The *Drosophila* Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis

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In a genetic screen for mutations that restrict cell growth and organ size, we identified a new tumor suppressor gene, *dMST*, which encodes the *Drosophila* homolog of the mammalian Ste20 kinase family members MST1 and MST2. Loss-of-function mutations in *dMST* result in overgrown tissues containing more cells of normal size. *dMST* mutant cells exhibit elevated levels of Cyclin E and DIAPI, increased cell growth and proliferation, and impaired apoptosis. *dMST* forms a complex with Sav and Wts, two tumor suppressors also implicated in regulating both cell proliferation and apoptosis, suggesting that they act in common pathways.

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In animal development, cell number and organ size are tightly controlled by mechanisms that regulate cell proliferation and cell death. Deregulation of these mechanisms has been implicated in cancers (Hanahan and Weinberg 2000). Although many genes controlling either cell proliferation or cell death have been identified, few genes have been implicated in regulating both processes. One exception is the pathway defined by the two genes *salvador* (*sav*) and *warts* (*wts*) (*large tumors* (*lats*), which encode WW domain-containing protein and Ser/Thr kinase, respectively (Justice et al. 1995; Xu et al. 1995; Tapon et al. 2002). Mosaic animals carrying *sav* or *wts* mutant clones exhibit tissue overgrowth phenotypes. Both *sav* and *lats* mutant cells contain elevated levels of Cyclin E and *Drosophila* inhibitor of apoptosis 1 (DIAP1), leading to increased cell proliferation and impaired apoptosis (Tapon et al. 2002). Sav binds Wts in vitro, and they genetically interact, suggesting they act in a common pathway to regulate cell proliferation and cell death. How the *sav/wts* pathway is regulated and the mechanisms by which it controls cell proliferation and apoptosis are not known.

**Results and Discussion**

To identify novel tumor suppressor genes, we systematically screened the *Drosophila* genome for mutations that cause tissue overgrowth phenotypes (see Materials and Methods). From this screen, we identified three alleles of a gene we named *dMST* (*Drosophila* homolog of MST1 and MST2). We used *dMSTBF33* for most analyses described in this study, as the molecular nature of *dMSTBF33* suggests that it is likely to be a null allele (see below).

*dMST* mutations result in tumor-like growth

Compared with wild-type eyes, *dMST* mosaic eyes are significantly larger and often protrude out in folds (Fig. 1A,B). Tumorous outgrowths were also observed when *dMST* clones were induced in other places, including the thorax, wing, and haltere (Fig. 1C–G). Hence, *dMST* is generally required for restricting tissue growth and organ size.

To determine how *dMST* controls organ size, we generated labeled *dMST* clones in wing discs and compared cell size and clone size between mutant clones and twin spots. *dMST* mutant cells do not exhibit discernible changes in cell size (Fig. 1J–K). Moreover, *dMST* mutant cells differentiated into wing margin bristles of normal size (Fig. 1H). However, *dMST* clones occupy significantly larger areas and contain more cells than do wild-type twin spots (Fig. 1I). As mutant clones and twin spots were derived from mitotic sister cells born at the same developmental stages, the increase in cell numbers and tissue mass of *dMST* mutant clones over twin spots suggests that *dMST* mutant cells grow and proliferate faster than do wild-type cells. Hence, the increase in size of *dMST* mosaic organs is caused by an increase in cell number but not cell size.

*dMST* regulates both cell proliferation and apoptosis

To further explore cell proliferation defects caused by *dMST* mutations, we focused on eye development. In wild-type eye discs, a single stripe of cells, referred to as the second mitotic wave (SMW), enters S phase synchronously posterior to the morphogenetic furrow (MF), and little bromodeoxyuridine (BrdU) labeling is present posterior to the SMW (Fig. 2A; Wolff and Ready 1993). In contrast, *dMST* mosaic discs exhibit extensive BrdU incorporation posterior to the SMW (Fig. 2B). To determine if extra mitosis also occurs in *dMST* mutant eye discs, we used the anti-phosphohistone H3 (pH3) antibody to label cells in M phase. In wild-type eye discs, few cells posterior to the SMW exhibit pH3 staining (Fig. 2C). In contrast, *dMST* mutant discs contain increased number of cells in M phase posterior to the SMW (Fig. 2D), suggesting that *dMST* mutations increase cell proliferation.

Cyclin E is an important regulator of S-phase initiation and progression in imaginal disc development (Richardson et al. 1995). In wild-type discs, Cyclin E is up-regulated at the SMW (Fig. 2E). We found that *dMST* mutant clones accumulate high levels of Cyclin E in a cell-autonomous fashion (Fig. 2F–I), which is consistent with the increased cell proliferation in *dMST* mutant discs.

In wild-type eyes, excessive cells between differentiated ommatidias are eliminated by a wave of apoptosis at

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early pupal stage so that a single layer of cells exists between two adjacent ommatidia [Fig. 2G; Wolff and Ready 1993]. In contrast, the dMST mutant disc contains multiple layer interommatidial cells [Fig. 2H]. The persistence of excessive interommatidial cells in dMST mutant discs implies that apoptosis could be compromised. To test this, we examined cell death in wild-type and dMST mosaic pupal retina 38 h after pupa formation [APF] and found that apoptosis was diminished in dMST mutant cells [Fig. 2I–I′]. Hence, dMST is required for apoptosis during development.

In Drosophila, apoptosis is triggered by death inducers, including head involution defective [hid], Abrams 1999]. Expressing GMR-hid induces precocious cell death in larval eye discs [Fig. 2J], which is blocked in dMST mutant cells [Fig. 2K,K′]. As a consequence, adult eyes derived from GMR-hid-expressing discs that also contain dMST mutant clones are larger than those derived from wild-type discs expressing GMR-hid [Fig. 2L,M]. In Drosophila, death promoters induce apoptosis in part by down-regulating the levels of the death inhibitor DIAP1 [Ryo et al. 2002; Yoo et al. 2002]. We found that dMST mutant cells exhibit higher levels of DIAP1 than do wild-type cells [Fig. 2N–O′], suggesting that dMST promotes cell death at least in part by down-regulating the levels of DIAP1. We also found that the expression of a diap1-lacZ reporter gene is elevated in dMST mutant cells [Fig. 2Q,Q′], indicating that dMST inhibits the transcription of diap1.

Molecular cloning of dMST

The dMST mutations were mapped by high-resolution meiotic recombination [Fig. 3A; see Materials and Methods]. We sequenced several candidate genes for molecular lesions present in mutagenized chromosomes containing dMST alleles. Both dMSTJM1 and dMSTWTS1 in-
introduced point mutations in the open reading frame (ORF) of an annotated gene **CG11228**, which encodes the *Drosophila* homolog of mammalian Ste20 kinase family members MST1 and MST2 (Creasy and Chernoff 1995a,b). A full-length cDNA corresponding to **CG11228** was placed under the control of a *tubulin* promoter to generate **tub-dMSTf**. Both **dMSTJM1** and **dMSTBF33** homozygotes expressing one copy of **tub-dMSTf** are viable, morphologically normal, and fertile, demonstrating that **CG11228** is **dMST**. **dMSTJM1** introduced a single amino acid substitution at a conserved residue (G181E), whereas **dMSTBF33** introduced a stop codon at amino acid 174 (Fig. 3B), suggesting that **dMSTBF33** is likely to be a null allele.

Like its mammalian counterparts, **dMST** contains an N-terminally situated kinase domain and C-terminally located regulatory domains [Fig. 3B,C]. The **dMST** kinase domain exhibits 84% and 83% identity to that of **hMST1** and **hMST2**, respectively. The C-terminally situated dimerization and kinase inhibitory domains present in **MST1** and **MST2** (Creasy et al. 1996) are also highly conserved in **dMST** [Fig. 3C].

**dMST interacts with Sav and Wts**

The **dMST** mutant phenotypes closely resemble those caused by **sav** or **wts** mutations [see Fig. 5I, I′, L′, O′, O, below; Tapon et al. 2002, data not shown]. It has been shown that Sav and Wts physically and genetically interact, suggesting that they may act in common pathways (Tapon et al. 2002). To determine if **dMST** could act in the same pathways, we examined if it physically interacts with Sav and Wts by using coimmunoprecipitation assay. We first determined if **dMST** binds Sav. S2 cells were transfected with DNA constructs expressing HA-tagged Sav and Flag-tagged full-length **dMST** ([**dMSTf**]), its N-terminal fragment containing the kinase domain (**dMSTn**), or its C-terminal fragment containing the regulatory domains (**dMSTc**; Fig. 4A). As shown in Figure 4B, both **dMSTf** and **dMSTc**, but not **dMSTn**, were coimmunoprecipitated with Sav, suggesting that **dMST** binds Sav through its C-terminal regulatory region. The dimerization domain at the C terminus of **dMST** appears to be essential for interaction as deletion of this domain from **dMSTc** abolished its ability to bind Sav [data not shown].

To define the domain in Sav that binds **dMST**, we gen-

![Figure 3. **dMST** encodes the homolog of the mammalian Ste20-like kinases MST1 and MST2. (A) The genomic region of the **dMST** locus. The positions of two of the P-element insertions used for mapping—**l(2)k06323** and **l(2)k00705**—are indicated. (B) Functional domains of **dMST**, and human MST1 (**hMST1**) and MST2 (**hMST2**). The kinase domains are shaded in gray. The black and hatched boxes indicate the dimerization and kinase inhibitory domains, respectively. The arrows indicate the positions of **dMST** and **dMST** mutations. (C) Sequence alignment among **dMST**, **hMST1**, and **hMST2**. The kinase domain, kinase inhibitory domain, and dimerization domain are underlined by a single solid line, a broken line, and a double solid line, respectively. The asterisk indicates K71, which is changed to R in **dMSTn**-R mutant. The arrowhead marks the boundary between **dMSTn** and **dMSTc**.

![Figure 4. **dMST** interacts with Sav and Wts. (A) Schematic drawing of Sav and **dMST** constructs used for transfection. The WW and coiled-coil (C-C) domains of Sav are indicated by shaded and black boxes, respectively. The Sav constructs contain two copies of HA tag, whereas the **dMST** constructs have a Flag tag at the N terminus. (B-D) S2 cells were transfected in indicated DNA constructs. (Top and middle panels) Cell extracts were immunoprecipitated (IP) with indicated antibodies, followed by Western blot (WB) with corresponding antibodies, as indicated. (Bottom panels) Cell lysates were also directly subjected to Western blot with indicated antibodies to determine the expression levels of individual constructs. The Wts constructs contain six copies of Myc tag at the N terminus. The asterisks indicate the positions of proteins expressed from the transfected constructs (bottom panels) or coimmunoprecipitated proteins (top panels). The arrows indicate immunoglobin G. Of note, **dMST1** and **dMSTc** appear to be much larger than their predicted sizes.](https://genesdev.cshlp.org/content/genesdev/article-lookup/doi/10.1101/gad.599108.109)
erated a series of truncated forms of Sav [Fig. 4A]. Both C-terminal fragments, SavC1 and SavC2, bind dMST. In contrast, all the C-terminally truncated fragments, including SavΔC1, SavΔC2, and SavΔC3, fail to bind dMST [Fig. 4C], suggesting that dMST binds the C-terminal region of Sav and the coiled-coil domain of Sav is essential.

We next examined if dMST binds Wts. S2 cells were transfected with DNA constructs expressing Myc-tagged Wts and Flag-tagged dMSTi, dMSTn, or dMSTc. As shown in Figure 4D, Wts binds dMSTi and dMSTn, but not dMSTc, suggesting that Wts interacts with the N-terminal region of dMST. The interaction between Wts and dMST is not affected by Sav, as coexpression of Sav does not increase the amount of dMST coimmunoprecipitated with Wts [data not shown]. However, it remains possible that Sav might regulate dMST/Wts interaction in vivo at physiological concentration. Taken together, these results suggest that dMST, Wts, and Sav form a complex in which Wts and Sav bind the kinase and regulatory domains of dMST, respectively.

When overexpressed in larval eye discs by using the GMR-gal4 driver, dMSTn, but not its kinase dead form (dMSTnK-R), induced ectopic apoptosis [Fig. 5E,P], leading to the formation of small rough eyes [Fig. 5B]. Although expressing GMR-Wts alone does not cause any significant defect [Fig. 5C], coexpressing GMR-Wts with dMSTn enhanced the defects caused by expressing dMSTn alone. For example, although flies expressing GMR-Gal4/dMSTn are viable, coexpressing dMSTn alone. For example, although flies expressing GMR-gal4/dMSTn are viable, coexpressing dMSTn with GMR-gal4/dMSTn causes flies to die as pharate adults that have smaller eyes than those of flies expressing GMR-gal4/dMSTn alone [Fig. 5D]. Similar phenotypic enhancement was obtained when two copies of UAS-dMSTn were expressed under GMR-gal4 [data not shown]. In contrast, the defects caused by dMSTn are not enhanced by GMR-Sav [data not shown]. These genetic interactions are consistent with our findings that dMSTn binds Wts but not Sav.

To determine the genetic epistasis, we overexpressed dMSTn with GMR-gal4 in eye discs that contain wts or sav mutant clones. dMSTn induces ectopic Drice activation in sav mutant cells, as in the case of wild-type cells [Fig. 5G,G′]. In contrast, the ectopic Drice activation induced by dMSTn is diminished in wts mutant cells [Fig. 5H,H′]. Consistently, dMSTn blocks the up-regulation of DIAP1 in sav, but not in wts, mutant cells [Fig. 5J–K′]. In addition, we found that dMSTn blocks the up-regulation of Cyclin E in sav but not in wts mutant cells [Fig. 5M′,M′′,P–P′]. These results suggest that dMST acts downstream of sav but upstream of or in parallel with wts to restrict cell proliferation and promote apoptosis.

Conclusion and perspective

The MST subfamily of Ser/Thr kinases is classified as putative mitogen-activated protein kinase kinase kinase [MAP4K; Dan et al. 2001]. Although numerous studies have been carried out to address their biochemical properties and regulations in cultured cells [Creasy and Chernoff 1995b; Creasy et al. 1996; Hirai et al. 1997; Graves et al. 1998; Lee et al. 1998; Glantschnig et al. 2002; Lee and Yonehara 2002; Cheung et al. 2003], their physiological roles remain elusive. In this study, we demonstrated that dMST plays a pivotal role in controlling cell number and organ size in Drosophila development. We showed that dMST fulfills such a role both by restricting cell growth and proliferation and by promoting cell death. The cell-autonomous elevation of Cyclin E and DIAP1 levels in dMST mutant clones suggest that dMST regulates cell proliferation and cell death cell autonomously. We provide biochemical evidence that dMST, Sav, and Wts form a complex. Our genetic epistasis study suggests sav acts upstream of dMST whereas wts downstream of or in parallel with dMST. Sav could regulate the formation or activity of dMST/Wts kinase complex. Alternatively, Sav could act to bridge the dMST/Wts kinase complex to its substrates, a function that can be bypassed by excess amounts of dMSTn.

It should be noted that sav, dMST, and wts may not
simply act in a linear pathway, as wts mutant phenotypes appear to be more severe than those caused by sav or dMST mutations [data not shown]. In addition, sav wts double-mutant phenotypes are stronger than sav or wts single-mutant phenotypes [Tapon et al. 2002]. Hence, although our results suggest that dMST, Sav, and Wts act in common pathways, they may exist in multiple complexes and have independent functions.

It has been shown that human Sav is mutated in several cancer cell lines, and mice lacking a homolog of wts/lats develop hyperplasia and tumors in several tissues [St. John et al. 1999; Tapon et al. 2002], raising the possibility that mammalian MST1 and MST2 may also function as tumor suppressors. In support of this notion, MST1 and MST2 also regulate cell proliferation in mammals. Given the functional conservation between many insect and mammalian pathways, we speculate that the mammalian MST/Sav/Lats pathway may also function as tumor suppressors.

Materials and methods

Mutations and transgenes

The scheme for genetic screening using the eyFLP system has been described [Newsome et al. 2000; Amanai and Jiang 2001]. For 2R and 3R screen, isogenic FRT-containing males (FR{T2D and FR{T2B) were mutagenized with 25 mM ethylmethanesulfonate (EMS). After 24 h recovery, the males were mated with female virgins with corresponding eyFLP/FR{T y w eyFLP, FR{T2D w y [2] clR1CyO [y+] for 2R or y w eyFLP2 glass-lacZ, FR{T2B w clR3/TM6B, y for 3R. The F2 progeny containing enlarged eyes were back-crossed to the eyFLP/FR{T stock for verifying the phenotypes and germline transmission. The F2 progeny that retained the overgrowth phenotypes were crossed to the balancer stock, y w Sp/CyO[y+], TM2/TM6B to establish lines. Three alleles of dMST (dMST{T, dMST{M, and dMST{R), two alleles of sav (sav{T and sav{R), and three alleles of wts (wts{T, wts{R, and wts{R)) were isolated. By using a combination of meiotic recombination and complementation test with the 2R deficiency kit, dMST mutations were mapped to a gap (56C11–56E5) between two nonoverlapping deficiencies D(2R)P34 (55E2–56C11) and D(2R)P17 (56F5–56F15). Fine mapping was achieved by high-resolution meiotic recombination by using several P-element insertion lines in this region. Specifically, dMST is 0.062% (8/12898) distal to 1/2P06323 (891001619), 0.059% (10/17011) distal to 1/2P07005 (89100485), and 0.072% (12/14488) distal to 1/2P06121 (89100607). dMST{R is placed distal to 1/2P06323 and 1/2P06121 because the majority of the recombinant chromosomes between dMST and the P elements retained FR{T2D. Several candidate genes situated 30–100 kb distal to 1/2P06323 insertion site were chosen for sequencing. dMST alleles were balanced over a GFP balancer. Both dMST{T and dMST{R homozygous flies at early larval stages. Genomic DNA was prepared from homozygous embryos and amplified by PCR, followed by direct sequencing. Both dMST{T and dMST{R, but not dMST{R, contained a point mutation in the coding region of CG11228 (Fig. 3). dMST full-length cDNA was obtained from Research Genetics (GH10354) and was verified by sequencing. The coding sequence for the full-length, the N-terminal fragment (1–342 amino acids), and the C-terminal fragment (343–669 amino acids) of dMST was PCR-amplified and subcloned into the pUAST vector to generate UAS-dMST{, UAS-dMST{n, and UAS-dMST{c. All constructs contain a Flag tag fused in frame to the N terminus. UAS-dMST{KR contains a single amino acid substitution at position 71 [K71R] generated by PCRamplified site-directed mutagenesis. tub{UAS-dMST contains a 2.6-kb tubulin{A promoter fused to dMST full-length cDNA in Casper vector [Basler and Struhl 1994]. sav full-length cDNA was obtained from Research Genetics (SD10307). The coding sequence corresponding to various regions of Sav was PCR-amplified and fused in frame with two copies of HA tags, followed by subcloning into the pUAST vector to generate UAS-HA-Sav (amino acids 1–608), UAS-HA-Sav{C (amino acids 439–608), UAS-HA-Sav{C2 (amino acids 507–608), UAS-HA-Sav{WW (amino acids 440–506), UAST-HA-Sav{C (amino acids 1–591), UAST-HA-Sav{C2 (amino acids 1–507), and UAST-HA-Sav{C3 (amino acids 1–439). UAS-Myc-Wts contains six copies of Myc tag at the N terminus of Wts. Other transgenes and stocks used in this study are dia-pia-lacZ (Tapon et al. 2002), hs-Myc-GFP (Jiang and Struhl 1998), hs-C2D (Jiang and Struhl 1995), GMR-Gal4 (Freeman 1996), GMR-hid (Stowers and Schwarz 1999), GMR-sav, and GMR-wts (Tapon et al. 2002). sav{R is a strong allele [Tapon et al. 2002]; wts{R is a strong allele isolated in a previous screen using the hsFLP/FR{T system (Jiang and Struhl 1995).

Generation of clones of mutant cells

dMST mutant clones were generated using FLP/FR{T-mediated mitotic recombination as described previously [Jiang and Struhl 1995]. The genotypes for generating dMST mosaic eyes with eyFLP, eyFLP2/ or y; FR{T2D w y [2] clR1FR{T2D dMST, or eyFLP2/ or y; FR{T2D hs-Myc-GFP/FR{T2D dMST. The genotype for generating dMST clones with hs-FLP: hs-flp/[+]/ or y; FR{T2D hs-Myc-GFP/FR{T2D dMST or hs-flp/[+]/ or y; FR{T2D hs-C2D yv/FR{T2D dMST. For generating dMST mutant eyes expressing hid, the following genotype is used: eyFLP/GMR-hid; FR{T2D hs-Myc-GFP/FR{T2D dMST. To generate sav or wts mosaic eyes with dMSTn expression, the following genotypes were used: eye-flp/+ or y; GMR-hid(UAS-dMSTn); FRT82B hs-Myc-GFP/FR{T2B hs-Myc-GFP/FR{T2B sav{R (wts{R).

Immunohistochemistry

Standard protocols for immunofluorescence staining, phalloidin staining, BrDU labeling, and TUNEL assay were used [Jiang and Struhl 1998; Wolfl 2000]. Primary antibodies used in this study are rat anti-CycE [Richardson et al. 1995], mouse anti-phosphohistone H3 (Upstate Biotechnology), mouse and rabbit anti-Drice (Yoo et al. 2002), rabbit anti-Dec-200 [Yoo et al. 2002], rabbit anti-Diap1 (Ryoo et al. 2002; Yoo et al. 2002), rabbit anti-Drice [Cappello], mouse anti-Myc (Santa Cruz Biotechnology), rabbit anti-GFP [Clone Tech], and mouse anti-Arm [Jiang and Struhl 1998]. Nuclear dye, 7-AAD, is from Molecular Probes.

Cell culture and immunoprecipitation

S2 cells were cultured in the Schneider’s Drosophila Medium [Hartogten] with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Transfection was carried out by using the Calcium Phosphate Transfection Kit [Speciality Media] according to manufacturer’s instructions. To express dMST and Sav in S2 cells, corresponding UAS constructs were cotransfected with an ub-Gal4-expressing construct. Immunoprecipitation and Western blot analysis were performed by using standard protocols as previously described [Robbins et al. 1997]. Antibodies used are mouse anti-Myc (Santa Cruz), mouse anti-HA, F7 (Santa Cruz), and mouse anti-Flag, M2 (Sigma).

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