

***Drosophila* transcription factor AP-2 in proboscis, leg and brain central complex development**

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SUMMARY

We report loss- and gain-of-function analyses that identify essential roles in development for *Drosophila* transcription factor AP-2. A mutagenesis screen yielded 16 lethal point mutant alleles of *dAP-2*. Null mutants die as adults or late pupae with a reduced proboscis, severely shortened legs (~30% of normal length) lacking tarsal joints, and disruptions in the protocerebral central complex, a brain region critical for locomotion. Seven hypomorphic alleles constitute a phenotypic series yielding hemizygous adults with legs ranging from 40–95% of normal length. Hypomorphic alleles show additive effects with respect to leg length and viability; and several heteroallelic lines were established. Heteroallelic adults have moderately penetrant defects that include necrotic leg joints and ectopic growths (sometimes supernumerary antennae) invading medial eye territory. Several *dAP-2* alleles with DNA binding domain missense mutations are null in hemizygotes but have

dominant negative effects when paired with hypomorphic alleles. In wild-type leg primordia, dAP-2 is restricted to presumptive joints. Ectopic dAP-2 in leg discs can inhibit but not enhance leg elongation indicating that functions of *dAP-2* in leg outgrowth are region restricted. In wing discs, ectopic dAP-2 cell autonomously transforms presumptive wing vein epithelium to ectopic sensory bristles, consistent with an instructive role in sensory organ development. These findings reveal multiple functions for dAP-2 during morphogenesis of feeding and locomotor appendages and their neural circuitry, and provide a new paradigm for understanding AP-2 family transcription factors.

Key words: *Drosophila*, Transcription factor AP-2, *dAP-2* alleles, Limb, Leg, Joint, Proboscis, Labellum, Brain central complex, Protocerebrum, Locomotion

INTRODUCTION

AP-2 transcription factors in humans and mice are encoded by a small multigene family that includes *AP-2 α* , *AP-2 β* and *AP-2 γ* (*Tcfap2a*, *Tcfap2b* and *Tcfap2c* – Mouse Genome Informatics; Bosher et al., 1996; McPherson et al., 1997; Mitchell et al., 1987; Moser et al., 1995; Oulad-Abdelghani et al., 1996; Williams et al., 1988). In contrast, there is only a single AP-2 family gene in *Drosophila* (Bauer et al., 1998; Monge and Mitchell, 1998). These proteins share a highly conserved DNA binding and dimerization domain in their C-terminal halves, and proline- and glutamine-rich transcriptional activation segments in their less conserved N-terminal halves (Williams et al., 1988). Murine AP-2 family genes have overlapping expression patterns with neural crest cells, the central nervous system, facial and limb mesenchyme, and various epithelia being principal sites of embryonic expression (Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1995; Moser et al., 1997b). *AP-2 α ^{-/-}* mice generated by targeted mutation die perinatally with severe craniofacial, nervous

system, limb and ventral body-wall defects (Schorle et al., 1996; Zhang et al., 1996) (reviewed by (Morris-Kay, 1996)). In *AP-2^{-/-}* embryos, increased cell death in the hindbrain, midbrain and proximolateral mesenchyme of the first branchial arch at stage E9 coincides with cranial closure failure and the onset of branchial arch and cranial ganglia hypoplasia (Schorle et al., 1996). *AP-2 β ^{-/-}* mice die postnatally of polycystic kidney disease associated with extensive apoptosis in collecting duct and distal tubular epithelia (Moser et al., 1997a).

In humans, AP-2 family transcription factors have been implicated as tumor suppressors in breast cancer and melanoma (Gee et al., 1999; Huang et al., 1998; Jean et al., 1998); and mutations in *AP-2 β* cause Char Syndrome, a dominant congenital disease characterized by facial and hand anomalies and patent ductus arteriosus (Satoda et al., 2000). Although a variety of gene promoters have been proposed as targets for regulation by AP-2 based on gene transfection studies with cultured cells, the genetic pathways where AP-2 family members function in the organism are not well

understood and analysis in vertebrates is complicated by redundancy issues (Maconochie et al., 1999).

We have identified the *Drosophila* homolog of AP-2 by degenerate PCR-mediated cDNA cloning (Monge and Mitchell, 1998), in order to use *Drosophila* genetics to outline regulatory pathways where AP-2 family genes function during development. Originally named *DAP-2*, we recently renamed this gene *dAP-2* to avoid a connotation of dominance with regard to mutant *dAP-2* alleles. *dAP-2* is expressed in the maxillary segment, protocerebrum and ventral nerve cord during embryogenesis and in the brain, optic lobes, ventral nerve cord and leg, antennal and labial imaginal discs (primordia of adult legs, antennae, and distiproboscis) during larval development (Monge and Mitchell, 1998; labial disc expression shown in this paper).

We show that *dAP-2* has essential roles in leg and proboscis outgrowth, leg joint formation and development of the adult brain central complex, a higher order center for regulation of locomotor activity. Analyses of hypomorphic and dominant negative *dAP-2* alleles in different heteroallelic combinations reveal that incremental increases in *dAP-2* activity rescue leg outgrowth, viability, and joint and locomotor defects in that order. Gain-of-function experiments indicate that large clones of *dAP-2*-expressing cells that span more than one leg segment partly phenocopy *dAP-2* loss-of-function mutations by causing shortening of affected segments. In contrast, narrow clones that span multiple segments frequently cause joint necrosis and ectopic partial joints in tarsi without interfering with leg outgrowth. In the wing where *dAP-2* is normally not expressed, ectopic *dAP-2* cell autonomously transforms vein epithelium to ectopic sensory bristles. This suggests that *dAP-2* function in leg development could involve regulatory pathways involved in sensory neurogenesis. In developing wings and legs of *Drosophila*, presumptive wing vein and leg joint territories represent limb segment boundaries with growth regulating properties that are associated with discrete activation of the Notch signaling pathway (Milan and Cohen, 2000), a cell:cell communication pathway best understood in the context of neurogenesis (Artavanis-Tsakonas et al., 1999). Loss- and gain-of-function effects of *dAP-2* on leg development are similar to loss- and gain-of-function effects of Notch signaling components in the leg (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). Our analyses indicate that *dAP-2* is an essential player in the growth organizing properties associated with leg segment boundaries, and may act in regulatory pathways that coordinate limb-growth with development of local and higher order aspects of limb-specific neural circuitry.

MATERIALS AND METHODS

Mutagenesis screen to isolate *dAP-2* mutant alleles

The following chromosomes were used: *y w*; *P[y+J73C FRT80B]* (Xu and Rubin, 1993; B. Dickson), *TM3 Sb P[w+]* (B. Dickson) and *Df(3L)XS-5R* (Karim et al., 1996), hereafter referred to as *Df(3L)5R*. Adult males 2-4 days old were starved for 8 hours and then transferred to bottles containing filters soaked with 1% sucrose + ethylmethanesulfonate (EMS) (Sigma) and allowed to feed for 14-16 hours. The optimal EMS concentration was determined in a pilot experiment by crossing males fed different concentrations of EMS to compound XX females and comparing the male:female ratios of

progeny obtained. 31 μ M EMS, the concentration chosen for the subsequent mutagenesis, resulted in approximately one lethal hit per X-chromosome. Based on chromosome size, about twice as many lethal hits are estimated to have occurred on the third chromosome at this EMS concentration. After EMS feeding, males were allowed to recover for 24 hours in normal culture bottles, and then were mated to equivalent numbers of *y w*; *TM3 Sb P[w+]/D* females (see Fig. 1 for subsequent crosses). Of 13,000 F₁ males tested, 87% were fertile and 147 carried lethal mutations that failed to complement *Df(3L)5R*. Balanced stocks were established for 141 of these (*y w*; *P[y+J73C*FRT80B/TM3 Sb P[w+]*).

dAP-2 antibody preparation and immunostaining of embryos and imaginal discs

The peptide CLDKSKIDNEKK (D1) containing the last 11 amino acids of *dAP-2* was synthesized (Macromolecular Resource Facility, Colorado State University), coupled to KLH (Sigma), and used to generate and affinity purify anti-*dAP-2* rabbit polyclonal antisera. Anti-*dAP-2* immunostaining of *Drosophila* embryos and dissected larval tissues revealed specific localization of *dAP-2* protein in sites essentially identical to those previously shown to express *dAP-2* RNA (Monge and Mitchell, 1998). To immunostain embryos from mutant stocks, embryos were collected and processed following standard procedures (Therianos et al., 1995), and incubated overnight at 4°C in primary antibody mix containing pre-absorbed anti-*dAP-2* (1:100) and 2A12 monoclonal antibody (the latter detects a tracheal antigen and provides an internal positive control) (1:5) (N. Patel, courtesy of M. Affolter, University of Basel). For detection of *dAP-2* in imaginal discs, 2A12 antibody was omitted. Biotinylated secondary antibodies, Vectastain and DAB staining reagents were used according to the manufacturer's recommendations (Vector Laboratories). Staging of embryos was according to (Campos-Ortega and Hartenstein, 1997).

DNA sequencing of *dAP-2* mutant alleles

Genomic DNA was extracted from wild-type and hemizygous *dAP-2* mutant adults (*dAP-2*Df(3L)1118*). *Df(3L)1118* (Fig. 1A) (courtesy of F. Karim) lacks the entire *dAP-2* locus as determined by quantitative Southern blotting (data not shown). *dAP-2*-specific primers were used to amplify four overlapping genomic DNA fragments by polymerase chain reaction (PCR). Gel-purified fragments were sequenced on both strands by dye terminator cycle DNA sequencing (Perkin Elmer) using an ABI PRISM 310 Genetic Analyzer. All sequences were confirmed by sequencing samples from at least two independent PCR reactions.

Scanning electron microscopy (SEM) and light microscopy

Adult flies stored in acetone were dehydrated, critical point dried, mounted on aluminum stubs and sputter-coated with gold/palladium for examination with a Hitachi-4000 scanning electron microscope. Prothoracic legs dissected from anesthetized flies were processed as follows for light microscopy: 70% ethanol for 1 minute, 100% ethanol for 1 minute twice and xylene for 1 minute twice, then mounted in Permount:xylene (2:1) under a coverslip. Legs from at least five adult females (<1 day old) of each genotype were examined and length measurements were made using photographs of 100 \times magnification views. For head preparations, anesthetized flies with wings removed were cleared in 10% KOH at 95°C for 30 minutes, rinsed in tap water, and equilibrated overnight in 9:1 mixture of glycerol and 1 M Tris HCl pH 7.5. Heads were mounted under a coverslip bridge.

Generation of *SE-tau-lacZ* and *UAS-dAP2* transgenic flies

The *SE-tau-lacZ* transformation vector was prepared as follows: the *tau- β -galactosidase* fusion gene with SV40 3' sequences from *tau-lacZ* (Callahan and Thomas, 1994) was inserted downstream of the ~340 bp hsp70 promoter/leader in transformation vector pKB256 (courtesy of K. Basler). A polylinker was inserted into an *Xba*I site

upstream of the promoter; and a 4 kb *SpeI/EcoRI* genomic fragment (SE in Fig. 2) containing *dAP-2* exons 1b-6 was cloned in antisense orientation into the polylinker. For gain-of-function experiments using the GAL4/UAS system, a transformation vector for generating UAS-*dAP2* transgenic lines was prepared by cloning the *dAP-2* cDNA (Monge and Mitchell, 1998) into pUAST (Brand and Perrimon, 1993). Each of the above transformation vectors, together with pUChsp Δ 2,3 P-element helper plasmid, was injected into *y w* embryos and *w+* transformants were selected (Spradling, 1986). Multiple transgenic lines were obtained for each construct, and homozygous lines were established for UAS-*dAP2* on chromosomes 1, 2 and 3, and for *SE-tau-lacZ* on chromosomes 2 and 3.

Ectopic *dAP-2* expression in leg and wing discs using GAL4/UAS system

Ectopic *dAP-2* expression was activated using UAS-*dAP2* lines and GAL4 driver lines *Tub>y+>GAL4* (a 'flip-out' line, courtesy of K. Basler; Basler and Struhl, 1994) and *p339-GAL4* (enhancer trap line expressing GAL4 in imaginal discs, courtesy of G. Halder; Halder et al., 1995). The *Tub>y+>GAL4* driver line (*y w*, *hsp70-flp*, *Tub α 1>y+, GFP>GAL4* on the X chromosome) allows generation of randomly located *y* clones that constitutively express GAL4 following FLP-mediated recombination. *Tub>y+>GAL4/+; UAS-dAP2/+* staged embryos or larvae were heat shocked at 36°C for 30-60 minutes to induce FLP. Ectopic *dAP-2* expression between 0-24 hours of development caused mainly lethal effects. Activation between 24-48 hours (1st-2nd instar larvae) resulted in increased survival of adults with leg and wing defects of varying severity. Leg defects were scored for cell autonomy based on the presence of *y* sensory bristles in affected (cell-autonomous) or adjacent (non-autonomous) leg areas. Opposite side normal legs and legs from heat-shocked parental stock flies served as controls. In experiments with driver *p339*, *dAP-2* immunostaining was used to correlate location of ectopic *dAP-2* in 3rd instar wing discs with wing defects in adults.

Embryonic brain analysis with *dAP-2* null and *dAP-2* mis-expressing embryos

*dAP-2*² and *dAP-1919* null alleles balanced over *TM3*, *Ubx-lacZ* were analyzed. The wild type was Oregon-R. Double-immunostainings for HRP, 22C10, FAS II, REPO/RK2 and ELAV (Campbell et al., 1994; Fujita et al., 1982; Grenningloh et al., 1991; Jan and Jan, 1982; Robinow and White, 1991) were carried out. Primary antibodies were rabbit anti-HRP (FITC-conjugated) 1:100 (Jackson Immunoresearch), rabbit anti- β -gal 1:400 (Milan Analytika), mouse anti- β -gal 1:100 (Developmental Studies Hybridoma Bank, DSHB), mouse anti-fasciclin II 1:5 (van Vactor et al., 1993), rat anti-ELAV 1:30 (DSHB), rat anti-RK2 1:1250 (Campbell et al., 1994) and mouse anti-22C10 (1:5) (Fujita et al., 1982). Alexa 568-conjugated secondary antibodies (Molecular Probes) were used (1:150). *dAP-2*^{-/-} embryos were identified by absence of *Ubx-lacZ*. The *SE3-tau-lacZ* reporter line was used to document that the *dAP-2* genomic SE fragment (Fig. 2) directs *tau-lacZ* expression in *dAP-2*-expressing cells in the medial protocerebrum. To analyze the fates and axonal projections of these cells in *dAP-2*^{-/-} mutant embryos, *SE3-tau-lacZ* flies were crossed to *dAP-2*²/*TM3*, *Ubx-lacZ* and *dAP-2*¹⁹/*TM3*, *Ubx-lacZ* flies and *sb+* F₁ progeny were crossed inter se to produce embryos for immunostaining. *dAP-2*^{-/-} embryos were identified by lack of *dAP-2* immunoreactivity. For misexpression of *dAP-2* in embryonic neural tissue, UAS-*dAP2* flies were crossed to *sca-GAL4* (Klaes et al., 1994), *1407-GAL4* (Broadie et al., 1995), and *C155 elav-GAL4* flies (Lin and Goodman, 1994), and embryos were analyzed using markers and antibodies mentioned above. Ectopic *dAP-2* expression was confirmed by *dAP-2* immunostaining. Embryos were mounted in Vectashield H-1000 (Vector) and examined using a Leica TCS SP laser confocal microscope. Optical sections ranging from 0.9 to 2 μ m were recorded in line average mode with picture size of 512 \times 512 pixels. Captured images from optical sections were arranged and processed using IMARIS (Bitplane).

Adult brain sectioning

Wild-type and *dAP-2* mutant adult flies were fixed, dehydrated, embedded in paraffin, oriented and cut into 7 μ m sections as described (Heisenberg et al., 1985). Sections were mounted on coated glass slides and visualized by autofluorescence.

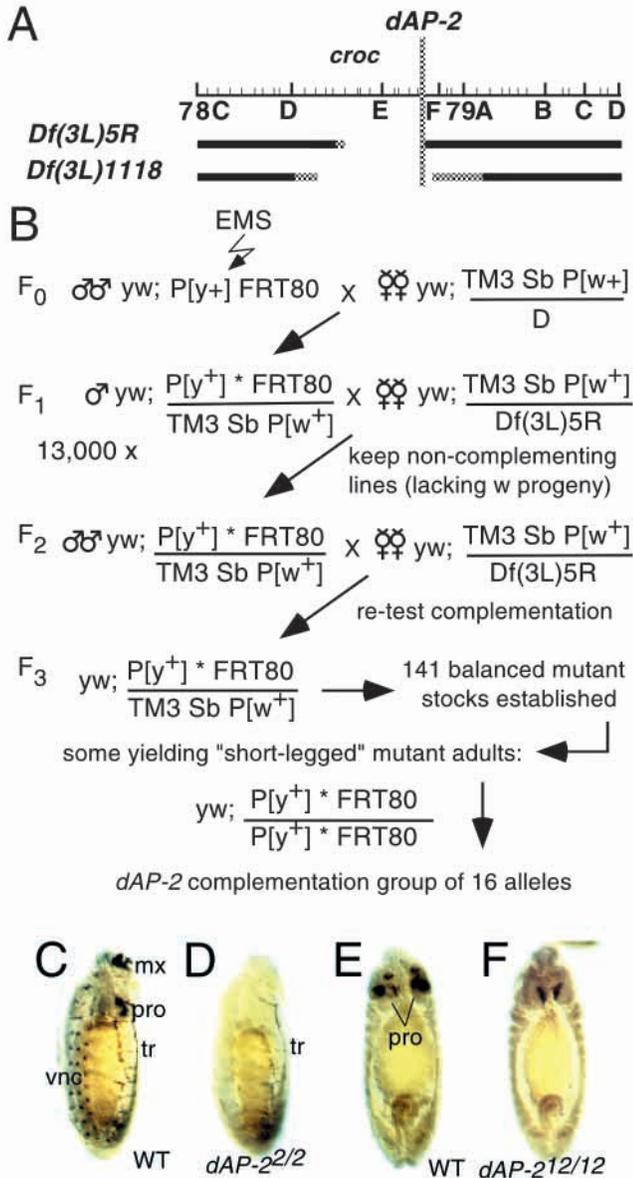
RESULTS

Mutagenesis screen to generate *dAP-2* point mutant alleles

We searched for candidate *dAP-2* mutations by mapping the approximate chromosomal location of *dAP-2* and examining deficiency lines and mutant complementation groups noted in the *Drosophila* database to be affected in this region on the third chromosome (78E-79A). Quantitative Southern blotting was performed on genomic DNA from available deficiency lines using *dAP-2* and control gene probes (data not shown). *Df(3L)5R*, the smallest deficiency tested, (lacking ~250 kb) was found to have a proximal breakpoint in the middle of *dAP-2* (Fig. 1A). Homozygous *Df(3L)5R* mutants failed to produce *dAP-2* RNA, but survived until larval development with no obvious defects, except a delay in head involution that is probably associated with loss of *croc*, a forkhead family gene that maps at 78E6 and is required for development of larval pharyngeal structures (data not shown; Häcker et al., 1995). Several complementation groups mapping in the vicinity of 78E-79A were eliminated as *dAP-2* candidates, based on positive complementation tests with *Df(3L)5R*. A search for insertion mutations in *dAP-2* from among a collection of third chromosome P-elements (Deak et al., 1997) also failed to yield any candidates (K. Kaiser, Edinburgh; data not shown).

Given the lack of *dAP-2* mutations for analysis, we carried out a mutagenesis screen to generate an allelic series of *dAP-2* point mutations (Fig. 1B). ~13,000 EMS-mutagenized third chromosomes were tested for lethal mutations failing to complement *Df(3L)5R*. The screen yielded 141 heterozygous mutant stocks (recessive lethal mutation over *TM3*, *Sb*, *P[w+]*), several of which produced homozygous mutant adults (*sb+*, *w*) or pharate pupae with extremely short, nonfunctional legs. Short-legged adults that managed to eclose were unable to walk or fly, and died within a day. As *dAP-2* is expressed in leg imaginal discs (Monge and Mitchell, 1998) and *AP-2 α* ^{-/-} mice have limb defects (Schorle et al., 1996; Zhang et al., 1996), the 'short-legged' lines were top candidates for *dAP-2* mutations. Complementation analysis with these identified a complementation group of 16 alleles, all adult lethal, among the 141 mutant stocks.

In order to test whether the 'short legged' complementation group represented *dAP-2*, an antibody generated against the extreme C terminus of *dAP-2* (see Materials and Methods) was used to immunostain embryos from mutant stocks in anticipation that lethal EMS mutations in *dAP-2* would include nonsense or splicing mutations causing loss of the C-terminal epitope. Of 14 'short-legged' lines tested, six yielded *dAP-2*-negative embryos and two yielded embryos with region-specific losses in *dAP-2* expression (Table 1) at frequencies consistent with Mendelian ratios of homozygous mutants. Immunostained wild-type and mutant embryos from two of these lines are shown in Fig. 1C,D. The immunostaining analysis provided fairly conclusive evidence



that the 'short-legged' complementation group represented mutations in *dAP-2*.

DNA sequencing confirms *dAP-2* complementation group

DNA sequencing of 11 *dAP-2* alleles was carried out and single point mutations were identified in all (Table 1). Genomic locations of these are shown in Fig. 2. The 16 *dAP-2* alleles are ordered in Table 1 with respect to severity of leg shortening in hemizygous mutants (leg shortening is a salient, graded external feature of the mutant phenotype; more below). Based on their hemizygous phenotypes and DNA sequence changes, nine alleles are classed as null and seven as hypomorphic (partial loss of function) alleles. The null alleles all yielded the same extreme leg shortening phenotype when paired with a *dAP-2* deficiency or another EMS-induced *dAP-2* null allele. Six of the null alleles yielded unstained embryos in *dAP-2* immunostaining experiments

Fig. 1. EMS mutagenesis scheme to isolate lethal mutant alleles of *dAP-2*. (A) The location of *dAP-2* on the left arm of the third chromosome is shown with respect to an adjacent gene, *crocodile* (*croc*) and two chromosomal deficiencies relevant to the analyses of *dAP-2* alleles. The 5' half of the *dAP-2* gene is deleted in *Df(3L)5R*. The entire *dAP-2* locus is deleted in *Df(3L)1118*. (B) Overview of the mutagenesis screen and identification of *dAP-2* mutant alleles. Progeny of EMS-mutagenized males were screened for failure to complement *Df(3L)5R*. Balanced mutant stocks were established for these mutant chromosomes (*, lethal mutation). Several of these stocks yielded short-legged homozygous mutant adults, and were subsequently found to belong to a complementation group of 16 alleles. (C-F) Identification of *dAP-2* immunonegative homozygous mutant embryos. Embryos from selected mutant stocks were double-stained with anti-*dAP-2* and 2A12, an internal control antibody that recognizes a tracheal antigen present after stage 13. Data for two lines representing the 'short-legged' complementation group are shown. (C,D) Stage 15 embryos, ventrolateral views. The *dAP-2*^{2/2} embryo (D) shows only 2A12-specific tracheal staining (tr) and has lost all sites of *dAP-2* expression seen in wild-type embryos (C) (mx, maxillary segment; pro, protocerebrum; vnc, ventral nerve cord). (E,F) Stage 13 embryos, dorsal views. The *dAP-2*^{12/12} embryo (F) expresses *dAP-2* in the medial brain, one of several brain regions where expression occurs in wild-type embryos (E) (2A12 does not stain stage 13 embryos).

consistent with loss of the C-terminal portion (minimally) of *dAP-2*. Five of these alleles have been sequenced. Three have mutations affecting splice sites (*dAP-2*², *dAP-2*³ and *dAP-2*¹⁵), and two have nonsense mutations (*dAP-2*¹⁴ and *dAP-2*¹⁹). The T to A transversion in *dAP-2*³ creates a new splice acceptor that probably causes out-of-frame termination after exon 1. The nonsense mutation in *dAP-2*¹⁴ would lead to protein lacking the entire DNA-binding and dimerization domain. In *dAP-2*², the splice acceptor mutation in exon 5 probably causes exon 4 to be spliced to exon 6 in *dAP-2* RNA resulting in out-of-frame termination in exon 6 (exon skipping is a common outcome when consensus splice sites are mutated (Mitchell et al., 1986)). In *dAP-2*¹⁵, the exon 7 splice donor mutation would result in failure to splice exon 8 encoding the final 36 amino acids.

Three *dAP-2* null alleles have missense mutations causing amino acid substitutions in the DNA-binding domain (Arg243His in *dAP-2*⁵, Arg243Cys in *dAP-2*¹³ and Ser273Phe in *dAP-2*⁴). Homozygous mutant embryos for these alleles were *dAP-2* immunopositive indicating that they disrupt *dAP-2* function without affecting protein stability. These alleles each yield a null phenotype when hemizygous, but show dominant negative effects when paired with hypomorphic *dAP-2* alleles (more later).

Three hypomorphic *dAP-2* alleles have been sequenced. Missense mutations in two of these (Gly287Arg in *dAP-2*¹⁰ and Arg306Ile in *dAP-2*¹⁸) and in the three null/dominant negative alleles mentioned above all cause substitutions within an 84 amino acid segment of the DNA-binding domain, which is 96% conserved between flies and humans. The arginine residue mutated in *dAP-2*¹⁸ corresponds to human *AP-2β* Arg288 which is immediately adjacent to the Arg289Cys mutation that causes Char Syndrome in a Scottish family (Satoda et al., 2000). *dAP-2*⁸ has a nonsense mutation in exon 2. Legs of hemizygous *dAP-2*⁸ mutants are slightly longer than those of hemizygous null mutants. When combined with other *dAP-2*

Table 1. EMS-induced *dAP-2* mutant alleles

Allele	Immunoreactivity*	Phenotype‡	Leg length§	Mutation¶
2	Negative	Null	30%	Exon 5 splice acceptor AG to AA
3	Negative	Null	30%	Intron 1, new splice acceptor, TG to AG
4	Positive	Null**	30%	C to T, Ser273Phe
5	Positive	Null**	30%	G to A, Arg243His
13	Positive	Null**	30%	C to T, Arg243Cys
14	Negative	Null	30%	C to T, Gln145Stop
15	Negative	Null	30%	Exon 7 splice donor GT to GA
16	Negative	Null	30%	
19	Negative	Null	30%	C to T, Gln342Stop
8	Weak positive	Hypomorphic	40%	C to T, Gln90Stop
12	Medial brain	Hypomorphic	40%	
11	Medial and lateral brain	Hypomorphic	45%	
17	n.d.	Hypomorphic	60-70%	
18	n.d.	Hypomorphic	85-90%	G to T, Arg306Ile
10	Positive	Hypomorphic	90-95%	G to A, Gly287Arg
9	Positive	Hypomorphic	90-95%	intron 2, new splice acceptor, GG to AG

*Status of dAP-2 protein in stage 9-17 homozygous mutant embryos as assessed by immunostaining with antibody against the C-terminal 11 amino acids of dAP-2.

‡Relative severity of mutant phenotype in hemizygous adults (mutant allele over *dAP-2* deficiency *Df(3L)1118*). Null, presumed complete loss of function (phenotype of allele over *dAP-2* deficiency is identical to that of allele over any other *dAP-2* null allele: all leg segments maximally shortened, tarsal joints lacking, legs nonfunctional); Null**, alleles that are null when hemizygous but have weak or moderate dominant negative effects when paired with wild-type or hypomorphic alleles; hypomorphic, partial loss of dAP-2 activity based on reduced severity of leg shortening compared with null alleles. Hypomorphic alleles show additive effects on leg length in heteroallelic adults.

§Leg length: total length of mutant prothoracic leg as % of wild-type (+*Df*) prothoracic leg length.

¶Single base change in *dAP-2* mutant allele relative to wild-type *dAP-2*. Numbering of amino acids is relative to dAP-2 isoform using exon 1b (see Fig. 2).

alleles, *dAP-2*⁸ causes a slight reduction of leg shortening associated with the other allele (Fig. 3B,C). *dAP-2*⁸ also partially rescues lethality of the mildest hypomorphic alleles *dAP-2*⁹ and *dAP-2*¹⁰ (more later). Consistent with these findings, homozygous *dAP-2*⁸ mutant embryos are weakly dAP-2 immunopositive, indicating that the mutation has not completely abolished production of dAP-2 protein (Table 1). It is not known whether the trace remaining protein results from an alternative RNA transcript that omits exon 2 or initiates downstream of it.

***dAP-2* is required for leg outgrowth and joint formation**

Shortened legs are a prominent feature of the *dAP-2* mutant phenotype. The degree of leg length reduction is allele-dependent, but in each case, all legs and all leg segments are affected. Hemizygous and homozygous null alleles result in maximally shortened legs (prothoracic legs ~30% of wild-type

length; Fig. 3B,E). In null mutant legs, the proximodistal order of segments is not grossly affected as landmarks for the coxa, femur, tibia, and first and last (5th) tarsal segments (sex comb and claws, respectively) are present in the correct order. However, no tarsal joints are evident (tibia and five tarsi are fused into one segment); and rows of misoriented sensory bristles in several leg regions indicate that proximodistal polarity is locally perturbed (see *dAP-2*¹⁷/*Df* tibia in Fig. 3G; higher magnification not shown). These stunted, nonfunctional legs show no nervous activity except for a faint twitching of the claws and of cuticle over the femur-tibia junction. In contrast, the abdominal muscles of mutant adults contract vigorously.

*dAP-2*⁸ (Fig. 3C) and two other hypomorphic alleles, *dAP-2*¹¹ (Fig. 3F) and *dAP-2*¹² (not shown) result in legs that are 40-45% of wild-type length. These have regained tibia-tarsal joints, but lack most or all tarsal joints. Four milder hypomorphic alleles (*dAP-2*¹⁷, *dAP-2*¹⁰, *dAP-2*⁹ (Fig. 3G-I) and *dAP-2*¹⁸ (not shown)) result in legs that are 70-95% of wild-type length. All joints are present in these legs; however, a high frequency of necrotic femur-tibia and trochanter joints (mainly in prothoracic and metathoracic legs) suggests joint development is abnormal (Fig. 3H, other data not shown). With regard to locomotion of hemizygous adults, *dAP-2*⁹ mutants (Fig. 3I) walk in an uncoordinated, halting fashion, drag their posterior legs, often stumble, and have difficulty righting. *dAP-2*¹⁰ mutants (Fig. 3H) stand with difficulty, and rarely but occasionally take feeble steps. *dAP-2*¹⁷ (Fig. 3G) and *dAP-2*¹⁸ (not shown) mutants have severely ataxic legs and are generally unable to stand, although newly eclosed adults can thrash their legs vigorously when supine.

Proboscis reduction in *dAP-2* mutant adults

Proboscis shortening is evident for all hemizygous *dAP-2* mutant alleles, and roughly correlates with the severity of leg

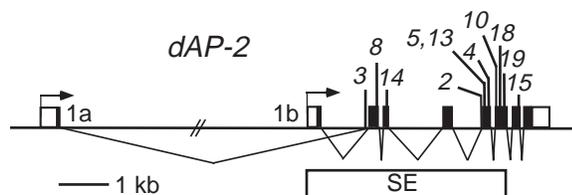


Fig. 2. Genomic locations of point mutations in *dAP-2* alleles. Mutations identified for 11 alleles are shown. *dAP-2* exons (boxed) are noted, including two alternative first coding exons, exon 1a and exon 1b, present in embryonic *dAP-2* cDNAs (Bauer et al., 1998; Monge and Mitchell, 1998, respectively). These alternative exons have different leader sequences (white boxes) and encode different N-terminal segments of 15 and 19 amino acids, respectively.

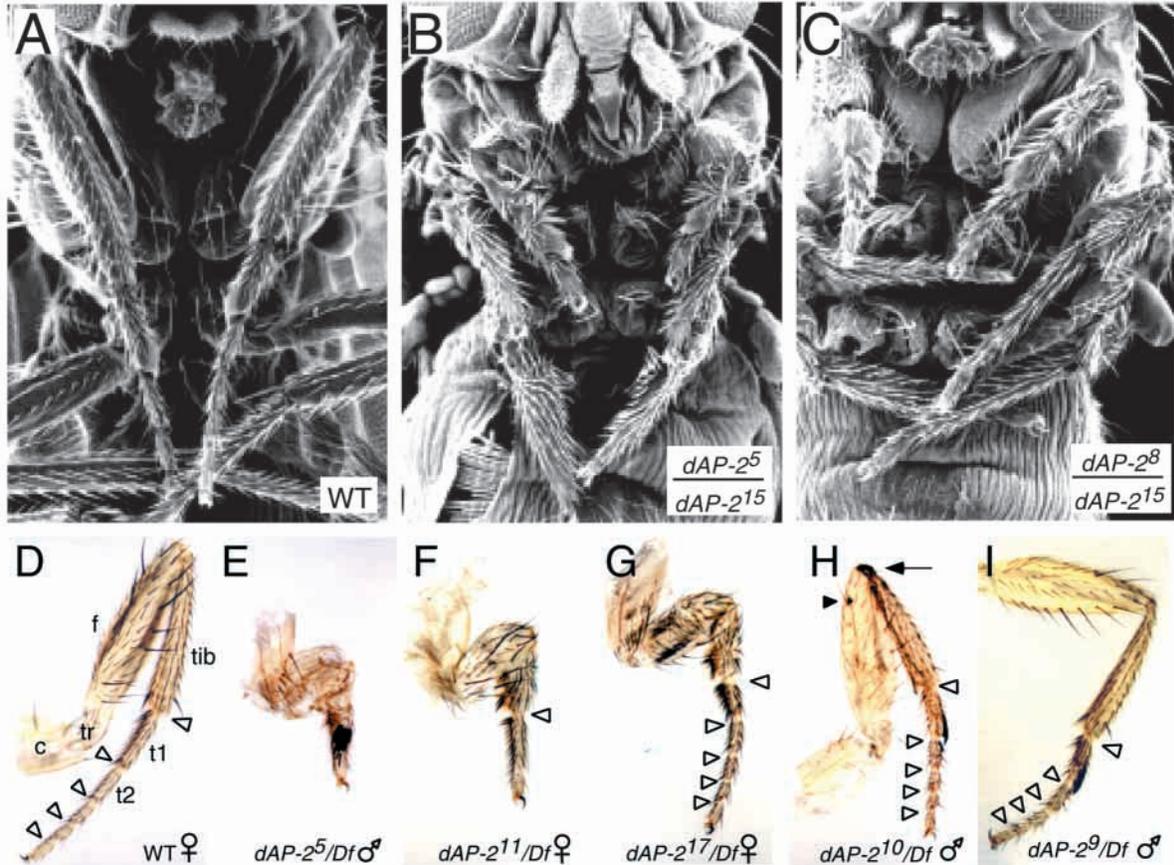


Fig. 3. Severe leg shortening and loss of joints in *dAP-2* mutant adults. (A-C) Scanning electron microscope ventral views of wild-type (A), *dAP-2⁵/dAP-2¹⁵* (B) and *dAP-2⁸/dAP-2¹⁵* (C) adult legs. (D-I) Light microscope views of prothoracic legs (forelegs), posterior side, except E where the anterior side is shown to note the male sex comb on the first tarsal. All legs are shown at the same magnification. Locations of joints distal to the tibia are marked by white arrowheads. (D) Wild-type (*dAP-2^{+/+}*) leg segments are noted, from proximal to distal: c, coxa; tr, trochanter; f, femur; tib, tibia; t1, first tarsal; t2, second tarsal; t5, fifth tarsal. (E-I) Legs of hemizygous mutants (*dAP-2ⁿ/Df(3L)1118*). The severely shortened *dAP-2⁵/Df* null mutant leg (E) lacks all joints between tibia and claw. (F-I) Legs of four hypomorphic mutants. Legs of *dAP-2¹¹/Df* mutants (F) are slightly longer than those of null mutants, and have regained the joint between the tibia and first tarsal. Legs of *dAP-2¹⁷/Df* (G) mutants are ~60-70% of wild-type length and have regained all joints. The mildly shortened *dAP-2¹⁰/Df* leg (H) has a necrotic femur-tibia joint (arrow), and muscle is detached from the dorsal side of the joint and retracted proximally (black arrowhead notes necrotic, retracted part). This defect is ≥80% penetrant in prothoracic and metathoracic legs of *dAP-2¹⁰/Df* adults, and ~30% penetrant in prothoracic legs of *dAP-2⁹/Df* adults (I).

shortening. Null mutants show a substantial reduction in length and width of the labellum (distiproboscis or labial palp) and number of pseudotracheal rows (chemosensory sensilla; Fig. 4B,E,E'). The prestomal cavity is still present and newly eclosed null mutants usually can move the proboscis and can drink if provided with water. The clypeus, maxillary palps and antennae (derivatives of the clypeolabral and antennal imaginal discs) lack overt external defects; however, increased spacing between antennae in null mutants suggests undergrowth of proximal antennal segments at the expense of head cuticle (Fig. 4A,B). The proboscis of *dAP-2⁹* mutants is nearly normal (not shown), while that of *dAP-2¹⁰* mutants is shortened but normal in width (Fig. 4C,F,F').

Proboscis and leg defects in *dAP-2* mutants correlate with *dAP-2* expression in imaginal disc primordia of these structures. In third instar wild-type larvae, *dAP-2* is expressed in several radial stripes in labial discs (paired primordia of the distiproboscis) and in concentric rings in leg imaginal discs (Fig. 5). Upon leg disc eversion, the rings of *dAP-2*-expressing

cells correspond to presumptive joint regions (Monge and Mitchell, 1998; Kerber et al., 2001). *dAP-2* null and partial-loss phenotypes indicate that *dAP-2* is required for joint development and also for elongation of leg segments. Wings and halteres of *dAP-2* mutants develop normally consistent with lack of *dAP-2* expression in imaginal disc primordia of these (data not shown).

***dAP-2* heteroallelic combinations rescue lethality and leg shortening and reveal effects of dominant negative alleles**

dAP-2 mutant alleles were tested in pairwise combinations to assess heteroallelic phenotypes. Hypomorphic *dAP-2* alleles show additive positive effects in that leg and proboscis defects are much reduced and lethality can be rescued in heteroallelic flies. *dAP-2^{8/9}*, *dAP-2^{8/10}* and *dAP-2^{10/17}* heteroallelic mutant lines have been established. Adults of these lines have normal or nearly normal length legs, and moderate incidences of necrotic joints and ectopic cuticular elements that invade

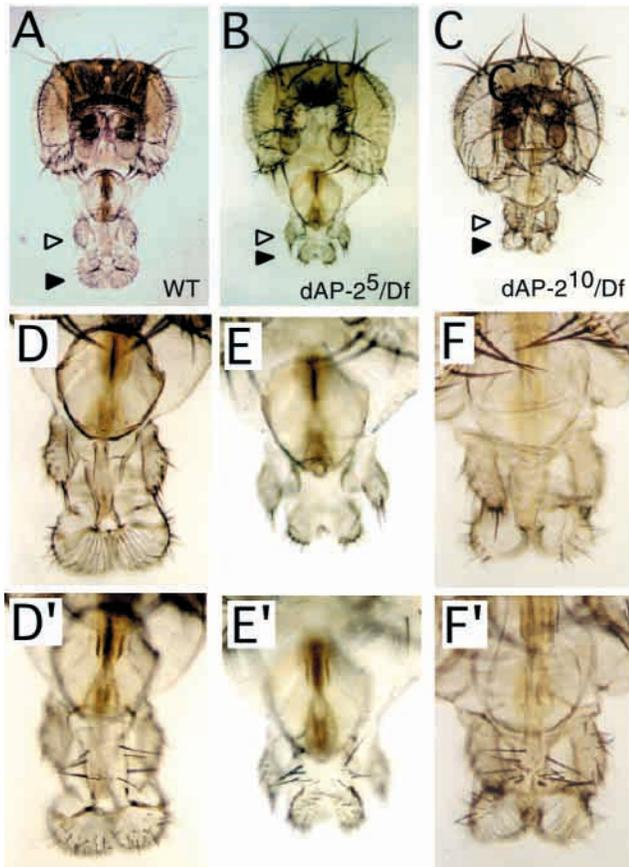


Fig. 4. Proboscis reduction in *dAP-2* mutants. (A-C) Wild-type (A) and *dAP-2* hemizygous mutant adult heads (*dAP-2⁵/Df(3L)1118*) viewed from the front at the same magnification. The right maxillary palp (white arrowhead) and the labellum (labial palp, black arrowhead) of the mediproboscis and distiproboscis, respectively, are noted. (D-F) Higher magnification frontal views of wild-type and mutant heads from clypeus (basiproboscis) to labellum. (D'-F') Views of the proboscis back-side, focusing on the long bristles of the distiproboscis prementum. Relative to wild type (A,D,D'), the distiproboscis of *dAP-2⁵* (B,E,E') and other *dAP-2* null mutants (not shown) is severely reduced in length and width. The proboscis of *dAP-2¹⁰* (C,F,F') mutants is moderately shortened along the proximodistal axis; the labellum is normal in width but abnormal in projection (pseudotrachea rows are visible on the back rather than front of the labellum).

medioventral eye territory. The latter growths include one or more types of sensory bristles (Fig. 6A), and are occasionally seen in mutant adults hemizygous for hypomorphic *dAP-2* alleles (as in Fig. 6B,C). In rare instances, supernumerary antennae develop and the adjacent eye is greatly reduced as in the *dAP-2^{8/10}* mutant shown in Fig. 6D,D'. Interestingly, extra antennae are never seen in *dAP-2* null mutants. This duplication defect is therefore somewhat reminiscent of the limb duplication that occur in *AP-2 α* chimeric mice but not in *AP-2 α ^{-/-}* mice (Nottoli et al., 1998). The ectopic antennae may indicate that the boundary in the eye-antenna disc between antennal-territory (where *dAP-2* is expressed) and eye-territory (where *dAP-2* is not expressed) is unstable when *dAP-2* is limiting. Femur-tibia joint necrosis coincident with detachment of the tibial levator muscle from the joint occurs at high

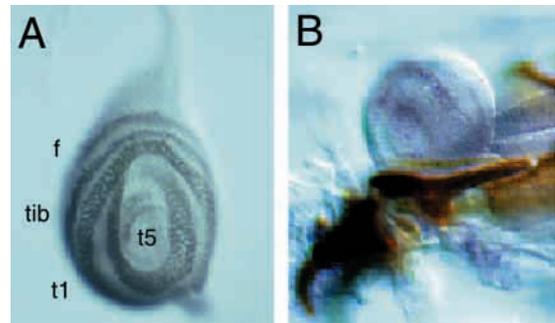


Fig. 5. *dAP-2* expression in labial and leg imaginal discs of third instar larvae. (A) Anti-*dAP-2* immunostained leg disc revealing restricted distribution of *dAP-2*-expressing cells in each presumptive leg segment excluding the most distal one (t5). f, femur; tib, tibia; t1, first tarsal, t5, fifth tarsal. (B) In the labial disc, several broad stripes of *dAP-2* expressing cells are seen.

penetrance in hemizygous *dAP-2¹⁰* adults (Fig. 3H and Fig. 6B), but only rarely in *dAP-2^{8/10}* and *dAP-2^{8/9}* flies (Fig. 6D and data not shown), indicating that, like leg shortening and lethality, joint defects are ameliorated by increased *dAP-2* activity.

Three null alleles (*dAP-2⁴*, *dAP-2⁵* and *dAP-2¹³*) were candidate dominant negative alleles based on the nature of their DNA-binding domain mutations and their immunopositive status in embryo staining experiments (Table 1). The effects of these alleles on wild-type and *dAP-2* hypomorphic alleles were examined. Legs of *dAP-2¹³/+* (Fig. 6F) and *dAP-2⁴/+* (not shown) adults were found to be (on average) slightly shorter (~3-5%) than those of *dAP-2⁺/Df(3L)1118* flies (Fig. 6A) and other *dAP-2* heterozygous flies (data not shown). Furthermore, when *dAP-2¹³* and *dAP-2⁴* alleles were combined with *dAP-2* hypomorphic alleles, such as *dAP-2⁹*, there was a significant average reduction (7-15%) in leg length compared with adults carrying the hypomorphic allele alone (Fig. 6G-I and other data not shown). Remarkably, the Arg243 residue mutated in both *dAP-2⁵* and *dAP-2¹³* corresponds to Arg225 in human *AP-2 β* , which is mutated identically to *dAP-2¹³* (Arg to Cys) in a recently studied Char Syndrome family (B. Gelb, Mount Sinai School of Medicine, personal communication). Studies of the DNA binding and dimerization activities of Char mutant *AP-2 β* protein in vitro support the idea that these proteins function dominantly by forming non-DNA binding heterodimers with wild type *AP-2 β* . The more conservative Arg243His mutation in *dAP-2⁵* had a weaker dominant negative effect on wild-type and hypomorphic *dAP-2* alleles than *dAP-2¹³* and *dAP-2⁴* (data not shown), suggesting that it may have a less deleterious effect on heterodimeric DNA binding than on homodimeric DNA binding.

UAS/GAL4 mediated mis-expression of *dAP-2* in leg and wing imaginal discs

In order to gain additional insight into developmental functions of *dAP-2*, we examined the effects of expressing *dAP-2* outside its normal expression domain in wild-type leg discs using the UAS/GAL4 system (Brand and Perrimon, 1993). UAS-*dAP2* flies were crossed to flies carrying the 'flip-out' GAL4 driver *Tub<y+>GAL4* (Basler and Struhl, 1994) (Fig. 7A), and random clones of cells constitutively expressing *dAP-2* were generated in imaginal discs of larval progeny by heat shock

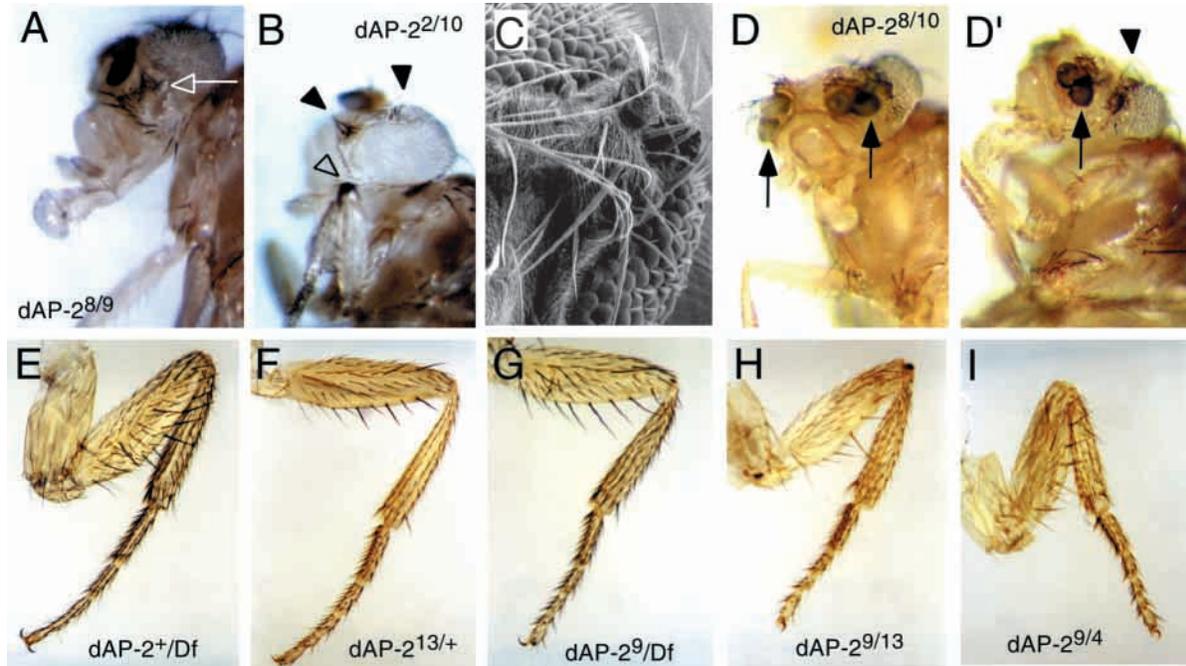


Fig. 6. Defects in *dAP-2* heteroallelic adults with hypomorphic and dominant negative alleles. (A) Ectopic bristled cuticular element (arrow) at the medioventral perimeter of the eye in a *dAP-2⁸/dAP-2⁹* adult. (B) Milder ectopic elements (black arrowheads) in *dAP-2¹⁰/dAP-2²* adult. The necrotic femur-tibia joint typical of *dAP-2¹⁰* mutants is noted (open arrowhead). (C) SEM view of a larger ectopic bristled growth on medial aspect of a *dAP-2¹⁰/dAP-2²* adult eye. (D) Two pairs of double-antennae (arrows) in a *dAP-2⁸/dAP-2¹⁰* adult. (D') Lateral view of left set of double antennae (arrow) showing partial third antenna (arrowhead). (E-I) Prothoracic legs from female adults, all at same magnification. *Df*, *Df(3L)1118*. (E) *dAP-2⁺/Df* leg. (F) *dAP-2⁺/13* leg (~5% shorter than *dAP-2⁺/Df*). (G) *dAP-2⁹/Df* leg (~10% shorter than *dAP-2⁺/Df*). (H) Heteroallelic *dAP-2⁹/13* (~7% shorter than *dAP-2⁹/Df*). (I) Heteroallelic *dAP-2⁹/4* (~14% shorter than *dAP-2⁹/Df*). *dAP-2¹³* and *dAP-2⁴*, both null alleles when hemizygous (Table 1 and data not shown), have dominant negative effects on hypomorphic alleles such as *dAP-2⁹* causing enhanced leg shortening and increased incidence of joint necrosis (femur-tibia and trochanter-coxa in H, trochanter-coxa in I).

induction (see Material and Methods). The surviving adult progeny displayed leg and wing defects, many of which involved dismorphic undergrowth of affected limbs. The size and location of *dAP-2*-expressing clones in legs were scored by the presence of *y* sensory bristles (Fig. 7B). These experiments revealed that large clones of *dAP-2*-expressing cells spanning one or more leg segments significantly inhibited outgrowth of the affected segments (Fig. 7D,E). Notably, *y*⁺ and *y* areas of shortened leg segments were fairly symmetrically affected. The large *y* clone shown in Fig. 7E has caused shortening of all segments by about 30% and fusion of the distal femur to the proximal tibia. In tarsi of this leg, ectopic *dAP-2* has interfered with normal joint formation and induced supernumerary, ectopic partial joints (Fig. 7E' and other focal planes, not shown). In Fig. 7H, the *y* clone begins in the distal tibia and extends distally into the tarsi of this metathoracic leg. The tibia, although slightly bowed, is not significantly shortened. However, the tibia-tarsal joint is abnormal, the second tarsal has three partial joints, and tarsal segments are shortened by about 30% (compare Fig. 7H,H' with wild-type leg in Fig. 7G). In contrast to the effects of large, segment-spanning clones, narrow clones of *dAP-2*-expressing cells spanning multiple leg segments frequently resulted in necrotic, abnormal joints without substantially reducing segment length (data not shown). These joint defects suggest that constitutive or elevated *dAP-2* expression or shifting of the boundary between *dAP-2*-expressing and non-expressing cells may be

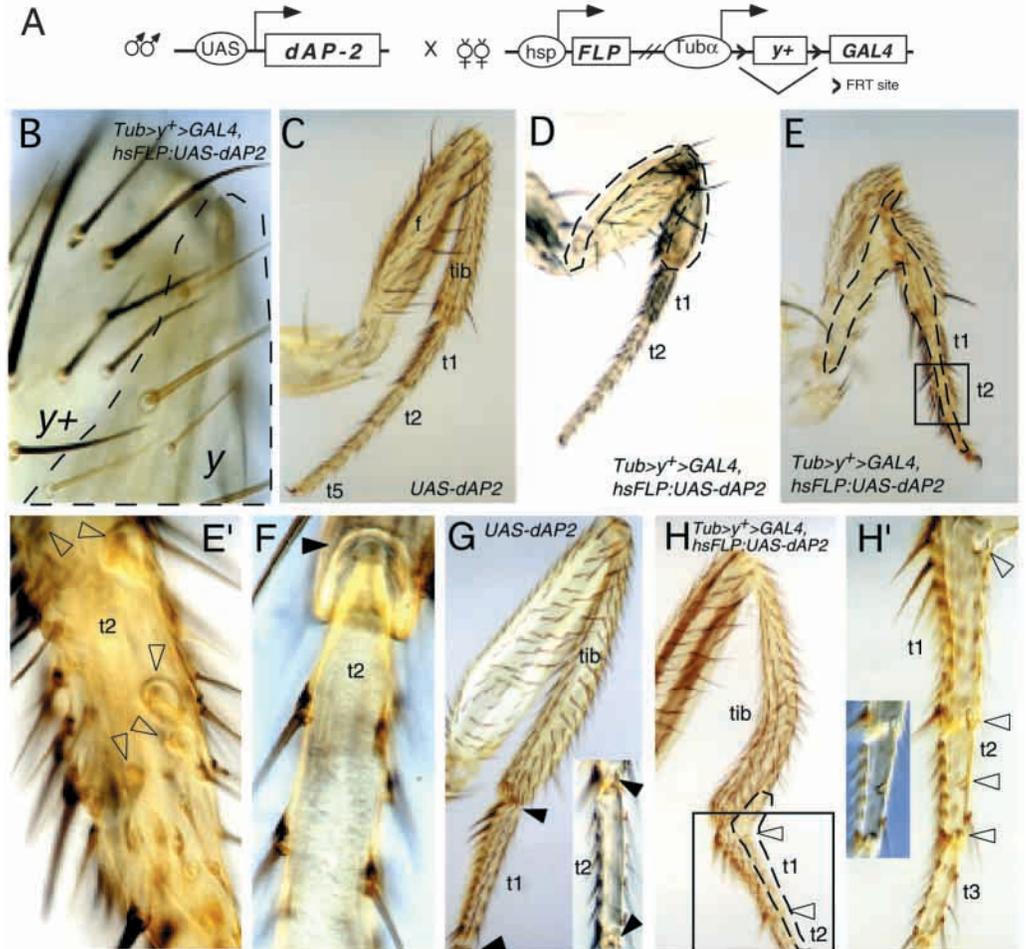
incompatible with normal joint development. In view of the known *dAP-2* expression pattern and loss-of-function phenotypes, the ability of ectopic *dAP-2* to induce supernumerary partial joints in tarsal *y* clones strongly suggests that *dAP-2* functions cell-autonomously in joint formation. In contrast, the role of *dAP-2* in leg segment outgrowth has a large non-autonomous component, as, in addition to requiring *dAP-2* expressing cells distally, segment outgrowth also requires substantial numbers of *dAP-2* non-expressing cells proximally in the leg segment primordium.

dAP-2 is not expressed in wing imaginal discs of 3rd instar larvae (data not shown). We examined the effects of ectopically expressing *dAP-2* in the wing disc using *GAL4* drivers *p339-GAL4* (Halder et al., 1995) and *Tub>y+>GAL4*. Ectopic *dAP-2* driven by *p339-GAL4* resulted in ataxic adults with abnormal labellums, misoriented notal bristles (not shown), wings with non-adherent ventral and dorsal surfaces, and two rows of ectopic sensory bristles on both the dorsal and ventral sides of the wing blade (Fig. 8D-F). The latter bristles appeared to represent transformations of the distal portions of veins 3 and 4. Anti-*dAP-2* immunostaining of *p339-GAL4; UAS-dAP2* wing discs revealed ectopic *dAP-2* in the wing pouch (future wing blade) in a pattern consistent with the location of ectopic sensory bristles on the adult wing (Fig. 8B). Similar ectopic sensory bristles were also observed when *Tub<y+<GAL4* was used to drive ectopic *dAP-2* expression in the wing (Fig. 8G). These data indicate that

Fig. 7. dAP-2 misexpression in leg imaginal discs. (A) Genetic cross to generate larvae for random activation of *UAS-dAP2* in imaginal discs by heat shock induction of FLP-mediated recombination (see Materials and Methods). (B-H') Legs of adult females derived from heat-shocked larvae; genotypes are noted. Cells in *y* clones constitutively express *dAP-2*; locations of *y* clones are outlined by the broken lines. (B) High magnification view of a *y* clone on the distal femur to demonstrate use of the *y* marker (*y* sensory bristles are pale yellow). (C-E) Prothoracic legs at same magnification.

(C) Normal leg from *UAS-dAP2* heat-shocked control; leg segments are labeled. f, femur; tib, tibia; t1, 1st tarsal, t2, 2nd tarsal; t5, 5th tarsal. (D) *y* clone causing shortening of femur and tibia. (E-E') *y* clone causing shortening of all leg segments. Boxed area in E is shown at higher magnification in E', revealing multiple, partial ectopic joints (open arrowheads) in the shortened 2nd tarsal.

(F) Normal 2nd tarsal at same magnification as E' to show ball and socket joint between t1 and t2. (G) Normal metathoracic leg showing femur, tibia, and 1st tarsal. Inset shows 2nd tarsal at higher magnification. Tarsal joints are noted with filled arrowheads. (H) Abnormal metathoracic leg with *y* clone causing bowing of tibia and shortening of 1st and 2nd tarsals. Boxed area is upper half of H'. Abnormal joints are noted with open arrowheads. (H') Higher magnification view of leg in H shows disruption of tibia/t1, t1/t2 and t2/t3 joints, and induction of ectopic partial joint in middle of t2. Inset shows t2 ectopic joint viewed with greater DIC contrast; magnification is same as inset in G.



dAP-2 can cell autonomously transform wing vein epithelium into ectopic sensory bristles.

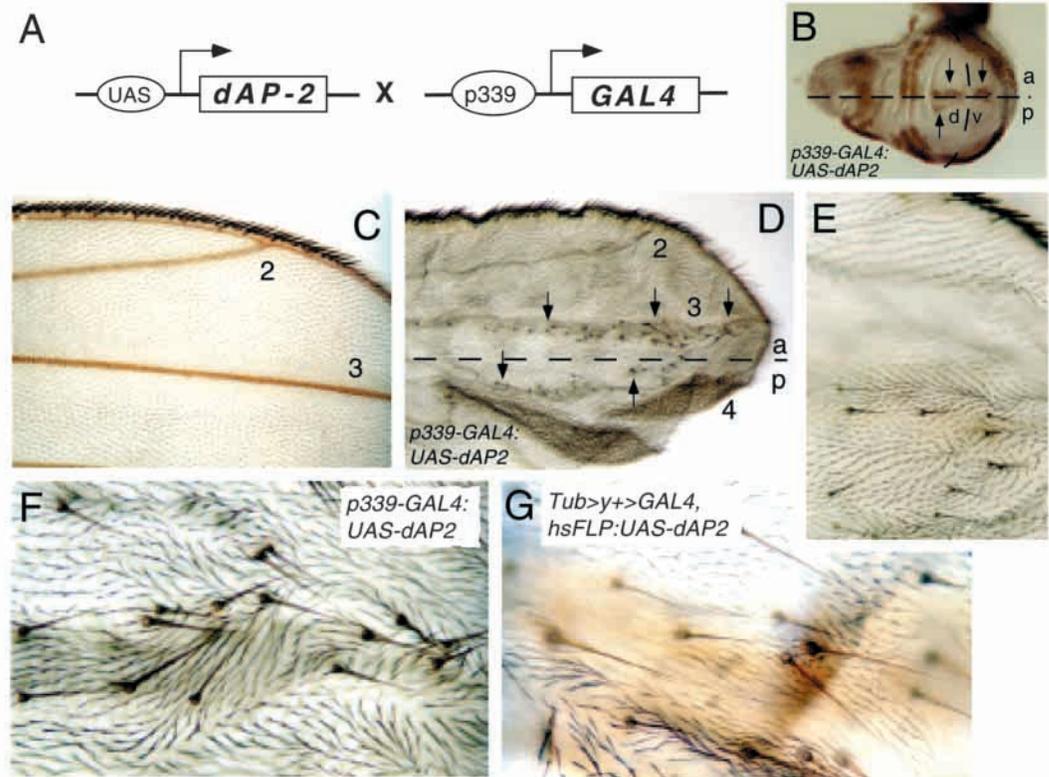
dAP-2 mutants lack gross defects in the embryonic brain

Although *dAP-2* is expressed in the embryonic maxillary segment and in the embryonic and larval central nervous system in wild-type (Monge and Mitchell, 1998), *dAP-2* mutants survive embryogenesis and larval development, indicating that zygotic *dAP-2* is not essential during these stages. Nonetheless, we examined *dAP-2* mutant embryos for morphological defects in the CNS and maxillary cephalic ganglia to look for changes that might have occurred in these without grossly affecting viability. Double-immunostaining and confocal microscopy were used to compare wild-type embryos with *dAP-2²* and *dAP-2¹⁹* homozygous null mutant embryos (Materials and Methods). Patterning, morphogenesis and axon pathway formation as well as neuronal and glial cell fate specification appeared grossly normal in *dAP-2* mutant embryos with respect to all markers tested (data not shown). In addition, no abnormalities were observed in the maxillary cephalic ganglia (Schmidt-Ott et al., 1994).

We examined specific *dAP-2*-expressing cells in the embryonic CNS in more detail using the *SE-tau-lacZ* reporter line (see Materials and Methods). In this line, *tau-lacZ* expression is regulated by a 3 kb enhancer element from the *dAP-2* gene (SE fragment in Fig. 2). Double-staining with anti- β -gal and anti-dAP-2 antibodies showed that *SE-tau-lacZ* is expressed in a subset of *dAP-2* expressing neurons located medially in the b1 neuromere (Hirth et al., 1995) of the protocerebrum. Transport of tau- β -gal into axons revealed that these cells send axons along the established preoral commissure by stage 13 and terminate contralaterally within the posterior medial part of b1 (Fig. 9). In a *dAP-2*-null mutant background, *SE-tau-lacZ*-expressing cells were still positioned correctly and made axonal projections that were indistinguishable from wild type (data not shown). Thus, loss of dAP-2 did not apparently affect the survival, migration or axonal path finding of these neurons.

Gain-of-function experiments were also performed to mis-express *dAP-2* using several embryonic brain-specific GAL4 drivers (see Materials and Methods). No gross morphological changes were seen in the developing embryonic CNS in response to *dAP-2* mis-expression and subsequent larval

Fig. 8. dAP-2 induces ectopic sensory bristles in wing blade epithelium. (A) Genetic cross to activate ectopic *dAP-2* expression using the *p339-GAL4* driver. (B) Anti-dAP-2 immunostained *p339-GAL4; UAS-dAP2* 3rd instar wing disc. The approximate anterior-posterior (a, p) and dorsal-ventral (d, v) compartment boundaries are shown (broken line). Arrows identify dAP-2 expressing cells which likely generate the ectopic bristles on the wing blades of *p339-GAL4; UAS-dAP2* adults. (C) Portion of wild-type wing showing anterior margin and veins 2 and 3. (D) In the *p339-GAL4; UAS-dAP2* wing, veins 3 and 4 are indistinct and replaced by ectopic sensory bristles (arrows). The approximate anterior-posterior compartment boundary (broken line) is noted for reference to imaginal disc view in B. The wing is misshapen and ruffled because of lack of adhesion between dorsal and ventral epithelia. (E,F) Higher magnification views of ectopic bristles on *p339-GAL4; UAS-dAP2* wings. (G) *Tub>y+>GAL4; UAS-dAP2* wing (same magnification as F) showing similar dAP-2 induced ectopic bristles. These bristles are largely if not exclusively *y* (compare to *y+* bristles in F), indicating cell autonomous induction.



metamorphosis was undisturbed (data not shown). Taken together, the data suggest that dAP-2 does not critically regulate specification and patterning of the embryonic brain.

Adult brain central complex is disrupted in *dAP-2* mutant adults

Loss of dAP-2 activity in the central nervous system could potentially have critical consequences for adult viability and other adult functions. To begin to address this question, we examined brains of *dAP-2* mutant adults in frontal paraffin sections to identify morphological changes. This analysis revealed a major defect that was reproducibly present in both null and hypomorphic *dAP-2* mutants. The defect entailed a disruption of the central complex, a prominent central neuropil region in the protocerebrum. Abnormalities in nerve tracts of the optic lobes (antenna-glomerular tract, antennal nerve and median bundle) and an unusual number of large cell somata around the neuropil were also noted. The central complex is comprised of four substructures, namely the protocerebral bridge, the fan shaped body, the paired noduli and the ellipsoid body. In *dAP-2* mutants, the fan shaped body is bisected (Fig. 10D,E) instead of being continuous across the midline as in wild type (Fig. 10A,B). The ellipsoid body and associated substructures are also disrupted (Fig. 10F) relative to wild type (Fig. 10C). Lesions and mutations (such as *no-bridge*¹, *central complex*¹ and *ocelliless*¹) that disrupt the central complex are associated with loss of locomotor activity (Strauss and Heisenberg, 1993; Martin et al., 1999). In preliminary locomotion studies, *dAP-2* heteroallelic flies displayed reduced

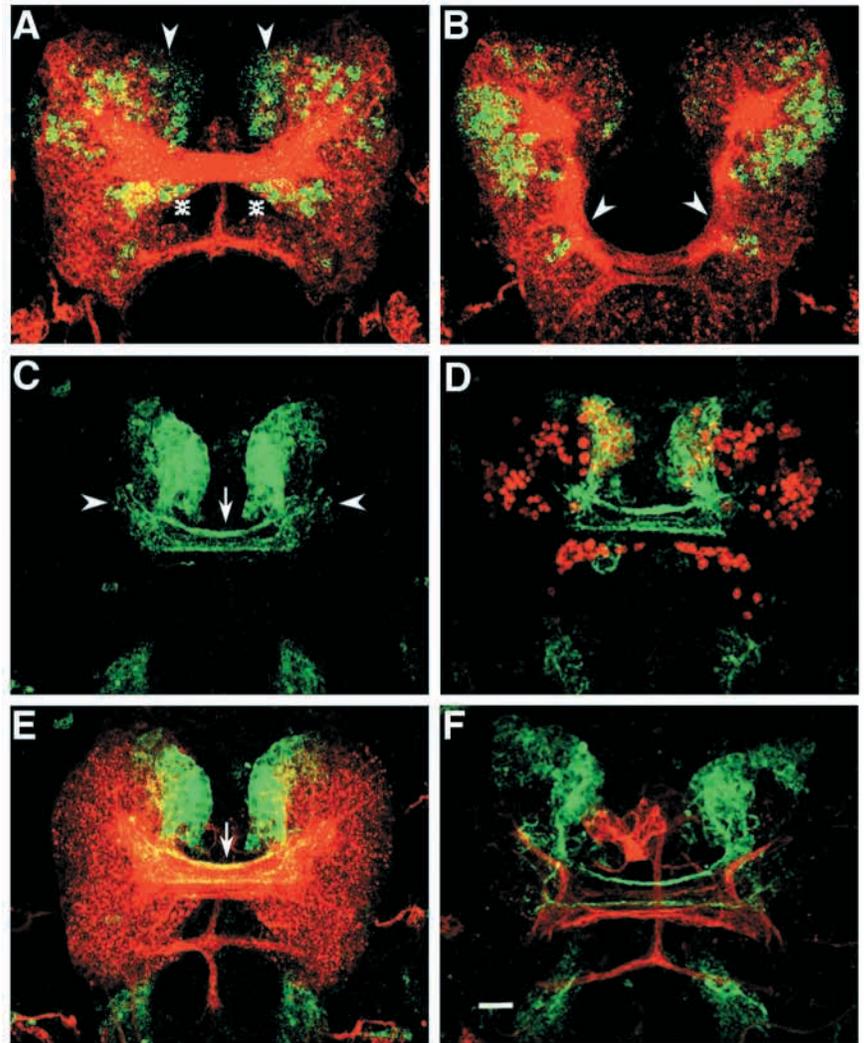
walking activity compared with wild-type flies, raising the possibility that dAP-2 is required for some aspect of central complex development crucial for locomotion (P. J. M. and K. Han, unpublished).

DISCUSSION

Parallels in mutant phenotypes of *Drosophila dAP-2* and murine *AP-2α*

Fundamental parallels between the leg and proboscis outgrowth defects paralleled here in *dAP-2* mutant flies, and the limb and orofacial defects that we and others have identified in *AP-2α* mutant mice (Schorle et al., 1996; Zhang et al., 1996) underscore an ancient role for AP-2 in development of locomotor and feeding appendages that pre-dates the separation of arthropod and chordate species. The major head defect in *dAP-2* null flies is undergrowth of the labellum (labial palps or distiproboscis), the prominent feeding appendage derived from the paired labial imaginal discs. Orofacial defects in *AP-2α*^{-/-} mice are mainly caused by severe undergrowth of the first branchial arch (Schorle et al., 1996), the largest and most anterior of the paired serially homologous branchial arches that constitute, together with the frontonasal prominence, the major primordia of the face and neck. The first branchial arch and the emigrant cranial neural crest cells which populate it constitute the primordia of structures that include the upper and lower jaws, anterior tongue, and the trigeminal ganglia (Sperber, 1989). Second branchial arch derivatives are

Fig. 9. *SE3-tau-lacZ* reporter marks subset of *dAP-2* expressing neurons in the embryonic protocerebrum. Stage 14 wild-type (A,B) and *SE3-tau-lacZ* transgenic (C-F) embryos, dorsal views; reconstructions of laser confocal optical sections. (A,B) Double labeling with anti-*dAP-2* (yellow/green) and anti-HRP (red). (C) *SE3-tau-lacZ* reporter gene expression labeled with anti- β -gal (green). (D) Double labeling with anti-*dAP-2* (red) and anti- β -gal (yellow/green). (E) Double labeling with anti-HRP (red) and anti- β -gal (yellow/green). (F) Double labeling with anti-FAS II (red) and anti- β -gal (yellow/green). Endogenous *dAP-2* expression occurs in the b1 neuromere (A, arrowheads) and in cell clusters adjacent to the preoral commissure (A, asterisks) as well as in a small subset of cells in the b2 neuromere (B, arrowheads). *tau-lacZ* reporter gene expression driven by the *dAP-2* enhancer fragment SE (Fig. 2) mimics endogenous *dAP-2* expression in the medial part of the b1 neuromere (C, D; compare to A,B) and reveals that from stage 13 onward these cells project axons contralaterally along the HRP and FAS II positive preoral commissure (E, arrow; F) and terminate within the posterior medial part of b1 (C, arrowheads). Scale bar: 10 μ m.

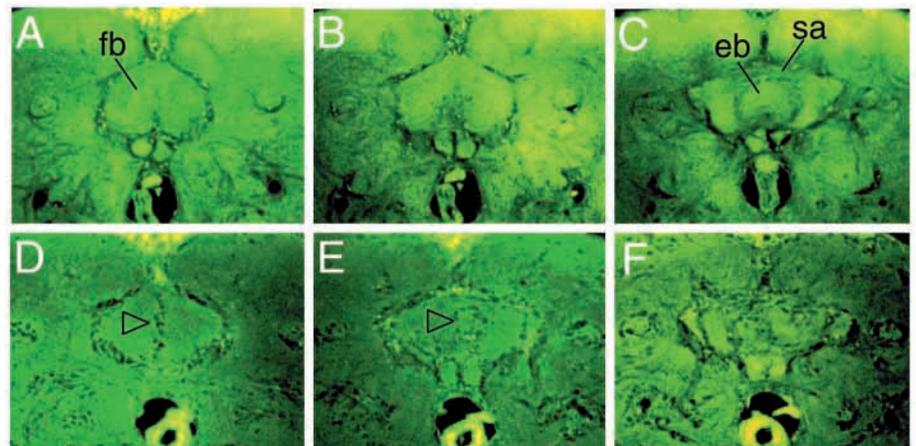


also underdeveloped in *AP-2*^{-/-} mice, and a role for AP-2 family transcription factors in regulating *Hoxa2* expression in neural crest cells of the second arch has been proposed, based on studies in transgenic mice (Maconochie et al., 1999). *Hoxa2* is a mammalian ortholog of *Drosophila proboscipedia* (*pb*), the *HOM-C* gene that specifies adult labial and maxillary palp fates, and transforms legs to palps when ectopically expressed in leg discs (Aplin and Kaufman, 1997).

dAP-2 loss- and gain-of-function phenotypes indicate that *dAP-2* acts region-specifically and at least partly non-autonomously to regulate limb outgrowth. Phenotypes associated with different combinations of wild-type,

hypomorphic and dominant negative *dAP-2* alleles reveal that as *dAP-2* activity is incrementally increased, limb and proboscis outgrowth is restored before viability and locomotor coordination. The *dAP-2* dominant negative alleles are particularly interesting because they are analogous to *AP-2 β* alleles that cause Char Syndrome in humans, and thus represent a *Drosophila* model for this congenital syndrome.

Fig. 10. Brain central complex defects in *dAP-2* mutant adults. Frontal sections of paraffin embedded brains of wild type (A-C) and *dAP-2*^{219/Df(3L)1118} mutants (D-F). The fan-shaped body (fb) is bisected in mutants (D,E) (open arrowhead) relative to wild type (A,B). The ellipsoid body (eb) and superior arch (sa) are also severely disorganized (F) relative to wild type (C).



Somewhat paradoxically, full loss of *AP-2 β* in mice causes a lethal kidney defect but no overt craniofacial or limb defects, and kidney defects have not been noted in Char Syndrome families (B. Gelb, personal communication). Perhaps the developmental anomalies in Char Syndrome represent novel effects of reduced *AP-2 β* activity that are somewhat analogous to the head cuticle defects seen in *dAP-2* hypomorphs but not in *dAP-2* null mutants. Alternatively, it is possible that Char Syndrome is caused, at least in part, by inhibition of *AP-2 α* or other *AP-2* family members, as it is known that *AP-2 β* and *AP-2 α* can heterodimerize in vitro (Moser et al., 1995).

Limb defects in *AP-2 α ^{-/-}* mice include reduction in limb length, loss of the radius and loss or transformation of digit 1 (thumb in primates) (Schorle et al., 1996). As *AP-2.2* is expressed earlier than *AP-2 α* in the limb buds (Chazaud et al., 1996), it is conceivable that loss of both factors could have more dire consequences on limb development that approach those seen in *dAP-2* null flies. Both *dAP-2* and murine *AP-2 α* act in growth-promoting zones of the limb. Further genetic analysis of *dAP-2* in *Drosophila* leg development will probably shed light on regulatory pathways within which *AP-2* family members function in the vertebrate limb.

A role for *dAP-2* in central nervous system development is revealed by the disruption of multiple substructures in the protocerebral central complex of *dAP-2* mutants. This brain region is a higher control center for larval and adult locomotor behavior, and is involved in the initiation and organization of behavior and the integration of visual and olfactory input from the two brain hemispheres (Martin et al., 1999; Strauss and Heisenberg, 1993). The central complex neuropil is generally less dense in *dAP-2* mutants than in wild-type brains, suggesting that neurons belonging to the central complex may fail to send projections and/or establish arborizations within and across the midline in *dAP-2* mutants. That the defect is caused by mis-routing of axons seems less likely, as no additional or enlarged structures are seen, such as those that occur as a consequence of axonal mis-routing in the mushroom body mutant *mbd^{KS65}* (Heisenberg et al., 1985). As structural disruption of the central complex is known to inhibit locomotor activity, it is possible that central complex defects play a part in the locomotor inhibition seen in *dAP-2* deficient adults with normal length legs. Immunostaining analysis with an antibody that detects both *AP-2 α* and *AP-2 β* reveals that one or both are expressed in the cerebellum, optic tectum and accessory olfactory bulb in E18.5 wild-type mice (P. J. M., unpublished). In adult vertebrates, these regions have important roles in organizing, coordinating and fine-tuning locomotive behaviors in response to sensory input.

Although *dAP-2* is apparently not crucial for gross aspects of regionalization and axonal pathfinding in the embryonic brain, it remains possible that *dAP-2*-expressing neurons crucially require *dAP-2* subsequent to migration and axonal pathfinding. That *AP-2* transcription factors have important late roles in some types of differentiated neurons is suggested by studies of murine *AP-2 α* in luteinizing hormone-releasing hormone (LHRH) neurons (also known as gonadotropin-releasing hormone neurons). *AP-2 α* begins to be expressed in differentiating LHRH neurons as they migrate into the forebrain during embryogenesis (Kramer et al., 2000a; Kramer et al., 2000). In *AP-2 α ^{-/-}* embryos, LHRH neurons migrate normally but, upon arrival at their destination in the brain,

eventually stop producing LHRH and appear to regress to a less differentiated state than wild-type LHRH neurons (Kramer et al., 2000a; Kramer et al., 2000b).

dAP-2 null and partial loss of function phenotypes, together with *dAP-2* mis-expression experiments reveal important roles for *dAP-2* in several aspects of limb morphogenesis and function, including limb outgrowth, joint formation, and possibly local sensory innervation. The requirement for *dAP-2* in brain central complex development suggests an evolutionarily expedient link between growth of limbs and elaboration of their higher order neural circuitry. An ability to couple morphological evolution of body parts to evolution of neural circuits that innervate those parts using shared transcription factors could be an important feature of gene expression networks that is currently under-appreciated. A potential role for *dAP-2* in local neurogenesis in limbs is suggested by the observation that ectopic *dAP-2* can cell autonomously transform wing vein epithelium into ectopic sensory organs. In *Drosophila*, presumptive wing vein and leg joint territories represent limb segment boundaries with growth regulating properties that require discrete activation of the Notch signaling pathway (Milan and Cohen, 2000). We have recently shown that *dAP-2* is activated by Notch signaling in leg imaginal discs (Kerber et al., 2001). While roles for Notch signalling in lateral inhibition during neurogenesis have been widely studied in both *Drosophila* and vertebrate systems (Artavanis-Tsakonas et al., 1999), requirements for the Notch signaling pathway in joint formation and leg segment outgrowth in *Drosophila*, and in formation of the dorsal-ventral boundary in vertebrate limbs have only recently been demonstrated (Bishop et al., 1999; de Celis et al., 1998; Laufer et al., 1997; Rauskolb and Irvine, 1999; Rodriguez-Esteban et al., 1997). The studies we have presented here provide important groundwork and resources for further studies to elucidate the genetic pathways where *dAP-2* functions in limb and nervous system development.

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REFERENCES

- Aplin, A. C. and Kaufman, T. C. (1997). Homeotic transformation of legs to mouthparts by proboscipedia expression in *Drosophila* imaginal discs. *Mech. Dev.* **62**, 51-60.
- Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.
- Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature* **368**, 208-214.
- Bauer, R., McGuffin, M. E., Mattox, W. and Tainsky, M. A. (1998). Cloning and characterization of the *Drosophila* homologue of the *AP-2* transcription factor. *Oncogene* **17**, 1911-1922.

- Bishop, S. A., Klein, T., Arias, A. M. and Couso, J. P. (1999). Composite signalling from *Serrate* and *Delta* establishes leg segments in *Drosophila* through *Notch*. *Development* **126**, 2993-3003.
- Bosher, J. M., Totty, N. E., Hsuan, J. J., Williams, T. and Hurst, H. C. (1996). A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. *Oncogene* **13**, 1701-1707.
- Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
- Broadie, K., Prokop, A., Bellen, H. J., O'Kane, C. J., Schulze, K. L. and Sweeney, S. T. (1995). Syntaxin and Synaptobrevin function downstream of vesicle docking in *Drosophila*. *Neuron* **15**, 663-673.
- Callahan, C. A. and Thomas, J. B. (1994). Tau- β -galactosidase, an axon-targeted fusion protein. *Proc. Natl. Acad. Sci. USA* **91**, 5972-5976.
- Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q. and Tomlinson, A. (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development* **120**, 2957-2966.
- Campos-Ortega, J. A. and Hartenstein, V. (1997). *The Embryonic Development of Drosophila melanogaster*. Heidelberg: Springer.
- Chazaud, C., Oulad-Abdelghani, M., Bouillet, P., Decimo, D., Chambon, P. and Dolle, P. (1996). AP-2.2, a novel gene related to AP-2, is expressed in the forebrain, limbs and face during mouse embryogenesis. *Mech. Dev.* **54**, 83-94.
- de Celis, J. F., Tyler, D. M., de Celis, J. and Bray, S. J. (1998). Notch signalling mediates segmentation of the *Drosophila* leg. *Development* **125**, 4617-4626.
- Deak, P., Omar, M. M., Saunders, R. D., Pal, M., Komonyi, O., Szidonya, J., Maroy, P., Zhang, Y., Ashburner, M., Benos, P. et al. (1997). P-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: correlation of physical and cytogenetic maps in chromosomal region 86E-87F. *Genetics* **147**, 1697-1722.
- Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A. and Shotwell, S. L. (1982). Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci. USA* **79**, 7929-7933.
- Gee, J. M., Robertson, J. F., Ellis, I. O., Nicholson, R. I. and Hurst, H. C. (1999). Immunohistochemical analysis reveals a tumour suppressor-like role for the transcription factor AP-2 in invasive breast cancer. *J. Pathol.* **189**, 514-520.
- Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57.
- Häcker, U., Kaufmann, E., Hartmann, C., Jürgens, G., Knochel, W. and Jäckle, H. (1995). The *Drosophila* fork head domain protein crocodile is required for the establishment of head structures. *EMBO J.* **14**, 5306-5317.
- Halder, G., Callaerts, P. and Gehring, W. J. (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* **267**, 1788-1792.
- Heisenberg, M., Borst, A., Wager, S. and Byers, D. (1985). *Drosophila* mushroom body mutants are deficient in olfactory learning. *J. Neurogenet.* **2**, 1-30.
- Hirth, F., Therianos, S., Loop, T., Gehring, W. J., Reichert, H. and Furukubo-Tokunaga, (1995). Developmental defects in brain segmentation caused by mutations of the homeobox genes *orthodenticle* and *empty spiracles* in *Drosophila*. *Neuron* **1995**, 769-778.
- Huang, S., Jean, D., Luca, M., Tainsky, M. A. and Bar-Eli, M. (1998). Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis. *EMBO J.* **17**, 4358-4369.
- Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.
- Jean, D., Gershenwald, J. E., Huang, S., Luca, M., Hudson, M. J., Tainsky, M. A. and Bar-Eli, M. (1998). Loss of AP-2 results in up-regulation of MCAM/MUC18 and an increase in tumor growth and metastasis of human melanoma cells. *J. Biol. Chem.* **273**, 16501-16508.
- Karim, F., Chang, H. C., Therrien, M., Wassarman, D. A., Laverty, T. and Rubin, G. M. (1996). A screen for genes that function downstream of Ras I during *Drosophila* eye development. *Genetics* **143**, 315-329.
- Kerber, B., Monge, I., Mueller, M., Mitchell, P. J. and Cohen, S. M. (2001). The AP-2 transcription factor is required for joint formation and cell survival in *Drosophila* leg development. *Development* **128**, (TO COME, in this issue)
- Klaes, A., Menne, T., Stollewerk, A., Scholz, H. and Klambt, C. (1994). The Ets transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS. *Cell* **78**, 149-160.
- Kramer, P., Guerrero, G., Krishnamurthy, R., Mitchell, P. J. and Wray, S. (2000a). Ectopic expression of luteinizing hormone-releasing hormone and peripherin in the respiratory epithelium of mice lacking transcription factor AP-2alpha. *Mech. Dev.* **94**, 79-94.
- Kramer, P., Krishnamurthy, R., Mitchell, P. J. and Wray, S. (2000b). Transcription factor activator protein-2 is required for continued luteinizing hormone-releasing hormone expression in the forebrain of developing mice. *Endocrinology* **141**, 1823-1838.
- Laufer, E., Dahn, R., Orozco, O. E., Yeo, C. Y., Pisenti, J., Henrique, D., Abbott, U. K., Fallon, J. F. and Tabin, C. (1997). Expression of Radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* **386**, 366-373.
- Lin, D. M. and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13**, 507-523.
- Maconochie, M., Krishnamurthy, R., Nonchev, S., Meier, P., Manzanares, M., Mitchell, P. J. and Krumlauf, R. (1999). Regulation of Hoxa2 in cranial neural crest cells involves members of the AP-2 family. *Development* **126**, 1483-1494.
- Martin, J. R., Raabe, T. and Heisenberg, M. (1999). Central complex substructures are required for the maintenance of locomotor activity in *Drosophila melanogaster*. *J. Comp. Physiol. A* **185**, 277-288.
- McPherson, L. A., Baichwal, V. R. and Weigel, R. J. (1997). Identification of ERF-1 as a member of the AP-2 transcription factor family. *Proc. Natl. Acad. Sci. USA* **94**, 4342-4347.
- Milan, M. and Cohen, S. M. (2000). Subdividing cell populations in the developing limbs of *Drosophila*: do wing veins and leg segments define units of growth control? *Dev. Biol.* **217**, 1-9.
- Mitchell, P. J., Urlaub, G. and Chasin, L. (1986). Spontaneous splicing mutations at the dihydrofolate reductase locus in Chinese hamster ovary cells. *Mol. Cell. Biol.* **6**, 1926-1935.
- Mitchell, P. J., Wang, C. and Tjian, R. (1987). Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* **50**, 847-861.
- Mitchell, P. J., Timmons, P. M., Hébert, J. M., Rigby, P. W. J. and Tjian, R. (1991). Transcription factor AP-2 is expressed in neural crest lineages during mouse embryogenesis. *Genes Dev.* **5**, 105-119.
- Monge, I. and Mitchell, P. J. (1998). DAP-2, the *Drosophila* homolog of transcription factor AP-2. *Mech. Dev.* **76**, 191-195.
- Morriss-Kay, G. (1996). Craniofacial defects in AP-2 null mutant mice. *BioEssays* **18**, 785-788.
- Moser, M., Imhof, A., Pscherer, A., Bauer, R., Amselgruber, W., Sinowatz, F., Hofstadter, F., Schule, R. and Buettner, R. (1995). Cloning and characterization of a second AP-2 transcription factor: AP-2 beta. *Development* **121**, 2779-2788.
- Moser, M., Pscherer, A., Roth, C., Becker, J., Mucher, G., Zerres, K., Dixkens, C., Weis, J., Guay-Woodford, L., Buettner, R. et al. (1997a). Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2beta. *Genes Dev.* **11**, 1938-1948.
- Moser, M., Ruschoff, J. and Buettner, R. (1997b). Comparative analysis of AP-2 alpha and AP-2 beta gene expression during murine embryogenesis. *Dev. Dyn.* **208**, 115-124.
- Nottoli, T., Hagopian-Donaldson, S., Zhang, J., Perkins, A. and Williams, T. (1998). AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. *Proc. Natl. Acad. Sci. USA* **95**, 13714-13719.
- Oulad-Abdelghani, M., Bouillet, P., Chazaud, C., Dolle, P. and Chambon, P. (1996). AP-2.2: a novel AP-2-related transcription factor induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Exp. Cell Res.* **225**, 338-347.
- Rauskolb, C. and Irvine, K. D. (1999). Notch-mediated segmentation and growth control of the *Drosophila* leg. *Dev. Biol.* **210**, 339-350.
- Robinow, S. and White, K. (1991). Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. *J. Neurobiol.* **22**, 443-461.
- Rodriguez-Esteban, C., Schwabe, J. W., De La Pena, J., Foy, B., Eshelman, B. and Belmonte, J. C. (1997). Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* **386**, 360-366.
- Satoda, M., Zhao, F., Diaz, G. A., Burn, J., Goodship, J., Davidson, H. R., Pierpont, M. M. and Gelb, B. D. (2000). Mutations in *TFAP2B* cause Char syndrome, a familial form of patent ductus arteriosus. *Nat. Genet.* **25**, 42-46.
- Schmidt-Ott, U., González-Gaitán, M., Jaekle, H. and Technau, G. M. (1994). Number, identity, and sequence of the *Drosophila* head segments as revealed by neural elements and their deletion patterns in mutants. *Proc. Natl. Acad. Sci. USA* **91**, 8363-8367.

- Schorle, H., Meier, P., Buchert, M., Jaenisch, R. and Mitchell, P. J.** (1996). Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* **381**, 235-238.
- Sperber, G. H.** (1989). *Craniofacial Embryology*. Oxford: Wright.
- Spradling, A.** (1986). P element-mediated transformation. In *Drosophila: A Practical Approach*, (ed. D. B. Roberts), pp. 175-197. Oxford: IRL Press.
- Strauss, R. and Heisenberg, M.** (1993). A higher control center of locomotor behavior in the *Drosophila* brain. *J. Neurosci.* **13**, 1852-1861.
- Therianos, S., Leuzinger, S., Hirth, F., Goodman, C. S. and Reichert, H.** (1995). Embryonic development of the *Drosophila* brain: formation of commissural and descending pathways. *Development* **121**, 3849-3860.
- van Vactor, D., Sink, H., Fambrough, D., Tsoo, R. and Goodman, C. S.** (1993). Genes that control neuromuscular specificity in *Drosophila*. *Cell* **73**, 1137-1153.
- Williams, T., Admon, A., Lüscher, B. and Tjian, R.** (1988). Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev.* **2**, 1557-1569.
- Xu, T. and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.
- Zhang, J., Hagopian-Donaldson, S., Serbedzija, G., Elsemore, J., Plehn-Dujowich, D., McMahon, A. P., Flavell, R. A. and Williams, T.** (1996). Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature* **381**, 238-241.