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# The AP-2 transcription factor is required for joint formation and cell survival in *Drosophila* leg development

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## SUMMARY

**Flies mutant for the *Drosophila* homologue of the mammalian transcription factor AP-2 show a severe reduction in leg length and fail to develop joint structures. Presumptive joint cells express dAP-2 in response to Notch signaling. dAP-2 is required for joint cell differentiation and can induce formation of supernumerary joints when misexpressed. Although dAP-2 is expressed only in presumptive joint cells, its activity is required to support cell survival in the entire leg segment. Taken together, our data indicate that dAP-2 is an important mediator of Notch activity in leg development.**

Key words: AP-2, Limb development, Joint formation, Notch, *Drosophila*

## INTRODUCTION

The transcription factor AP-2 (activator protein 2) was first isolated as a positive regulator of SV40 virus early transcription (Mitchell et al., 1987). Homologues of AP-2 have subsequently been cloned from various organisms including mouse, chicken, *Xenopus* and *Drosophila* (Mitchell et al., 1991; Snape et al., 1991; Shen et al., 1997; Bauer et al., 1998; Monge and Mitchell 1998). They show a great similarity in the overall protein structure, in particular in the DNA-binding domain. The developmental role of AP-2 has so far been best studied in the mouse (Mitchell et al., 1991; Schorle et al., 1996; Zhang et al., 1996; Nottoli et al., 1998). Three AP-2 family genes, AP-2a, AP-2b and AP-2.2 (AP-2g), have been found in the mouse. The proteins encoded by these are very similar and can form heterodimers, which may contribute to their ability to regulate a wide variety of target genes (Mitchell et al., 1991; Williams and Tjian 1991; Moser et al., 1995; Chazaud et al., 1996; Oulad-Abdelghani et al., 1996).

AP-2 family genes are often co-expressed during mouse development. For example, all three are expressed in neural crest cells, which migrate from the dorsal neural tube and give rise to much of the peripheral nervous system, and to specific bones and connective tissues in the head. In addition to craniofacial regions, AP-2 family genes are all expressed in the skin, urogenital tract and central nervous system, and AP-2a and AP-2.2 are both expressed in the mesenchyme of the outgrowing limb bud.

AP-2a and AP-2b knockout mice have been studied. The phenotype of AP-2a knockout mice is the most severe. The most prominent defects in these mice are craniofacial defects and exencephaly (failure of the brain region of the neural tube to close dorsally; Schorle et al., 1996; Zhang et al., 1996). AP-2a null mutant embryos show reduced growth of facial primordia and increased cell death in specific regions of craniofacial mesenchyme and brain tissue. By late gestation, AP-2a mutant mice exhibit severe defects in the jaws, eyes, nose, mouth, ears, and cranial ganglia. Subsequent studies have shown that AP-2 factors regulate the expression of the *Hoxa2* gene in a subset of cranial neural crest cells (Maconochie et al., 1999). However, *Hoxa2* cannot be directly responsible for the majority of craniofacial defects in AP-2a mutant mice, because these defects are found in regions anterior to the *Hoxa2* expression domain. When the function of AP-2a was addressed in more detail in chimaeric mice, it was found that defects caused by loss of AP-2a, including neural tube, face, eye, body wall and limb defects, each represent independent, local roles for AP-2a in these tissues (Nottoli et al., 1998; West-Mays et al., 1999). Highly penetrant limb defects in AP-2a knockout mice include loss of the radius bone and loss or transformation of the first digit of the forelimb. In contrast, chimaeric mice show a duplication of limb structures (Schorle et al., 1996; Zhang et al., 1996; Nottoli et al., 1998). The latter patterning defects are probably an outcome of interactions between AP-2a wild-type and null mutant cells in the chimaeric limb, because they are not seen in AP-2a null mutant mice.

The requirement for AP-2b during development does not seem to be as strict as for AP-2a. AP-2b mutant mice die postnatally because of kidney failure caused by increased cell death in the kidney (Moser et al., 1997). Thus, although AP-2a and AP-2b proteins show extensive overlap in their expression

patterns, their mutant phenotypes indicate that AP-2b has redundant or relatively subtle roles in many sites where AP-2a is crucial during development. The function of AP-2.2 in mouse development is so far not known.

Only one AP-2 gene family homolog exists in *Drosophila* (Bauer et al., 1998; Monge and Mitchell 1998). *dAP-2* produces two different mRNAs that use different first exons and encode proteins that differ at their N termini (Bauer et al, 1998; Monge et al., 1998). Alternative first coding exons are also used by murine AP-2 (Meier et al., 1995). dAP-2 displays a great degree of similarity with AP-2 proteins from other organisms, and is slightly more similar to murine AP-2a than to other murine AP-2 family members. The DNA-binding domain is the most conserved part of the protein, and dAP-2 binds to the same DNA sequence as its mammalian counterparts (Bauer et al., 1998). The embryonic expression pattern of dAP-2 has been described in detail previously and it has been noted that the gene is also expressed in rings in the leg imaginal disc (Bauer et al., 1998; Monge and Mitchell 1998). Here we report that *Drosophila dAP-2* mutants are defective in leg development. We present evidence that *dAP-2* is an important mediator of Notch signaling in joint formation. dAP-2 is expressed in the presumptive joint cells under control of Notch signaling. dAP-2 is required for joint formation and can induce supernumerary joints when ectopically expressed. Previous studies have shown that Notch activity is required in the presumptive joint cells to support development of the entire segment (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine 1999), suggesting that the joint cells can exert a non-autonomous effect on nearby cells. Our results suggest that dAP-2 acts downstream of Notch, perhaps to regulate the genes required for production of this signal.

## MATERIALS AND METHODS

### Fly strains

The *dAP-2$^{stummelbein}$* insertion was isolated in 1991 during the characterization of the Mcp element (Isolation number 47.29.1; referred to as w#14.29 by Muller et al., 1999). Excision of the P-element reverts the phenotype to wild type, indicating that the P-element is the cause of the mutation. The *croc$^{2}$* deficiency was induced in an ethylmethane sulphonate (EMS) mutagenesis for the *crocodile* locus (Hacker et al., 1995). The extent of the *croc$^{2}$* deletion was determined in preparations of salivary gland chromosomes of *croc$^{2}$*/+ animals (not shown). *big brain-lacZ* and *E(spl)-CD2* are described by de Celis et al. (de Celis et al., 1998). *Su(H)$^{SF8}$* allele was used to test for Notch signaling dependence of dAP-2 (Schweisguth and Posakony 1992). UAS-Notch$^{intra}$ was used to test for ectopic induction of dAP-2 expression (Struhl and Adachi, 1998). The *dAP-2$^{2}$* and *dAP-2$^{15}$* alleles were isolated in an EMS-mutagenesis screen (see Monge et al., 2001). Neither allele expresses dAP-2 protein at detectable levels (data not shown). Molecular characterization demonstrating that these are null alleles is presented elsewhere (Monge et al., 2001). Flies were reared in standard cornmeal molasses medium, at 25°C unless stated otherwise.

### Antibodies

Antibody to a dAP-2 peptide was raised in rabbits (Monge et al., 2001). Specificity of anti-dAP2 antibody was verified by labeling mutant discs and clones of mutant cells. No labeling was detected in mutant tissue (not shown). Rat-anti-Distal-less, mouse-anti-Dachshund and rabbit-anti-Homothorax were used as described (Wu and Cohen, 1999). Anti-β-galactosidase (Cappel) and anti-CD2 (Serotech) were used to visualize the expression of the *big brain-lacZ* and *E(spl)CD2* transgenes. TUNEL staining was performed as described (Milan et al., 1997). Confocal microscopy was performed using a Leica TCS SP confocal microscope. A Zeiss Axiophot was used for Normarski microscopy. Images were processed using Adobe Photoshop.

### Clonal analysis

Mutant clonal analysis was performed with the FLP/FRT system (Xu and Rubin, 1993). All clones were induced at 48+/-12 hours after egg laying by heat shock treatment at 38°C for one hour to induce expression of the FLP transgene. *Su(H)* mutant clones were induced in *hsFlp;FRT40A armlacZ/FRT Su(H)$^{SF8}$* larvae. *dAP-2* clones were induced in *y hsFLP; FRT80B y$^{+}$/FRT80B dAP-2$^{15}$* larvae. Thus, the *dAP-2* mutant clones were marked by the absence of y$^{+}$. Joint formation was affected in all cases where dAP-2 mutant clones crossed a joint. Gal4-expressing clones were induced in *act>CD2stop>Gal4/hsFlp; UAS-Notch$^{intra}$/UASGFP* larvae.

### Ectopic expression of dAP-2

We used the UAS/Gal4 system (Brand and Perrimon, 1993) to ectopically express dAP-2. *UAS-dAP-2* transgenic lines are described in detail elsewhere (Monge et al., 2001). Several independent lines were tested and all gave similar results. *patched$^{GAL4}$* was used to drive ectopic expression of dAP-2. Ectopic dAP-2 expression was confirmed by antibody staining (data not shown). The crosses were incubated at 25°C and pharate adults were dissected out of the pupal cases for preparation of leg cuticle. N$^{ts}$; ptc$^{Gal4}$/UASdAP-2 larvae were reared at 18°C for 3 days and then shifted to 25°C for the rest of development. dpp$^{Gal4}$ and UAS-Notch$^{intra}$ were recombined onto the dAP-2 mutant chromosome by standard procedures. Flies were incubated at 29°C.

## RESULTS

### '*Stummelbein*'

An allele of *dAP-2* was isolated as a viable mutation causing a severe reduction in leg length (Fig. 1C). Because of this phenotype, we originally called this mutation *stummelbein* (German for 'short leg'). The *stummelbein* mutation is caused by integration of a P-element into the *dAP-2* locus (Fig. 1A). Excision of the P-element reverts the *stummelbein* phenotype to wild type, indicating that the P-element is indeed the cause of the *stummelbein* mutation (data not shown). We mapped the P-element by in situ hybridization to polytene chromosomes to the band 78F1-2-79A1 on the left arm of the third chromosome (data not shown). In situ hybridization to polytene chromosomes has previously shown that dAP-2, the *Drosophila* homologue of the AP-2 transcription factor, maps to the *stummelbein* region. In order to test whether *stummelbein* might indeed affect the *dAP-2* transcription unit we cloned sequences flanking the *stummelbein* P-element by inverse PCR. We found that the P-element is integrated 87 base pairs upstream of *dAP-2* (Fig. 1A). *dAP-2* has two alternative first exons predicted to produce proteins, which differ in about the first 20 amino acids (Bauer et al., 1998; Monge and Mitchell 1998). The *stummelbein* P-element is inserted close to the first exon (Monge and Mitchell, 1998) (Fig. 1A). The alternative first exon is located about 9kb upstream of the P-element insertion site.

To verify that the *stummelbein* P-element insertion is a mutant in *dAP-2*, we crossed *stummelbein* mutant flies with flies carrying two different EMS induced mutations in *dAP-2*

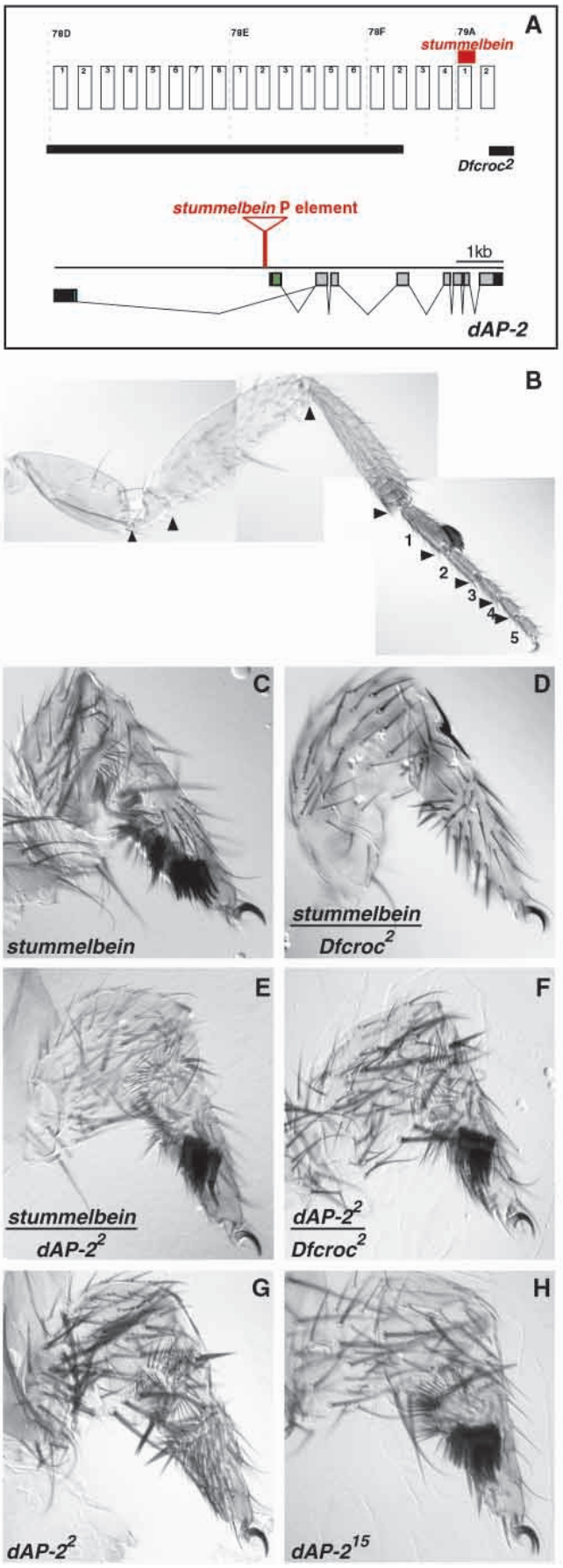


**Fig. 1.** The legs of *dAP-2* mutants are severely shortened and do not have joints. (A) Genomic organization of the *dAP-2* locus. (B) Wild type foreleg of a male fly. Arrowheads indicate the joints. (C-H) *stummelbein* is allelic to *dAP-2*. (C) Homozygous *stummelbein* mutant leg. The sex combs as well as the transverse rows (out of focus) are in the appropriate position, but the joints are missing. The first leg is most strongly affected. The second and third legs are slightly longer, but they also fail to form joint structures (data not shown; Monge et al, 2001). (D) The *stummelbein* mutant phenotype is as severe over a deficiency – Df*croc*$^{2}$ – that removes the *stummelbein* locus, indicating that the *stummelbein* mutant is a null allele. The extent of the deficiency has been determined and is indicated in A. (E) Transheterozygous animal *stummelbein*/*dAP-2*$^{2}$ shows a *stummelbein* phenotype, indicating that *stummelbein* is allelic to *dAP-2*. Two independent *dAP-2* alleles *dAP-2*$^{2}$ and *dAP-2*$^{15}$ were tested and gave the same result. All three alleles do not express dAP-2 protein anymore (data not shown). The molecular characterization of the *dAP-2* EMS alleles is described in Monge et al., 2001. (F) The *dAP-2* mutants used are also null alleles as they give a similar phenotype over the *croc*$^{2}$ deficiency. (G-H) Phenotype of the homozygous *dAP-2* mutants. (G) *dAP-2*$^{2}$, (H) *dAP-2*$^{15}$.

(Monge et al., 2001). The leg defects in the homozygous *dAP-2* mutants and the *dAP-2/stummelbein* flies were indistinguishable (Fig. 1C,E,G,H), indicating that *stummelbein* is defective in *dAP-2* activity. The defects in homozygous *stummelbein* or *dAP-2* mutants were comparable in severity with the defects produced when these alleles were heterozygous with a deletion that removes the *dAP-2* gene (Df*croc*$^{2}$; Fig. 1A,D,F). This suggests that the *stummelbein* and *dAP-2* mutants behave genetically as strong loss-of-function mutations in the leg. We were unable to detect dAP-2 protein expression in *stummelbein* mutant leg discs or in *dAP-2*$^{2}$ or *dAP-2*$^{15}$ mutant embryos and discs by antibody labeling (data not shown).

## Primary subdivision of the leg is normal in *dAP-2* mutant leg discs

*dAP-2* mutants are often pupal lethal and most of the homozygous flies die before eclosion. Comparison with wild-type legs shows that *dAP-2* mutant legs are severely truncated

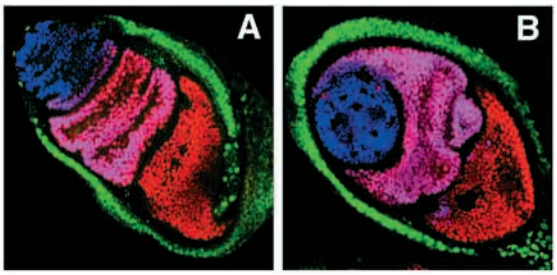


**Fig. 2.** Proximal-distal regionalization of the leg in *dAP-2* mutants. (A) Wild-type leg disc from a white prepupa stained with Homothorax (Hth, green), Dachshund (Dac, red) and Distal-less (Dll, blue; overlap between Dll and Dac appears pink). Only Dll is expressed at the very distal tip; more proximally the Dll expression domain overlaps with Dac. In the femur region, only Dac is expressed. The proximal-most cells express Hth. (B) Disc of a corresponding stage from *dAP-2*$^{2}$ mutant, stained as in A. The disc is smaller, but shows the appropriate expression domains of Dll, Dac and Hth. This indicates that the major patterning events occur correctly in *dAP-2* mutants. Similar results were obtained for the *dAP-2*$^{15}$ mutant.

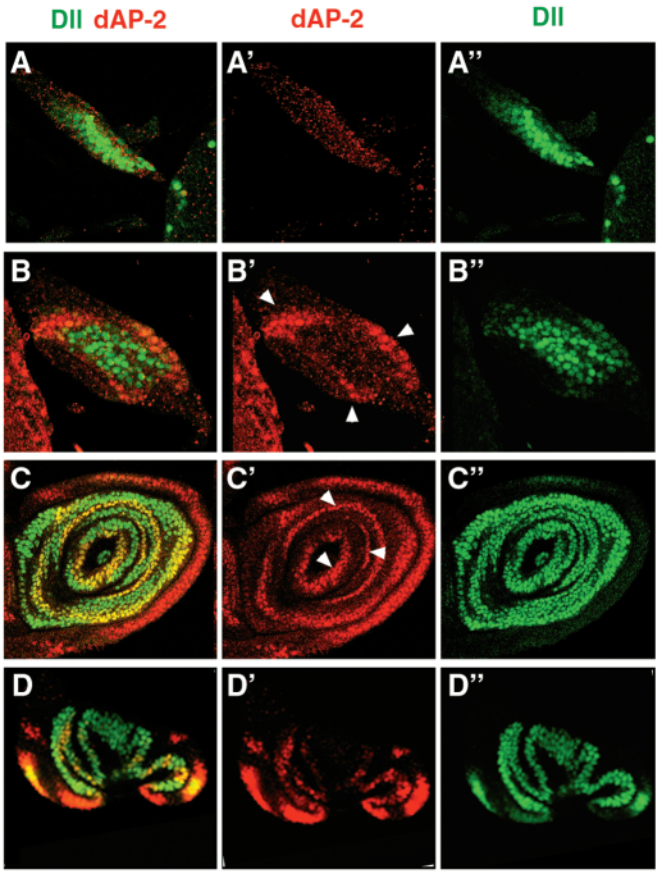


**Fig. 3.** dAP-2 expression in the leg disc. Double stainings with Dll (green) and dAP-2 (red, overlap appears yellow). (A-D) overlay, (A'-D') dAP-2 alone, (A''-D'') Dll alone. (A-A'') dAP-2 is not expressed in second instar leg discs, whereas Dll expressing cells can be detected. (B-B'') dAP-2 expression can be detected in mid-third instar discs at the proximal border of the Dll expression domain. dAP-2 expression is initiated shortly after Dac expression (not shown). (C-C'') dAP-2 is expressed in rings along the proximal-distal axis in mature third instar discs. (D-D") Optical cross section. The dAP-2 expression remains in rings until pupal development (see below).

along the proximal-distal axis and show fusions of leg segments (Fig. 1B, compare with Fig. 1C-H). To better understand the basis for the defects in *dAP-2* mutant legs, we examined the expression of genes that reflect the primary subdivision of the leg imaginal disc along the proximal-distal axis (Cohen et al., 1989; Mardon et al., 1994; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). Distal-less (Dll), Dachshund (Dac) and Homothorax (Hth) proteins are expressed in broad, partially overlapping domains along the proximal-distal axis of the leg (Fig. 2A). Dll and Dac are required in the region of the leg affected in *dAP-2* mutants. Although the *dAP-2* mutant discs are smaller, we found that the overlapping pattern of Dll and Dac expression was unaffected in the *dAP-2* mutant discs (Fig. 2B). This suggests that dAP-2 is unlikely to be involved in the early stages of axial patterning of the leg.

### *dAP-2* is expressed in presumptive joints

To find out when and where dAP-2 might be required in the leg disc, we followed its expression throughout leg disc development. dAP-2 protein is not expressed in second instar larval discs (Fig. 3A). dAP-2 is first detectable at the beginning of third instar (Fig. 3B), slightly later than the onset of Dac expression (data not shown). dAP-2 expression starts as a ring outside the early Dll domain. In mature third instar discs, dAP-2 is expressed in a series of rings along the proximal distal axis (Fig. 3C,D; see Monge and Mitchell, 1998). These rings coincide with the expression domains of the Notch targets *big brain* and *Enhancer of split* (*E(spl)*; Fig. 4).

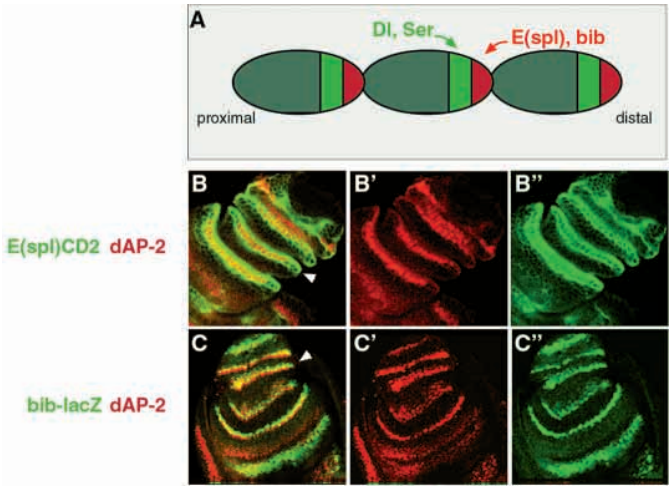


**Fig. 4.** dAP-2 is expressed in presumptive joints. (A) Components of the Notch signaling pathway during joint development. Cells expressing the Notch ligands Delta (Dl) and Serrate (Ser) signal to the neighboring cells. These cells respond by expressing *Enhancer of split* (*E(spl)*) and *big brain* (*bib*) as described (de Celis et al., 1998). (B-C) dAP-2 expression coincides with E(spl) and bib. dAP-2 protein is shown in red, bib-lacZ and E(Spl)CD2 are shown in green. (B'-C') dAP-2 alone, (B''-C'') markers alone. (B-B") dAP-2 is co-expressed with E(spl) (arrowhead). (C-C") dAP-2 is also coexpressed with bib (arrowhead). The presumptive tarsal regions are shown. Expression in more proximal regions is broader and partially overlapping.

Notch signaling has been implicated in formation of the joints between segments in the leg (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine 1999). Cells close to the end of each tarsal segment express elevated levels of the Notch ligands Delta and Serrate (summarized in Fig. 4A). Signaling by these ligands through Notch induces the expression of the *big brain-lacZ* reporter gene in distally adjacent cells. The observation that dAP-2, *E(spl)-CD2* and *big brain-lacZ* expression patterns coincide prompted us to ask whether dAP-2 expression also depends on Notch signaling activity. To test this, we generated clones of cells in which Notch signaling was impaired or overactivated (Fig. 5). Suppresser of Hairless (Su(H)) is required to activate targets of the Notch pathway (Schweisguth and Posakony 1992; Schweisguth, 1995). dAP-2 is not expressed in the tarsal rings in *Su(H)* mutant clones induced 48±12 hours after egg laying (Fig. 5A), indicating that Notch signaling activity is required. Conversely, dAP-2 expression is ectopically induced in cells expressing a constitutively active form of Notch in the disc epithelium (Fig. 5B). These results indicate that dAP-2 is expressed in presumptive joint cells under the control of the Notch signaling pathway.

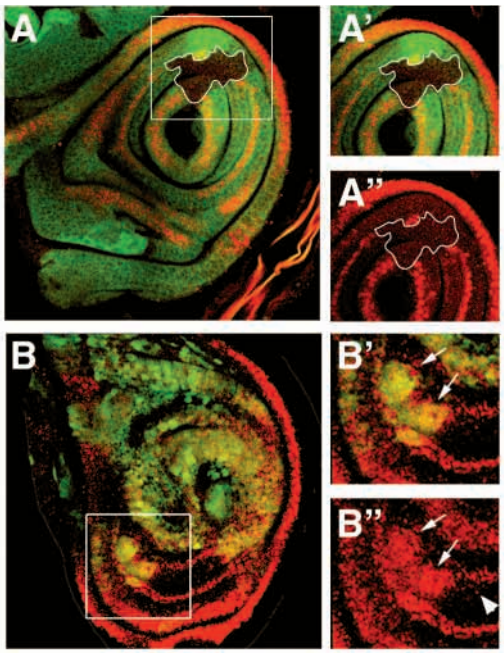


**Fig. 5.** dAP-2 is expressed in response to Notch signaling. A-A'' Su(H) mutant clones do not express dAP-2. The mutant clone (white line) is marked by the absence of β-gal staining (green, A' and A'' detail of the clone area). dAP-2 rings outside the clone are not affected. (B-B'') Clones expressing a constitutively active form of Notch ($Notch^{intra}$) in the disc epithelium ectopically activate dAP-2. $Notch^{intra}$ expressing cells are marked by GFP (green), dAP-2 is in red (B'-B'' are magnifications of the boxed area in B). The arrowheads point to two endogenous dAP-2 expressing rings. The $Notch^{intra}$-expressing clone between these two rings expressed dAP-2. Note that the optical section cuts across the folded epithelium, so that some of the $Notch^{intra}$ expressing cells in the center of the disc are not from the epithelial layer. These cells do not induce dAP-2 and appear green.

## dAP-2 and joint formation

In order to test whether dAP-2 is required for joint formation, we induced clones of *dAP-2* mutant cells (Fig. 6). *dAP-2* mutant clones that are located in the central 'interjoint' region of the segment do not show a phenotype (Fig. 6B), indicating that dAP-2 is not required for normal growth, survival or differentiation of leg cells. However, in all cases when the clones cross between tarsal segments they affect joint formation (Fig. 6C, 12/12 clones examined). Although *dAP-2* mutant cells appear to be unable to participate in joint formation (Fig. 6C'), wild-type cells adjacent to the clone can form the joint (Fig. 6C''. Note that clones cannot include the entire circumference of the leg because they are restricted to A or P compartments and so cannot include the entire joint). The length of the leg segment is normal, indicating that partial loss of dAP-2 expression can be compensated by the wild-type cells that contribute to forming the inter-segmental joint. Very large *dAP-2* mutant clones can show a reduction in leg length, as has previously been reported for clones lacking Notch activity (4/12 clones examined; de Celis et al., 1998). These observations suggest, that dAP-2 functions as a mediator of Notch activity in joint formation.

Ectopic activation of the Notch pathway can induce leg repatterning, outgrowths and ectopic joint structures in tarsal segments (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine 1999). As dAP-2 is required downstream of Notch for joint formation we asked whether ectopic expression of dAP-2 would be sufficient to induce ectopic joints. dAP-2 was misexpressed in a stripe of cells along the proximal-distal axis of the leg using the $patched^{Gal4}$ driver (i.e. crossing the endogenous dAP-2 rings). Legs from $patched^{Gal4}$/*UAS-dAP-2* flies contained many ectopic joints in the tarsal segments (Fig. 6D). We note that dAP2 did not produce the other pattern abnormalities associated with expression of activated Notch. The ectopic joints induced by dAP2 in the tarsal region have wild-type morphology. We note that the supernumerary joints tend to be clustered together and not uniformly distributed along the segment. The significance of this observation is unclear. To ask whether dAP-2 is sufficient to mediate all of the activities of Notch in joint formation we expressed *UAS-dAP-2* using $patched^{Gal4}$ in a $Notch^{ts}$ mutant background. Under these conditions, joints do not form (data not shown). This indicates that, while dAP-2 is required for joint formation it is not able to induce joints in the absence of Notch activity. To further test the requirement for dAP-2, we expressed $Notch^{intra}$ with the $dpp^{Gal4}$ driver in dAP-2 mutant larvae. In the absence of dAP-2 activity, activated Notch was not able to rescue joint formation (data not shown). Together, these observations indicate that whereas dAP-2 expression is regulated by Notch signaling, dAP-2 is not the only mediator of Notch activity in joint formation and the requirement for dAP-2 function cannot be overcome by constitutive activation of Notch. We suggest that some other Notch-dependent activity may be required to define a region in which joint formation is possible when dAP-2 is expressed.

## dAP-2 activity is required in the joints for survival of cells in the interjoint region

dAP-2 is expressed in joint cells. In addition to being required for joint formation, dAP-2 activity appears to be required to support normal development of the intervening 'interjoint' tissue. We can infer that this requirement is indirect because clonal analysis showed that *dAP-2* mutant cells contribute to normal development of interjoint tissue (e.g. Fig. 6B). Development of the interjoint region was compromised in cases where *dAP-2* mutant clones were large enough to remove joints. The small size of the segments in the *dAP-2* mutant legs could be due to reduced growth or increased cell death. We did not observe a difference in the amount of cell division in *dAP-2* mutant and wild-type leg imaginal discs labeled with antibody to the phosphorylated form of histone H3 (which labels mitotic cells; Hendzel et al., 1997; data not shown). In contrast, *dAP-2* mutant leg imaginal discs show a considerable increase in the amount of cell death, as visualized by acridine orange and TUNEL labeling (Fig. 7A-D and data not shown). Double labeling for TUNEL and β-galactosidase on *dAP-2* mutant discs that carried the *big brain-lacZ* reporter, revealed that much of the cell death occurred in the interjoint region (Fig. 7D,F). We note that dAP-2 activity is not required for *big brain-lacZ* expression in the presumptive joints. These observations indicate that although dAP-2 is required for joint formation, it is not required for expression of the other known Notch targets in the presumptive joints. These genes are expressed in well-resolved rings in third instar. This indicates that the loss of tissue due to cell death does not compromise the ability of Notch ligands to activate Notch signaling and target gene expression in the mutant disc. By pupal stages, the interjoint regions appear to have been lost or reduced so that the tarsal rings

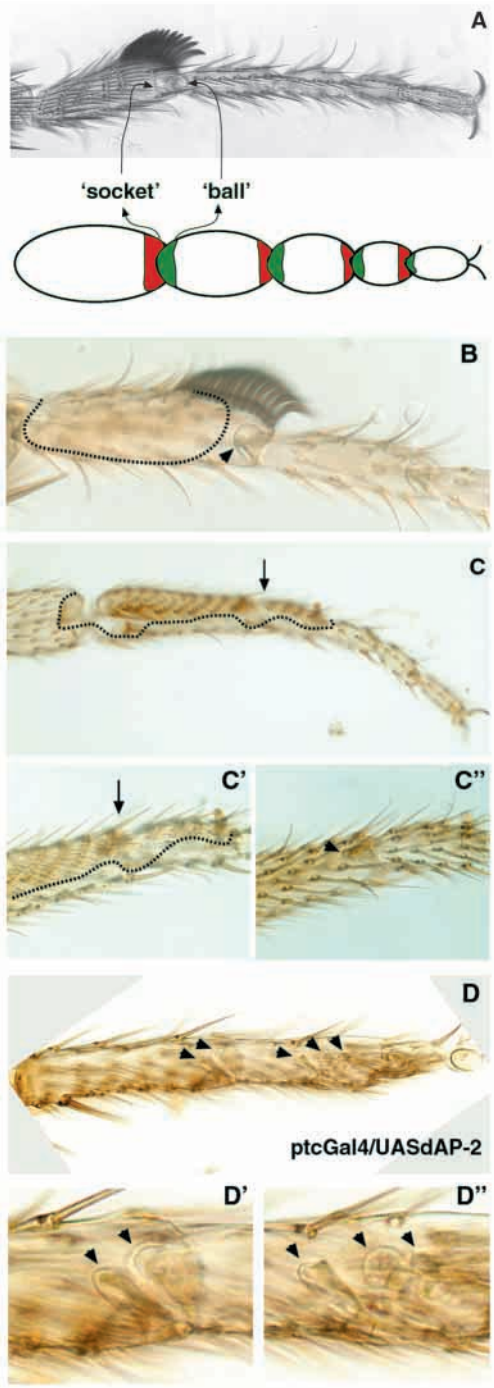


**Fig. 6.** dAP-2 is required for joint formation and can be instructive for joint formation. (A) Distal part of a wild type leg illustrating the 'ball and socket' structure of the joints in the tarsus. (B) *dAP-2* mutant clones confined to the interjoint region do not perturb leg development. The broken line outlines the clone border, the arrowhead points to the joint. The $y^-$ *dAP-2* mutant clone is located in the first tarsal segment and does not cross the joints. (C) Leg with a *dAP-2* mutant clone that crosses the tibia/first tarsal and first/second tarsal joints and both joints show defects. The clone reaches laterally from the distal tip of the tibia until the end of the second tarsal segment (broken line). Note that the clone includes only part of the circumference of the leg. The joint between tibia and first tarsal segment forms in the wild-type tissue but is absent or abnormal in the mutant tissue. Because of the complicated morphology of this joint it is not possible to determine with certainty whether some mutant cells are able to contribute to formation of this joint. The joint between the first/second tarsal segment is simpler in morphology. Higher magnifications show that dAP-2 mutant cells do not seem to be able to form the tarsal joint (C'), whereas the neighboring wild-type cells form a joint (C", arrowhead). (D) Ectopic expression of dAP-2 with ptcGal4 induces ectopic joints (arrowheads). These are located close to the endogenous joints and are morphologically indistinguishable from wild type joints. D' and D" show magnifications of the more proximally (D') and the more distally located (D") ectopic joints.

of *E(spl)-CD2* expression fuse into a continuous band of expression (Fig. 7E,F). Taken together, these observations indicate that dAP-2 activity is required in joint cells both for joint formation and to support cell survival in the interjoint region.

## DISCUSSION

### dAP-2 mediates a subset of the functions of Notch in leg development

dAP-2 is expressed in the presumptive joints under control of the Notch signaling pathway. dAP-2 is required for formation of joints and is sufficient to induce supernumerary joints when ectopically expressed. Unlike Notch mutants, strong dAP-2 mutants are viable and produce flies with short legs. We have presented evidence that the activity of dAP-2 in the presumptive joints is required to support survival of cells in the interjoint region. On the basis of clonal analysis it has been inferred that Notch activity in the joints was required for development of the interjoint region and that joints are centers of growth control in the leg (de Celis et al., 1998). Our results suggest that Notch acts via dAP-2 to support survival of cells in the interjoint region of the leg segments. Clonal analysis has shown that dAP-2 activity is not required by the interjoint cells themselves, so we suggest that dAP-2 might control expression of a secreted factor that is produced by the joint cells and acts non-autonomously to support survival of nearby cells. These observations suggest that dAP-2 is an important mediator of Notch signaling in joint formation and leg segment development (Fig. 8).

Several observations indicate that dAP-2 does not mediate all of the effects on Notch in the leg. First, dAP-2 is not sufficient to induce joints in a Notch mutant leg. Second, other Notch-dependent target genes, including *big brain* and *E(spl)* are induced normally in *dAP-2* mutant leg discs. Third, ectopic activation of the Notch pathway produced supernumerary joints that were often associated with outgrowths of the leg (de Celis et al., 1998; Rauskolb and Irvine 1999). Like Notch, ectopic dAP-2 induced supernumerary joints, but did not cause outgrowths. These observations indicate that dAP2 mediates some, but not all of the activities of Notch in the leg (Fig. 8). For example, Fringe is expressed at high levels in the interjoint region. It has been shown that ectopic expression of Fringe can inhibit joint formation (Bishop et al., 1999; Rauskolb and Irvine 1999). It is possible that the presence of Fringe influences Notch activity to limit joint formation by dAP-2. This may provide an explanation for the clustering of ectopic joints when dAP-2 is misexpressed.

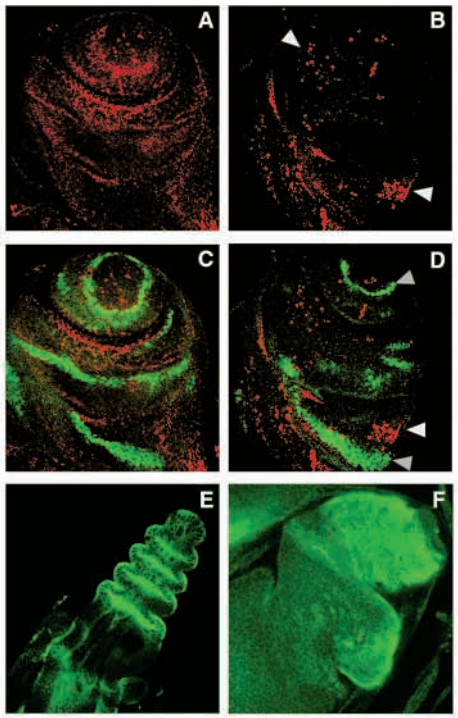


**Fig. 7.** dAP-2 is required for cell survival in the interjoint region. (A,C,E) wild-type and (B,D,F) *dAP-2* mutant. (A-B) TUNEL staining shows enhanced cell death in *dAP-2* mutants. (C,D) Double labeling for *big brain-lacZ* (green) reveals that cell death occurs mostly in the interjoint region (white arrowhead in D point to dying cells, gray arrowheads to the presumptive joint cells indicated by *big brain-lacZ*). Note that *big brain-lacZ* is expressed in the *dAP-2* mutant disc. (E) Expression of E(spl)-CD2 is normal in third instar *dAP-2* mutant discs. (F) At later stages, the rings of E(spl)-CD2 expressing cells have fused in *dAP-2* mutants.

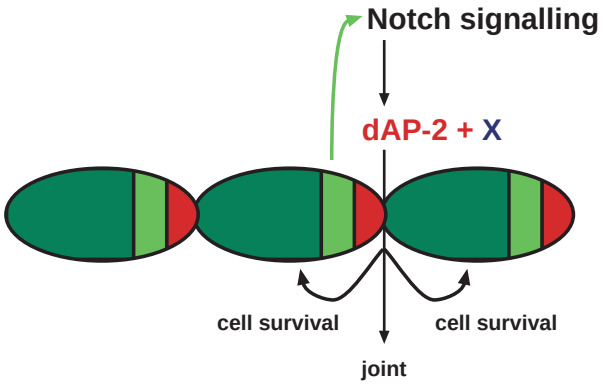


**Fig. 8.** Model for the role of dAP-2 in leg development. dAP-2 is required for joint formation, presumably together with other factors. dAP-2 is also needed to promote growth/cell survival in the interjoint region. We suggest that dAP-2 activity is required for expression of a secreted factor that influences cell survival in interjoint tissue.

## Boundary regions and growth control

Boundary regions have been implicated as centers of growth control in a variety of developmental processes (reviewed by Held, 1995; Irvine, 1999; Milan and Cohen, 2000). Compartment boundaries serve as sources of secreted signaling proteins required to support growth of the wings and legs. At later stages of development, additional subdivisions occur, including wing veins and leg segments. These too are implicated in growth control (de Celis et al., 1998; Milan and Cohen, 2000). Our observations provide some insight into the mechanism by which inter-segmental joints influence the growth of leg segments. *dAP-2* mutant flies show a severe reduction in the length of the leg, whereas clones of mutant cells in the interjoint region have no effect. We note that the extra joints induced by ectopic expression of dAP-2 do not cause overgrowth of the leg. Thus dAP-2 does not appear to produce a growth factor per se. One possibility is that dAP-2 expression is required in joint cells to produce a survival factor to support development of the leg segments. Alternatively, the cell death observed in *dAP-2* mutant leg discs might be a secondary consequence of pattern abnormalities, as has been observed in embryos mutant for segmentation genes.

## Do dAP-2 and vertebrate AP-2 genes have conserved functions?

Based on analyses of mouse, frog and chick AP-2 family members, vertebrate AP-2 transcription factors appear to play conserved roles in similar developmental contexts. The expression domains of AP-2 that seem most evidently conserved between fly and vertebrates are those in the nervous system, head and limbs (Mitchell et al., 1991; Winning et al., 1991; Chazaud et al., 1996; Schorle et al., 1996; Shen et al., 1997; Bauer et al., 1998; Monge and Mitchell, 1998). AP-2a mutant mice show a highly penetrant loss of the radius and transformation or loss of the first digit in the forelimb (Schorle et al., 1996; Zhang et al., 1996). AP-2a and AP2g are both expressed in the limb bud mesenchyme, with AP-2g showing an earlier onset than AP-2a (Mitchell et al., 1991; Chazaud et al., 1996). As limb bud outgrowth occurs, AP-2a is expressed in the distal limb bud (progress zone). Given the potential redundancy between AP2a and AP-2g in limb development, it is perhaps not surprising that the limb phenotype in AP-2 knockout mice is relatively mild. Interestingly, duplications of limb structures have been observed in AP-2a chimaeric mice. Since these are not seen in the null mutant mice, it appears they arise as a result of interactions between mutant and wild-type cells in the mosaic limbs. We have not observed limb duplications in *dAP-2* loss-of-function mutants. However, small outgrowths are sometimes seen in homozygous *dAP-2* mutant legs and the sex combs are sometimes expanded (e.g. Fig. 1C). This could be due to aberrant healing in areas where extensive cell death has occurred. Interestingly, we observe ectopic joints when dAP-2 is expressed ectopically, suggesting that in the fly the interaction between dAP-2 expressing and non-dAP-2 expressing cells might also be important. This could indicate a functional similarity between vertebrate AP-2 and dAP-2 in limb development.

The radius (bone) is sometimes missing and the axial skeleton is abnormal in AP-2 mutant mice – it is therefore possible that AP-2 plays a role in bone development. This is supported by the observation that ossification occurs more slowly in AP-2 mutant mice than in wild-type mice (Schorle et al., 1996). The Notch signaling pathway plays a role in endochondral bone development and thus indirectly in joint formation in the chicken limbs (Crowe et al., 1999). In this process, Notch signaling is required to regulate the differentiation of chondrocytes and to downregulate their

proliferative activity. As signaling pathways are often conserved between species, it is possible that vertebrate AP-2 factors are also regulated by the Notch signaling pathway.

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