Luciferase transient expression assays showed *hpo* promoter activity to mainly depend on DRE1 and DREF levels. Consistent with this, chromatin immunoprecipitation assays showed DREF to bind to the genomic region of the *hpo* gene containing the DREs, and the level of *hpo* mRNA was reduced in DREF knockdown S2 cells treated with *DREF*dsRNA. These data provide compelling evidence that DREF is a positive regulator of the *hpo* gene and its expression is directly activated by the DRE/DREF pathway. It has already been shown that DREF can simultaneously activate various apoptosis inducing genes for instance JNK pathway genes (Yoshioka *et al*. 2012) and p53 (Trong-Tue *et al*. 2010). The increase of hpo level by overexpression of DREF resulted in increased levels of inactive phospho-Yki and a concomitant decrease of the DIAP1 level to further enhance apoptosis in eye discs (Fig. 11). In fact a half dose reduction of the *hpo* gene extensively decreased the apoptosis signals in eye discs of DREF-overexpressing flies.

Coordination of positive and negative regulation for cell proliferation is essential to achieve appropriate organ formation. During normal development and regeneration of organs after surgical excision, the organs reach their proper size by precisely controlling cell proliferation and its arrest. The Hippo pathway restricts cell proliferation via cell cycle arrest and inducing apoptosis. The major effect of DREF-overexpression in eye discs has been for a long time thought to be induction of ectopic DNA synthesis (Hirose *et al*. 2001). However, in this thesis study here, re-examining the influence of DREF-overexpression in eye discs, suggests that the major effect is the induction of apoptosis accompanied with induction of *hpo* expression, and that the change in DNA synthesis appears to be only marginal. It should be noted that similarly strong induction of apoptosis was also observed when DREF was overexpressed in wing discs (Yoshida *et al*. 2001). Therefore it can be proposed that the Hippo pathway is one of the major targets of the DRE/DREF regulatory system. In addition to Hippo pathway-related genes, DREF positively regulates the *basket* gene, a *Drosophila* JNK (Yoshioka *et al*. 2012). JNK exerts a protective function for the genome and promotes apoptosis just like *p53*, which is also a target of DRE/DREF (Trong-Tue *et al*. 2010). In addition, the Hpo pathway and the JNK pathway cooperate in tissue growth and regeneration (Matsukage *et al*. 2008; Fujiwara *et al*. 2012). Thus DREF, in regulating both Hippo and JNK pathway-related genes appears to play a key role in coordination of these two important signal transduction systems (Fig. 11).

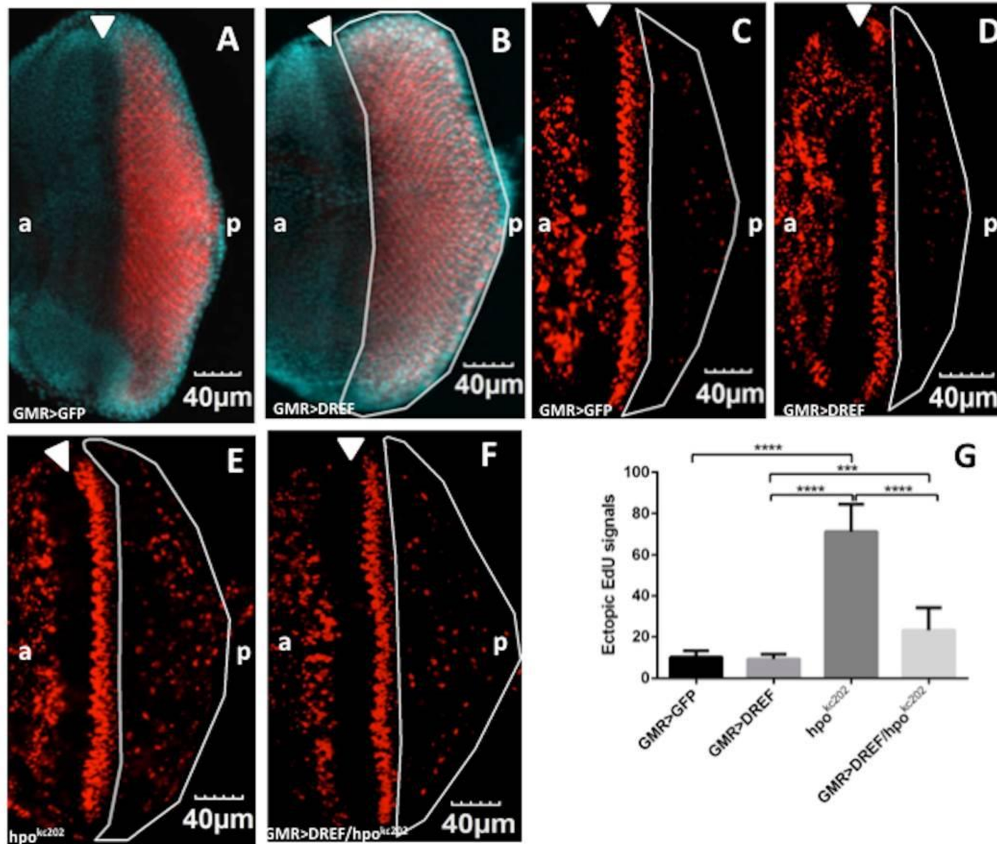


Fig. 1. Half reduction of the *hpo* gene dose enhances cell proliferation which can be suppressed by overexpression of DREF. (A) Eye imaginal discs of flies overexpressing *UAS-GFP* are stained with anti-GFP antibody (Red) and DAPI (Blue). (B) Eye discs expressing *UAS-DREF* are stained with anti-DREF antibody (Red) and DAPI (Blue). (For cell proliferation, eye imaginal discs were labeled with EdU (Red)). (C) *GMR-GAL4*/+; *UAS-GFP*/+; +. (D) *GMR-GAL4*/+; *UAS-DREF*/+; +. (E) *w**; +; hpo^{KC202}/*CyO*. (F) *GMR-GAL4*/+; *UAS-DREF*/+; hpo^{KC202}/+. (G) Quantification of the number of ectopic EdU cells in the posterior region of the eye discs. ***$p < 0.001$, ****$p < 0.0001$. White arrowheads indicate morphogenetic furrow (MF). The white border line indicates the posterior region. Scale bars are for 40 μm. a: anterior, p: posterior. The flies were reared at 28 ℃.

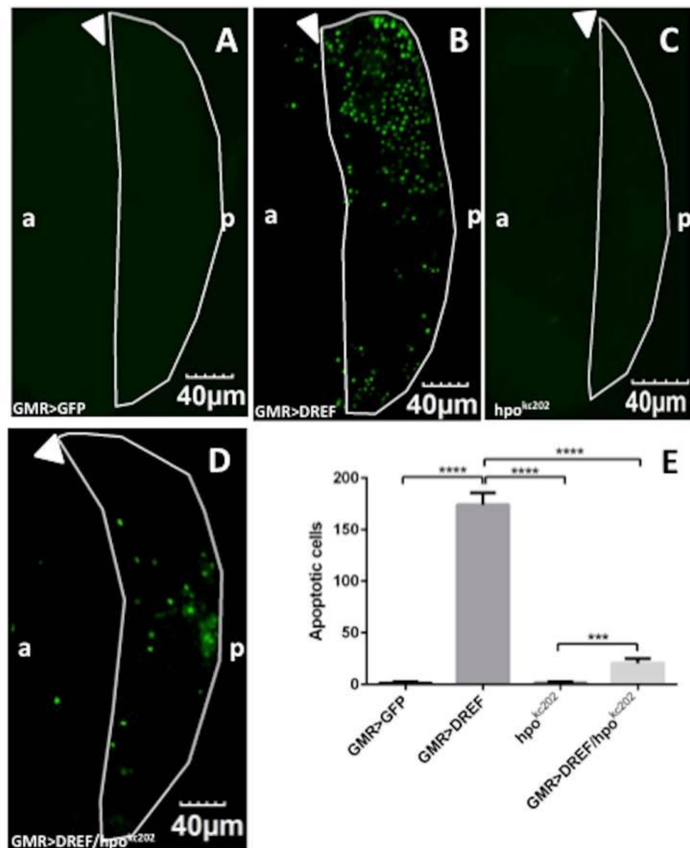


Fig. 2. Half dose reduction of the *hpo* gene reduces the cell death program in DREF overexpression flies. Cell Event Caspase-3/7 Green Detection Reagent assays were performed in eye imaginal discs (Green). (A) *GMR-GAL4*/+; *UAS-GFP*/+; +. (B) *GMR-GAL4*/+; *UAS-DREF*/+; +. (C) *w**; *hpo*^{KC202}/*CyO*; +. (D) *GMR-GAL4*/+; *UAS-DREF*/+; *hpo*^{KC202}/+. (E) Quantification of the number of apoptotic cells in the posterior region of the eye discs. ***$p < 0.001$, ****$p < 0.0001$. White arrowheads indicate the morphogenetic furrow (MF). The white border line indicates the posterior region. Scale bars are for 40 μm. a: anterior, p: posterior. The flies were reared at 28 ºC.

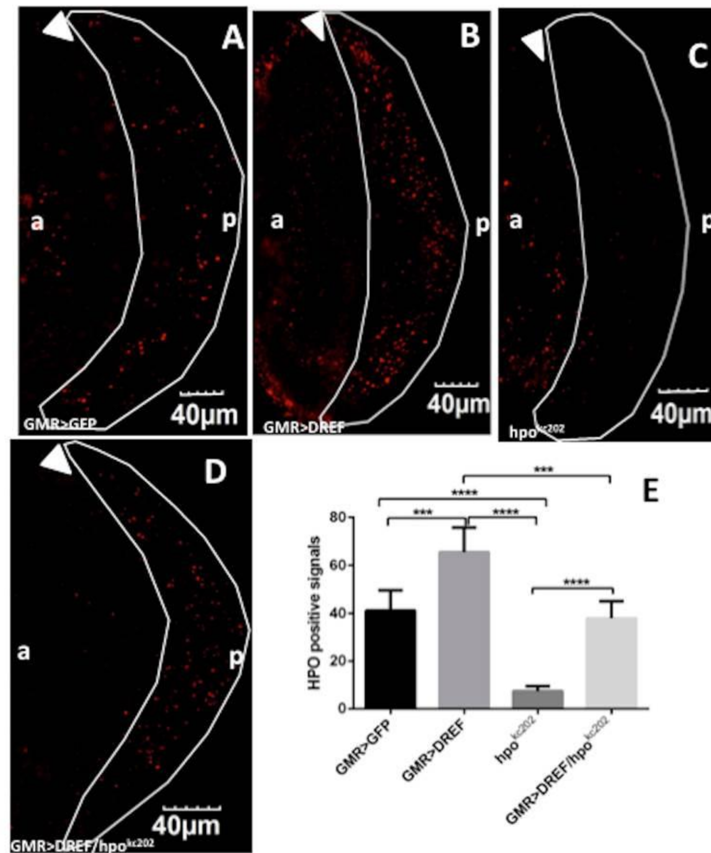


Fig. 3. Overexpression of DREF significantly enhances Hpo signals in eye imaginal discs. Eye imaginal discs were immunostained with anti-hpo antibodies (Red). (A) *GMR-GAL4*/+; *UAS-GFP*/+; +. (B) *GMR-GAL4*/+; *UAS-DREF*/+; +. (C) *w**; +; *hpo*^{KC202}/*CyO*. (D) *GMR-GAL4*/+; *UAS-DREF*/+; *hpo*^{KC202}/+. (E) Quantification of the number of Hpo positive signals in the posterior region of the eye discs. ***$p < 0.001$, ****$p < 0.0001$. White arrowheads indicate the morphogenetic furrow (MF). The white border line indicates the posterior region. Scale bars are for 40 μm. a: anterior, p: posterior. The flies were reared at 28 ºC.

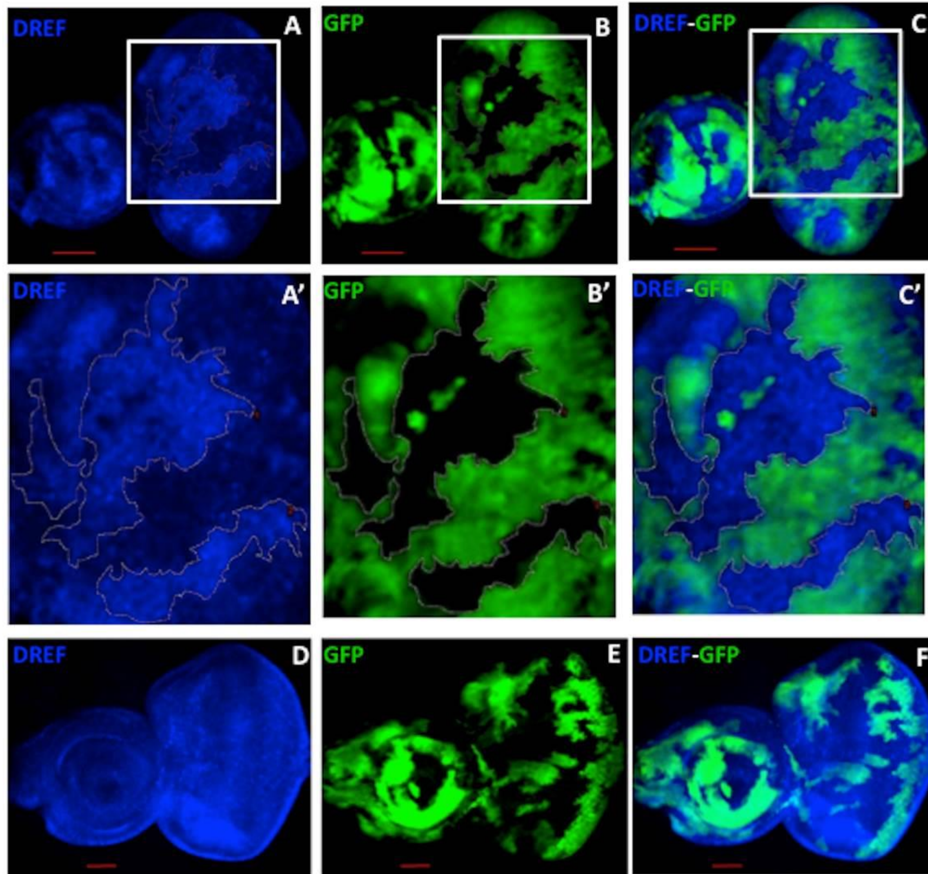


Fig. 4. Confirmation of DREF knockdown in flip-out experiments

(A) Eye imaginal discs of *hs-flp*; *Act5C>FRT y FRT>GAL4*, *UAS-DREFIR* flies are stained with anti-DREF antibody (Blue). (B) Cells expressing *DREF*dsRNA are marked with GFP (Green). (C) Merged image of anti-DREF and GFP signals in DREF knockdown eye discs. (A'), (B') and (C') are larger images of white box areas in (A), (B), and (C), respectively. In the control flies expressing GAL4 alone, the eye discs were stained with anti-DREF antibody (D) and anti-GFP antibody (E). (F) Merge image of anti-DREF and GFP signals in the control eye discs. The bars are for 40 μm.

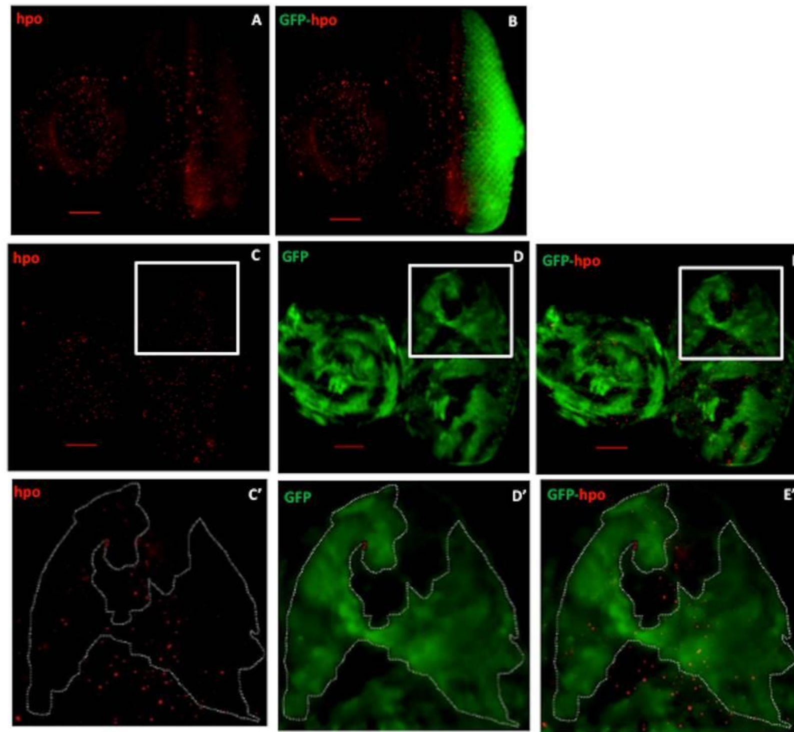


Fig. 5. Expression of *DREF* dsRNA reduces hippo levels in eye discs.

(A) Eye imaginal discs of *GMR>GFP* flies are stained with anti-hpo antibody (Red). (B) Merge image of anti-hpo and GFP signals (Green) of the flies overexpressing GFP in the posterior region. (C) Eye discs of the flip-out experiment are stained with anti-hpo antibody (Red). (D) Cells expressing *DREF*dsRNA are marked with GFP (Green). (E) Merged image of anti-hpo and GFP signals in *DREF* knockdown eye discs. (C'), (D') and (E') are larger images of white box areas in (C), (D), and (E), respectively. Scale bars are for 40 μm.

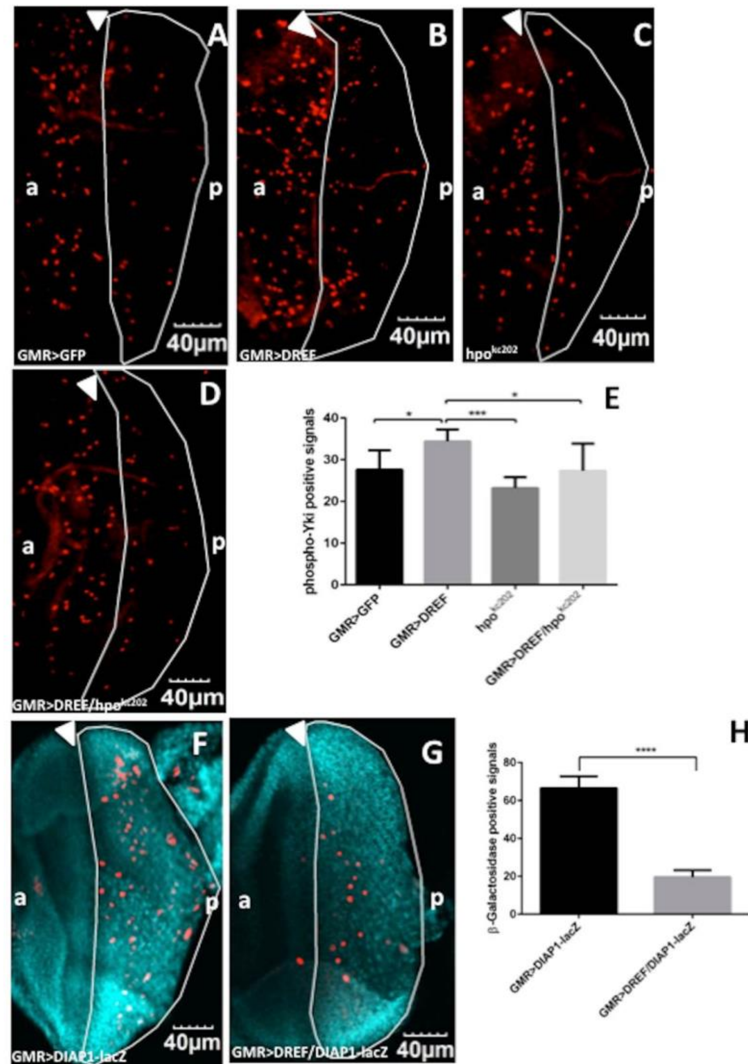
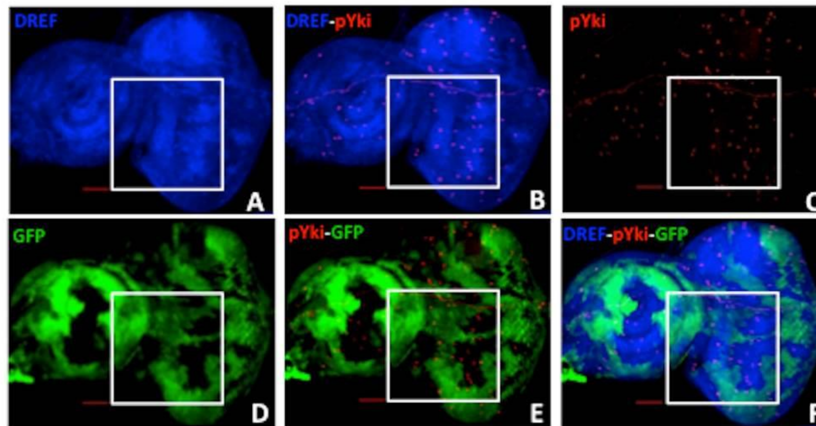


Fig. 6. Overexpression of DREF increases the phospho-Yki level and reduces *diap1-lacZ* signals in eye imaginal discs.

Eye imaginal discs were immunostained with anti-phospho-Yki antibodies. (Red) (A) *GMR-GAL4*/+; *UAS-GFP*/+; +. (B) *GMR-GAL4*/+; *UAS-DREF*/+; +. (C) *w**; + ; *hpo*^{KC202}/*CyO*. (D) *GMR-GAL4*/+; *UAS-DREF*/+; *hpo*^{KC202}/+. (E) Quantification of the number of positive phospho-Yki signals in the posterior region of the eye discs. To examine expression of the *diap1-lacZ* reporter, eye discs were stained with anti-β-galactosidase antibody (Red) and DAPI (Blue). (F) GMR-GAL4/+; +; *diap1-lacZ*/+. (G) *GMR-GAL4*/+; *UAS-DREF*/+; *diap1-lacZ*/+. (H) Quantification of the number of β-galactosidase positive signals in the region posterior to the morphogenetic furrow. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. White arrowheads indicate the morphogenetic furrow (MF). The white border line indicates the posterior region. Scale bars are for 40 μm. a: anterior, p: posterior. The flies were reared at 28 ºC.



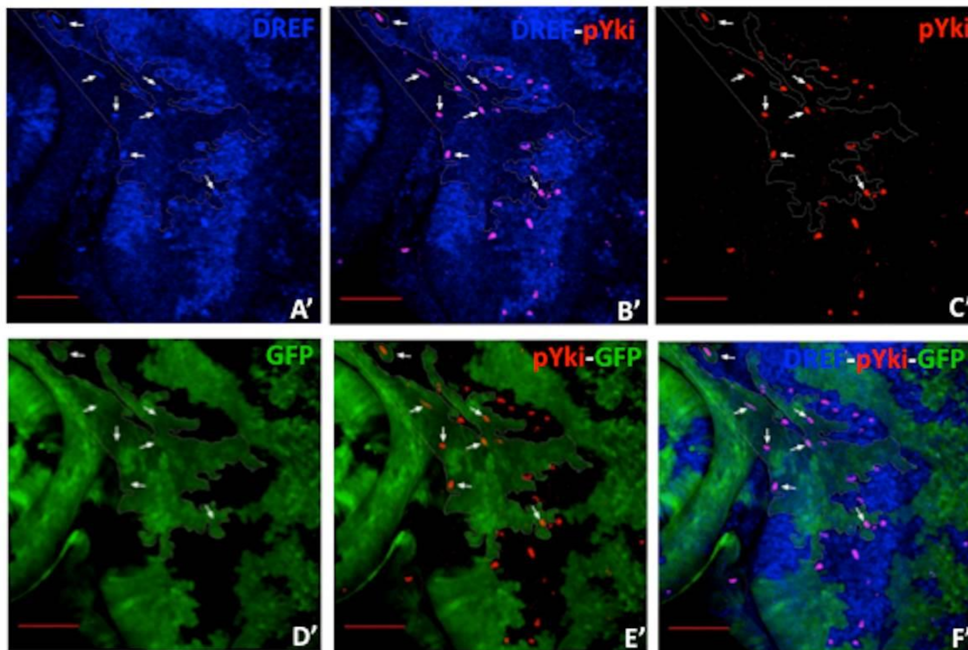


Fig. 7. Expression of *DREF* dsRNA reduces phospho-Yki levels in eye discs. Eye imaginal discs of *hs-flp*; *Act5C>FRT y FRT>GAL4*, *UAS-DREFIR* flies are stained with anti-DREF antibody (Blue) (A) and with anti-phospho Yki antibody (Red) (C). (B) Merged image of DREF and phospho-Yki signals in DREF knockdown eye discs. (D) Cells expressing *DREF*dsRNA are marked with GFP (Green). (E) Merged image of phospho-Yki and GFP signals. (F) Merged image of DREF, phospho-Yki and GFP signals. (A'-F') larger images of white box areas in (A-F). The white arrows indicate both DREF-positive and phospho Yki-positive cells in GFP clone area. The bars are for 40 μm.

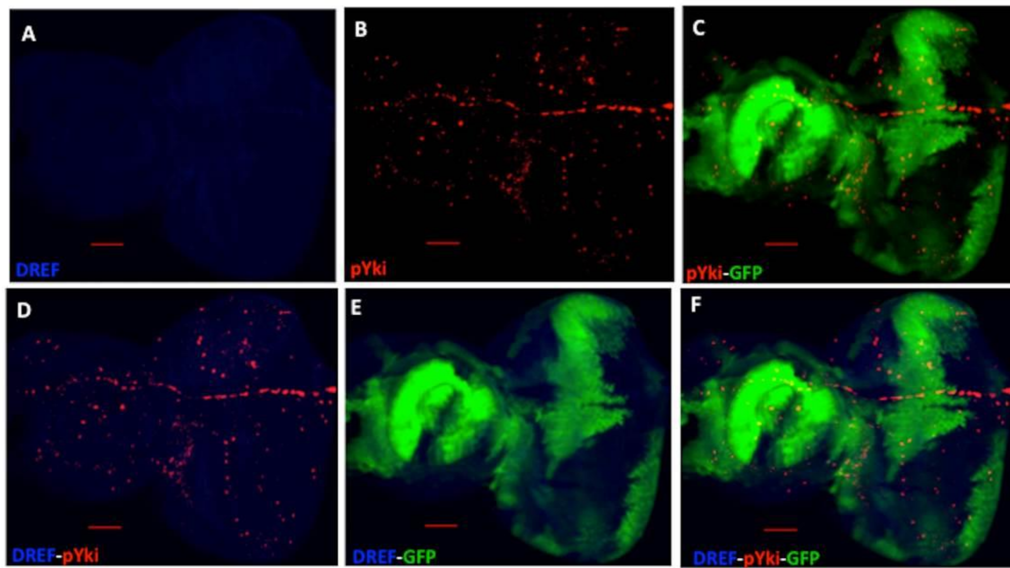


Fig. 8. Distribution of endogenous phospho-Yki in the control flip-out experiments. Eye imaginal discs of *hs-flp*; *Act5C>FRT y FRT>GAL4*, *UAS-GFP* flies are stained with anti-DREF antibody (Blue) (A) and with anti-phospho Yki antibody (Red) (B). (C) Merged image of phospho-Yki and GFP signals. (D) Merged image of DREF and phospho-Yki signals. (E) Merged image of DREF and GFP signals. (F) Merged image of DREF, phospho-Yki and GFP signals. The endogenous phospho-Yki signals scattered throughout the whole eye discs and there was no change in the level of DREF signals. The bars are for 40 μm.

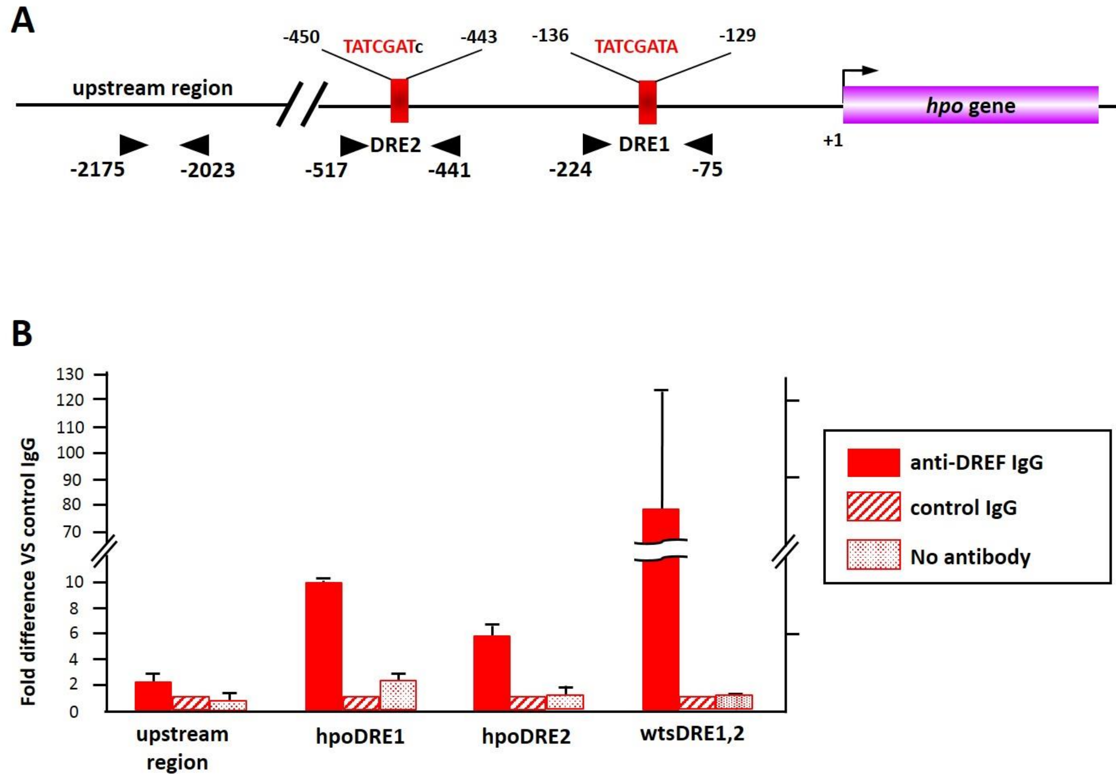


Fig. 9. The *hpo* gene carries DRE and DRE-like sequences in the 5'-flanking region. (A) Schematic of the 5'-flanking region of the *hpo* gene. The transcription initiation site is indicated by the arrow and designated as +1. The positions and nucleotide sequences of DRE1 and DRE2 are shown. Arrowheads indicate positions of the primers used for real-time PCR reactions. (B) Crosslinked chromatin of S2 cells was immunoprecipitated with either anti-DREF IgG or control rabbit IgG. Genomic regions containing hpoDRE1, hpoDRE2, and wtsDRE1, 2 were amplified by PCR, and compared with amplicons from immunoprecipitates with control rabbit IgG.

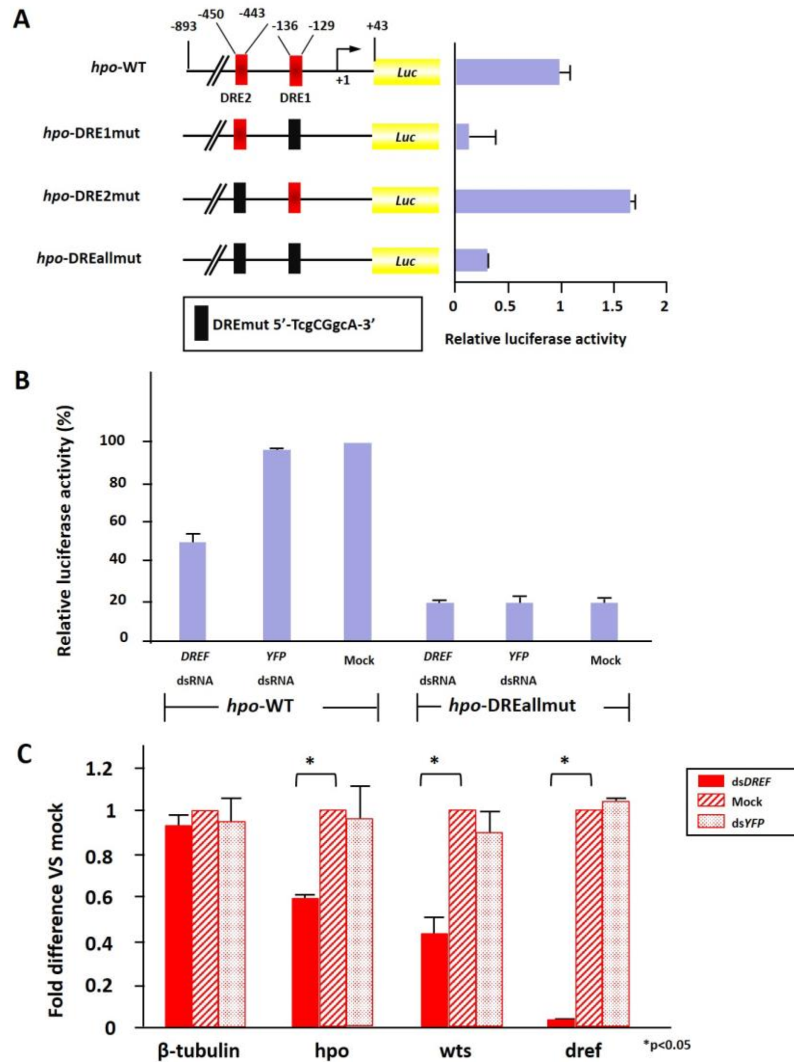


Fig. 10. DREF plays essential roles in *hpo* gene promoter activity in cultured S2 cells. (A) Schematic features of the *hpo* promoter-luciferase fusion plasmid *hpo*-WT-*luc* and its base-substituted derivatives (*hpo*-DRE1mut-*luc*, *hpo*-DRE2mut-*luc*, *hpo*-DREallmut-*luc*). DRE is represented by an open box and mutated DRE by a closed box. Plasmids were transfected into S2 cells and luciferase activities measured at 48 hours thereafter. Luciferase activity was normalized to Renilla luciferase activity and expressed relative to that of *hpo*-WT-*luc*. Mean activities with standard deviations from three independent transfections are shown. (B) Effects of *DREF*dsRNA treatment on *hpo* gene promoter activity in S2 cells. Mean activities with standard deviations from three independent transfections are shown. (C) *DREF*dsRNA treatment reduces endogenous *hpo* mRNA levels in S2 cells. *DREF* mRNA, *wts* mRNA and *hpo* mRNA in *DREF* dsRNA-treated cells were measured by quantitative RT-PCR and compared with the values for non-dsRNA treated cells (Mock). mRNA for β-tubulin was used as a negative control and mRNA for *wts* as a positive control. * $p < 0.05$.

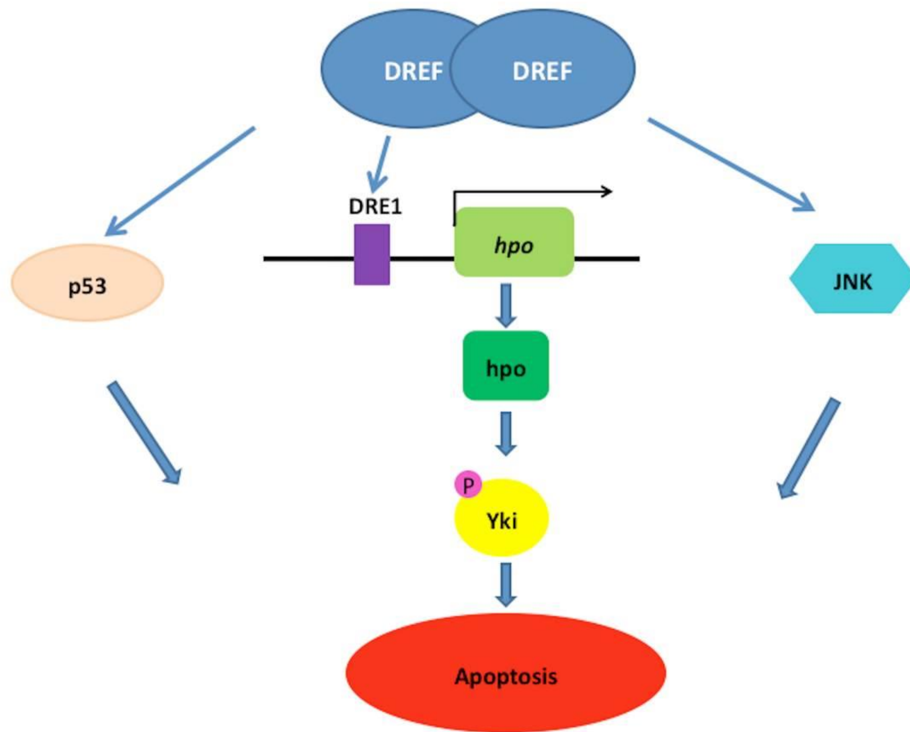


Fig. 11. A model of the role of DREF in regulation of Hippo, JNK and p53 pathways inducing apoptosis. DREF binds to DRE in the *hpo* promoter region which activates *hpo* gene transcription. DREF upregulates both JNK and p53 pathways to induce apoptosis (Trong-Tue *et al*. 2010; Yoshioka *et al*. 2012). Activation of the Hippo pathway by DREF inactivates Yorkie to further enhance apoptosis.

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