The EMBO Journal (2004) 23, 3538–3547 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
www.embojournal.org

THE EMBO JOURNAL

Coactivator MBF1 preserves the redox-dependent AP-1 activity during oxidative stress in *Drosophila*

Marek Jindra[1,2], Ivana Gaziova[1], Mirka Uhlirova[1], Masataka Okabe[2], Yasushi Hiromi[2,3] and Susumu Hirose[2,3,*]

[1]Department of Molecular Biology, University of South Bohemia and Institute of Entomology ASCR, Ceske Budejovice, Czech Republic, [2]Department of Developmental Genetics, National Institute of Genetics, Mishima, Japan and [3]Department of Genetics, SOKENDAI, Mishima, Japan

Basic leucine zipper proteins Jun and Fos form the dimeric transcription factor AP-1, essential for cell differentiation and immune and antioxidant defenses. AP-1 activity is controlled, in part, by the redox state of critical cysteine residues within the basic regions of Jun and Fos. Mutation of these cysteines contributes to oncogenic potential of Jun and Fos. How cells maintain the redox-dependent AP-1 activity at favorable levels is not known. We show that the conserved coactivator MBF1 is a positive modulator of AP-1. Via a direct interaction with the basic region of *Drosophila* Jun (D-Jun), MBF1 prevents an oxidative modification (S-cystenyl cystenylation) of the critical cysteine and stimulates AP-1 binding to DNA. Cytoplasmic MBF1 translocates to the nucleus together with a transfected D-Jun protein, suggesting that MBF1 protects nascent D-Jun also in *Drosophila* cells. *mbf1*-null mutants live shorter than *mbf1*$^+$ controls in the presence of hydrogen peroxide (H_2O_2). An AP-1-dependent epithelial closure becomes sensitive to H_2O_2 in flies lacking MBF1. We conclude that by preserving the redox-sensitive AP-1 activity, MBF1 provides an advantage during oxidative stress.

The EMBO Journal (2004) **23,** 3538–3547. doi:10.1038/sj.emboj.7600356; Published online 12 August 2004
Subject Categories: proteins; development
Keywords: AP-1; coactivator; *Drosophila*; MBF1; oxidative stress

Introduction

Organisms from the most primitive prokaryotes to mammals have evolved a number of mechanisms to maintain cellular redox balance and thus evade oxidative stress caused by naturally arising reactive oxygen species (ROS). These mechanisms include low-molecular radical scavengers and antioxidant enzymes. Studies in yeast and mammalian cell lines have identified regulatory pathways of antioxidant defense. These involve protein kinases, such as JNK and ERK, that activate transcription factors, which turn on stress response genes (Davis, 2000). JNK signaling is required for oxidative stress defense also in the fruit fly *Drosophila melanogaster*, which serves as a model to characterize it genetically (Stronach and Perrimon, 1999; Wang *et al*, 2003).

Among the key transcription factors activated by the JNK and ERK kinases are the basic region leucine zipper (bZIP) proteins of the Jun and Fos families (Karin, 1995). Related bZIP proteins combine through their leucine zippers to yield an array of DNA-binding dimers, known as AP-1 (activator protein-1). AP-1 activity can be induced by signals as diverse as growth factors, peptidic hormones and neurotransmitters, microbial infections and physical and chemical stresses. In response, AP-1 triggers a spectrum of cellular processes such as apoptosis or proliferation, differentiation and mobilization of defense against stress (Shaulian and Karin, 2002).

Jun and Fos not only activate the cellular defense against oxidative challenge, but also sense redox imbalances: their activity depends upon their redox state. This appears to be an important and universal principle, since also other transcription regulators, such as NF-κB or p53, are sensitive to oxidation (Marshall *et al*, 2000). The sensitivity is conferred by reactive sulfhydryl groups of cysteine residues. Abate *et al* (1990) have shown that specific cysteines within the basic regions of c-Jun and c-Fos are responsible for oxidative inhibition of AP-1 DNA-binding capacity *in vitro*, which can be restored with reducing agents. In contrast, Jun and Fos mutants with serine replacing the critical cysteine residues bind DNA regardless of redox conditions. Thus the hypersensitive cysteine introduces a new element of regulation into AP-1. That such regulation is indeed necessary for normal cell functioning is obvious from consequences of the critical cysteine mutation into serine. Exactly such substitution is found in the viral transforming protein v-Jun (Bohmann *et al*, 1987; Maki *et al*, 1987), where it contributes to the oncogenic activity synergistically with other mutations (Morgan *et al*, 1994). Similarly, the corresponding cysteine-to-serine substitution leads to a transforming activity of Fos (Okuno *et al*, 1993). In both cases, the mutant AP-1 forms apparently escape the redox regulation.

Paradoxically, while AP-1 mobilizes antioxidant defense, it is at the same time sensitive to oxidation. This suggests that during redox imbalances *in vivo*, something must protect Jun and Fos from oxidative damage. A nuclear protein Ref-1 (redox factor 1), also implicated in DNA repair, has been shown to reactivate AP-1 by a thioredoxin-independent reduction of the critical cysteine residues (Xanthoudakis and Curran, 1992; Xanthoudakis *et al*, 1992; Hirota *et al*, 1997; Ordway *et al*, 2003). While Ref-1 mediates a reparative reduction of once inactivated nuclear AP-1, it seems logical that some factor should also prevent oxidation of newly synthesized Jun and Fos, particularly during oxidative stress.

A good candidate to perform this protective role is the multiprotein bridging factor 1 (MBF1). MBF1, also known as endothelial differentiation-related factor 1 (EDF1), primarily

*Corresponding author. Department of Developmental Genetics, National Institute of Genetics, 1111, Yata, Mishima, Shizuoka-ken 411-8540, Japan. Tel.: +81 559 816771; Fax: +81 559 816776; E-mail: shirose@lab.nig.ac.jp

Received: 21 January 2004; accepted: 16 July 2004; published online: 12 August 2004

resides in the cytoplasm and can relocate to the nucleus upon external stimuli (Mariotti *et al*, 2000). MBF1 acts as a coactivator of the bZIP protein GCN4, the closest relative of Jun in the budding yeast. MBF1 interacts directly with both GCN4 and the TATA-binding protein (TBP), implying that it interconnects the bZIP factor with the basal transcription machinery (Takemaru *et al*, 1998). MBF1 has also been shown to bind human c-Jun (Kabe *et al*, 1999) and stimulate c-Jun-dependent transcription (Busk *et al*, 2003). Unlike other AP-1 interacting proteins, MBF1 is unique in that it binds to the basic region where the redox-sensitive cysteines are located.

We have chosen the fruit fly *Drosophila* to examine the relationships between AP-1 and MBF1. Compared to four Fos and three Jun paralogs in mammals, *Drosophila* is a simple model with only one D-Fos and one D-Jun protein (Kockel *et al*, 2001). In this study, we show that through a direct interaction with D-Jun, MBF1 protects the critical cysteine residue from oxidation and stimulates AP-1 binding to DNA. A mutation removing *mbf1* causes sensitivity to oxidative stress *in vivo* and compromises an AP-1-dependent process of epithelial tissue closure. Studies of MBF1 therefore open an avenue to learn more about AP-1 regulation and function.

Results

Critical cysteine residues confer D-Jun and D-Fos sensitivity to oxidation

Drosophila D-Jun and D-Fos proteins have cysteine residues in the same positions as the subunits of human AP-1, which rapidly loses DNA-binding activity upon oxidation (Figure 1A). To see whether *Drosophila* AP-1 also undergoes oxidative inactivation, we tested the binding of bacterially expressed D-Jun and D-Fos bZIP domains to an AP-1 site using electrophoresis mobility shift assays under disparate redox conditions. Both D-Jun and D-Fos were truncated such that each protein harbored a single cysteine within the basic region, and were designated J and F, respectively (Figure 1B). Their DNA-binding properties were compared with those of point mutants, J^S and F^S, in which the critical cysteine residues had been replaced with serine (Figure 1A). Like their human orthologs, J and F proteins were unable to bind DNA in the absence of dithiothreitol (DTT; Figure 2A, lane 1). Because neither protein bound to DNA alone in our conditions, no binding occurred also when the cysteine was mutated in either D-Fos (Figure 2A, lane 4) or D-Jun (not shown). Only when both proteins were mutated, their complex with the AP-1 site could be detected in the absence of DTT (lane 7). Addition of 1 mM DTT allowed weak binding of the J/F dimer (lane 2); the binding of the mutant proteins under the same conditions was stronger (lanes 5 and 8). Increasing DTT concentration to 10 mM enhanced the binding of dimers in which at least one protein contained cysteine (lanes 3 and 6). These results show that *Drosophila* Jun and Fos are readily inactivated by oxidation of the critical cysteine residues, and suggest existence of factor(s) that maintain the activity of these proteins under oxidative conditions.

MBF1 ensures AP-1 binding to DNA through interaction with D-Jun

To study whether *Drosophila* MBF1 supports AP-1 activity, we first examined the effect of MBF1 on the DNA-binding activity of AP-1 (Figure 2). When MBF1 was added to the electrophoresis mobility shift assay with bacterially produced J and F, it only mildly stimulated DNA binding (Figure 2B, compare lanes 5 and 7). MBF1 exerted a stronger effect when copurified with J from pooled bacterial cultures, each expressing only one protein (lane 9), and the strongest effect was observed when MBF1 was coexpressed with J in *Escherichia coli* using a bicistronic construct (lane 10). The increased DNA-binding activity was not due to a higher yield of the coexpressed proteins (Figure 1C). Thus, MBF1 had to be in contact with D-Jun already within the *E. coli* cells or at least during the purification steps in order to ensure robust AP-1 activity; later addition of MBF1 was not sufficient. MBF1 could also stimulate the DNA-binding activity of the mutant J^S/F^S complex (Figure 2B, lanes 6 and 11), which is only partially sensitive to oxidative condition (Figure 2A, lanes 7 and 8). This result suggests that MBF1 has a more general positive effect on D-Jun that is not entirely mediated by keeping the cysteines reduced.

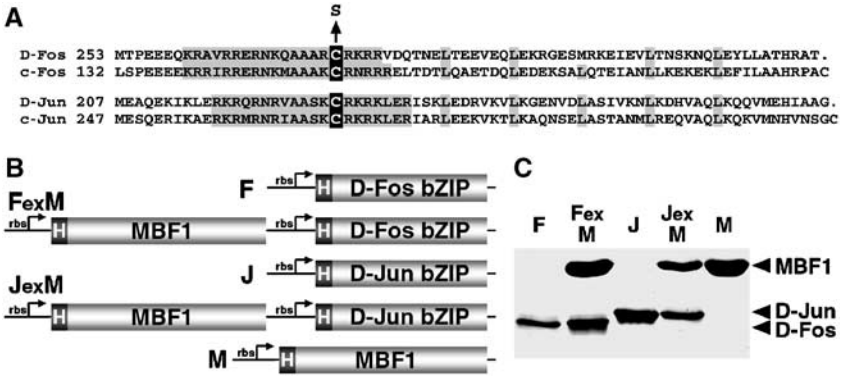

Figure 1 Recombinant D-Fos, D-Jun and MBF1 proteins used in this study. (**A**) *Drosophila* Fos and Jun truncated proteins, aligned with human c-Fos and c-Jun. Conserved basic regions and leucine residues of the bZIP domains are shaded; the critical cysteines are in black boxes. These cysteines were mutated to serine to produce redox-insensitive D-Fos and D-Jun forms (F^S and J^S). The cysteines C-terminal to the leucine zipper were replaced with stop codons in all D-Fos and D-Jun constructs. (**B**) His-tagged D-Fos (F), D-Jun (J) and MBF1 (M) were expressed in *E. coli* either separately or coexpressed from bicistronic plasmid constructs (FexM and JexM). rbs, ribosome-binding site. (**C**) AP-1 and MBF1 proteins were expressed from constructs shown in (B), affinity purified using the His tag, separated on a reducing SDS–polyacrylamide gel and stained with Coomassie blue.

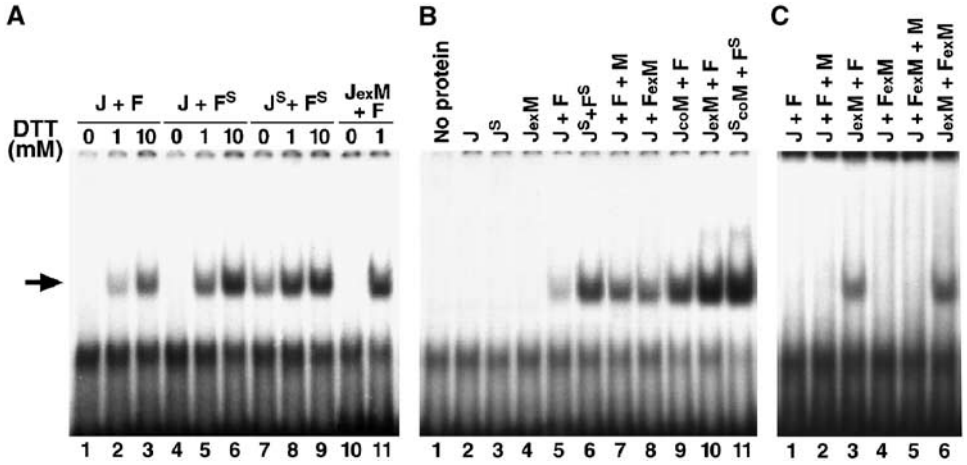

Figure 2 Effects of oxidation and MBF1 on the binding of D-Fos and D-Jun to an AP-1 site. Gel retardation assays were performed with Jun and Fos, each containing the single critical cysteine (J, F), and with the serine mutants (J^S, F^S). MBF1 was added separately (J + F + M), coexpressed with Jun (JexM) or Fos (FexM) from a bicistronic plasmid, or copurified from mixed *E. coli* cultures each expressing one protein (JcoM). The arrow shows the AP-1/DNA complex. (**A**) Binding of freshly purified proteins under oxidative (no DTT) or reducing conditions revealed that *Drosophila* AP-1 activity depends on the redox state of the critical cysteine residues in its DNA-binding domain. (**B**) Binding of freshly purified proteins in the presence of 1 mM DTT showed that weak AP-1 activity (lane 5) was greatly enhanced by coexpression of Jun with MBF1 (lane 10). Both Jun and Fos were required for the binding. (**C**) The assay conditions were as in (B) except that the proteins were aged for 5 days in solution at 4°C. No binding was observed unless MBF1 had been coexpressed with Jun.

To further test the function of MBF1 in preserving AP-1 activity, we artificially 'aged' purified proteins for several days at 4°C. Upon such treatment, AP-1 completely lost its DNA-binding activity, suggesting inactivation due to oxidation and/or denaturation (compare Figure 2B, lane 5, with Figure 2C, lane 1). However, when D-Jun was coexpressed with MBF1, it was able to form active AP-1 even after the aging treatment (Figure 2C, lane 3). Once lost, the AP-1 activity could not be restored by subsequent addition of MBF1 (Figure 2C, lane 2). MBF1 showed its protective effect only on D-Jun; aged AP-1 proteins failed to bind DNA when MBF1 was either added or coexpressed with D-Fos (Figure 2C, lanes 2, 4 and 5). However, D-Fos was still functional, because no DNA binding occurred without it in our assays (Figure 2B, lanes 2–4). These results indicate that MBF1 prevents the deterioration of AP-1 activity specifically through acting on D-Jun.

MBF1 binds the basic region of D-Jun and protects the critical cysteine from oxidation

The enhanced DNA-binding activity of AP-1 in the presence of MBF1 suggests that MBF1 may protect AP-1 from oxidation through a direct contact. To test for interaction between MBF1 and AP-1 proteins, we performed GST pull-down assays with the hexahistidine-tagged D-Jun and D-Fos bZIP domains (Figure 1) and a GST-MBF1 fusion. Figure 3A shows that MBF1 specifically bound the D-Jun but not the D-Fos bZIP region. The failure to bind D-Fos likely was not due to D-Fos deterioration, since this protein was active in our electrophoresis mobility shift assays. Although we have not mapped the exact amino acids required for MBF1 binding in the D-Jun bZIP peptide, we surmise that they include the basic residues near the critical cysteine (Figure 3B), because these basic residues in GCN4 are required for yeast MBF1 binding (Takemaru *et al*, 1998). A very similar basic motif in the nuclear receptor Ftz-F1 (Figure 3B) is necessary for the binding of insect MBF1 (Takemaru *et al*, 1997).

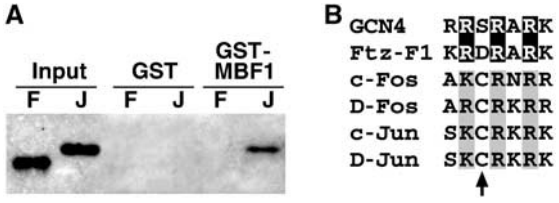

Figure 3 MBF1 binds a conserved basic motif. (**A**) GST pull-down assay showed a direct interaction of a GST-MBF1 fusion protein with the His-tagged D-Jun bZIP domain (J) but not with the same region of D-Fos (F). (**B**) Alignment of basic regions in the yeast bZIP factor GCN4, a nuclear receptor Ftz-F1, and the human and *Drosophila* AP-1 family members. All proteins except D-Fos have been shown to bind MBF1. The black boxes indicate arginine residues in GCN4 and Ftz-F1, known to be required for MBF1 binding; the corresponding basic residues in Fos and Jun are shaded. The arrow points to the critical redox-sensitive cysteine in AP-1 proteins.

To test whether MBF1 prevents oxidation of the redox-sensitive cysteine of D-Jun, the D-Jun bZIP domain expressed in *E. coli* either alone or with MBF1 (Figure 1C) was subjected to MALDI-TOF mass analysis. Figure 4 shows that D-Jun coexpressed with MBF1 remained in the reduced state. In contrast, when expressed alone, a majority of D-Jun increased its mass by 222.6 Da, an increment corresponding to S-cystenyl cystenylation (i.e. cystenyl cyteine attached to D-Jun via a mixed disulfide bond). Consistently, the modified form retained a single reactive SH group per molecule as revealed by monoalkylation with iodoacetamide (data not shown). To confirm that S-cystenyl cystenylation indeed concerned the critical cysteine residue, we show that no such modification occurred in the D-Jun bZIP domain harboring the cysteine-to-serine substitution (J^S), expressed in the absence of MBF1 (Figure 4). MBF1 therefore functions to protect the critical cysteine from oxidative modification.

MBF1 expression and nuclear translocation with D-Jun

Since oxidative stress can occur at any time, MBF1 should be expressed constantly in order to prevent AP-1 oxidation. We

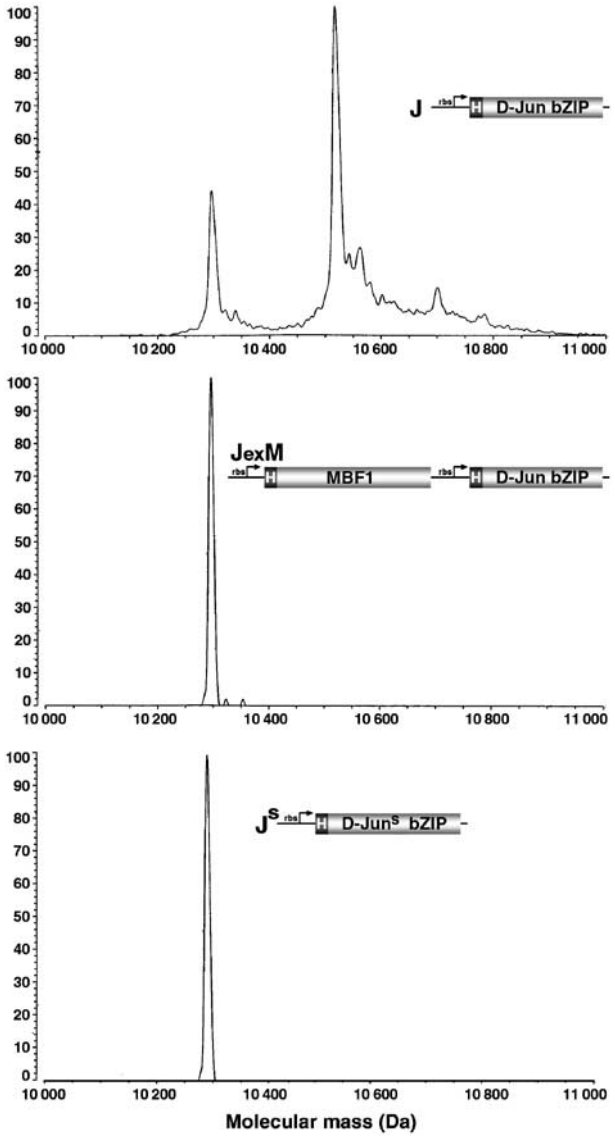

Figure 4 MBF1 prevents an oxidative modification of D-Jun. The His-tagged bZIP domain of D-Jun (J) was expressed in *E. coli* either alone (top) or coexpressed with MBF1 (JexM); its cysteine-to-serine mutant (J^S) was expressed alone (bottom). The purified proteins (see Figure 1C) were subjected to MALDI-TOF mass analysis. D-Jun expressed alone shows a mass increment of 222.6 Da, corresponding to S-cystenyl cystenylation. The mass of His-tagged MBF1 is around 18 kDa.

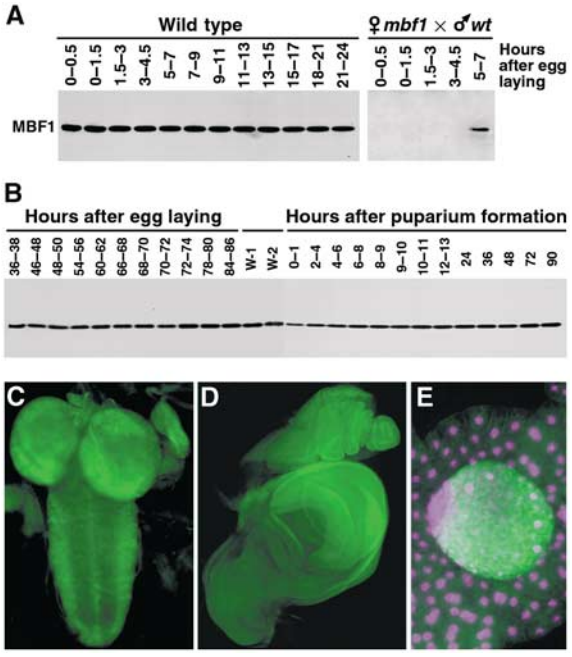

Figure 5 Expression pattern of MBF1 in *Drosophila*. (**A**) Western blot showing constitutive MBF1 expression during embryogenesis. Embryos from *mbf1* mutant mothers were devoid of all maternal MBF1 protein. Zygotic expression from the paternal *wild-type* chromosome began between 5 and 7 h after egg laying (right panel). (**B**) Western blot showing MBF1 expression throughout the postembryonic life. W-1 and W-2, wandering stages of larvae. (**C–E**) Sites of high MBF1 expression during larval life were the central nervous system (C, second instar), imaginal discs (D, late third instar) and the testis (E, center), but not the fat body (E, surrounding tissue). MBF1 was detected with a specific polyclonal antibody, and DAPI was used for DNA staining in (E). Magenta is used as colorblind friendly (http://jfly.iam.u-tokyo.ac.jp/color/text.html).

determined the developmental profile of MBF1 expression using a specific polyclonal antibody that detected a single band of the expected size (16 kDa) on Western blots. The presence of the MBF1 protein started in the embryo with a strong maternal contribution and was maintained throughout embryogenesis, with zygotic translation ensuing 5–7 h after egg laying (Figure 5A). Expression continued for the entire postembryonic life without temporal fluctuations (Figure 5B). Among tissues exhibiting high MBF1 levels were the central nervous system, imaginal discs and gonads, but not the fat body (Figure 5C–E).

Although MBF1 interacts with nuclear partners, previous data (Kabe *et al*, 1999; Mariotti *et al*, 2000; Liu *et al*, 2003) have shown that MBF1 is primarily a cytoplasmic protein, suggesting that MBF1 may cotransport with interacting transcription factors to the nucleus. We have tested whether MBF1 translocates to the nucleus with D-Jun in *Drosophila* cells. As shown in Figure 6, the MBF1 protein resides predominantly in the cytoplasm of both embryonic (S2) and imaginal disc ($Cl.8^+$) cells, cultured under control conditions. In contrast, MBF1 moved to the nucleus in S2 cells that had been transfected with a plasmid expressing D-Jun-His, the entire D-Jun protein with a C-terminal hexahistidine tag (Figure 6A). As revealed with an antibody against His tag, the D-Jun-His protein also accumulated in the nucleus. Co-immunoprecipitation of MBF1 and D-Jun-His from these transfected cells with the His-tag antibody (Figure 6B) suggested that MBF1 translocated to the nucleus upon interaction with D-Jun. Such an interaction likely occurs through MBF1 binding to the bZIP domain of D-Jun, demonstrated in the GST pull-down assay (Figure 3). Transfection of $Cl.8^+$ (Figure 6C) or S2 cells (not shown) with only the His-tagged bZIP domain of D-Jun confirmed that this domain alone was sufficient for the nuclear translocation of MBF1. These data suggest that MBF1 migrates to the nucleus in complex with the newly synthesized D-Jun protein.

Null mbf1 mutants are sensitive to oxidative stress

To study the role of MBF1 *in vivo*, we generated deletions in the *Drosophila mbf1* gene through P-element transposon

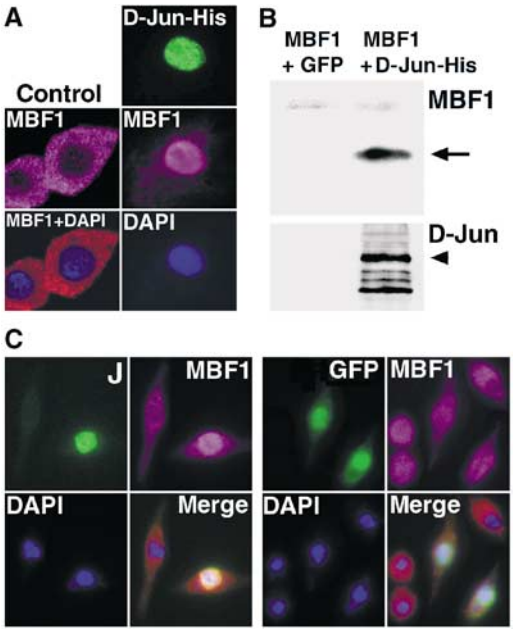

Figure 6 MBF1 and D-Jun form a complex and cotranslocate to the nucleus in *Drosophila* cells. (**A**) *Drosophila* S2 cells showed translocation of the endogenous cytoplasmic MBF1 protein (control) to the nucleus upon misexpression of the whole His-tagged D-Jun protein (right column). (**B**) Upon cotransfection of S2 cells with MBF1 and D-Jun-His (but not GFP), MBF1 was recovered from the cell lysate together with the D-Jun-His protein by using an anti-His-tag monoclonal antibody. The Western blot was first probed with anti-MBF1, then stripped and re-probed with a D-Jun antiserum (bottom). (**C**) Expression of a His-tagged bZIP domain of D-Jun in Cl.8$^+$ cells was sufficient for nuclear translocation of the MBF1 protein (left panel); expression of nuclear GFP had no effect on MBF1 localization. Cells were fixed and stained 36 h after transfection. Staining with anti-His tag was visualized with DTAF (FITC)-conjugated secondary antibody. Anti-MBF1 was detected with a Cy3-conjugated secondary antibody, shown as magenta that is friendly to colorblind people (http://jfly.iam.u-tokyo.ac.jp/color/). GFP was visualized with direct fluorescence.

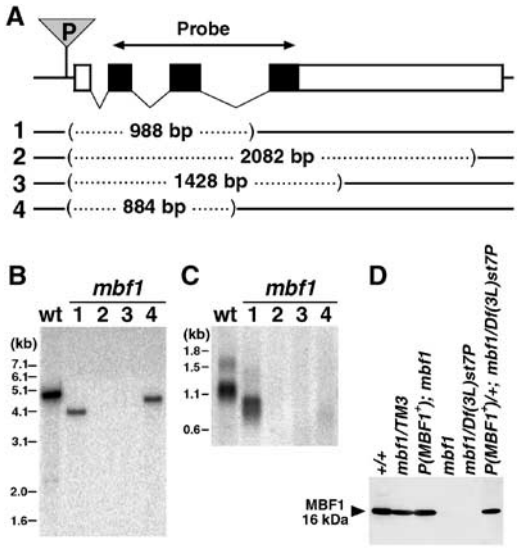

Figure 7 *mbf1* mutant flies are molecularly null. (**A**) Map of the *mbf1* gene located on chromosome 3L with a P-element insertion (P) 21 bp upstream of the first exon and four imprecise P excisions (1–4). Black boxes denote coding exons of *mbf1*, used as a hybridization probe in (B, C); untranslated regions are open. The dotted lines represent DNA deleted in *mbf1* alleles *mbf1*^{1} to *mbf1*^{4} (1–4). Centromere is to the right. (**B**) Southern blot of genomic DNA from *wild-type* (*wt*) and homozygous *mbf1* mutant flies shows that *mbf1*^{2}and *mbf1*^{3} lack the coding region of *mbf1*. (**C**) Northern hybridization of mRNA shows the complete loss of both transcripts (1.6 and 1.1 kb) in *mbf1*^{2} to *mbf1*^{4} homozygotes. (**D**) Western blot analysis of the MBF1 protein from adult flies confirms that *mbf1*^{2} is a null allele. A transgenic construct *P(mbf1*$^+$*)* restores the production of the MBF1 protein. *TM3* is a third chromosome balancer; *Df(3L)st7P* is a deficiency including *mbf1*.

insertion and its subsequent imprecise excision (Figure 7A). The molecular lesions of four deletion alleles (*mbf1*^{1} through *mbf1*^{4}) spanned from the original P-element insertion site toward the coding region, affecting *mbf1* but no other gene (Figure 7A and B). Except for the *mbf1*^{1} allele that had a shortened mRNA, all alleles failed to produce the *mbf1* transcript (Figure 7C). All four alleles, either homozygous or hemizygous over a *Df(3L)st7P* deficiency that includes *mbf1*, were totally devoid of the MBF1 protein (e.g. *mbf1*^{2}; Figure 7D). Despite the complete absence of the MBF1 protein, all four alleles were viable and fertile under standard laboratory conditions. We used *mbf1*^{2}, which had the longest deletion of 2082 bp, in all experiments described hereafter. A rescue construct that includes a 4.6 kb genomic *mbf1* fragment in a P-element vector restored production of the MBF1 protein at all stages examined (Figure 7D and data not shown).

The ability of MBF1 to prevent oxidation of D-Jun suggests that the *mbf1* gene might have an important function during stress, when AP-1 triggers various stress responses. To test the possibility that the loss of *mbf1* affects oxidant tolerance in *Drosophila*, we compared the survival of the *mbf1*^{2} mutants with *mbf1*$^+$ animals (*P(w*$^+$*mbf1*$^+$*)/P(w*$^+$*,mbf1*$^+$*); mbf1*^{2}) in the presence of hydrogen peroxide (H_2O_2) as a source of oxidative stress. When placed on diet containing 0.1 or 0.3% H_2O_2 as first instar larvae, *mbf1*^{2} animals reached adulthood about 3.5 times less frequently than the *mbf1*$^+$ strain (Figure 8A). To test oxidative stress tolerance in adults, we exposed males of equal size and age to 0.5% H_2O_2 and percent surviving was scored at regular intervals (Figure 8B). The median survival time of the *mbf1*^{2} homozygotes was 67 h, compared to 93 h for the *mbf1*$^+$ strain. *mbf1*$^-$ hemizygotes obtained from *Df(3L)st7P/+* mothers, crossed with *mbf1*^{2} males, lived on average 64 h on 0.5% H_2O_2. The sensitivity was therefore not caused by another mutation on the *mbf1*$^-$ chromosome or by a maternal effect. Flies possessing four doses of *mbf1*$^+$ (two endogenous and two transgenic) were more resistant to H_2O_2 than animals with two copies (Figure 8B). When catalase activity was inhibited prior to H_2O_2 treatment by feeding flies with 5 mM aminotriazole, the effect of H_2O_2 was greatly enhanced; the lifespan of *mbf1* mutants was less than 60% that of the rescued flies (Figure 8B). A similar enhancement was observed when flies were pretreated with 5 mM buthionine-sulfoximine, a drug that reduces the free radical scavenging capacity by depleting the endogenous pool of glutathione (data not shown). Together, these results show that the loss of MBF1 renders animals sensitive to ROS.

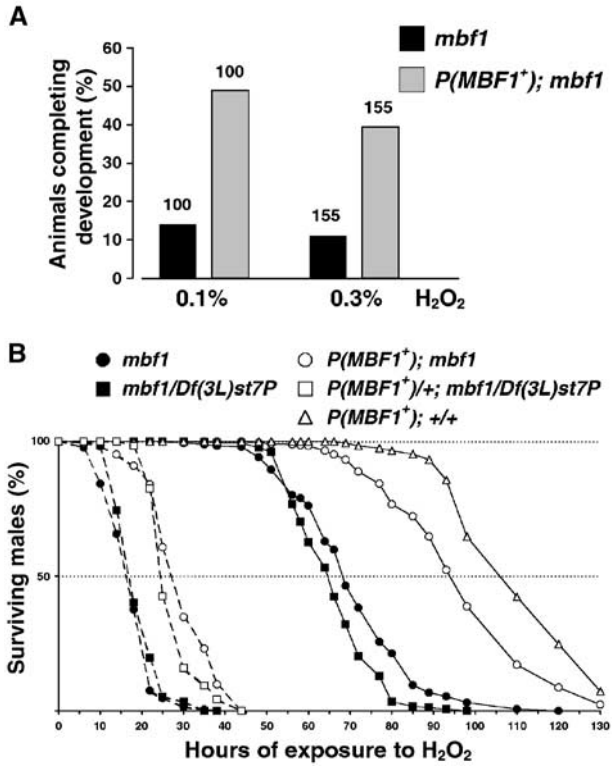

Figure 8 MBF1-deficient mutants are sensitive to oxidative stress. (**A**) Equal numbers of first instar larvae of the *mbf1* mutant and rescued lines were placed on diet containing indicated concentrations of H_2O_2 and numbers of emerging adults were counted. Values above bars indicate the numbers of larvae. (**B**) Adult males (30 per vial) were exposed to H_2O_2 either directly (solid lines) or after reducing their catalase activity by feeding 5 mM aminotriazole for 60 h (broken lines), and their lifetime was recorded. The open symbols denote flies possessing at least one copy of $mbf1^+$. Line $P(mbf1^+)/P(mbf1^+)$; +/+ contains four copies of $mbf1^+$. The numbers of flies tested per genotype ranged between 120 and 540, and the resistance of each genotype was tested at least three times.

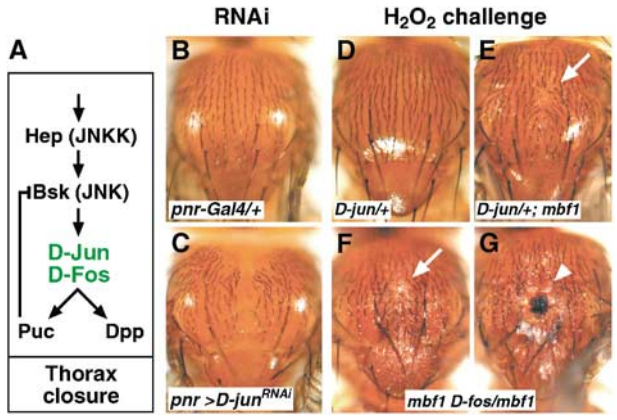

Figure 9 Genetic interaction between MBF1 and AP-1 during thorax closure. (**A**) The JNK cascade is required for *Drosophila* thorax closure (after Kockel *et al*, 2001). Hemipterous (Hep) phosphorylates a Jun N-terminal kinase Basket (Bsk). The redox-sensitive JNK substrates (AP-1) are in green. A JNK phosphatase Puckered (Puc) and a TGFβ signal Dpp (Decapentaplegic) are putative targets of AP-1. Incomplete function of Hep or D-Fos causes cleft adult thorax; Puckered is a negative regulator of the thorax closure. (**B, C**) RNAi knockdown of D-Jun, targeted to the dorsal epidermis using *pnr-Gal4*, prevents complete fusion of the thorax; a control expressing the driver alone has normal thorax (B). (**D–G**) Animals of indicated genotypes were challenged with H_2O_2 at the onset of metamorphosis. Emerging adults doubly mutant for *mbf1* and either *D-jun* (E) or *D-fos* (F,G) occasionally displayed partially cleft thorax with naked cuticle (arrows), sometimes accompanied with necrosis (arrowhead).

An AP-1-dependent developmental process becomes sensitive to oxidative stress in mbf1 mutant background

In addition to triggering antioxidant responses, AP-1 has various developmental functions including formation of the adult thorax (reviewed by Kockel *et al*, 2001). D-Fos is required for fusion of the wing imaginal discs at the dorsal midline (Riesgo-Escovar and Hafen, 1997a; Zeitlinger and Bohmann, 1999). To see whether also D-Jun is required for thorax closure, we induced an RNA interference (RNAi) knockdown of D-Jun in the dorsal epithelium using the *pnr-Gal4* driver (Heitzler *et al*, 1996). This resulted in mild to severe defects of thorax fusion in 23% of the $UAS\text{-}D\text{-}Jun^{RNAi}/+$; *pnr-Gal4/+* flies (Figure 9C).

The AP-1 proteins (Zeitlinger and Bohmann, 1999) and MBF1 (Figure 5D) are coexpressed in the wing imaginal discs. To examine whether the ability of MBF1 to protect D-Jun from oxidation is important for development, we tested whether *mbf1* interacts genetically with *D-jun*/*D-fos* under an H_2O_2 challenge (Figure 9D–G). Animals that were heterozygous for the recessive lethal mutations in *D-jun* or *D-fos* did not show any defects when treated with H_2O_2 at the onset of metamorphosis, indicating that a single dose of *D-jun* or *D-fos* is sufficient to support normal development under the oxidative challenge. However, in *mbf1* mutant background, *D-jun/+* and *D-fos/+* animals often produced adults with defects in the thorax, manifest as a depressed patch of naked cuticle at the dorsal midline (Figure 9E and F). In some *mbf1 D-fos/mbf1* flies, a necrosis occurred at the site of the wound (Figure 9G). The thorax defects in $mbf1^-$ mutants were similar to the phenotypes resulting from compromised function of several components of the D-JNK cascade (Figure 9A) including D-Jun (Figure 9C) and D-Fos. These results thus suggest that as *in vitro*, the lack of MBF1 destabilizes AP-1 under oxidative conditions *in vivo*.

Discussion

MBF1 in the redox regulation of AP-1

Sensitivity of AP-1 to oxidation requires a mechanism to protect AP-1 activity. We introduce MBF1 as a new player that allows cells to maintain adequate AP-1 activity under oxidative stress. *Drosophila* AP-1 components D-Jun and D-Fos undergo oxidative inactivation via the same cysteine residues as the human orthologs. MBF1 prevents this oxidation and preserves the DNA-binding activity. In *mbf1* mutants, an AP-1-dependent developmental process becomes hypersensitive to oxidative stress, suggesting that MBF1 also protects D-Jun from an oxidative modification *in vivo*. The protection is unlikely to be complete because it relies on the binding of MBF1 to Jun. Thus, the AP-1 action may be in an equilibrium between acceleration by the MBF1 protection of Jun and brake by the oxidative inactivation of Jun.

The mechanism by which MBF1 ensures the activity of AP-1 is different from that of the nuclear protein Ref-1, which reactivates oxidized AP-1 by reduction (Xanthoudakis and

Curran, 1992). MBF1 was a much stronger enhancer of AP-1 activity when coexpressed and copurified with D-Jun from *E. coli* than when it was added to the DNA-binding assay separately. Unlike Ref-1, MBF1 was unable to restore AP-1 activity once lost. Thus, rather than reactivating AP-1, MBF1 protects it from oxidation in a preventive manner. Protection from oxidation is however one of several stabilizing effects that MBF1 exerts on AP-1, because MBF1 can stimulate DNA binding even of mutant AP-1 proteins, possessing serine instead of the redox-sensitive cysteine residues.

MBF1 enhanced the DNA-binding activity of AP-1 selectively through D-Jun. Since MBF1 bound D-Jun but not D-Fos in a direct interaction assay, we propose that the selectivity is based on an exclusive contact between MBF1 and D-Jun. This was unexpected as human MBF1 was shown to bind a GST-c-Fos fusion (Kabe *et al*, 1999). On the other hand, D-Jun and c-Jun share more similarity than the Fos orthologs; in particular, the critical cysteine context KCR reads RCR in D-Fos.

Although AP-1 regulation via the redox-sensitive cysteine residues was postulated more than a decade ago (Abate *et al*, 1990), the nature of the cysteine modification remained unknown. The prediction is that a regulatory oxidation may involve a reversible formation of sulfenic acid or a disulfide bond (Marshall *et al*, 2000). To examine how the critical cysteine is modified, we determined the molecular mass of the bacterially expressed D-Jun used in DNA-binding assays. The *E. coli* system allowed us to express D-Jun in the absence of endogenous MBF1. Surprisingly, we identified a previously undescribed modification of the critical cysteine, S-cystenyl cystenylation. In a striking contrast, no such modification occurred in D-Jun coexpressed with MBF1 or in D-Jun lacking the critical cysteine residue. S-glutathiolation of the cysteine, a similar modification that was known to prevent binding of c-Jun to an AP-1 site (Klatt *et al*, 1999), was not observed in D-Jun despite GSH:GSSG is an abundant redox system in *E. coli* (Sundquist and Fahey, 1989). Whether S-cystenyl cystenylation is only a product of the prokaryotic expression system or whether it represents true physiological regulation of AP-1 activity remains to be tested. However, our aim was to disclose the role for MBF1, and the ability of MBF1 to avert S-cystenyl cystenylation shows that this role is to protect D-Jun.

While our data illuminate the role of MBF1 in the protection of the redox-sensitive cysteine in D-Jun, MBF1 also stimulated DNA binding of the serine mutant (Figure 2). Thus the effect of MBF1 on D-Jun is not limited to protecting the critical cysteine but includes a more general stabilizing effect on the basic region. This is consistent with the observation that yeast MBF1 enhanced DNA binding of GCN4, which harbors a serine in the position of the oxidation-sensitive cysteine (Takemaru *et al*, 1998). Analysis of yeast MBF1 and GCN4 indicates that this serine resides within the region contacted by MBF1 (Takemaru *et al*, 1998). We speculate that it is this evolutionarily ancient function of MBF1 to support the activity of bZIP proteins that permitted the acquisition of the redox regulation of AP-1 by oxidation of the critical cysteine; in the absence of MBF1, such mutation (serine to oxidation-sensitive cysteine) would be prone to the total destruction of the AP-1 activity even under mild oxidative conditions. Interestingly, the yeast counterpart of AP-1 (yAP-1) is also required for antioxidant defense and is accordingly regulated by the redox state, albeit at the level of nuclear export (Kuge *et al*, 1997; Yan *et al*, 1998; Toone *et al*, 2001). The metazoan AP-1 may have introduced redox sensing at the DNA-binding step since it is directly involved in transcriptional regulation compared with the nuclear export.

MBF1 supports AP-1 functioning during oxidative stress in Drosophila

Despite the fact that evolutionary conservation of MBF1 suggests an essential role for the protein, null mutants lacking MBF1 proved to be viable in *Drosophila* (this study) and yeast (Takemaru *et al*, 1998) under laboratory conditions. Strikingly, however, in both organisms, MBF1 is essential during stress situations encountered in the real world: *Drosophila mbf1* mutants are sensitive to oxidative stress induced by H_2O_2, and yeast MBF1 mutants are unable to overcome nutritional stress due to their inability to maintain the activity of GCN4, a regulator of amino-acid synthesis (Takemaru *et al*, 1998). A comparative advantage provided by MBF1 under stress conditions is thus the likely cause of its evolutionary conservation. We propose that in both yeast and *Drosophila*, MBF1 achieves these functions via the same mechanism, through binding a bZIP transcription factor.

The interaction between MBF1 and D-Jun, documented in this study, provides a molecular basis of the H_2O_2 sensitivity of *mbf1* mutants. This is supported by the recently published evidence that JNK signaling is indeed required for oxidant resistance in *Drosophila* (Wang *et al*, 2003). A developmental defect that can occur in *mbf1* mutants under oxidative stress is the failure to form a continuous cuticle at the dorsal midline. The cell shape changes of epithelia that occur at the dorsal closure during embryogenesis and adult morphogenesis are regulated by the JNK signaling pathway, culminating in the phosphorylation of D-Jun (Glise *et al*, 1995; Sluss *et al*, 1996; Glise and Noselli, 1997; Hou *et al*, 1997; Kockel *et al*, 1997; Riesgo-Escovar and Hafen, 1997a, b; Zeitlinger *et al*, 1997; Agnes *et al*, 1999; Zeitlinger and Bohmann, 1999; Martin-Blanco *et al*, 2000). Using a knockdown experiment, we show here that also D-Jun is directly involved in the adult thorax closure. Because MBF1 exhibits a genetic interaction with AP-1 subunits under H_2O_2 challenge, it is likely that D-Jun requires its partner MBF1 to be protected from oxidation during its function in thorax closure. Necrotic wounds in *mbf1 D-fos/mbf1* flies are a newly observed phenomenon, which may be connected with the exposure to H_2O_2 and may reflect a specific requirement for Fos in wound healing (Martin and Nobes, 1992). Another phenotype that *mbf1* mutants display is the reduced longevity when challenged with H_2O_2. Since AP-1 is known to trigger antioxidant defense, we favor the idea that H_2O_2 hypersensitivity of *mbf1* mutants is also due to their failure to protect *Drosophila* AP-1 activity during oxidative condition. For either phenotype function, the possibility remains that MBF1 also supports functions of other transcription factors.

Dual mode of coactivator action

MBF1 was first described as a coactivator that bridges bZIP transcription factors and the basal transcriptional machinery. Yeast MBF1 supports GCN4-dependent activation of the *HIS3* gene (Takemaru *et al*, 1998) and *Drosophila* MBF1 serves as a coactivator of a bZIP protein Tracheae defective/Apontic during morphogenesis of the tracheal and nervous systems

(Liu *et al*, 2003). In either case, MBF1 facilitates the formation of a ternary complex consisting of the bZIP protein, MBF1 and the general transcription factor TBP. MBF1 has been recently shown to interact also with human AP-1 proteins (Kabe *et al*, 1999) and function as a novel transcriptional coactivator of c-Jun in a human cell line (Busk *et al*, 2003).

Results presented here suggest a new function for coactivators. We demonstrate that MBF1 can prevent an oxidative modification of D-Jun produced in bacteria, and that MBF1 activity becomes important under oxidative environmental conditions *in vivo*. Association of MBF1 with D-Jun in *Drosophila* cells and the D-Jun-dependent nuclear localization of MBF1 suggest that endogenous Jun, once synthesized, is quickly bound by MBF1. Thus it is possible that transcriptional coactivators may exert a stabilizing or protective effect on their partner transcription factors even before they engage in transcription, and that the formation of the ternary complex is a two-step phenomenon involving a preformed complex and TBP.

Materials and methods

Expression and purification of recombinant proteins

DNA regions encoding amino acids from Met_{253} to Thr_{324} in D-Fos and from Met_{207} to Gly_{279} in D-Jun (Perkins *et al*, 1990) were N-terminally fused with a hexahistidine tag (6H) in the pET-28a vector (Novagen). Both proteins were terminated by converting the TGC codons of Cys_{325} in D-Fos and Cys_{280} in D-Jun to TGA. To produce redox-insensitive AP-1 forms, the single remaining Cys_{275} in D-Fos and Cys_{229} in D-Jun were substituted with serine (AGC) using PCR-based mutagenesis (Abate *et al*, 1990). The entire coding region of *Drosophila* MBF1 (Liu *et al*, 2003) was also cloned behind the 6H tag in pET-28a. For coexpression, bicistronic pET-28a vectors with tandemly cloned 6H-tagged MBF1 and D-Jun (or MBF1 and D-Fos) sequences (Figure 1) were constructed according to Li *et al* (1997). All proteins were expressed in *E. coli* strain BL21-CodonPlus (Stratagene) and recovered under native conditions. Bacteria at $OD_{600} = 0.5$ were induced with 1 mM IPTG and grown for 4–6 h at 25°C. Harvested cells were sonicated in buffer A (20 mM Tris–HCl (pH 7.2), 500 mM KCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100) on ice and centrifuged (38 000 *g*, 20 min at 4°C). The supernatant was loaded onto an Ni-NTA agarose affinity column (Qiagen), and proteins were eluted with 250 mM imidazole in buffer A and stored in this solution at 4°C or frozen at −80°C.

Electrophoresis mobility shift assay

DNA-binding assays were carried out in 12.5. mM Hepes–KOH (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 4 mg/ml bovine serum albumin (BSA) and DTT (1 mM unless specified otherwise). Combinations of recombinant Jun, Fos and MBF1 proteins (each approximately 100 ng/20 µl reaction) were preincubated in this buffer for 30 min at 30°C, then 20 µg of poly(dI-dC) and 25 fmol of the ^{32}P-labeled probe were added and the reaction was incubated for another 30 min at 30°C. The probe AP-1 site has been described previously (Perkins *et al*, 1990; Eresh *et al*, 1997). Complexes were resolved on 6% polyacrylamide gels in Tris–borate–EDTA (pH 8.3) at room temperature. Contrary to findings of others (Perkins *et al*, 1990; Eresh *et al*, 1997), we have not observed binding of D-Jun or D-Fos homodimers to the same AP-1 site under any conditions. This could be due to truncations, designed to eliminate a second cysteine C-terminal to the leucine zipper in both proteins.

GST pull-down assay

The entire MBF1 protein, N-terminally fused with GST, was expressed in *E. coli* using the pGEX-4T-3 vector and purified from a soluble fraction on Glutathione Sepharose 4B beads (Pharmacia). Interaction assay with the purified His-tagged D-Jun and D-Fos bZIP domains was performed as described (Takemaru *et al*, 1998) and the bound proteins were detected on Western blots with an anti-polyhistidine monoclonal antibody (Sigma) diluted 1:5000.

MALDI-TOF analysis

The bZIP region of D-Jun was expressed alone or coexpressed with MBF1 in *E. coli* and the 6H-tagged proteins were affinity purified as described above. The purified proteins were desalted on C18ZipTip and analyzed on a MALDI-TOF mass spectrometer Axima-CFR (Shimazu, Kyoto, Japan) using alpha-cyano-4-hydroxycinnamic acid as matrix.

MBF1 antibody, immunoblot and tissue staining

The entire 6H-tagged MBF1 protein was prepared as described above, then purified on Mono S column (Pharmacia) and used for immunization of rabbit. The polyclonal serum was diluted 1:50 000 for immunoblots of total *Drosophila* extracts, and 1:10 000 for tissue staining. For Western blots, embryos, larvae, pupae or flies were homogenized directly in a denaturing sodium dodecylsulfate (SDS) buffer, and proteins (15 µg per lane) were separated on an SDS–15% polyacrylamide gel. Detection was with a goat HRP-conjugated anti-rabbit antibody (1:3000) and a chemiluminescent substrate. For tissue staining, larvae were dissected in a phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 1 h. After permeabilization with 0.3% Triton X-100 in PBS and blocking in 5% normal goat serum, the tissues were incubated for 24 h with anti-MBF1, washed and then stained overnight with a Cy3-conjugated goat anti-rabbit antibody (Amersham) diluted 1:2000. DNA counterstaining was with DAPI (200 ng/ml).

Transfection, immunostaining and immunoprecipitation in Drosophila cultured cells

S2 cells were grown in Shields and Sang medium (Sigma) with 10% FBS (Invitrogen). Cl.8$^+$ cells were kept in Shields and Sang medium supplemented with 2% FBS, 2.5% fly extract and 0.125 IU/ml insulin. Cells (10^6/ml) were transfected using calcium phosphate precipitation with 0.5 µg of the pIE1^{hr}/PA plasmid (a gift from Dr Bock), expressing either MBF1, the entire D-Jun protein with a C-terminal hexahistidine tag (D-Jun-His), the 6H-tagged bZIP domain of D-Jun or the green fluorescent protein (GFP) under the IE baculoviral promoter. For immunoprecipitation, cells were lysed in 25 mM Hepes (pH 7.4), 60 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4 and mixed protease inhibitors (Sigma). Dynabeads Protein G (Dynal) magnetic beads bound with a monoclonal anti-polyhistidine antibody (Sigma) were incubated with 100 µl of the cell extract and washed as described (Liu *et al*, 2003). Co-immunoprecipitated D-Jun-His and MBF1 proteins were detected with anti-MBF1 and anti-D-Jun (Bohmann *et al*, 1994) rabbit polyclonal sera. For immunostaining, cells were fixed for 10 min with 3.7% formaldehyde and permeabilized for 10 min in 0.2% Triton X-100 in PBS. After blocking with 2.5% skim milk and 2.5% BSA in TBST (25 mM Tris–HCl (pH 7.5), 136 mM NaCl, 2.68 mM KCl, 0.1% Triton X-100), cells were incubated with the anti-MBF1 (1:10 000) and the anti-polyhistidine (1:800) antibodies in blocking solution for 2 h. After washing in TBST, MBF1 was visualized with anti-rabbit Cy3 (Amersham) and 6H-tagged D-Jun with anti-mouse DTAF (Jackson Immunoresearch).

Preparation and genetic rescue of mbf1 mutants

The P[*lacW*$^+$] element sited in the first exon of the *argos* gene (*sty*^{p2}; Okano *et al*, 1992), about 100 kb to the left of *mbf1* on the 3L chromosome, was mobilized in the presence of the P transposase Δ2-3. Close to 4500 males representing new insertion lines were individually mated with *TM3*-balanced virgins. The males were captured 4 days later, and in pools of 10 were prepared for PCR analysis (Gloor *et al*, 1993). P[*lacW*$^+$] insertions near *mbf1* were screened using PCR with one primer identical to the P-element 31-bp terminal repeat and two antisense *mbf1*-specific primers. All three primers were combined in standard reactions (1 min at 94°C, 1 min at 68°C, 3 min at 72°C, 30 cycles) with 1 µl of the fly extract and the ExTaq DNA polymerase (Takara). A single line with P[*lacW*$^+$] around 20 bp upstream of the first *mbf1* exon was isolated. Genomic DNA from homozygous flies was sequenced to determine the P[*lacW*$^+$] position and to confirm that its original insertion site in *argos* was now wild type. Expression of *mbf1* mRNA and protein products was unaffected by the P-element insertion. *mbf1*-null mutants were generated by remobilizing P[*lacW*$^+$]. Imprecise excisions were detected among 500 males using PCR as described above, except that two primers flanking the *mbf1* gene were used. Sequencing of the PCR products determined the extent of deletions in the resulting alleles. The absence of the

genomic DNA, mRNA and protein in homozygous *mbf1* mutants was confirmed with standard hybridization methods using the coding region of *mbf1* as a probe, and by Western blot. Genetic rescue, which fully restored MBF1 expression, was carried out as described previously (Liu *et al*, 2003), except that the genomic fragment containing $mbf1^+$ was placed on the second chromosome.

Fly stocks and RNAi

The *mbf1*-null mutants were used either as homozygotes or as hemizygotes over a *Df(3L)st7P* deficiency including *mbf1*. Loss of *mbf1* was combined with recessive embryonic lethal alleles of *D-jun* ($cn^1 Jra^{IA109} bw^1 sp^1$) and *DJNK* ($bsk^2 cn^1 bw^1 sp^1$) on the second chromosome (Nüsslein-Volhard *et al*, 1984). The recessive lethal allele of *D-fos* ($ru^1 h^1 th^1 st^1 cu^1 sr^1 e^s kay^1 ca^1$) (Jürgens *et al*, 1984) was recombined with $mbf1^2$ to produce $mbf1^2 sr^1 e^s kay^1 ca^1$, referred to in the text as *mbf1 D-fos*. RNAi silencing of *D-jun* was performed using the UAS/Gal4 system (Kennerdell and Carthew, 2000). The UAS transgenic line expressing a hairpin-loop *D-jun* RNA was a kind gift from Dr Yanicostas (Inst. Monod, Paris); the *pannier* (*pnr*) Gal4 driver was described previously (Calleja *et al*, 1996).

Oxidant resistance tests

H_2O_2 (0.5%) was added to 1.3% sucrose in 1% low melting point agarose at 40°C. The medium was dispensed into glass vials and allowed to solidify. Catalase (3-amino-1,2,4-triazole; Sigma) and GSH synthase (L-buthionine-[*S*,*R*]-sulfoximine; Sigma) inhibitors in aqueous solutions were applied the same way. Adult males 3–5 days old were placed in vials (30 per vial) and their survival was monitored. At least 120 flies were tested per genotype, over 500 for the $mbf1^-$ and rescued lines. Resistance of larvae was tested by placing first instar larvae on a sucrose–yeast medium containing 0.1 or 0.3% H_2O_2. Animals at the larval–pupal transition were challenged with H_2O_2 vapors using a tissue paper soaked with 30% H_2O_2 and placed into vials with wandering and freshly pupariating larvae.

Acknowledgements

We thank Shigeo Hayashi, Keith Yamamoto and Jean-Antoine Lepesant for discussions, Dino Yanicostas for $D\text{-}jun^{RNAi}$ flies and Dirk Bohmann for anti-D-Jun antibody and D-Jun cDNA. Advice provided on oxidant sensitivity tests by Herve Tricoire and on the use of aminotriazole by Pedro Oliveira is appreciated. We acknowledge receiving fly stocks from the Bloomington center. This work was supported by 204/04/1371 from the Czech Grant Agency to MJ, who was also supported by a Center of Excellence Program of Japan, and by a Grant-in-aid for Scientific Research from MEXT of Japan to SH.

References

Abate C, Patel L, Rauscher FJ, Curran T (1990) Redox regulation of Fos and Jun DNA-binding activity *in vitro*. *Science* **249:** 1157–1161

Agnes F, Suzanne M, Noselli S (1999) The *Drosophila* JNK pathway controls the morphogenesis of imaginal discs during metamorphosis. *Development* **126:** 5453–5462

Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R (1987) Human protooncogene c-Jun encodes a DNA-binding protein with structural and functional properties of transcription factor AP-1. *Science* **238:** 1386–1392

Bohmann D, Ellis MC, Staszewski LM, Mlodzik M (1994) *Drosophila* Jun mediates Ras-dependent photoreceptor determination. *Cell* **78:** 973–986

Busk PK, Wulf-Andersen L, Strom CC, Enevoldsen M, Thirstrup K, Haunso S, Sheikh SP (2003) Multiprotein bridging factor 1 cooperates with c-Jun and is necessary for cardiac hypertrophy *in vitro*. *Exp Cell Res* **286:** 102–114

Calleja M, Moreno E, Pelaz S, Morata G (1996) Visualization of gene expression in living adult *Drosophila*. *Science* **274:** 252–255

Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103:** 239–252

Eresh S, Riese J, Jackson DB, Bohmann D, Bienz M (1997) A CREB-binding site as a target for decapentaplegic signalling during *Drosophila* endoderm induction. *EMBO J* **16:** 2014–2022

Glise B, Bourbon H, Noselli S (1995) *hemipterous* encodes a novel *Drosophila* MAP kinase kinase, required for epithelial-cell sheet movement. *Cell* **83:** 451–461

Glise B, Noselli S (1997) Coupling of Jun amino-terminal kinase and decapentaplegic in *Drosophila* morphogenesis. *Genes Dev* **11:** 1738–1747

Gloor GB, Preston CR, Johnsonschlitz DM, Nassif NA, Phillis RW, Benz WK, Robertson HM, Engels WR (1993) Type-i repressors of P-element mobility. *Genetics* **135:** 81–95

Heitzler P, Haenlin M, Ramain P, Calleja M, Simpson P (1996) A genetic analysis of *pannier*, a gene necessary for viability of dorsal tissues and bristle positioning in *Drosophila*. *Genetics* **143:** 1271–1286

Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K, Yodoi J (1997) AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci USA* **94:** 3633–3638

Hou XS, Goldstein ES, Perrimon N (1997) *Drosophila* Jun relays the Jun amino-terminal kinase signal transduction pathway to the decapentaplegic signal transduction pathway in regulating epithelial cell sheet movement. *Genes Dev* **11:** 1728–1737

Jürgens G, Wieschaus E, Nüsslein-Volhard C, Kluding H (1984) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster* 2. Zygotic loci on the 3rd chromosome. *Wilhelm Roux's Arch Dev Biol* **193:** 283–295

Kabe Y, Goto M, Shima D, Imai T, Wada T, Morohashi K, Shirakawa M, Hirose S, Handa H (1999) The role of human MBF1 as a transcriptional coactivator. *J Biol Chem* **274:** 34196–34202

Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein- kinases. *J Biol Chem* **270:** 16483–16486

Kennerdell JR, Carthew RW (2000) Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nat Biotechnol* **18:** 896–898

Klatt P, Molina EP, De Lacoba MG, Padilla CA, Martinez-Galisteo E, Barcena JA, Lamas S (1999) Redox regulation of c-Jun DNA binding by reversible S-glutathiolation. *FASEB J* **13:** 1481–1490

Kockel L, Homsy JG, Bohmann D (2001) *Drosophila* AP-1: lessons from an invertebrate. *Oncogene* **20:** 2347–2364

Kockel L, Zeitlinger J, Staszewski LM, Mlodzik M, Bohmann D (1997) Jun in *Drosophila* development: redundant and nonredundant functions and regulation by two MAPK signal transduction pathways. *Genes Dev* **11:** 1748–1758

Kuge S, Jones N, Nomoto A (1997) Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J* **16:** 1710–1720

Li C, Schwabe JWR, Banayo E, Evans RM (1997) Coexpression of nuclear receptor partners increases their solubility and biological activities. *Proc Natl Acad Sci USA* **94:** 2278–2283

Liu QX, Jindra M, Ueda H, Hiromi Y, Hirose S (2003) *Drosophila* MBF1 is a co-activator for Tracheae defective and contributes to the formation of tracheal and nervous systems. *Development* **130:** 719–728

Maki Y, Bos TJ, Davis C, Starbuck M, Vogt PK (1987) Avian-sarcoma virus-17 carries the jun oncogene. *Proc Natl Acad Sci USA* **84:** 2848–2852

Mariotti M, De Benedictis L, Avon E, Maier JAM (2000) Interaction between endothelial differentiation-related factor-1 and calmodulin *in vitro* and *in vivo*. *J Biol Chem* **275:** 24047–24051

Marshall HE, Merchant K, Stamler JS (2000) Nitrosation and oxidation in the regulation of gene expression. *FASEB J* **14:** 1889–1900

Martin P, Nobes CD (1992) An early molecular-component of the wound-healing response in rat embryos—induction of c-Fos protein in cells at the epidermal wound margin. *Mech Dev* **38:** 209–216

Martin-Blanco E, Pastor-Pareja JC, Garcia-Bellido A (2000) JNK and decapentaplegic signaling control adhesiveness and cytoskeleton dynamics during thorax closure in *Drosophila*. *Proc Natl Acad Sci USA* **97:** 7888–7893

Morgan IM, Havarstein LS, Wong WY, Luu P, Vogt PK (1994) Efficient induction of fibrosarcomas by v-Jun requires mutations in the DNA-binding region and the transactivation domain. *Oncogene* **9:** 2793–2797

Nüsslein-Volhard C, Wieschaus E, Kluding H (1984) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster* 1. Zygotic loci on the 2nd chromosome. *Wilhelm Roux's Arch Dev Biol* **193:** 267–282

Okano H, Hayashi S, Tanimura T, Sawamoto K, Yoshikawa S, Watanabe J, Iwasaki M, Hirose S, Mikoshiba K, Montell C (1992) Regulation of *Drosophila* neural development by a putative secreted protein. *Differentiation* **52:** 1–11

Okuno H, Akahori A, Sato H, Xanthoudakis S, Curran T, Iba H (1993) Escape from redox regulation enhances the transforming activity of Fos. *Oncogene* **8:** 695–701

Ordway JM, Eberhart D, Curran T (2003) Cysteine 64 of Ref-1 is not essential for redox regulation of AP-1 DNA binding. *Mol Cell Biol* **23:** 4257–4266

Perkins KK, Admon A, Patel N, Tjian R (1990) The *Drosophila* Fos-related AP-1 protein is a developmentally regulated transcription factor. *Genes Dev* **4:** 822–834

Riesgo-Escovar JR, Hafen E (1997a) Common and distinct roles of DFos and DJun during *Drosophila* development. *Science* **278:** 669–672

Riesgo-Escovar JR, Hafen E (1997b) *Drosophila* Jun kinase regulates expression of decapentaplegic via the ETS-domain protein Aop and AP-1 transcription factor DJun during dorsal closure. *Genes Dev* **11:** 1717–1727

Shaulian E, Karin M (2002) AP-1 as a regulator of cell life and death. *Nat Cell Biol* **4:** E131–E136

Sluss HK, Han ZQ, Barrett T, Davis RJ, Ip YT (1996) A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*. *Genes Dev* **10:** 2745–2758

Stronach BE, Perrimon N (1999) Stress signaling in *Drosophila*. *Oncogene* **18:** 6172–6182

Sundquist AR, Fahey RC (1989) Evolution of antioxidant mechanisms: thiol-dependent peroxidases and thioltransferases among procaryotes. *J Mol Evol* **29:** 429–435

Takemaru K, Harashima S, Ueda H, Hirose S (1998) Yeast coactivator MBF1 mediates GCN4-dependent transcriptional activation. *Mol Cell Biol* **18:** 4971–4976

Takemaru K, Li FQ, Ueda H, Hirose S (1997) Multiprotein bridging factor 1 MBF1 is an evolutionarily conserved transcriptional coactivator that connects a regulatory factor and TATA element-binding protein. *Proc Natl Acad Sci USA* **94:** 7251–7256

Toone WM, Morgan BA, Jones N (2001) Redox control of AP-1-like factors in yeast and beyond. *Oncogene* **20:** 2336–2346

Wang MC, Bohmann D, Jasper H (2003) JNK signaling confers tolerance to oxidative stress and extends lifespan in *Drosophila*. *Dev Cell* **5:** 811–816

Xanthoudakis S, Curran T (1992) Identification and characterization of Ref-1, a nuclear-protein that facilitates AP-1 DNA-binding activity. *EMBO J* **11:** 653–665

Xanthoudakis S, Miao G, Wang F, Pan Y-CE, Curran T (1992) Redox activation of Fos–Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J* **11:** 3323–3335

Yan C, Lee LH, Davis LI (1998) Crm1p mediates regulated nuclear export of a yeast AP-1-life transcription factor. *EMBO J* **17:** 7416–7429

Zeitlinger J, Bohmann D (1999) Thorax closure in *Drosophila*: involvement of Fos and the JNK pathway. *Development* **126:** 3947–3956

Zeitlinger J, Kockel L, Peverali FA, Jackson DB, Mlodzik M, Bohmann D (1997) Defective dorsal closure and loss of epidermal decapentaplegic expression in *Drosophila fos* mutants. *EMBO J* **16:** 7393–7401