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A Genetic Screen Targeting the Tumor Necrosis Factor/Eiger Signaling Pathway: Identification of Drosophila TAB2 as a Functionally Conserved Component

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ABSTRACT

Signaling by tumor necrosis factors (TNFs) plays a prominent role in mammalian development and disease. To fully understand this complex signaling pathway it is important to identify all regulators and transduction components. A single TNF family member, Eiger, is encoded in the Drosophila genome, offering the possibility of applying genetic approaches for pursuing this goal. Here we present a screen for the isolation of novel genes involved in the TNF/Eiger pathway. On the basis of Eiger's ability to potently activate Jun-N-terminal kinase (JNK) and trigger apoptosis, we used the Drosophila eye to establish an assay for dominant suppressors of this activity. In a large-scale screen the Drosophila homolog of TAB2/3 (dTAB2) was identified as an essential component of the Eiger-JNK pathway. Genetic epistasis and biochemical protein-protein interaction assays assign an adaptor role to dTAB2, linking dTRAF1 to the JNKKK dTAK1, demonstrating a conserved mechanism of TNF signal transduction in mammals and Drosophila. Thus, in contrast to morphogenetic processes, such as dorsal closure of the embryo, in which the JNK pathway is activated by the JNKKK Slipper, Eiger uses the dTAB2-dTAK1 module to induce JNK signaling activity.

LIGANDS of the tumor necrosis factor (TNF) family regulate fundamental processes in humans, such as apoptosis, cell survival, differentiation, proliferation, and inflammation. Deregulation of TNF signaling pathways is associated with many diseases, including autoimmune disorders and cancer. The study of TNF signaling mechanisms in mammalian systems is complicated by the existence of numerous ligands and receptors and at least three different intracellular signaling pathways (Wallach *et al.* 1999; Locksley *et al.* 2001). Recently it became apparent that there is a single TNF ligand (Eiger) encoded in the Drosophila genome (Igaki *et al.* 2002; Moreno *et al.* 2002; Kauppila *et al.* 2003), raising the prospect of investigating conserved principles underlying this signaling system by simple genetic means.

Like a subset of the mammalian TNF proteins, Eiger is a potent inducer of apoptosis. Unlike its mammalian counterparts, however, the apoptotic effect of Eiger does not require the activity of the caspase-8 homolog DREDD, but it completely depends on its ability to activate the Jun-N-terminal kinase (JNK) pathway and subsequent activation of the Drosophila apoptosome (DARK + DRONC) (Moreno *et al.* 2002). Although the JNK pathway is used multiple times in Drosophila development, Eiger is the only known extracellular protein that triggers its activation. Of particular interest is therefore the interface between the cell membrane and the core JNK cassette [consisting of the JNKK Hemipterous (Hep), the JNK Basket (Bsk), and the transcription factors Jun and Fos]. Apart from Wengen (Wgn), the presumptive TNF receptor homolog in Drosophila (Kanda *et al.* 2002; Kauppila *et al.* 2003), no other components have been convincingly implicated in JNK activation upstream of the candidate JNKKK dTAK1 (Vidal *et al.* 2001).

Here we designed and performed a genetic screen to isolate rate-limiting components in mediating Eiger-induced apoptosis. We report the identification of >100 mutations that weaken the Eiger-JNK pathway. While some of these mutations affect already known components, such as Bsk and dTAK1, and thus validate the screen, we further show that one group of alleles inactivates a previously uncharacterized Drosophila gene, *CG7417*. By genetic and biochemical means we demonstrate that it functions as the TAB2/3 homolog. TAB2/3 have been demonstrated to link TNF receptor-associated factor (TRAF) proteins to TAK1 in mammalian interleukin- and TNF-signal transduction (Takaesu *et al.* 2000; Ishitani *et al.* 2003). We propose that the Drosophila TAB2/3 homolog provides, together with dTRAF1, an adaptor function enabling the presumptive JNKKKK Misshapen (Msn) to activate the JNKKK dTAK1, which in turn advances the Eiger signal to the JNKK Hep and its JNK substrate Bsk.

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MATERIALS AND METHODS

Fly stocks: The Bloomington Drosophila Stock Center (BDSC) provided *bsk*^{1}, *Df(2R)P34*, and the Exelixis deficiency collection. The following reagents have been described previously: *UAS-egr* (Moreno *et al.* 2002), *dTAK1* alleles and their rescuing transgene (Vidal *et al.* 2001), *UAS-hep*^{CA} (Adachi-Yamada *et al.* 1999), and *GMR-Gal4* (Hay *et al.* 1994).

Stocks carrying the *UAS-Drosophila homolog of TAB2/3* (*dTAB2*) and *tubulinα1-dTAB2* transgene were obtained by standard *P*-element-induced transformation. The *dTAB2* full-length cDNA (LD40663) was cloned into pUAST (Brand and Perrimon 1993) or into a vector containing the *tubulinα1* promoter (Basler and Struhl 1994), respectively.

EMS mutagenesis: Drosophila males carrying the *GMR-Gal4* insertion were starved for 8 hr before mutagenesis. These males were then kept for 24 hr in a bottle containing a filter paper soaked with 0.4% EMS in sugar solution (1 g/100 ml). After a recovery phase of another 24 hr on normal food, the mutagenized males were mated at 25° with virgins carrying the *UAS-egr* transgene.

Genetic distance to *GMR-Gal4* insertion: Chromosomes carrying the *GMR-Gal4* insertion and a suppressor mutation were allowed to recombine with a wild-type chromosome in females. Such virgins were crossed to *UAS-egr* males. The number of *GMR-Gal4* progeny with a suppressed eye phenotype in relation to the number of *GMR-Gal4* progeny with a small eye phenotype reflects the genetic distance between the suppressor mutation and the *GMR-Gal4* insertion.

Generation and analysis of *ey-flp* mosaics: For genes that did not dominantly suppress the Eiger-induced small eye phenotype, such as *djun* and *dTRAF1*, *ey-flp* clones were generated to obtain animals with eyes composed largely of homozygous mutant cells (Newsome *et al.* 2000). In this background the ability of Eiger to induce apoptosis was analyzed. Such analysis was uninformative, however, for the *dTRAF1*^{ex1} allele (Cha *et al.* 2003) as mosaic eyes exhibited a distorted pattern already in the absence of Eiger expression.

Sequencing: Genomic DNA was amplified by PCR using evenly spaced primers in the *CG7417*, *bsk*, and *dTAK1* coding regions. PCR products were analyzed by standard sequencing.

***Drosophila* cell culture and transfection:** Schneider (S2) cells were cultured in Schneider's Drosophila medium (Invitrogen, San Diego) supplemented with 10% fetal calf serum and 1% penicillin/streptomysin at 25°. Cells were transfected with expression vectors, using Cellfectin (Invitrogen) according to the manufacturer's protocol.

Expression vectors: Full-length *dTRAF1* and *dTRAF2* coding sequences were amplified by PCR, using ESTs RE63023 and RE19938 as templates, respectively. These PCR fragments were inserted into the triple-HA-containing vector pMZ55 and subcloned into pUAST along with the 3× HA tag. The *dTAK1*-FLAG construct was amplified by PCR from transgenic flies harboring *UAS-dTAK1* (gift from Makoto Nakamura) and inserted into pUAST. *FLAG-dTAB2*, *HA-dTAB2-N*, *HA-dTAB2-C*, and *HA-dTAB2-Δcc* were amplified by PCR from a *HA-dTAB2* construct and cloned into pUAST. The *UAS-Wengen* plasmid was a gift from E. Moreno.

Immunoprecipitation and immunoblotting: S2 cells (0.75×10^6 cells/well) were seeded into a 12-well plate. One day after seeding cells were transfected with the indicated expression vectors. Forty-eight hours after transfection the cells were harvested and lysed in lysis buffer containing 150 mm NaCl, 50 mm Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholic acid, and protease inhibitors (Complete Mini; Roche, Indianapolis). Lysates were mixed either with an anti-HA antibody and 25 μl of Protein-A sepharose beads or with 25 μl of anti-FLAG agarose beads (Sigma, St. Louis) and allowed to rotate at 4° overnight. The beads were then collected and washed with the lysis buffer four times. Proteins were eluted from the beads and resolved on a 4–12% NUPAGE gel system (Invitrogen) and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with either anti-HA antibody (3F10, Roche) or anti-FLAG M2 antibody (Sigma) followed by appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). Signals were detected with ECL reagents (Amersham, Arlington Heights, IL).

Double-stranded RNA production: Double-stranded RNA (dsRNA) was prepared as described by the Dixon lab (Clemens *et al.* 2000). Briefly, using PCR products as templates, the MEGASCRIPT T7 transcription kit (Ambion, Austin, TX) was used to produce RNA according to the manufacturer's protocol. RNA products were ethanol precipitated and resuspended in DEPC-treated water. dsRNA was generated by annealing at 65° for 30 min followed by slow cooling to room temperature. The following sets of forward (FP) and reverse primers (RP) were used: *Basket* (FP 5′ cgccgcaaaggaacttgg 3′; RP 5′ tcagcatcatac cacacg 3′), *dTAK1* (FP 5′ gatgaccaacaatcgcgg 3′; RP 5′ ggcgct gagtggcctcagc 3′), *msn* (FP 5′ atggcgcaccagcagcaacaac 3′; RP 5′ ccatctccagagcggtgatgc 3′), and *dTAB2* (FP 5′ atggcggctacaccac caatgc-3′; RP 5′ gtcgctgctggcgctgcataatc 3′).

LPS treatment: S2 cells were treated with dsRNA (15 μg/10^6 cells) as indicated in Figure 5F. The cells were then split into two. One-half was left untreated and one-half was treated with lipopolysaccharide (LPS) (Sigma) at a concentration of 50 μg/ml for 10 min. The cells were then lysed in lysis buffer. The lysates were analyzed by immunoblotting to detect phosphorylated JNK (Promega, Madison, WI) and JNK (Santa Cruz Biotechnologies, Santa Cruz, CA).

Luciferase assay: S2 cells (0.4×10^6 cells/well) were seeded into a 24-well plate. One day after seeding cells were transfected with an AP1-luciferase reporter plasmid along with the indicated expression vector. The total DNA concentration (1 μg) was kept constant by supplementing with empty vector. Forty-eight hours after transfection, cells were harvested, lysed in passive lysis buffer, and luciferase activity was measured using the dual luciferase assay system (Promega). The values shown reflect the relative luciferase activity: the ratio of firefly (AP1 luciferase) and *tub-renilla* luciferase activity of one representative experiment in which each transfection was made in duplicate.

RESULTS

A dominant modifier screen to identify new components of the Eiger-JNK pathway: Forced expression of Eiger in the developing compound eye of Drosophila triggers massive apoptosis and results in a small eye phenotype (Figure 1, A and B; Igaki *et al.* 2002; Moreno *et al.* 2002; Kauppila *et al.* 2003). A reduction in copy number of genes encoding core JNK pathway components partially rescues this phenotype (Figure 1C; Igaki *et al.* 2002; Moreno *et al.* 2002). Complete elimination of *bsk* (encoding Drosophila JNK) or *djun* in mosaic animals by genetic means or suppression of Bsk activity by forced expression of the JNK-phosphatase Puckered (Martin-Blanco *et al.* 1998) reverted the Eiger-induced small eye phenotype (data not shown and Moreno *et al.* 2002). Hence, all apoptosis-inducing activity of the Eiger pathway is apparently transduced by the JNK pathway (Figure 1E). Animals heterozygous for *dTAK1* dominant-negative alleles (Figure 1D) or hemizygous

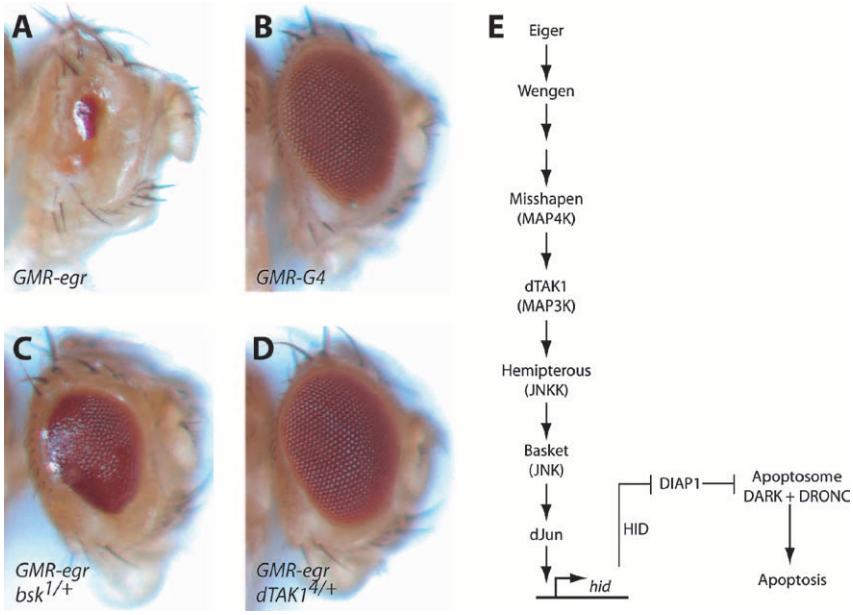

FIGURE 1.—The small eye phenotype caused by *eiger (egr)* overexpression in the eye provides a sensitized system to screen for new components. (A) *GMR-Gal4* UAS-*egr*/+. Overexpression of *egr* in the Drosophila compound eye leads to a small eye phenotype due to massive induction of apoptosis. (B) *GMR-Gal4*/+ control eye. (C) *GMR-Gal4 UAS-egr*/*bsk*^{1}. Removing a single copy of the Drosophila JNK gene *bsk* dominantly suppresses the small eye phenotype. (D) *dTAK1*^{4}/+ ; *GMR-Gal4 UAS-egr*/+. *dTAK1*^{4} is a dominant-negative allele of *dTAK1*, which fully suppresses the small eye phenotype in a dominant fashion. (E) Schematic representation of the current model of Eiger signaling.

mutant for *dTAK1* (null allele, Figure 4F) also show a complete suppression of the small eye phenotype. Thus dTAK1 appears to provide the most relevant JNKKK function in the Eiger pathway, as none of the other five putative JNKKK homologs encoded in the Drosophila genome (STRONACH 2005) can substitute for dTAK1. Indeed, removing one copy of *slipper*, which codes for a JNKKK involved in JNK activation during the morphogenetic process of dorsal closure (STRONACH and PERRIMON 2002), does not suppress the small eye phenotype (data not shown).

The above described assay was used as a basis for a screening system to identify genes required for Eiger signaling. Adult males carrying a *GMR-Gal4* transgene were mutagenized with EMS and mated to *UAS-eiger* females (*UAS-egr*; see crossing scheme in Figure 2A). The progeny was scored for suppression of the small eye phenotype. Candidate suppressors were isolated, retested, and mapped to individual chromosomes by virtue of visible markers on chromosomes 2 and 3 (Figure 2A). After screening ~55,000 animals, 117 stocks with suppressor mutations were established (Figure 2B), each categorized in one of three phenotypic classes on the basis of the extent of the rescue: "complete" (not shown), "intermediate" (Figure 2C), and "weak" (Figure 2D).

Validation of the screen: In our screen, the genome of GMR-Gal4 animals was exposed to the EMS mutagen. Since Gal4 activity is vital for the small eye phenotype, we expected some of the suppressors to harbor mutations in the *Gal4* driver transgene. Twenty-one such events were indeed identified on the basis of the following criteria: (i) lack of recombination separating the suppressor mutation and the *GMR-Gal4* transgene insertion and (ii) suppression of unrelated Gal4-dependent overexpression phenotypes, such as the *UAS-Inr*-driven big eye phenotype (BROGIOLO *et al.* 2001). Most members of this class show a full reversion of the Eiger-induced small eye phenotype and thus accounted for the vast majority of the complete suppressors.

As described above, JNK activity is critical for the transduction of the Eiger signal. One prediction for our screen would therefore be that it leads to the isolation of new *basket* (*bsk*) alleles (Figure 2E). All suppressor mutations that mapped to the second chromosome were subjected to a complementation analysis with a previously described allele of *bsk* (RIESGO-ESCOVAR *et al.* 1996; SLUSS *et al.* 1996). Ten mutations failed to complement *bsk*^{1} and subsequent sequence analysis revealed that all of them carry molecular lesions in the *bsk* coding region (Figure 2J, Table 1).

Only one mutation (*G14*) mapped to the X chromosome (Figure 2F). This mutation is homozygous viable and completely suppresses the Eiger-induced small eye phenotype when hemizygous (not shown). Since this behavior reflects exactly that of known *dTAK1* alleles (Figure 4F), we expected, and also found, a mutation in the *dTAK1* coding region of *G14*. To confirm that the detected mutation (Thr221 → Iso) is indeed responsible for the observed suppression of Eiger signaling, rescue experiments with a genomic *dTAK1* transgene were performed (VIDAL *et al.* 2001). *G14* animals carrying the *dTAK1* rescue construct displayed a reduced suppression of the small eye phenotype (Figure 2G). The known *dTAK1* allele *dTAK1*^{4} shows the same behavior (Figure 2H, compare with Figure 1D). The presumed dominant-negative nature of these two *dTAK1* alleles (*4* and *G14*) may explain why the eye phenotype is not completely reverted to "small." Indeed, the suppression activity of a presumed *null* allele of *dTAK1* is fully inhibited by the *dTAK1* transgene rescue construct

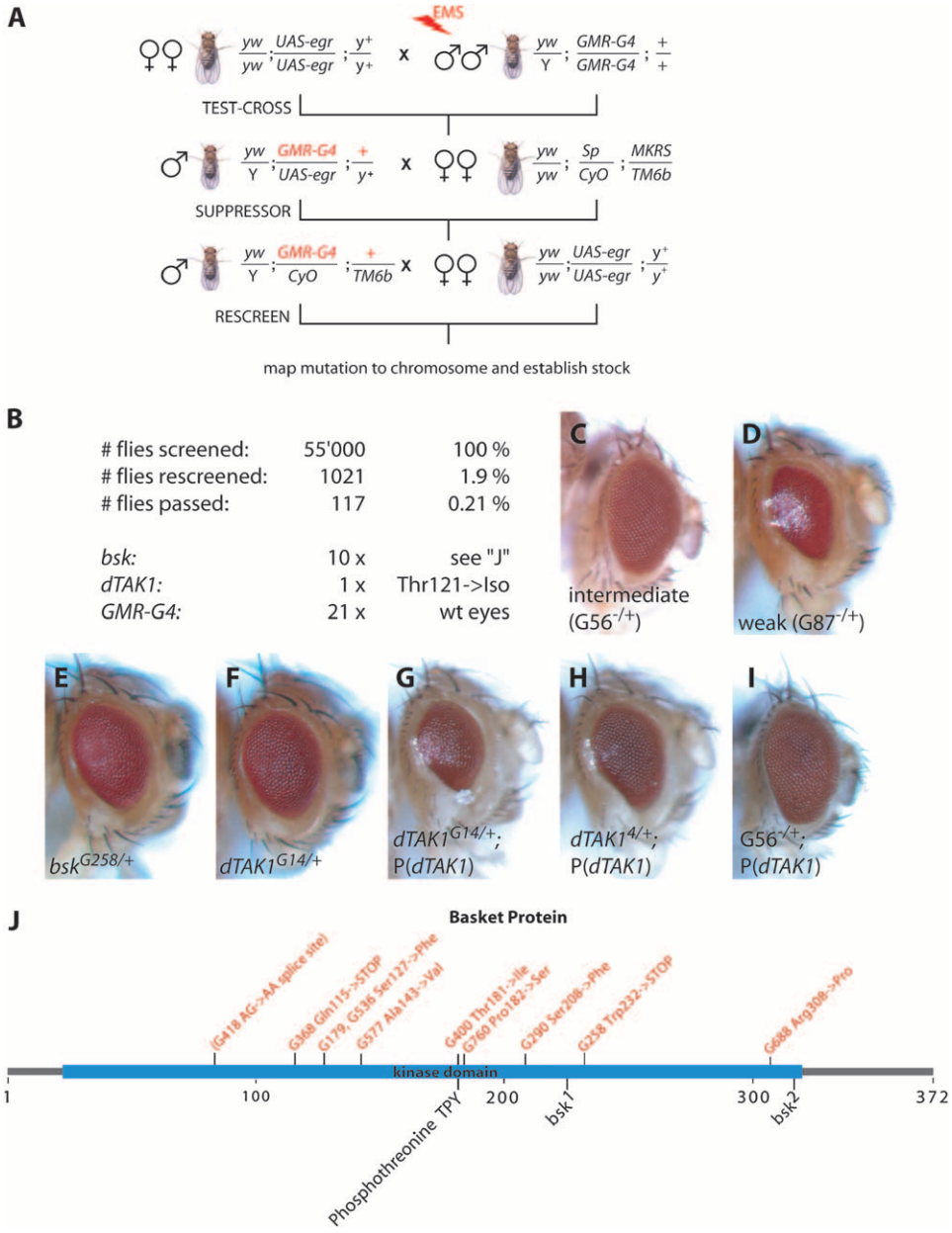

FIGURE 2.—A dominant modifier screen to identify new components of the Eiger pathway in Drosophila. (A) Crossing scheme. Males carrying a *GMR-Gal4* transgene are mutagenized with EMS and crossed to virgins carrying a *UAS-egr* transgene. Suppressors were rescreened, balanced, and mapped to a chromosome by virtue of visible dominant markers. The second chromosome is marked by the w^+ (dark red) included in the *GMR-Gal4* insertion. The w^+ of the *UAS-egr* insertion displays an orange eye color. The third chromosome is marked by the absence of a P[y^+] insertion present on the non-EMS-treated chromosome. Lethal mutations on the X chromosome escaped our detecting system. Since the X chromosome was not marked, viable mutations, which were obtained only when female suppressors were selected, could not be followed and therefore were obtained only rarely. (B) Numbers of animals that were screened and rescreened and stocks that were established, with the number of alleles identified for positive controls. C–I are in a *GMR-Gal4 UAS-egr*/+ background. (C) *GMR-Gal4 UAS-egr*/*G56*. An example of a suppressor belonging to the "intermediate" class is shown. (D) *GMR-Gal4 UAS-egr*/*G87*. An example of a suppressor belonging to the "weak" class is shown. (E) *GMR-Gal4 UAS-egr*/bsk^{G258}. *G258*, one of the new *bsk* alleles identified in the screen, dominantly suppresses the small eye phenotype. (F) $dTAK1^{G14}$/+; *GMR-Gal4 UAS-egr*/+. *G14*, the *dTAK1* allele identified in the screen, dominantly suppresses the small eye phenotype. (G) $dTAK1^{G14}$/+; *GMR-Gal4 UAS-egr*; *P(dTAK1)*/+. (H) $dTAK1^{4}$/+; *GMR-Gal4 UAS-egr*/+; *P(dTAK1)*/+. (I) *GMR-Gal4* UAS-*egr*/*G56*; *P(dTAK1)*/+. A *dTAK1* genomic rescue transgene partially reverts the suppression of the small eye phenotype caused by heterozygous loss of $dTAK1^{G14}$ or $dTAK1^{4}$ but not the suppression caused by the unrelated suppressor mutation *G56*. The suppression of the small eye phenotype is not completely reverted by the *dTAK1* rescue construct due to the dominant-negative nature of the $dTAK1^{G14}$ and $dTAK1^{4}$ alleles. Both carry amino acid substitutions in the kinase domain of dTAK1 (VIDAL *et al.* 2001), which leads to dominant-negative proteins. (J) Molecular lesions found in the 10 novel *bsk* mutants.

(Figure 4G), while an unrelated suppressor mutation (*G56*) from our screen showed the same extent of suppression irrespective of the presence or absence of the *dTAK1* rescue construct (Figure 2I, compare with Figure 2C).

Mapping and molecular cloning of a novel Eiger suppressor: Complementation analysis revealed that, with the exception of our *bsk* alleles and one thus far uncharacterized complementation group, most of the mutations isolated in our screen are homozygous viable like mutations in the *dTAK1* or *eiger* (*egr*) genes (VIDAL *et al.* 2001; IGAKI *et al.* 2002). To group our second chromosomal suppressors by other means, we mapped them by recombination analysis relative to the *GMR-Gal4* insertion (see MATERIALS AND METHODS). In parallel, we screened a large collection of deficiencies for dominant suppressors of the Eiger-induced small eye phenotype. These deficiencies are molecularly mapped and uncover ~56% of the Drosophila genome (PARKS *et al.* 2004). Interestingly, one group of our EMS-induced mutations mapped to the same chromosomal region as deficiency *Df(2R)Exel6069*, which behaved as a suppressor

TABLE 1

Molecular lesions identified in *basket* (*bsk*)

Allele	DNA level	Protein level
G418	3′ splice site intron 3 A**G** → A**A**	—
G368	**C**AG → **T**AG	Gln115 → Stop
G179 (independent of G536)	T**C**C → T**T**C	Ser127 → Phe
G536 (independent of G179)	T**C**C → T**T**C	Ser127 → Phe
G577	G**C**A → G**T**A	Ala143 → Val
G400	A**C**C → A**T**C	Thr181 → Ile
G760	**C**CC → **T**CC	Pro182 → Ser
G290	T**C**C → T**T**C	Ser208 → Phe
G258	TG**G** → TG**A**	Trp232 → Stop
G688	C**G**G → C**C**G	Arg308 → Pro

Alleles are ordered according to their position in the protein. Nucleotide changes are in bold face type. In a *GMR-Gal4 UAS-egr* background, all alleles display an "intermediate" strength of suppression.

of Eiger signaling (Figure 3B). *Df(2R)Exel6069* uncovers only 20 genes at cytological position 56B5–56C11. By using overlapping deficiencies and sequence analysis (Figure 3A), we identified in 39 suppressors molecular lesions in gene *CG7417* (Figure 3C, Table 2). We interpreted these results as an indication that *CG7417* may encode a component critically required for Eiger signaling.

CG7417 encodes the Drosophila homolog of TAB2: The full open reading frame (represented by cDNA *LD40663*) of *CG7417* encoded an uncharacterized protein of 831 amino acids with a CUE, a coiled-coil, and a zinc-finger domain (Figure 3C). These domains are found together only in human TAB2 and TAB3 and in homologs of these proteins in other organisms. Human TAB2 and TAB3 are almost identical and were identified as binding partners of TAK1 (Takaesu *et al.* 2000; Ishitani *et al.* 2003; Cheung *et al.* 2004; Jin *et al.* 2004). On the basis of the conserved domain architecture we propose CG7417 as the Drosophila homolog of TAB2 and TAB3 and hereafter refer to *CG7417* as *dTAB2*.

Each of the 39 *dTAB2* alleles displays a similar degree of suppression of the Eiger-induced small eye phenotype (Figure 4A). Removing both copies of *dTAB2* does not fully revert the small eye phenotype to wild type (Figure 4B), indicating that even in complete absence of dTAB2 a slight activation of the pathway can occur (see discussion). To verify that the suppression activity of our alleles is indeed caused by the mutations detected in

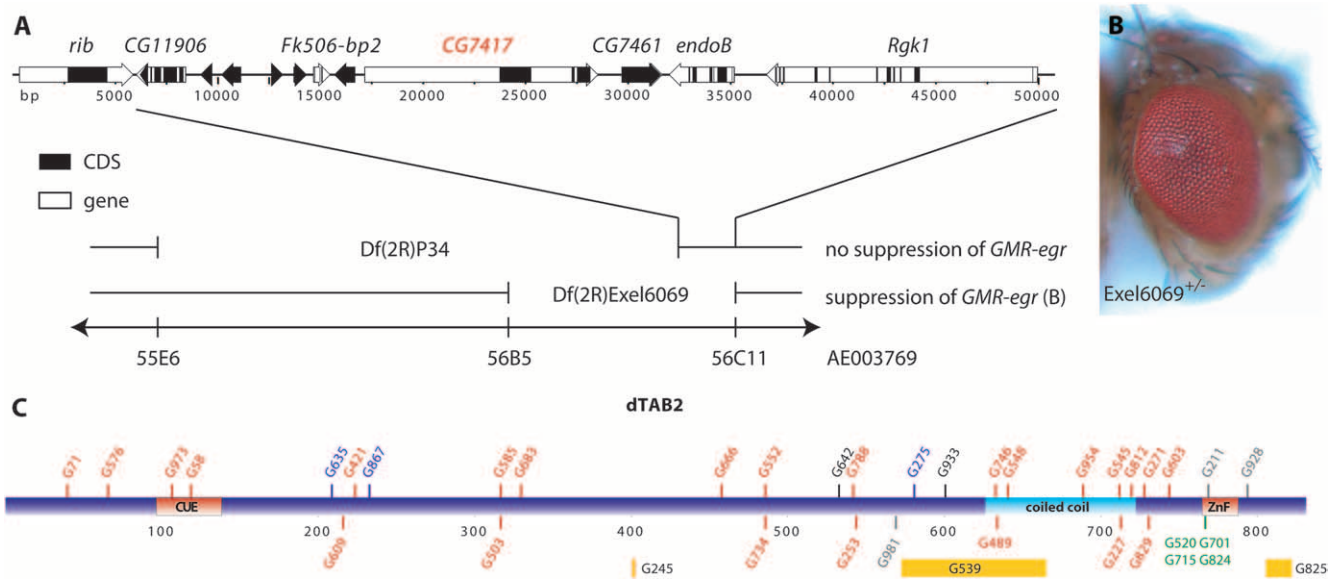

Figure 3.—Identification of *dTAB2 (CG7417).* (A) The two overlapping deficiencies *Df(2R)Exel6069* and *Df(2R)P34* narrow down the region of interest to 11 genes. The distal breakpoint of *Df(2R)P34* was placed between *CG11906* and *ribbon* on the basis of the fact that *Df(2R)P34* and *ribbon* fail to complement each other (Bradley and Andrew 2001). The *CG7417* coding region has a mutation in 39 of the established suppressor stocks. (B) *GMR-Gal4 UAS-egr/Df(2R)Exel6069. Df(2R)Exel6069* dominantly suppresses the small eye phenotype whereas *Df(2R)P34* does not. (C) Domain architecture and mutations identified in the dTAB2 protein. Red, Gln → Stop. Blue, Arg → Stop. Green, Trp → Stop. Gold, deletions. Black, splice sites mutated. Gray, amino acid substitutions.

TABLE 2

Molecular lesions identified in *dTAB2* (*CG7417*)

Allele	DNA level	Protein level
G71	**C**AG → **T**AG	Gln40 → Stop
G576	**C**AG → **T**AG	Gln66 → Stop
G973	**C**AG → **T**AG	Gln107 → Stop
G58	**C**AA → **T**AA	Gln119 → Stop
G635	**C**GA → **T**GA	Arg209 → Stop
G609	**C**AA → **T**AA	Gln216 → Stop
G421	**C**AG → **T**AG	Gln224 → Stop
G867	**C**GA → **T**GA	Arg233 → Stop
G503 (independent of G585)	**C**AA → **T**AA	Gln317 → Stop
G585 (independent of G503)	**C**AA → **T**AA	Gln317 → Stop
G683	**C**AG → **T**AG	Gln330 → Stop
G245	7-bp Del bp 1203–1209 of CDS	Premature Stop at AA422
G666	**C**AG → **T**AG	Gln458 → Stop
G552 (independent of G734)	**C**AG → **T**AG	Gln486 → Stop
G734 (independent of G552)	**C**AG → **T**AG	Gln486 → Stop
G642	5′ splice site intron 2 G**T** → G**A**	—
G788	**C**AG → **T**AG	Gln542 → Stop
G253	**C**AG → **T**AG	Gln544 → Stop
G981	C**T**G → C**A**G + **G**AC → **A**AC	Leu570 → Gln + Asp592 → Asn
G539	284-bp Del bp 1711–1994 of CDS + CA insertion at breakpoint → in frame!	Thr571 → His, Glu572 → Gln, AA 573–666 deleted
G275	**A**GA → **T**GA	Arg581 → Stop
G933	3′ splice site intron 3 A**G** → A**A**	—
G746	**C**AA → **T**AA	Gln633 → Stop
G489	**C**AG → **T**AG	Gln634 → Stop
G548	**C**AG → **T**AG	Gln641 → Stop
G954	**C**AG → **T**AG	Gln689 → Stop
G545	**C**AG → **T**AG	Gln712 → Stop
G227	**C**AG → **T**AG	Gln713 → Stop
G812	**C**AG → **T**AG	Gln720 → Stop
G271	**C**AG → **T**AG	Gln728 → Stop
G829	**C**AG → **T**AG	Gln731 → Stop
G603	**C**AG → **T**AG	Gln744 → Stop
G520 (independent of G715)	T**G**G → T**A**G	Trp767 → Stop
G715 (independent of G520)	T**G**G → T**A**G	Trp767 → Stop
G701 (independent of G824)	TG**G** → TG**A**	Trp767 → Stop
G824 (independent of G701)	TG**G** → TG**A**	Trp767 → Stop
G211	**T**GC → **G**GC + AA**C** → AA**A**	Cys769 → Gly + Asn770 → Cys
G928	G**G**T → G**A**T	Gly794 → Asp
G825	53-bp Del bp 2437–2489 of CDS	Frameshift leads to a longer protein

Alleles are ordered according to their position in the protein. Nucleotide changes are in boldface type. In a *GMR-Gal4 UAS-egr* background, all alleles display an "intermediate" strength of suppression.

the *dTAB2* coding region, rescue experiments with a *tubulinα1* promoter-driven *dTAB2* transgene were carried out. The predicted suppression caused by heterozygosity for *dTAB2* could be overcome by expression of a *tubulinα1-dTAB2* transgene (Figure 4C). Importantly, the *tubulinα1-dTAB2* construct had no effect on the suppression of the Eiger eye phenotype brought about by an unrelated suppressor mutation (*G56*, Figure 4D).

dTAB2 functions upstream of Hep and dTAK1: Expression of a constitutively active form of Hep (*hep*^{CA}) (Adachi-Yamada *et al.* 1999) causes a reduction in eye size (Figure 4H). This effect is mediated by JNK activation as it is completely inhibited by coexpression of Puc (not shown). The Hep^{CA} small eye phenotype is suppressed by reducing *bsk* activity (Figure 4I), but not by reducing *dTAB2* activity (Figure 4J), indicating that Bsk and dTAB2 act downstream and upstream of Hep, respectively. The Hep^{CA} phenotype is not suppressed in males hemizygous for *dTAK1*, placing dTAK1, like dTAB2, upstream of Hep (not shown). To address where dTAB2 and dTAK1 act relative to each other, epistasis experiments were performed in S2 cells, using a JNK luciferase

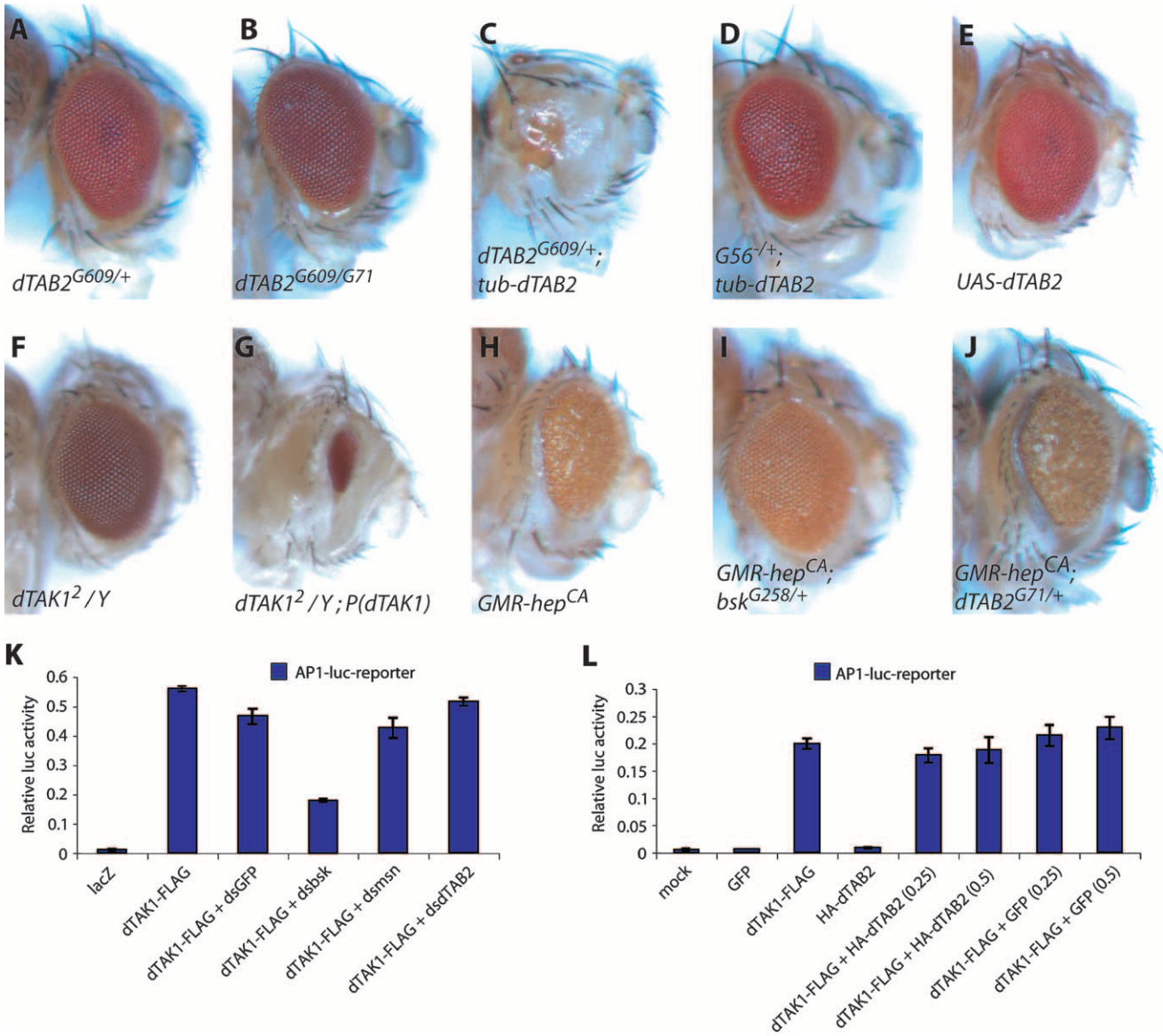

Figure 4.—dTAB2 functions upstream of Hep and dTAK1. A–G are in *GMR-Gal4 UAS-egr/+* background. (A) *GMR-Gal4 UAS-egr/dTAB2*^{G609}. *dTAB2* alleles dominantly suppress the small eye phenotype. (B) *GMR-Gal4 UAS-egr dTAB2*^{G609}*/dTAB2*^{G71}. The small eye phenotype is not completely suppressed in *dTAB2* homozygous mutant flies. (C) *GMR-Gal4 UAS-egr/dTAB2*^{G609}; *tub-dTAB2*. (D) *GMR-Gal4 UAS-egr/G56*; *tub-dTAB2*. A *tub-dTAB2* rescue transgene reverts the suppression of the small eye phenotype caused by heterozygous loss of *dTAB2*, but not the suppression brought about by the unrelated suppressor mutation *G56*. (E) *GMR-Gal4 UAS-egr/UAS-dTAB2*. Overexpression of wild-type *dTAB2* has a dominant-negative effect on Eiger signal transduction. (F) *dTAK1*^{2}/Y; *GMR-Gal4 UAS-egr/+*. A hemizygous null allele of *dTAK1* completely suppresses the small eye phenotype. *dTAK1*^{2} has an early stop mutation (Vidal *et al.* 2001). (G) *dTAK1*^{2}/Y; *GMR-Gal4 UAS-egr/+*; *P(dTAK1)/+*. In contrast to dominant-negative alleles of *dTAK1* (*G14*, *4*), the suppression of the small eye phenotype brought about by a null allele of *dTAK1* can be completely reverted by introducing a *dTAK1* genomic rescue transgene. (H) *GMR-Gal4 UAS-hep*^{CA}*/+*. (I) *GMR-Gal4* UAS-*hep*^{CA}*/bsk*^{G258}. (J) *GMR-Gal4 UAS-hep*^{CA}*/dTAB2*^{G71}. The small eye phenotype caused by overexpression of a constitutive active form of Hep is dominantly suppressed by removing one copy of *bsk* but not by removing one copy of *dTAB2*. (K) RNAi against *bsk* but not against *msn* or *dTAB2* suppresses the dTAK1-FLAG-induced activation of the AP1-luc-reporter in S2 cells. (L) Overexpression of *UAS-HA-dTAB2* does not activate the AP1-luc-reporter. Wild-type dTAB2 does not exert a dominant-negative effect on dTAK1-mediated activation of the JNK pathway. Numbers in parentheses indicate amounts of plasmid in micrograms.

reporter system in combination with RNAi. Expression of dTAK1 strongly activated a JNK luciferase reporter. Reporter activity was reduced by RNAi against *bsk* (Figure 4K) or *djun* (not shown), but not by RNAi against *msn* or *dTAB2* (Figure 4K), although RNAi against *msn* and *dTAB2* strongly reduces their protein levels (data not shown). These experiments place Msn and dTAB2 upstream of dTAK1.

dTAB2 links dTRAF1 to dTAK1: In contrast to Hep^{CA} and dTak1, overexpression of dTAB2—either in S2 cells

(Figure 4L) or *in vivo* (not shown)—does not activate the JNK pathway, indicating that dTAB2 does not function as a direct activator of dTAK1 but possibly provides an adaptor function. Consistent with this notion, we find that overexpression of dTAB2 exerts a dominant-negative effect on Eiger signal transduction, as it suppresses the Eiger-induced small eye phenotype (Figure 4E).

To explore the molecular nature of such an adaptor function we carried out protein-protein interaction assays with candidate partners of dTAB2. We first found that N-terminally HA-tagged dTAB2 can immunoprecipitate C-terminally FLAG-tagged dTAK1 from Drosophila S2 cell lysates, and vice versa (Figure 5A). The N-terminal half of dTAB2 (aa 1–450), which includes the CUE domain, did not bind to dTAK1, but the C-terminal half (aa 451–831), which includes coiled-coil and Zn-finger domains, was sufficient to interact with dTAK1 (Figure 5B). Removal of amino acids 451–749 severely impaired its interaction with dTAK1 (Figure 5B). This indicates that dTAB2, like TAB2/3 in mammalian systems, interacts with dTAK1 most likely through its coiled-coil domain.

On the basis of the proximal placement of dTAB2 in the Eiger pathway (see above), we also analyzed its interaction with the Drosophila homologs of the TRAF proteins. FLAG-dTAB2 was coexpressed either with an HA-dTRAF1 or with HA-dTRAF2 in S2 cells and was immunoprecipitated with an anti-HA antibody. Western blot analysis of the immune complexes with an anti-FLAG antibody revealed that dTRAF1 (the homolog of hTRAF2) and dTRAF2 (the homolog of hTRAF6) precipitated dTAB2 (Figure 5C). The weaker binding of dTAB2 to dTRAF1 compared to dTRAF2 might be explained by the lack of a RING-finger domain in dTRAF1. The interaction of TAB2/3 with TRAF2/6 in mammals is dependent in part on ubiquitination, which is mediated by the ring-finger domain (Takaesu *et al.* 2000; Ishitani *et al.* 2003; Kanayama *et al.* 2004). The intact ZnF domain of TAB2/3 is required for binding to polyubiquitin chains (Kanayama *et al.* 2004).

We also tested the binding of the presumptive Eiger receptor Wengen for its interaction with Drosophila TRAF proteins. Wengen was expressed with either HA-dTRAF1 or HA-dTRAF2 in S2 cells. The dTRAFs were precipitated with anti-HA and the precipitates were analyzed with an anti-Wengen antibody (Figure 5D). In agreement with the result of Kauppila *et al.* (2003) we found that Wengen can interact with dTRAF2. In addition, we find that Wengen also interacts with dTRAF1 (Figure 5D).

Next, we asked whether dTRAF1 interacts with dTAK1 directly or via dTAB2. S2 cells were cotransfected with dTAK1-FLAG and either HA-dTRAF1 or HA-dTRAF2. We could detect a very weak binding of both TRAFs with dTAK1, perhaps mediated by endogenous dTAB2. Upon coexpression of dTAB2, significantly increased amount of dTAK1 was precipitated (Figure 5E). This result suggests that dTAB2 can act as an adaptor molecule to link dTRAFs to dTAK1.

dTAB2 mediates JNK activation also upon LPS stimulation: LPS-induced JNK phosphorylation reflects JNK activation during an innate immune response (Sluss *et al.* 1996; Boutros *et al.* 2002). Treatment of S2 cells with LPS indeed dramatically increases JNK phosphorylation (Figure 4F). RNAi targeting *dTAK1* or *dTAB2* (Figure 4F), but not *eiger*, *wengen*, or *msn* (not shown), prevents this increase in JNK phosphorylation. From this we conclude that dTAB2 mediates dTAK1 activation not only in the Eiger pathway but also in response to other stimuli. Furthermore these results suggest that dTAB2, like dTAK1 (Vidal *et al.* 2001), may also play an important role in innate immunity.

DISCUSSION

Here we describe an effective genetic modifier screen for the identification of components of the primordial TNF-JNK-pathway in Drosophila. The isolation of mutations in *bsk* and *dTAK1* validated the specificity of the screen. In addition, the identification of *dTAB2* alleles demonstrates that this screening system will also lead to the discovery of other novel components, which so far have escaped detection by genetic means. Together with the low redundancy of its genome, our findings indicate that Drosophila serves as a suitable system to genetically dissect the TNF pathway. Identification of new evolutionarily conserved components of the TNF pathway may shed light on as-yet unknown aspects of this signaling system that plays numerous roles in human disease.

The screen: The high number of alleles identified for *bsk* and *dTAB2* suggests that we have reached saturation for dosage-sensitive components, at least for the second and third chromosome. The allele frequencies for the loci analyzed differ considerably. The fact that we found only a single *dTAK1* allele can be explained by the genetic setup, in which only a small fraction of mutagenized X chromosomes are recovered (see Figure 2A legend). While the allele frequencies for the *bsk* and *Gal4* genes are roughly proportional to the size of their coding regions (*bsk*, 1 kb—10 alleles; *Gal4*, 2.6 kb—21 alleles), we isolated a surprisingly high number of *dTAB2* alleles (the *dTAB2* coding region is ~2.5 kb, with 39 molecularly confirmed alleles). This high number of *dTAB2* alleles is particularly surprising when the low degree of sequence conservation is taken into consideration. Only short domains with sequence similarities to its mammalian homologs can be identified (Figure 3C: CUE, coiled coil, and ZnF), consistent with a role as an adaptor protein. The most effective way to abolish the function of an adaptor is to disconnect the two protein interaction domains, which genetically is best achieved by the introduction of a stop codon between these domains. EMS induces primarily G/C → A/T mutations (Ashburner 1989). Codons of only three

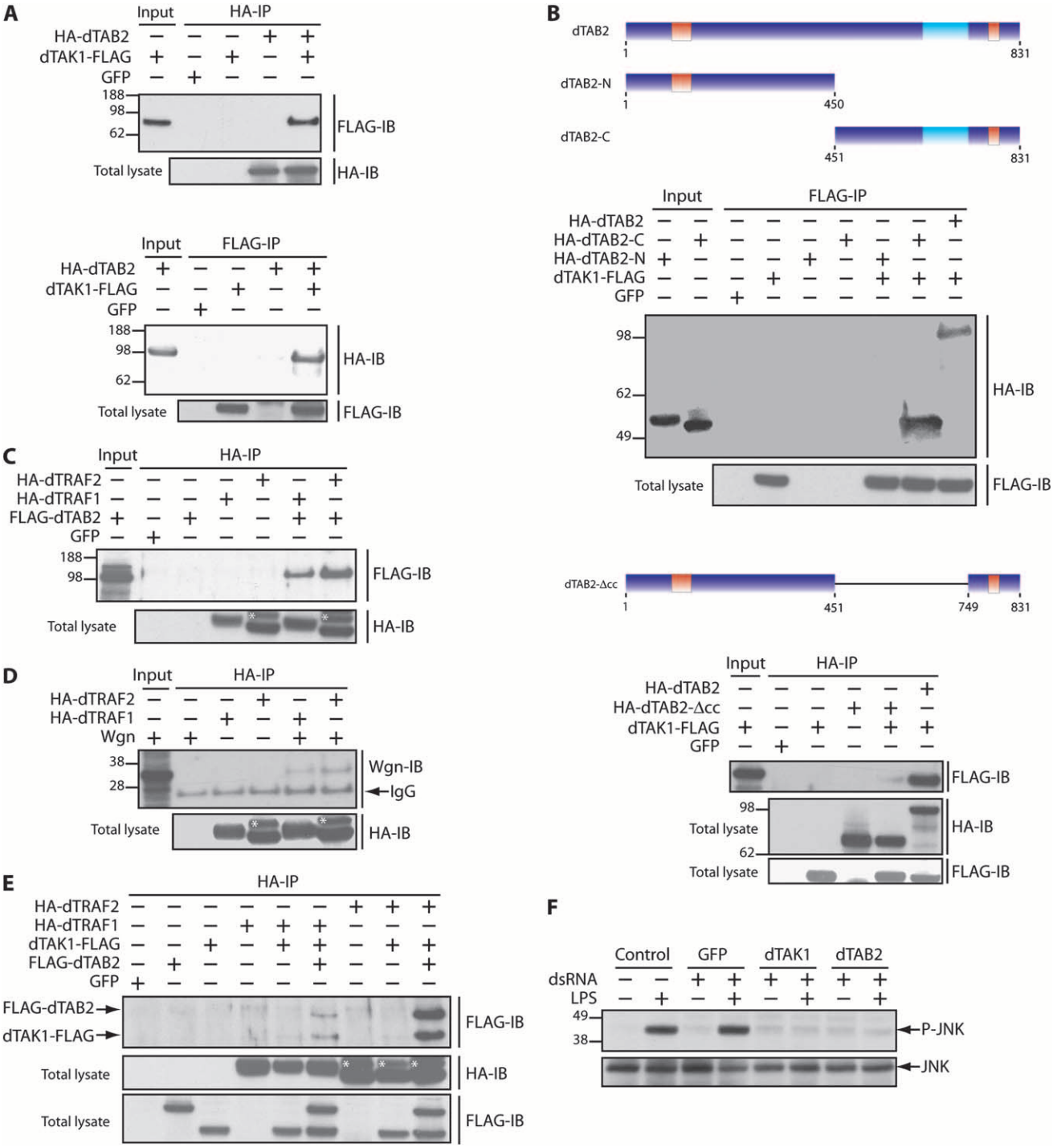

FIGURE 5.—dTAB2 is in a complex with dTRAF1/2 and dTAK1. (A) dTAB2 interacts with dTAK1. S2 cells were transfected with plasmids encoding *UAS-HA-dTAB2* and *UAS-dTAK1-FLAG* together with *ptub-GAL4*. Samples that immunoprecipitated with anti-FLAG antibody were immunoblotted with anti-HA antibody and vice versa. (B) dTAB2 interacts with dTAK1 most likely through its coiled-coil domain. S2 cells were transfected with plasmids encoding *UAS-dTAK1-FLAG* and *UAS-HA-dTAB2* or *UAS-HA-dTAB2-N* or *UAS-HA-dTAB2-C* or *UAS-HA-dTAB2-Δcc* together with *ptub-GAL4*. Anti-FLAG immunoprecipitated samples were immunoblotted with anti-HA antibody or vice versa. (C) dTAB2 associates with both dTRAF1 and dTRAF2. S2 cells were transfected with plasmids encoding *UAS-FLAG-dTAB2* and *UAS-HA-dTRAF1* or *UAS-HA-dTRAF2* together with *ptub-GAL4*. Samples immunoprecipitated with the anti-HA antibody were detected with anti-FLAG antibody. (D) The presumptive Eiger receptor Wengen interacts with both dTRAF1 and dTRAF2. S2 cells were transfected with plasmids encoding Wengen and *UAS-HA-dTRAF1* or *UAS-HA-dTRAF2* together with *ptub-GAL4*. Anti-HA antibody immunoprecipitated samples were immunoblotted with an anti-Wengen antibody. (E) dTAB2 links dTRAF1 and dTRAF2 to dTAK1 and forms a triple complex. S2 cells were transfected with plasmids encoding *UAS-FLAG-dTAB2*, *UAS-dTAK1-FLAG*, and *UAS-HA-dTRAF1* or *UAS-HA-dTRAF2* together with *ptub-GAL4*. Anti-HA antibody-immunoprecipitated samples were immunoblotted with anti-FLAG antibody. (C–E) Asterisks (*) indicate nonspecific bands in the HA-dTRAF2 lysates. (F) dTAB2 is also required for LPS-induced JNK activation. RNAi against dTAK1 or dTAB2 but not against GFP abolishes LPS-mediated phosphorylation of JNK in S2 cells.

amino acids can be mutated to stops by this means: Gln (CAA, CAG), Trp (TGG), and Arg (only CGA). For Arg five other codons exist for which this is not the case, while all Gln and Trp codons can serve as substrates for EMS-induced nonsense mutations. Thus the frequency by which EMS causes premature chain terminations in a gene is largely a function of the Gln and Trp content of its product. It is interesting to note, therefore, that dTAB2 has a Gln content that exceeds the mean Gln frequency of the Drosophila proteome by more than a factor of 2.5 (13.7% *vs.* 5.1%). Indeed, molecular analysis of our *dTAB2* alleles revealed that 24 of the 39 alleles are nonsense mutations of Gln codons (Figure 3C, red alleles).

The pathway: A central issue concerning the TNF/JNK pathway relates to the question of how TAK1 is activated (Shibuya *et al.* 1996; Kishimoto *et al.* 2000; Sakurai *et al.* 2000). On the basis of previous studies and our genetic and biochemical analysis we propose a model for the Eiger pathway, in which dTAB2 and dTRAF1 function as adaptors between the JNKKKK Msn and the JNKKK dTAK1 and in this way may mediate activation of dTAK1 by Msn and the subsequent transduction of the signal via Hep and Bsk (Figure 6). The outline of the pathway is based on the following arguments:

1. Genetic studies have demonstrated the involvement of Msn and dTAK1 in Eiger signaling (Igaki *et al.* 2002; Moreno *et al.* 2002).
2. We identified dTAB2 as an additional component of the Eiger pathway (this article).
3. Epistasis experiments in S2 cells and *in vivo* place Msn and dTAB2 upstream of dTAK1 and Hep (Figure 4, H–K).
4. Liu *et al.* (1999) have shown that Msn interacts with dTRAF1.
5. dTAB2 also binds to dTRAF1 (Figure 5C). Although we failed to detect biochemical evidence for a triple complex Msn-dTRAF1-dTAB2 (data not shown), points 4 and 5 suggest that dTRAF1 may act as an adaptor to link Msn and dTAB2. It is possible that such a complex forms only transiently and is thus difficult to detect biochemically.
6. A triple complex consisting of dTRAF1-dTAB2-dTAK1 can form (Figure 5D), in which dTAB2 functions as a link between dTRAF1 and dTAK1. While each of the above arguments may also be compatible with other models, they collectively support a scenario (Figure 6) in which dTAB2 facilitates the phosphorylation of dTAK1 by Msn. The dominant-negative effect observed by expression of wild-type dTAB2 is an indication that dTAB2 protein levels are critical for proper complex formation (Figure 4E). Our observation that the Eiger-induced small eye phenotype is not entirely suppressed in animals homozygous mutant for dTAB2 suggests that even in the absence of dTAB2 Msn is able to activate dTAK1, although only inefficiently. In the wild-type situation, dTAB2 may function as an adaptor that stabilizes such a signaling complex for efficient transduction of the Eiger signal.

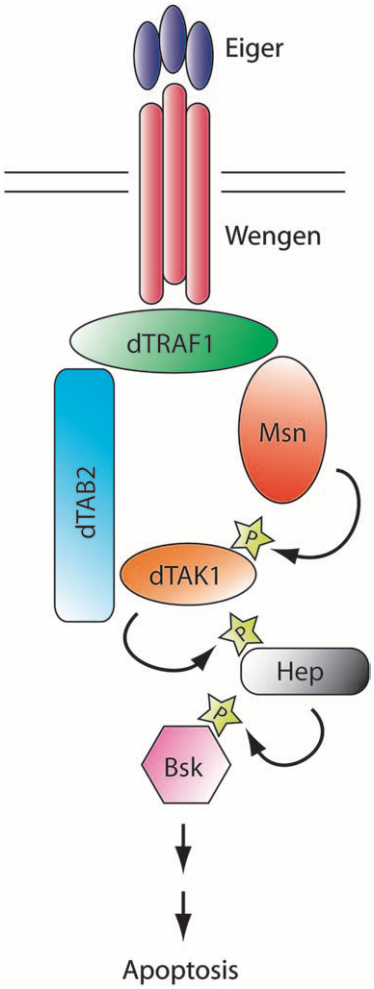

Figure 6.—Proposed model for Eiger signaling in Drosophila. Following the binding of Eiger to Wengen a signaling complex consisting of Msn-dTRAF1-dTAB2-dTAK1 is stabilized, which allows the phosphorylation and activation of dTAK1 by Msn. Subsequently dTAK1 activates the core JNKK-JNK module, consisting of the Drosophila homologs Hep and Bsk.

Even though dTRAF1 exhibited a weaker interaction than dTRAF2 toward dTAB2 and Wengen, we suggest that dTRAF1 rather than dTRAF2 functions as a component of this signaling complex on the basis of the following arguments: (1) Only dTRAF1 but not dTRAF2 binds to Msn (Liu *et al.* 1999); (2) loss-of-function and protein-interaction studies place *dTRAF1* in the JNK pathway and *dTRAF2* in the NF-κB pathway (Liu *et al.* 1999; Shen *et al.* 2001; Cha *et al.* 2003); and (3) males hemizygous mutant for *dTRAF2* do not suppress the Eiger-induced small eye phenotype (not shown; *dTRAF1* alleles are homozygous lethal and could not be properly analyzed—see materials and methods).

In mammalian systems it is not understood how TAK1 is activated. TAB1 is an activator of TAK1, but the

mechanism by which it activates TAK1 and the possible involvement of upstream kinases are not known (Shibuya *et al.* 1996; Sakurai *et al.* 2000). In Drosophila no functional TAB1 homolog has been identified so far. On the basis of our genetic epistasis data, its interaction with dTRAF1, and its homology to MAP4Ks, we propose that Msn functions as an upstream kinase of dTAK1. In mammals NIK and germinal center kinases are structural homologs of Msn. NIK has been demonstrated to act downstream of TAK1 (Ninomiya-Tsuji *et al.* 1999) in NF-κB activation. Several germinal center kinases have been involved in TRAF-mediated activation of JNK (Yuasa *et al.* 1998; Fu *et al.* 1999; Shi *et al.* 1999; Shi and Kehrl 2003). It will be interesting to determine whether one of them plays a role in TNF-induced activation of TAK1. The mapping of other suppressor mutations and the characterization of their corresponding gene products may unravel important aspects of this evolutionarily ancient signaling pathway that has been employed for prominent roles in mammalian development and homeostasis.

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