**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-2α*/*AP-2β* and *AP-2γ* (*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-2α*\(^{−/−}\) 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 (Morriss-Kay, 1996). In *AP-2α*\(^{−/−}\) 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-2β*\(^{−/−}\) 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-2β* 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; lobal 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+>73C FRT80B (Xu and Rubin, 1993; B. Dickson), TM3 Sb P[w+]/ Df(3L)1118 (courtesy of K. Basler). A polylinker was inserted into an DHFR site XbaI. The resulting construct was used to generate and affinity purify anti-dAP-2 rabbit polyclonal antisera. 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× 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-b-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 polynuker was inserted into an XbaI site.
upstream of the promoter; and a 4 kb SpeIEcoRI 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 pUCHspaA2.3 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; hs70-flp, TubB>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, dAP2 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' and dAP-199 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'TM3, Ubx-lacZ and dAP-2'99/TM3, Ubx-lacZ flies and sbw 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 μm were recorded in line average mode with picture size of 512×512 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 μm 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 chromosomes 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)IsR, 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)IsR 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)IsR. 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)IsR. 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-2α/− mice have limb defects (Scholer 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 cause substitutions within an 84 amino acid segment of the DNA-binding domain, which is 96% conserved between flies and humans. The arginine residue which is immediately adjacent to the Arg289Cys mutation that 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-213, 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\textsuperscript{2}, Arg243Cys in dAP-2\textsuperscript{13} and Ser273Phe in dAP-2\textsuperscript{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\textsuperscript{10} and Arg306Ile in dAP-2\textsuperscript{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\textsuperscript{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\textsuperscript{8} has a nonsense mutation in exon 2. Legs of hemizygous dAP-2\textsuperscript{8} mutants are slightly longer than those of hemizygous null mutants. When combined with other dAP-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 $dAP-2^7/Df$ 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, $dAP-2^{1/4}$ (Fig. 3F) and $dAP-2^{21/2}$ (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 ($dAP-2^{7/7}$, $dAP-2^{1/6}$, $dAP-2^9$ (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, $dAP-2^{29}$ 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.](image-url)
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 null mutants (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-28/9, dAP-28/10 and dAP-210/17 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. 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-29/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-21+/-) 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-210/Df adults (I).
Fig. 4. Proboscis reduction in dAP-2 mutants. (A-C) Wild-type (A) and dAP-2 hemizygous mutant adult heads (dAP-2°/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 elyptus (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-2° (B,E,E') and other dAP-2 null mutants (not shown) is severely reduced in length and width. The proboscis of dAP-2°/Df(3L)1118 (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).

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.

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^{28/10} 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 duplications that occur in AP-2α chimeric mice but not in AP-2α/−/− 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 penetrance in hemizygous dAP-2^{10} adults (Fig. 3H and Fig. 6B), but only rarely in dAP-2^{28/10} and dAP-2^{289} 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-2^4, dAP-2^5 and dAP-2^13) 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-2^{13/+} (Fig. 6F) and dAP-2^{2/+} (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,line when dAP-2^{13} and dAP-2^{4} alleles were combined with dAP-2 hypomorphic alleles, such as dAP-2^{2}, 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-2^{4} and dAP-2^{5} corresponds to Arg225 in human Char Syndrome family (B. Gelb, Mount Sinai School of Medicine, personal communication). Studies of the DNA binding and dimerization activities of Char mutant AP-2β protein in vitro support the idea that these proteins function dominantly by forming non-DNA binding heterodimers with wild type AP-2β. The more conservative Arg243His mutation in dAP-2^{5} had a weaker dominant negative effect on wild-type and hypomorphic dAP-2 alleles than dAP-2^{13} and dAP-2^{4} (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>+/>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
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

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**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-2+/dAP-2 adult. (B) Milder ectopic elements (black arrowheads) in dAP-2+/dAP-2 adult. The necrotic femur-tibia joint typical of dAP-210/2 mutant is noted (open arrowhead). (C) SEM view of a larger ectopic bristled growth on medial aspect of a dAP-2+/dAP-2 adult eye. (D) Two pairs of double-antennae (arrows) in a dAP-2+/dAP-2 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-2+/Df leg (~10% shorter than dAP-2+/Df). (H) Heteroallelic dAP-2+/13 (~7% shorter than Df). (I) Heteroallelic dAP-2+/4 (~14% shorter than dAP-2+/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-2+/ causing enhanced leg shortening and increased incidence of joint necrosis (femur-tibia and trochanter-coxa in H, trochanter-coxa in I).
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-2 2 and dAP-2 19 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-2-2 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

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**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.
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 (antenno-glomerular tract, antennal nerve and median bundle) and an unusual number of large cell somata around the neuropil were also noted. 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.

**DISCUSSION**

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

Fundamental parallels between the leg and proboscis outgrowth defects identified 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
also underdeveloped in \textit{AP-2}^{−/−} mice, and a role for AP-2 family transcription factors in regulating \textit{Hoxa2} expression in neural crest cells of the second arch has been proposed, based on studies in transgenic mice (Maconochie et al., 1999). \textit{Hoxa2} is a mammalian ortholog of \textit{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). \textit{dAP-2} loss- and gain-of-function phenotypes indicate that \textit{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 \textit{dAP-2} alleles reveal that as \textit{dAP-2} activity is incrementally increased, limb and proboscis outgrowth is restored before viability and locomotor coordination. The \textit{dAP-2} dominant negative alleles are particularly interesting because they are analogous to \textit{AP-2β} alleles that cause Char Syndrome in humans, and thus represent a \textit{Drosophila} model for this congenital syndrome.

\textbf{Fig. 9.} \textit{SE3-tau-lacZ} reporter marks subset of \textit{dAP-2} expressing neurons in the embryonic protocerebrum. Stage 14 wild-type (A,B) and \textit{SE3-tau-lacZ} transgenic (C-F) embryos, dorsal views; reconstructions of laser confocal optical sections. (A,B) Double labeling with anti-\textit{dAP-2} (yellow/green) and anti-\textit{HRP} (red). (C) \textit{SE3-tau-lacZ} reporter gene expression labeled with anti-\textit{β-gal} (green). (D) Double labeling with anti-\textit{dAP-2} (red) and anti-\textit{β-gal} (yellow/green). (E) Double labeling with anti-\textit{HRP} (red) and anti-\textit{β-gal} (yellow/green). (F) Double labeling with anti-\textit{FAS II} (red) and anti-\textit{β-gal} (yellow/green). Endogenous \textit{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). \textit{tau-lacZ} reporter gene expression driven by the \textit{dAP-2} enhancer fragment SE (Fig. 2) mimics endogenous \textit{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 \( \mu \text{m} \).

\textbf{Fig. 10.} Brain central complex defects in \textit{dAP-2} mutant adults. Frontal sections of paraffin embedded brains of wild type (A-C) and \textit{dAP-2^{−/−}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-2β 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-2β 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-2α or other AP-2 family members, as it is known that AP-2β and AP-2α can heterodimerize in vitro (Moser et al., 1995).

Limb defects in AP-2α−/− 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-2α is expressed earlier than AP-2β 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-2α 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 mbd865 (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-2α and AP-2β 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-2α in luteinizing hormone-releasing hormone (LHRH) neurons (also known as gonadotropin-releasing hormone neurons). AP-2α begins to be expressed in differentiating LHRH neurons as they migrate into the forebrain during embryogenesis (Kramer et al., 2000a; Kramer et al., 2000b). In AP-2α−/− 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; Lauffer 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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