

A *Drosophila* p38 orthologue is required for environmental stress responses

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The p38 mitogen-activated protein kinase (MAPK) cascade is an evolutionarily conserved signalling mechanism involved in processes as diverse as apoptosis, cell fate determination, immune function and stress response. Aberrant p38 signalling has been implicated in many human diseases, including heart disease, cancer, arthritis and neurodegenerative diseases. To further understand the role of p38 in these processes, we generated a *Drosophila* strain that is null for the *D-p38a* gene. Mutants are homozygous viable and show no observable developmental defects. However, flies lacking *D-p38a* are susceptible to some environmental stresses, including heat shock, oxidative stress and starvation. These phenotypes only partially overlap those caused by mutations in *D-MEKK1* and *dTAK1*, suggesting that the *D-p38a* gene is required to mediate some, but not all, of the functions ascribed to p38 signalling.

Keywords: *Drosophila*; p38 MAPK; stress signalling

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INTRODUCTION

p38/HOG1 was first identified in yeast as a gene required for osmotic maintenance (Brewster *et al*, 1993). p38 signalling has since been shown to be important in organisms as diverse as plants and humans, and in processes including apoptosis, cell fate determination, immunity and stress responses (Ono & Han, 2000; Johnson & Lapadat, 2002). Aberrant p38 signalling has been implicated in many human diseases, including heart disease, cancer, arthritis and neurodegenerative disease, making it an attractive target for pharmaceutical intervention (Obata *et al*,

2000). For this reason, defining the processes regulated by p38 *in vivo* is essential.

The mitogen-activated protein kinase (MAPK) signalling cascade consists of three components: MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). The main families of MAPKs are the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs (Kyriakis & Avruch, 1996; Ip & Davis, 1998; Pearson *et al*, 2001). Like all MAPKs, p38s are serine/threonine kinases, which contain a canonical TGY dual phosphorylation motif (Hanks & Hunter, 1995).

Four vertebrate p38 genes have been identified: α , β , δ and γ . However, the requirements for p38 in development and homeostasis remain the least clearly defined of the MAPKs. Murine p38 α knockout mice are embryonic lethal and show defects in angiogenesis (Adams *et al*, 2000; Allen *et al*, 2000; Tamura *et al*, 2000). Single gene knockouts for *MKK3* (Lu *et al*, 1999; Wysk *et al*, 1999) and *MKK6* (Tanaka *et al*, 2002) are viable and lack developmental defects, whereas the phenotype of double *MKK3* and *MKK6* knockouts mimics the p38 α mutant (Brancho *et al*, 2003), making detailed *in vivo* analysis difficult. Mutations that disrupt p38 pathway signalling in *Caenorhabditis elegans* result in mild defects in olfactory neuron cell fate determination (Sagasti *et al*, 2001; Tanaka-Hino *et al*, 2002), sensitivity to high metal ion concentrations and starvation (Koga *et al*, 2000), or pathogen resistance (Kim *et al*, 2002; Aballay *et al*, 2003).

Two *Drosophila* p38 MAPK genes, *D-p38a* and *D-p38b*, have been identified. D-p38 activity is increased in *Drosophila* cell lines in response to a variety of environmental stimuli, including osmotic shock, heat shock, oxidative stress, immune stimulation, serum starvation and UV radiation (Han SJ *et al*, 1998; Han ZS *et al*, 1998). The MAPKKs *D-MKK3/licorne* and *D-MKK4* have been shown to activate both p38 isoforms *in vitro* (Han SJ *et al*, 1998; Han ZS *et al*, 1998). A small deletion that removes several genes including *D-MKK3/licorne* results in embryos with axis determination defects, although interpretation of these data is complicated by the presence of other genes in the deletion (Suzanne *et al*, 1999). Additionally, the *Drosophila* genome contains two MAPKKK genes implicated in p38 signalling. Both *D-MEKK1* and *dTAK1* (TGF-Activated Kinase) mutants have been isolated, and are homozygous viable and fertile, with no observed developmental defects. *D-MEKK1* mutants show stress response

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phenotypes including sensitivity to heat shock and osmotic stress, but have a normal immune response (Inoue *et al*, 2001), whereas *dTAK1* mutants have a strong immune response deficiency phenotype (Vidal *et al*, 2001). Overexpression screens indicate that TGF- β /dTAK1 signalling during development may act through JNK, D-p38a or D-p38b, depending on the overexpression system and tissue used (Adachi-Yamada *et al*, 1999; Takatsu *et al*, 2000; Mihaly *et al*, 2001). The relationship between D-MEKK1 and the p38 kinases is complex; phosphorylation of p38 in response to heat shock and osmotic stress is reduced but not abolished in *D-MEKK1* null mutants, indicating that another MAPKKK is also partially responsible for p38 phosphorylation in the stress response (Inoue *et al*, 2001).

Importantly, no mutations in either *Drosophila p38* gene have been reported so far. In this paper, we report the phenotypes of a complete deletion of *D-p38a*. Mutants show only partial phenotypic overlap with *D-MEKK1*, sharing susceptibility to some environmental stresses but not to others. *D-p38a* mutants show no detectable developmental defects, suggesting that D-p38a and D-p38b may be partially redundant as mediators of at least some aspects of MAPKK/MAPKKK activity.

RESULTS

Generation of a *D-p38a* null strain

To address the role of p38 signalling *in vivo*, we generated a *D-p38a* null strain, *D-p38a*¹, using a series of P-element-mediated mutagenesis strategies (Fig 1A). We mobilized the EP(3)3637 P-element (820 base pairs (bp) upstream of the *D-p38a* locus;

Rörth *et al*, 1998), and recovered a new line that contained a second P-element insertion, EP(3)3637-2, 263 bp downstream of the *D-p38a* locus. These two elements were then simultaneously excised, creating a complete deletion of the *D-p38a* locus. The original EP(3)3637 was precisely excised, leaving both flanking genes intact, as determined by direct sequencing. Reverse transcription-PCR (RT-PCR) confirmed that this strain is null for the *D-p38a* locus (Fig 1B).

D-p38a mutant strain is viable

Unlike mouse *p38 α* mutants, which are embryonic lethal (Adams *et al*, 2000; Allen *et al*, 2000; Tamura *et al*, 2000), *D-p38a*¹ flies are viable and fertile, and show no developmental abnormalities. No gross defects in patterning or apoptosis were observed in developing embryonic or larval tissues, including the embryonic nervous system, or the leg, wing and eye imaginal discs, in contrast to mutants of the *Drosophila* EGFR/Ras/MAPK and JNK pathways. Expected mendelian ratios of viable progeny were observed from crosses of mutants to flies heterozygous for a chromosomal deficiency of the *D-p38a* region (48% *D-p38a*^{1/+} versus 52% *D-p38a*^{1/deficiency}, *n*=353). In addition, no interaction was observed when one allele of *jnk/bsk*¹ or *jnk/bsk*² was introduced into a *D-p38a*¹ background; deficiencies removing one copy of *D-p38b* or both *MKK3/lic* and *MKK7/hep* also had no effect. Reducing *D-p38b* activity by overexpression of a *D-p38b* antisense construct (Adachi-Yamada *et al*, 1999) in a wild-type or *D-p38a*¹ background similarly resulted in no developmental defects. Loss of one copy of *D-p38a* suppressed the ommatidial polarity phenotype induced by overexpression of the planar polarity gene *disheveled* (*dsh*), as previously reported (Paricio *et al*, 1999); however, removal of both copies of *D-p38a*¹ failed to modify the hypomorphic phenotype of *dsh*¹, making the significance of this observation unclear.

D-p38a mutants are susceptible to certain stresses

On the basis of previous work demonstrating the role of p38 signalling in environmental stress responses, we tested the susceptibility of *D-p38a* mutants to a variety of such stresses. Adult *D-p38a*¹ mutants showed reduced resistance to dry starvation (*n*=220; Fig 2A). In addition, *D-p38a*¹ mutant flies are susceptible to 37°C heat shock (*n*=480; Fig 2B). This susceptibility seems to be a specific defect in the heat-shock response, as the average lifespan of *D-p38a*¹ flies was unchanged compared with wild type at both 25 and 29°C (data not shown, *n*≥380). This finding is in contrast to the phenotype observed for adult *D-MEKK1* mutant flies, which were largely wild type in their response to heat shock. Lastly, *D-p38a*¹ mutants are susceptible to oxidative stress; flies placed on medium containing 1% H₂O₂ showed a reduced lifespan compared with both wild-type and *D-MEKK1* mutant flies (*n*=373; Fig 2C).

Curiously, unlike *D-MEKK1* mutant flies, *D-p38a*¹ mutants were not susceptible to osmotic shock. The viability of *D-p38a*¹ homozygous and heterozygous flies was equivalent when reared on food containing 0.2 M NaCl, whereas the viability of *D-MEKK1* homozygous flies was reduced (*n*=368), as previously reported (Inoue *et al*, 2001; Fig 2D). We observed a smaller reduction in *D-MEKK1*^{ur36} viability than previously reported (Inoue *et al*, 2001); the line has been subsequently outcrossed and this

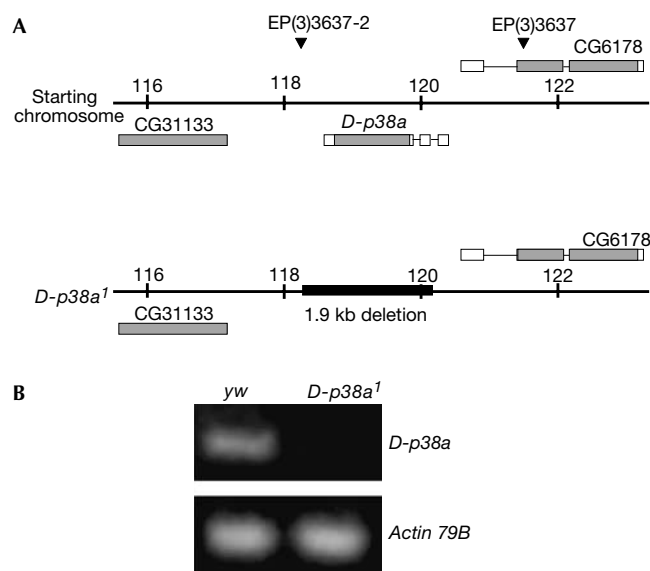


Fig 1 | Mutagenesis of the *D-p38a* locus. (A) Boxes indicate gene structure; shaded boxes indicate coding regions. Triangles indicate P-element insertion sites. EP(3)3637 was mobilized and the local hop EP(3)3637-2 recovered. Next, both P-elements were simultaneously excised, resulting in complete deletion of *D-p38a*. The black box indicates the size and position of the deletion, as determined by genomic DNA sequencing. Neither neighbouring gene was disrupted. (B) RT-PCR analysis confirms that *D-p38a*¹ is null for the locus. No *D-p38a* transcript is detected in mutants. Actin (control) levels are unchanged.

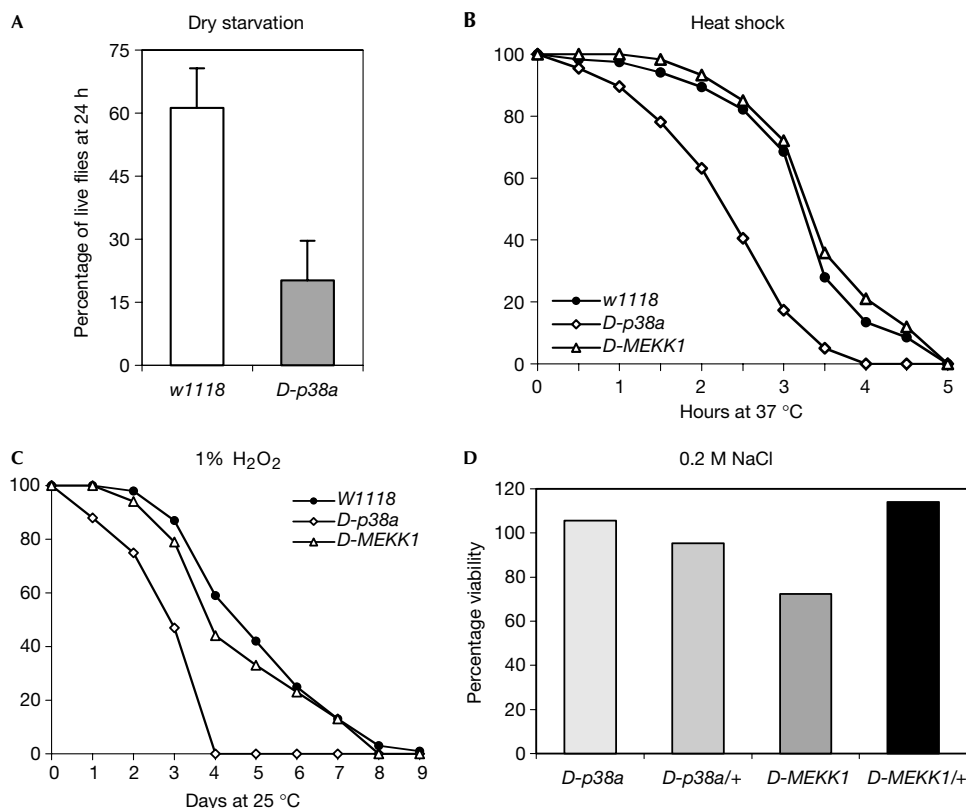


Fig 2 | *D-p38a* mutants are sensitive to some environmental stresses. (A) *D-p38a*¹ mutants show reduced resistance to dry starvation. Adult *Drosophila* were placed in empty culture vials, and placed at 25 °C. Live flies were counted twice daily. (B) *D-p38a*¹ flies are vulnerable to 37 °C heat shock, whereas *D-MEKK1* mutants are not. Adult *Drosophila* were placed in vials containing normal growth medium, and placed at 37 °C. Live flies were counted every 30 min. (C) *D-p38a*¹ mutants are susceptible to oxidative stress, whereas *D-MEKK1* mutants are not. Adult *Drosophila* were placed in vials containing a modified growth medium, consisting of 1.3% low-melting agarose, 1% sucrose and 1% H₂O₂, and placed at 25 °C. Live flies were counted daily. (D) *D-p38a*¹ flies were crossed to flies heterozygous for a deficiency in the region, and balanced *D-MEKK1*^{ur36} flies in two genetic backgrounds were crossed to each other. Flies were allowed to lay eggs on normal food containing 0.2 M NaCl, and the total numbers of homozygous and heterozygous offspring were counted.

disparity in sensitivity to osmotic shock may reflect a difference in the genetic background. Although *dTAK1* mutants were immune response deficient (Vidal *et al*, 2001), and overexpression of p38a *in vivo* impaired the innate immune response of larvae (Han ZS *et al*, 1998), no immune response deficiency was observed in *D-p38a*¹ mutant adults. Northern analysis showed that following septic injury, the inducible expression of five representative antimicrobial peptide genes, *diptericin*, *attacinA*, *cecropinA*, *defensin* and *drosomycin*, was similar in control and mutant flies (Fig 3A). Moreover, survival of *D-p38a* mutants following bacterial infection was not compromised (Fig 3B). Additionally, wound healing appeared normal in adults and embryos (data not shown; B. Stramer and P. Martin, personal communication). Finally, we used Affymetrix oligonucleotide arrays to provide a more global measure of gene regulation after infection. Microarray analysis indicated that antimicrobial peptide gene expression was comparable to controls, as was the expression of other genes important in immune and stress responses: although some variation in expression levels was observed between control and mutant flies, such variation is normal for immune response genes

(Fig 3C; also see supplementary information online for the full microarray data set).

One potential exception is induction of *attacinA*, which was consistently reduced on our microarrays for *D-p38a* flies. However, subsequent attempts to confirm this result using RT-PCR and northern analysis did not clearly confirm this result (Fig 3A). There are four *attacin* genes (*attacinA–D*) in the *Drosophila* genome, and cross-hybridization of the *attacinA* probe to these other genes makes it difficult to determine which (if any) of them is defective in the response to septic injury. Because the viability of the *D-p38a* flies after septic injury is normal, any defect in the induction of the single *attacinA* gene may not have a significant effect on the overall immune response of the mutants.

Overexpression of D-p38a alters the heat stress response

To demonstrate that the stress responses reported above are indeed due to loss of *D-p38a* function, we performed rescue experiments by expressing D-p38a on an inducible ubiquitous heat-shock promoter. Ectopic expression of D-p38a from a single copy of the transgene in a wild-type background did not change

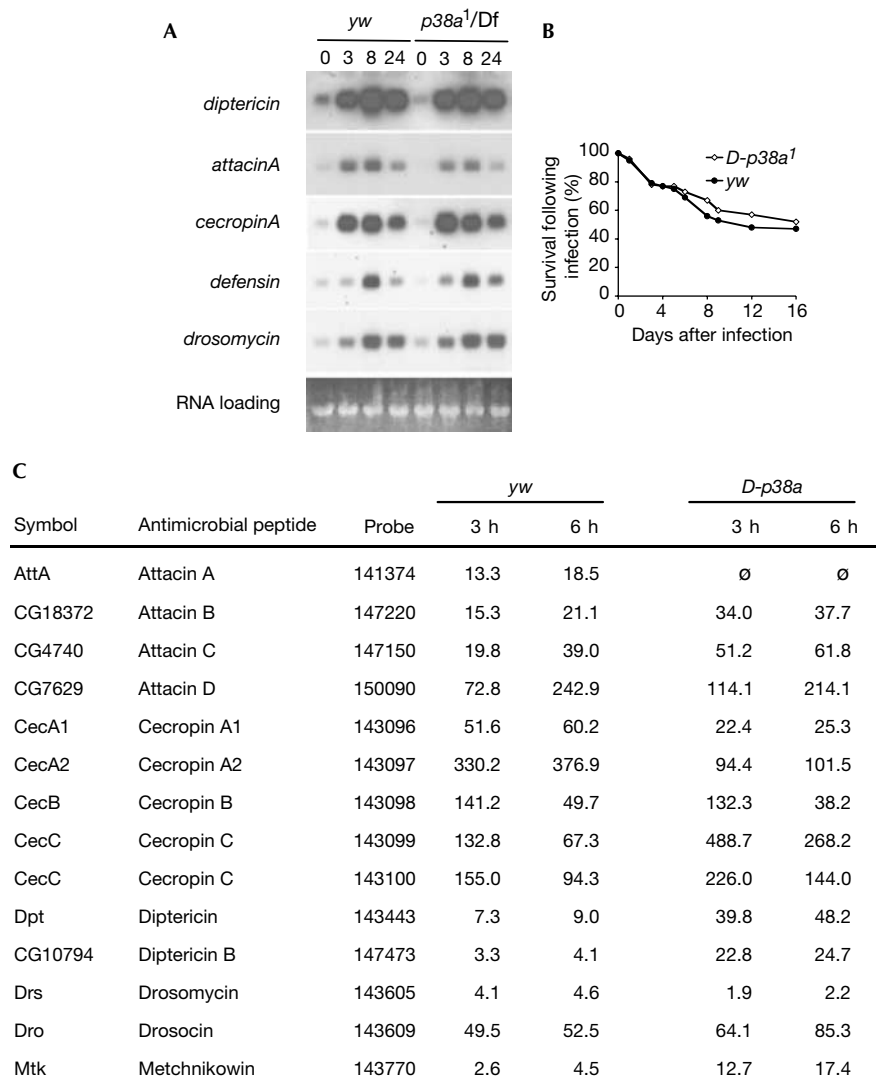


Fig 3 | *D-p38a* mutants are not sensitive to septic injury. (A) Following infection, there were no changes in RNA levels for *diptericin*, *cecropinA*, *defensin* and *drosomycin*, as assessed by northern analysis; *attacinA* showed at most a small decrease. (B) Survival following infection was also unaffected. Adult *Drosophila* were inoculated with a bacterial cocktail of *E. coli* 055:B5, *M. luteus* and *E. carotovora* 15, and allowed to recover at 25 °C. Live flies were counted every 24 h. (C) DNA microarray experiments confirm that the immune response profile of *D-p38a* mutants is largely normal.

survival of 37 °C heat shock (Fig 4). However, similar expression of D-p38a in a *D-p38a¹* mutant background was successful in rescuing mutant survival to 86.8% of wild-type levels.

Surprisingly, a further increase in expression derived from two copies of the transgene in a *D-p38a* mutant background resulted in a less robust rescue of heat stress, to 75.4% of wild-type levels. In addition, expressing D-p38a with two copies of the transgene in a wild-type background further reduced the survival rate to just 33.6% of control (Fig 4). This suggests that levels of D-p38a above a certain threshold can inhibit the ability of flies to adapt to heat shock. Therefore, although these experiments confirm that signalling through *D-p38a* mediates heat-shock response, it is also clear that tight regulation of D-p38a is required for appropriate stress response signalling.

DISCUSSION

Our genotypically null *D-p38a¹* strain lacks defects in development or apoptosis. *D-p38a* mutants are sensitive to some environmental stresses, such as heat shock, dry starvation and H₂O₂ exposure. *D-p38a* flies are not sensitive to high osmolarity or infection, distinguishing them from *D-MEKK1* and *dTAK1* flies, respectively. Another MAPK, such as D-p38b or JNK, may mediate these processes. Significantly, reduction of D-p38b or JNK did not show any additional phenotypes in a *D-p38a* null background, although we cannot rule out the ability of residual D-p38b or JNK to provide full function. Conversely, *D-p38a* mutants are more susceptible to acute heat shock and oxidative stress, whereas *D-MEKK1* mutants responded in a manner similar to wild type. This difference suggests that at least for some responses, D-p38a

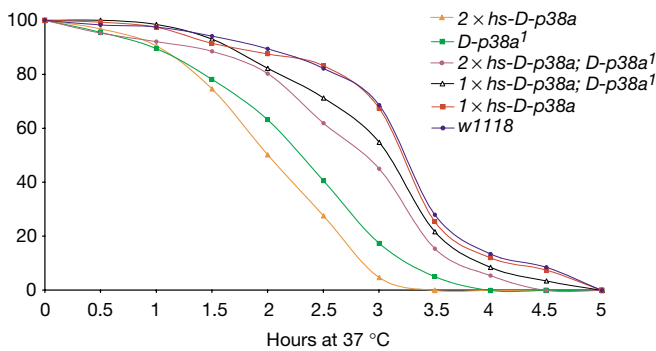


Fig 4 | Ectopic expression of p38a rescues heat-shock sensitivity. *D-p38a*¹ mutants are sensitive to 37 °C heat shock, as previously shown. Expression of *D-p38a* with one copy of the transgene in a wild-type background has no effect. Expression of *D-p38a* in one copy in a *D-p38a*¹ background rescues heat sensitivity to 86.8% of wild type, demonstrating that the *D-p38a*¹ heat-shock sensitivity phenotype is due to the loss of *D-p38a*. However, expression of *D-p38a* with two copies of the transgene in a *D-p38a*¹ background rescues heat sensitivity to only 75.4% of wild type. Expressing *D-p38a* with two copies of the transgene in a wild-type background reduced the survival rate to just 33.6% of control, suggesting that high levels of *D-p38a* also inhibit the ability of flies to adapt to heat shock. An average of 200 flies was tested for each genotype.

activity is independent of D-MEKK1. These phenotypes highlight the complexity of signalling in this pathway; phosphorylation of p38 in response to heat and osmotic stress is reduced but not abolished in *D-MEKK1* mutants, indicating that another MAPKKK may be partially responsible for phosphorylating the p38 MAPKs (Inoue et al, 2001). To assess the p38 pathway function more completely, mutations in the *D-MKK3/licorne*, *D-MKK4* and *D-p38b* loci will be required.

p38 signalling is a potential target for pharmacological intervention in many inflammatory diseases, including pulmonary disease, Crohn's disease and Alzheimer's disease. Clinical trials are presently underway to study the efficacy of p38 inhibitors in rheumatoid arthritis and asthma, among others. Given the chronic nature of these diseases, understanding the consequences of long-term loss of p38 signalling *in vivo* may be important in predicting potential negative outcomes in patients. Indeed, in one example, clinical development was abandoned after adverse neurological effects were seen in animal models, although it is unclear whether these effects were specific to the loss of p38 function or another unintended target (Kumar et al, 2003). We demonstrate that *in vivo* loss of *D-p38a* in *Drosophila* does not have any developmental consequences or affect longevity, but does confer sensitivity to specific environmental stresses.

METHODS

Fly strains. EP(3)3637, Df(3R)crbF894, CyOΔ23/EgrBc, *dsh*¹, *jnk/bsk*¹ and *jnk/bsk*² were obtained from the Bloomington Stock Center. Df(2L)b82a2 was obtained from the Umeå Stock Center. *UASp38b^{antisense}* and D-MEKK1^{ur36} flies were a gift from K. Matsumoto. *hs-p38a* has been described (Han ZS et al, 1998). *sevDsh* were provided by M. Mlodzik.

P-element excisions. EP(3)3637 flies were crossed to transposase-expressing lines, and male progeny carrying both elements were mated to w; TM3Sb/TM6BtB females. EP(3)3637-2 flies, containing a second P-element insertion 3' of the *D-p38a* locus, were then crossed to transposase-expressing lines, and male progeny carrying all three elements were collected, mated to w; TM3Sb/TM6BtB females, and progeny with white eyes were collected and analysed. The *D-p38a*¹ mutation was subsequently recombined onto the chromosome FRT82B to remove a lethal background mutation.

Genomic DNA was isolated as described (Liao et al, 2000). Insertion lines were screened by PCR for proximity to the original P-element insertion. Excisions of the tandem P-elements in EP(3)36372 were analysed by PCR using a series of primers that recognized genomic sequences flanking the *D-p38a* locus.

RT-PCR analysis of mutants. Total RNA was isolated from adult flies using TRI Reagent (Molecular Research Center Inc.). Complementary DNA was synthesized with random primers and SuperScript II Reverse Transcriptase RT-PCR System (Invitrogen) and analysed by PCR with the following primer sequences: p38a, 5'-TGGAAAAGATGTTGGAG-3' and 5'-TATCCTCGAAGCTGTGATCG-3', and Actin79B, 5'-CATCCGCAAGGATCTGTATG-3' and 5'-TTCCTTTGCATACGGTCAG-3'.

Stress tests. Adult flies of the genotypes shown below were aged 3–5 days and subjected to various environmental stresses, and live flies were counted, on the basis of movement following manipulation of the vial. All tests were performed at 25 °C unless otherwise stated.

w¹¹¹⁸
w; FRT82B, *D-p38a*¹/FRT82B, *D-p38a*¹
w; *hs-p38a*/+; FRT82B, *D-p38a*¹/FRT82B, *D-p38a*¹
w; *hs-p38a*/*hs-p38a*; FRT82B, *D-p38a*¹/FRT82B, *D-p38a*¹
w; *hs-p38a*+
w; *hs-p38a*/*hs-p38a*
w; D-MEKK1^{ur36-old}/D-MEKK1^{ur36-new}

Oxidative stress: Adult *Drosophila* were placed in vials containing a modified growth medium, consisting of 1.3% low-melting agarose, 1% sucrose and 1% H₂O₂, and live flies were counted daily (Monnier et al, 2002).

Dry starvation: Adult *Drosophila* were placed in empty culture vials, and live flies were counted twice daily (Kang et al, 2002).

Heat shock: Adult *Drosophila* were placed in vials containing normal growth medium, and placed in a 37 °C water bath. Live flies were counted every 30 min.

Osmotic shock: Progeny from the crosses w; FRT82B, *D-p38a*¹/FRT82B, *D-p38a*¹ × w; Df(3R)crb-F89-4, *st*¹e/TM3, Sb or D-MEKK1^{ur36-old}/TM3, SbSer × D-MEKK1^{ur36-new}/TM3, SbSer were grown from embryos on medium containing 0.2 or 0.5 M NaCl (Inoue et al, 2001). Genotypes were scored on eclosion. Viability was calculated by dividing the number of observed progeny by the number of predicted progeny for each genotype. χ^2 analysis was used to test for fit.

Infection experiments. Adult flies (*yw* and *yw*; *D-p38a*/Df(3R)crb-F89-4, *st*¹e¹, to control for background mutations) were aged 3–5 days at 18 °C. Bacterial infections were performed by pricking CO₂-anaesthetized flies in the thorax, using a needle dipped in a bacterial cocktail containing *Escherichia coli* 055:B5, *Micrococcus luteus* and *Erwinia carotovora* 15. Flies were allowed to recover at 25 °C, and live flies were counted daily.

Northern analysis. Following infection, adult flies were homogenized and total RNA was isolated using TRI Reagent (Molecular Research Center Inc.). Northern blots were performed as previously described (Han ZS *et al*, 1998).

DNA microarray analysis. Infections were performed and RNA was isolated as above. Double-stranded cDNA was prepared, and biotin-labelled cRNA made using the BioArray High Yield Kit according to the manufacturer's protocol (Affymetrix). cRNA (30 µg) was hybridized to *Drosophila* GeneChips (Affymetrix). Fluorescence intensity measurements and data analysis were carried out using GeneChip software. Baseline values were obtained from the 0 h condition for each genotype. Each condition was performed in duplicate. Transcripts that were changed at least twofold at two or more time points were considered significant.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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