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The 62E early-late puff of *Drosophila* contains D-spinophilin, an ecdysone-inducible PDZ-domain protein dynamically expressed during metamorphosis

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Summary

At the onset of metamorphosis in *Drosophila melanogaster*, the steroid hormone 20-OH ecdysone induces a small number of early and early-late puffs in the polytene chromosomes of the third-instar larval salivary gland whose activity is required for regulating the activity of a larger set of late puffs. Most of the corresponding early and early-late genes have been found to encode transcription factors that regulate a much larger set of late genes. In contrast, we describe here the identification of an ecdysone-regulated gene in the 62E early-late puff, denoted *D-spinophilin*, that encodes a protein similar to the mammalian protein spinophilin/neurabin II. The D-spinophilin protein is predicted to contain a highly conserved PP1-binding domain and adjacent PDZ domain, as well as a coiled-coil domain and SAM domain, and belongs to a family of related proteins from diverse organisms. Transcription of *D-spinophilin* is correlated with 62E puff activity during the early stages of metamorphosis and is ecdysone-dependent, making this the first member of this gene family shown to be regulated by a steroid hormone. Examination of the dynamic patterns of D-spinophilin expression during the early stages of metamorphosis are consistent with a role in central nervous system metamorphosis as well as a more general role in other tissues. As *D-spinophilin* appears to be the only member of this gene family in *Drosophila*, its study provides an excellent opportunity to elucidate the role of an important adaptor protein in a genetic model organism.

1. Introduction

The fruitfly *Drosophila melanogaster* provides a powerful model system for elucidating the molecular and genetic mechanisms by which steroid hormones regulate developmental processes. In *Drosophila*, changes in the concentration of the steroid hormone 20-OH ecdysone (hereafter referred to as ecdysone) regulate specific developmental events, the most dramatic of which is the metamorphosis from the larval to the adult form (Riddiford, 1993). Drastic changes in the body plan of the organism occur during metamorphosis, including morphogenesis and differentiation of imaginal tissues to give rise to adult structures (Fristrom & Fristrom, 1993) and elimination of larval tissues by histolysis. Other larval tissues such as the central nervous system (CNS) are extensively remodelled during metamorphosis (Truman *et al.*, 1993). Thus, *Drosophila* metamorphosis is an ideal experimental system for gaining insights into how different tissue responses are coordinated and executed in response to hormonal signals.

Underlying the different responses to ecdysone during metamorphosis are changes in gene expression first observed as the induction and regression of transcriptional puffs in the salivary gland polytene chromosomes of late third-instar larvae. Elegant experimental analysis of the changes in puff activity occurring in response to ecdysone under a variety of experimental conditions led to a hierarchical model for the genetic regulation of the late-larval ecdysone response in the salivary gland (Ashburner *et al.*, 1974). In this model, an ecdysone receptor : ecdysone complex directly activates a small set of early puffs. These early puffs were proposed to contain early genes that encoded regulatory proteins whose activity causes the regression of the early puffs themselves and the induction of a much larger set of late puffs. These late

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puffs would then contain late genes encoding effectors of the ecdysone response, the gene products directly required to mediate the cellular processes involved in metamorphosis.

Molecular-genetic analyses of the genes involved in this puff response have confirmed and extended this hierarchical model to include other ecdysone target tissues (see reviews by Bayer *et al*., 1996; Thummel, 1996; and Richards, 1997). The ecdysone receptor gene, *EcR*, encodes three protein isoforms (EcR-A, -B1, -B2), each of which is activated by heterodimerization with an orphan nuclear receptor, USP. These EcR isoforms are differentially distributed among the target tissues, and these differences are likely to contribute to the tissue specificity of the ecdysone response (Talbot *et al*., 1993; Robinow *et al*., 1993; Bender *et al*., 1997; Schubiger *et al*., 1998). Molecular analyses of the early genes *BR-C*, *E74* and *E75* responsible for the early puffs at 2B, 74EF and 75B showed that they encode, respectively, members of the C2H2 zinc finger, ETS and nuclear receptor families of transcription factors. Like *EcR*, these early genes encode multiple isoforms that exhibit distinct spatial and/or temporal expression patterns and provide critical regulatory functions during metamorphosis.

Excluded from the original hierarchical model were three characterized early-late puffs 46F, 62E and 78D, whose induction occurs 2–3 h after the early genes and requires both ecdysone and the products of one or more early genes (Ashburner & Richards, 1976). Molecular analyses of the early-late genes E78 and DHR3 corresponding to the early-late puffs at 78D and 46F, respectively, showed that both encode nuclear receptor transcription factors (Koelle *et al*., 1992; Stone & Thummel, 1993; Russell *et al*., 1996). Contrary to the results expected on the basis of the observed regulation of puffing, transcription of these early-late genes occurs as a primary response to hormone, although maximal transcript accumulation occurs in the presence of early gene products (Stone & Thummel, 1993; Huet *et al*., 1995). Genetic analysis shows that these genes, like the early genes, act as regulatory factors during metamorphosis (Russell *et al*., 1996; Lam *et al*., 1999). In particular, the *DHR3* gene has been shown to play a critical role in regulating gene expression during the transitional period between the late larval ecdysone pulse and the prepupal ecdysone pulse occurring some 10 h later, acting as a repressor of early gene expression and an activator of the prepupal competence factor βFTZF1 (Lam *et al*., 1997; White *et al*., 1997). Thus, the genes corresponding to two of the early-late puffs share several of the characteristics of early genes in the ecdysone genetic regulatory hierarchy.

Given the importance of the early and early-late genes identified within the previously characterized early and early-late puff loci, we set out to identify and characterize the ecdysone-regulated gene(s) corresponding to the 62E early-late puff. We report here the identification of an ecdysone-regulated gene within the 62E puff, denoted *D-spinophilin*, encoding a protein with similarity to mammalian spinophilin/neurabin II. The conserved protein domains found in *D-spinophilin* include a PDZ domain and a SAM domain, each of which has been shown previously to be involved in protein : protein interactions and targeting of specific proteins to specific targets on the cell membrane, and a protein phosphatase one (PP1)-binding domain involved in modulating the activity of target proteins, such as receptors and ion channels, through the activity of PP1 (for reviews see Shenolikar, 1994; Ponting *et al*., 1997; Kyba & Brock, 1998). Based on a preliminary analysis of the dynamic temporal and spatial patterns of protein expression observed during the late larval ecdysone pulse, we suggest that *D-spinophilin* is involved in modulating the activity of protein targets at cell membranes in a variety of tissue types. Specific expression in putative photoreceptor cells and the optic lobe further suggest a potential role for *D-spinophilin* in the remodelling of the CNS during metamorphosis. The identification of *D-spinophilin* as a member of the ecdysone genetic regulatory hierarchy creates a new entry point for understanding how steroid hormone responses and hormone-mediated genetic regulatory cascades are converted into specific cellular- and tissue-specific hormone responses.

2. Materials and methods

(i) *Fly culture and genetic analysis*

Flies were maintained on either a standard cornmeal, yeast and molasses medium or on instant fly medium (Carolina Biological) in uncrowded conditions at 25 °C. The *l(3)06911* stock was obtained from the Bloomington Stock Center. Deficiency stocks with proximal breakpoints in the 62D-F region were gifts from J. Mason (Wang *et al*., 1994). Balancer chromosome and other mutations used in this study are described in Lindsley & Zimm (1992).

Staging of late third-instar larvae was performed as described by Maroni & Stamey (1995). Briefly, larvae were cultured on standard or instant fly medium (Carolina Biologicals) containing 0·05% bromophenol blue and were staged on the basis of disappearance of the blue dye from larval guts. Large late third instar larvae whose guts were completely blue and had not yet begun wandering were picked out of the food. Most such larvae are developmentally more than 18 h before puparium formation (BPF). Larvae whose guts are completely blue and have begun wandering are approximately 12–18 h BPF;

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while those whose guts have cleared the blue dye following the cessation of feeding are approximately 2–4 h before pupariation.

The *l(3)06911* mutation is caused by a *P{PZ}*-type *P* element insertion in the 62E region that was localized by *in situ* hybridization (Spradling *et al*., 1999; Berkeley Drosophila Genome Project). This mutation is semi-lethal since homozygous stocks display reduced viability (data not shown). Genomic DNA flanking the insertion site was isolated by plasmid rescue essentially as described in Stowers *et al*. (2000).

(ii) *Construction of the 62E contig*

Purified DNA from yeast artificial chromosome DY402, containing 140 kb of genomic DNA from the 62E region (Garza *et al*., 1989), was used to screen genomic phage and cosmid libraries. Genomic clones containing primarily single-copy sequences were identified by Southern hybridization with DY402, and those producing overlapping restriction-digestion patterns were cross-hybridized to produce the 62E contig. *In situ* hybridization of selected clones to polytene chromosomes was carried out as described by Langer-Safer *et al*. (1982). Methods for purifying and manipulating DNA were essentially as described by Sambrook *et al*. (1989).

(iii) *Deficiency mapping*

Salivary glands squashes were carried out as described by Ashburner (1989). Thirteen chromosomal deficiencies with putative breakpoints in the 62E-F region originally isolated by Wang *et al*. (1994) were characterized. Each deficiency-bearing stock was first placed over a *TM6B*, *Tb Hu e* balancer chromosome to produce balanced stocks. Four deficiency stocks – *Df(3L)AG*32, *102*, *148*, and *156* – were non-complementing when crossed to stocks bearing lethal *P* element insertion mutations in the *misshapen* gene located in the proximal 62E region (Treisman *et al*., 1995), and each of these four also failed to form a visible 62E puff (not shown); only the *AG32* and *AG102* deficiencies were analysed further. Cytological analysis and *in situ* hybridization experiments were carried out using heterozygous *Df/+*, non-*Tubby* larvae produced by crossing *Df/TM6B*, *Tb* males to *Oregon-R* females.

(iv) *Identification and sequencing of cDNAs in the 62E puff region*

cDNAs were identified by screening a λgt10 phage library constructed from late third-instar larval tissues cultured in the presence of cycloheximide plus ecdysone (a gift from C. Thummel) and a directional cDNA library constructed from 0- to 2-h prepupae (Stowers *et al*., 1999). The resulting cDNAs were placed into independent transcription units based on the results obtained from hybridization to other cDNAs and to the genomic contig. The *c62E-5* cDNA was restriction digested with *Eco*RI and *Xho*I and the restriction fragments subcloned into *pBluescript KS* (Stratagene). The resulting recombinant plasmids were purified using the Wizard miniprep DNA purifications system (Promega) according to the manufacturer's instructions and sequences using an ABI 373A-stretch automated DNA sequencer (Applied Biosystems). DNA primers between 21 and 23 nucleotides in length were designed to allow sequence walking. Genomic restriction fragments containing *c62E-5*-derived exons were identified by hybridization with *c62E-5*-derived probes and subcloned into *pBluescript KS* (Stratagene).

(v) *Northern blot analysis*

Late third-instar larvae isolated prior to the late larval ecdysone pulse (approx. 18 h BPF) were hand-dissected in Grace's insect-tissue culture medium (Gibco-BRL) supplemented with 2% ethanol at 5:1 (Ashburner, 1972). Larval tissues (corresponding to 10 larvae per time point) were then incubated in 6-well tissue-culture plates with 1 ml of medium per well for 1 h in the absence of hormone. This medium was then removed and replaced with fresh medium with or without ecdysone at 5×10^{-6} M (Sigma) and incubated for the required period of time. Tissues were then collected by centrifugation and total RNA extracted for Northern blot analysis.

Total RNA was isolated essentially as described by Andres & Thummel (1995). Purified RNA was fractionated by formaldehyde agarose gel electrophoresis, transferred to Hybond N+ membranes (Amersham), and hybridized as described by Karim & Thummel (1992). A 1·7 kb *Eco*RI/*Xho*I fragment corresponding to the 5′ end of the *EL62-1* cDNA, and containing the PDZ domain, was used as a probe for Northern blot hybridizations. Preparation of radio-labelled cDNA probes was carried out essentially as described by Stowers *et al*. (1999). The *rp49* probe used as an RNA loading control is described in O'Connell & Rosbash (1984).

(vi) *Analysis of D-spinophilin protein expression*

A specific *Sac*I/*Xho*I c*62E-5* cDNA restriction fragment spanning the PP1-binding domain and the PDZ domain (amino acids nos. 1256–1408) was cloned into the *ptrcHis* expression vector (Invitrogen), and the

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resulting fusion protein expressed and purified using a nickel column (Qiagen) according to the manufacturer's instructions. Immunization of rabbits and isolation of rabbit serum was performed as described by Harlow & Lane (1998). Affinity purification of rabbit polyclonal antibodies was carried out using the amino-link kit (Pierce) according to the manufacturer's instructions.

Developmentally staged late third-instar larvae were collected and homogenized in 80 μl homogenization buffer (100 mM Tris-Cl pH 8·0, 150 mM NaCl, 2 mM EDTA, 2% SDS, 5% βME, 4 M urea) and stored at −20 °C. Samples were thawed on ice and 20 μl of 5 × loading buffer (78·15 mM Tris-Cl pH 6·8, 0·25% glycerol, 2·5% SDS, 6·25% βME, 0·03% bromophenol blue) was added and the samples boiled for 3–5 min. Approximately 1·5–2 larval protein equivalents were loaded per lane onto duplicate 10% SDS-PAGE gels and run for 2–4 h at 40 mA in running buffer (25 mM Tris, 250 mM glycine pH 8·3, 0·1% SDS). One gel was stained to evaluate protein loading and the other was electro-blotted to Hybond ECL membrane (Amersham) essentially as described in Sambrook *et al*. (1989). Molecular weight markers were the kaleidoscope markers (Biorad). Western blot membranes were blocked for 8–12 h in PBT supplemented with 5% non-fat dry milk at 4 °C, then rinsed with PBT for 1 h, 15 min and 2 × 5 min. The affinity-purified anti-D-spinophilin antibodies were incubated (1 : 10 dilution) with the blots for 8–12 h at 4 °C and washed as described above. Donkey anti-rabbit HRP-conjugated secondary antibodies (Amersham) were then incubated (1 : 5000 dilution) with the membranes for an additional 6–8 h, rinsed as described above, and visualized by chemiluminescence using the ECL detection system (Amersham) according to the manufacturer's instructions.

Staged animals were dissected in phosphate-buffered saline (Ashburner, 1989). Isolated larval tissues were fixed in 0·1 M PIPES pH 6·9, 1 mM EGTA pH 6·9, 1·0% Triton X-100, 2 mM $MgSO_4$, 1·0% formaldehyde for 30 min at 4 °C with gentle agitation, then rinsed once with wash buffer (50 mM Tris pH 6·8, 150 mM NaCl, 0·5% NP40, 1 mg/ml bovine serum albumin (BSA)). Tissues were incubated for 12 h at 4 °C in blocking buffer (50 mM Tris pH 6·8, 150 mM NaCl, 0·5% NP40, 5 mg/ml BSA). Blocking buffer was removed and primary antibodies were added (1 : 3 dilution in wash buffer) and the reaction incubated at 4 °C with gentle agitation for 15–20 h, followed by three successive rinses (10, 20 and 45 min) in wash buffer at 4 °C. Secondary mouse anti-rabbit fluorescein-conjugated antibodies were added at a 1 : 1000 dilution in wash buffer, and the tissues incubated for an additional 8–12 h, and rinsed as above. Tissues were mounted in Vectasheild (Vector Laboratories) and viewed using a Nikon Microphot-FX microscope or a Zeiss LSM 410 confocal microscope.

3. Results

(i) *Cytogenetic analysis of the 62E puff*

To identify ecdysone-response genes within the 62E puff we used a yeast artificial chromosome to identify genomic lambda and cosmid clones containing genomic DNA from the 62E region and constructed the genomic contig shown in Fig. 1. Since we were unsuccessful in identifying ecdysone-induced transcription units within the 62E contig by differential cDNA hybridization, we more accurately mapped the 62E puff in order to delineate the genomic region most likely to contain ecdysone-inducible transcription units.

First, we carried out *in situ* hybridization with genomic clones to chromosomes in which the 62E puff was active. Genomic sequences in *λ21* and *λ4* (Fig. 2*a*, *b*) are seen to hybridize within the centre of the puff region, while genomic sequences in *cos10* (Fig. 2*c*) and *cos14* (not shown; see Fig. 2*d*) lie at the distal and proximal edges, respectively, of the puffed region. This analysis led to the alignment of the cytological map (Fig. 1*a*) and the genomic contig (Fig. 1*c*). Second, we mapped chromosomal deficiencies having breakpoints in the 62E region (Wang *et al*., 1994) with respect to the 62E puff by *in situ* hybridization with genomic clones (Fig. 2*d–f*, Table 1), enabling us to align the cytological, deficiency and contig maps as shown (Fig. 1*a–c*). A number of the deficiency chromosomes having cytologically defined proximal breakpoints in 62D6-E1, including *Df(3L)AG124* and *Df(3L)AG105* listed in Table 1, were found to produce a normal 62E puff, and *in situ* hybridization confirmed that the proximal breakpoint of these deficiencies is distal to the central core of the puff region defined by the lambda clones (Table 1). This indicates that all genomic sequences necessary for the formation of the 62E puff are proximal to 62D6-E1. Conversely, chromosomes bearing the deficiency *Df(3L)AG169* do not produce the 62E puff and have deleted the genomic region corresponding to the centre of the 62E puff. In Fig. 2*d*, *in situ* hybridization with a *cos14* probe to polytene chromosomes from a *Df(3L)AG169/+* heterozygote shows that the corresponding genomic sequences are present on both chromosomes and delineate the proximal edge of the 62E puff. Other weaker *in situ* hybridization signals obtained with *cos14* result form the presence of a *fold-back* transposable element in the cloned DNA (D. Garza, unpublished). Fig. 2*e* shows that the genomic region corresponding to λ21 is in the middle of the 62E puff and is absent from the deficiency-bearing chromosome. Last, hybridization of a *cos14* probe, shown in Fig. 2*f*, shows that the corresponding

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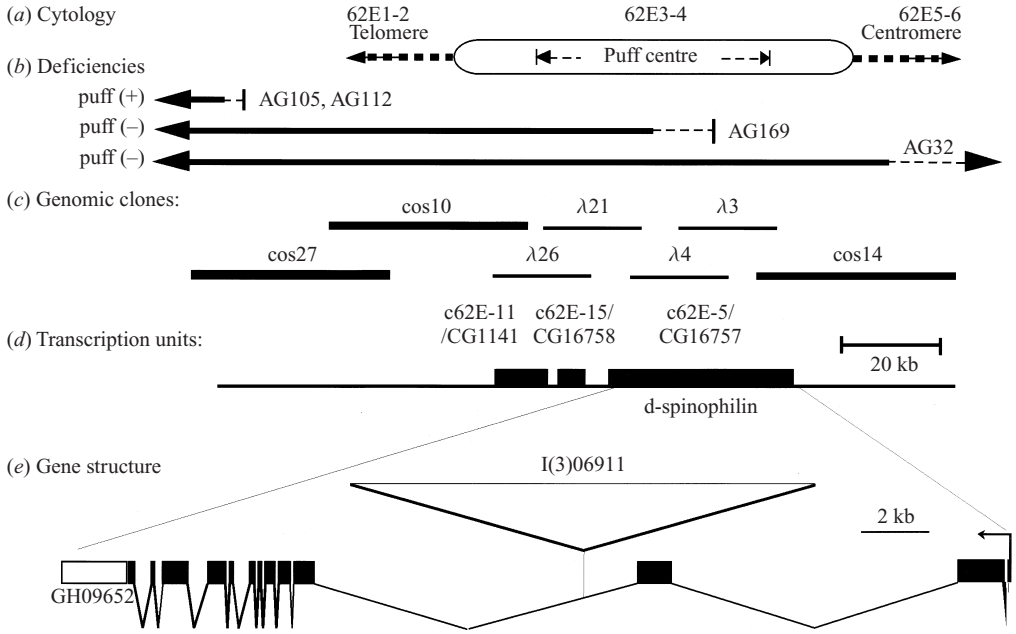

Fig. 1. Molecular genetics of the 62E puff region. Shown in (*a*) through (*d*) is an alignment of the cytological map (*a*), deficiency map (*b*), genomic clone map (*c*) and the genomic extent of the transcription units identified as being derived from the centre of the 62E puff (*d*). Shown in (*e*) is the gene structure of a composite *D-spinophilin* transcript and the relative position of the *P* element insertion mutation *l(3)06911* within the gene.

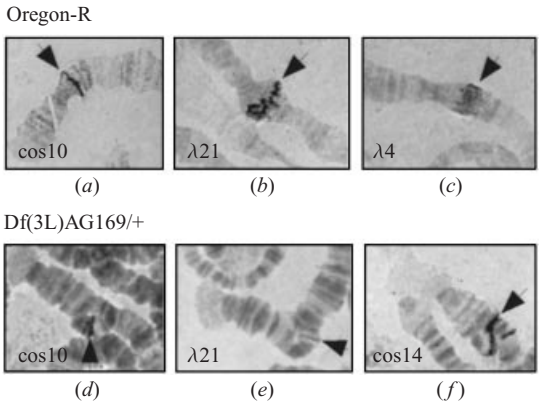

Fig. 2. Cytological analysis of the 62E puff region. Shown are representative results obtained by *in situ* hybridization of the indicated genomic DNA probes to salivary gland polytene chromosomes from either wild-type (*a–c*) or *Df(3L)AG169/+* (*d–f*) late third-instar larvae.

genomic DNA is at the distal edge of the 62E puff and is absent on the deficiency-bearing chromosome. In each case shown (Fig. 2*d–f*), the *Df(3L)AG169* deficiency chromosome does not form a visible 62E puff. Southern blot analysis further indicated that the proximal breakpoint of the *Df(3L)AG169* deficiency lies within a 10.3 kb *Eco*RI fragment spanning the region of overlap between λ3 and λ4 (data not shown). These results show that genomic sequences distal to the proximal breakpoint of *Df(3L)AG169* in λ3 are required for puffing. The results of our cytogenetic analysis of the 62E puff collectively suggest that the genomic region corresponding to the puff centre contains genomic sequences required for puffing, and is therefore likely to contain ecdysone-regulated transcription units corresponding to the 62E early-late puff.

Table 1. *Breakpoint and puffing data for all deficiency lines used in this study as characterized by polytene squash, Southern blotting and* in situ *hybridization*

Deficiency	Proximal breakpoint	*cos27*	*cos10*	*λ26*	*λ21*	*λ3*	*cos14*	62E puff
Df(3L)AG124	62D6-E1	+	+	*	+	*		Yes
Df(3L)AG105	62D6-E1	*	+	+	*	*		Yes
Df(3L)AG169	62E4	*	–	*	–	+	+	No
Df(3L)AG112	62E4-5	*	–	*	–	–		No
Df(3L)AG72	62F1-2	*	*	–	*	–		No
Df(3L)AG32	62F1-2	–	*	–	*	*		No

Breakpoints correspond to cytological positions on the third-instar polytene chromosome. *In situ* hybridization results: each column is a different genomic clone used as a probe and each row is the deficiency line being tested. *, not tested; –, no hybridization signal seen on the deficiency chromosome; +, hybridization signal seen on the deficiency chromosome.

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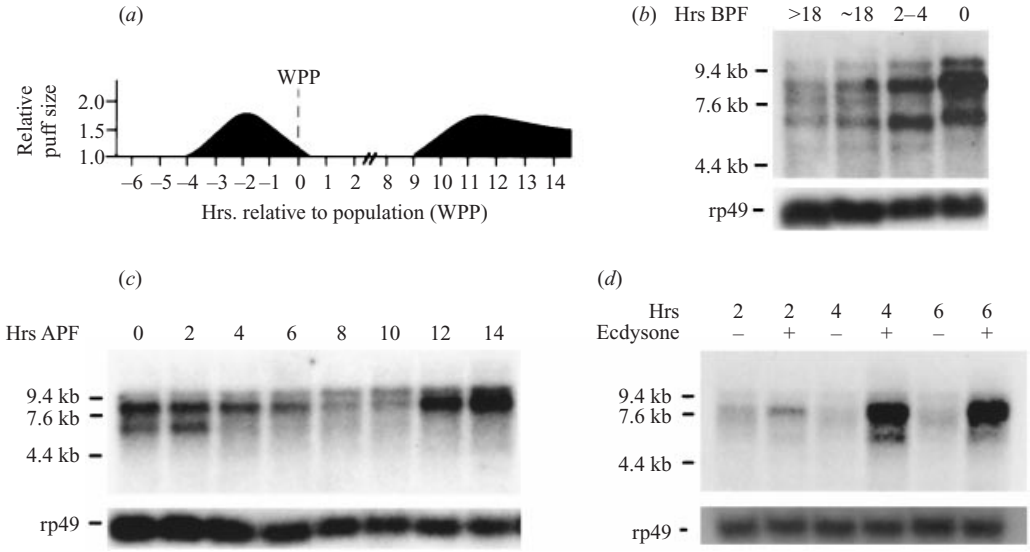

Fig. 3. Identification of transcripts whose abundance is correlated with 62E puff activity. Shown in (*a*) is the temporal pattern of the 62E early-late puff activity during the late-larval and prepupal stages (modified from Ashburner & Richards, 1976). The remaining panels show the results of hybridizing a *c62-5*-derived riboprobe to Northern blots prepared using total RNA isolated from developmentally staged late third-instar larvae (*b*), prepupae staged at 2 h intervals (*c*), and from larval tissues cultured either in the presence (+) or absence (−) of ecdysone at 5×10^{-6} M for 2, 4 and 6 h (*d*). The *rp49* gene encodes a ribosomal protein and is used as a probe to control for RNA loading. BPF, before puparium formation; APF, after puparium formation.

(ii) *Identification of an ecdysone-regulated gene within the 62E puff*

Based on the results above, individual genomic restriction fragments from the central region of the contig encompassing lambda clones λ26, λ21, λ4 and λ3 were used to identify more than 30 independent cDNAs corresponding to transcription units within the puff centre. These cDNAs were then placed into three independent transcription units whose genomic extent was determined and is shown in Fig. 1. Representative cDNAs were hybridized to Northern blots containing RNA isolated from developmentally staged late third-instar larvae and prepupae in order to identify cDNAs that hybridized to transcripts whose abundance increased and decreased in parallel with the previously defined induction, regression and reinduction of the 62E early-late puff (Fig. 3*a*). This Northern analysis led to the identification of a 4·2 kb cDNA, *c62E-5*, that hybridized with a number of transcripts whose abundance increased during the late-larval ecdysone pulse, when the 62E puff is initially induced (Fig. 3*b*). Transcript abundance then decreased following pupariation, a time when the 62E puff has regressed, and increased once again shortly after the 62E puff is reinduced by the prepupal ecdysone pulse, some 12–14 h later (Fig. 3*c*). We also note that there are differences in the specific size transcripts that increase in abundance during the late larval and prepupal ecdysone pulses.

To test whether the observed increases in transcript abundance are mediated by ecdysone, we next hybridized the *c62-5*-derived probe to Northern blots containing total RNA isolated form larval tissues cultured in the presence or absence of ecdysone (Fig. 3*d*). In the presence of ecdysone we see a dramatic increase in the accumulation of a number of transcripts. In particular, we see a dramatic increase in the same size transcript seen to increase by the greatest amount during the late larval ecdysone pulse *in vivo*. In contrast, we see a rapid decrease in the abundance of most or all of the hybridizing transcripts in the absence of ecdysone. Based on these results we conclude that the gene corresponding to the *c62E-5* cDNA is ecdysone-regulated, and that its transcriptional activation is likely to contribute to the formation of the 62E early-late puff.

While these results provide strong evidence that transcripts identified with the *c62E-5* cDNA correspond to those associated with induction of the 62E early-late puff, this does not exclude the possibility that transcriptional activation of other genes also contributes to puff formation. Our analysis of the *c62E-11* group of cDNAs within the puff region (corresponding to the *CG1141* gene: see Fig. 1) indicates that it is also ecdysone-regulated and may therefore contribute to the formation of the 62E puff (D. Garza, unpublished). Conversely, some aspects of *D-spinophilin* expression are likely to be ecdysone-independent, since *D-spinophilin* transcripts are observed prior the late larval ecdysone pulse (Fig. 2*a*, lane 1) and throughout development (J. Keegan and D. Garza, unpublished).

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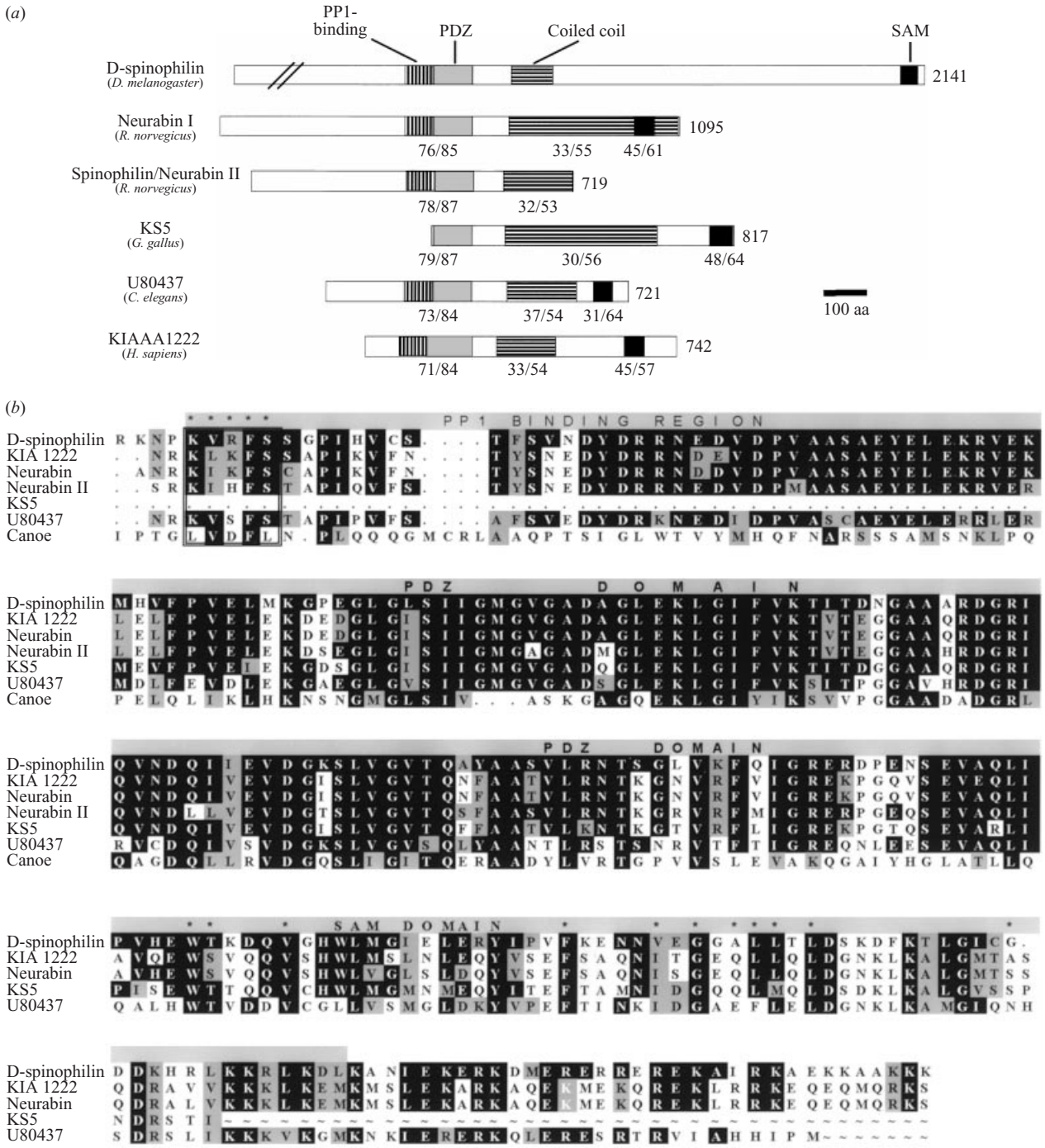

Fig. 4. *D-spinophilin* is a member of a gene family encoding a number of conserved protein domains. (*a*) Box diagram showing low-resolution protein alignment, including the relative size and position of the PP1-binding domain (boxes with vertical lines), the PDZ domain (solid grey boxes), the coiled-coil domain (boxes with horizontal lines) and SAM domain (solid black boxes). The percentage identity/similarity with respect to D-spinophilin is indicated under each conserved domain. Also indicated at the end of each protein is the total length in amino acid residues. (*b*) Amino acid alignment of the PP1-binding/PDZ domains and the SAM domain. Amino acids identical to those found in the putative D-spinophilin protein are shown as black boxes with white text, those that are similar as grey boxes with black text. Amino acid alignments and percentage identity/similarity were determined using Wisconsin Package version 10.1, Genetics Computer Group (GCG), Madison Wisconsin. GenBank accession numbers are as follows: D-spinophilin, AF285759; neurabin I, AAC53454; neurabin II/spinophilin, AAC05183; KS5, AAC69997; U80437, AAB37620; and KIAAA1222, BAA86536.

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(iii) c62-5 *identifies the* D-spinophilin *gene*

We next determined the DNA sequence of the *c62E-5* cDNA (AC# AF285759: see Section 2), and compared it with the *Drosophila* EST database (Berkeley Drosophila Genome Project, BDGP) and the *Drosophila* genome sequence (Adams *et al*., 2000). This led to the identification of *CG16757* as the gene corresponding to the *c62E-5* cDNA. Although there are a number of discrepancies between the proposed sequence and genomic structure of *CG16757* and the genomic structure based on the DNA sequence of c62E-5, they agree across most of their shared sequences. Given the large number of hybridizing bands observed on Northern blots, some of the observed differences may reflect differences in the structure of different mRNAs produced by differential promoter utilization and/or alternative splicing. We note in particular the absence of a C:G base pair in *c62E-5* that is found at position 5730 in the *CG16757* sequence. This difference allows the reading frame of *c62E-5* to continue, and results in the inclusion of a highly conserved SAM domain not predicted to be part of the *CG16757*-encoded protein. The *D-spinophilin* gene structure presented (Fig. 1*e*) is a composite of the gene structure for *c62E-5* cDNA, a 5′ extension based on the predicted gene structure of *CG16757*, and a 3′ extension based on *GH09652*, a cDNA contiguous with the 3′ end of *c62E-5* and coding for a 3′ UTR (BDGP). The resulting composite *c62E-5*/*CG16757*-derived transcript is 8288 bases in length, is comprised of 14 exons collectively spanning more than 32 kb of genomic DNA (Fig. 1), and is predicted to encode a protein of 2141 amino acids with a predicted size of 232 kDa. The central core region of the predicted protein encompasses 385 amino acids that include a number of conserved protein domains, and is most similar overall (46% identity and 61% similarity) to the mammalian protein spinophilin/neurabin II (Allen *et al*., 1997; Satoh *et al*., 1998).

Based on the observed conservation of amino acid sequence (Fig. 4), we hereafter refer to the *Drosophila* protein as D-spinophilin and the corresponding *Drosophila* gene as *D-spinophilin*. The putative D-spinophilin protein is part of a family of related proteins found in diverse species, including *Caenorhabditis elegans*, *Gallus gallus*, *Rattus norvegicus* and *Homo sapiens*. Within the core region of the protein are three previously defined conserved domains: a PDZ domain, a protein phosphatase 1 (PP1)-binding domain and a coiled-coil domain (Fig. 4*a*). PDZ domains were first identified in the proteins PSD95, DLG, and zo-1 and are known to target specific molecules to specific targets at the cell membrane (reviewed in Ponting *et al*., 1997). The PP1-binding domain of r-spinophilin/neurabin II has been shown to specifically bind PP1 and coiled-coil domains have been shown to mediate various protein:protein interactions (Allen *et al*., 1997; Hsieh-Wilson *et al*. 1999; McAvoy *et al*., 1999). We have also identified a conserved SAM domain near the carboxy-terminus of the D-spinophilin protein, also shown to be involved in protein:protein interactions, including self-association of proteins to form homodimers (Kyba & Brock, 1998).

Alignment of the conserved PP1-binding domain, PDZ domain and SAM domain between the different family members illustrates the remarkable sequence conservation among the family members from these diverse species (Fig. 4*b*). Within the 152 amino acid region encompassing the PP1-binding and PDZ domains, the percentage similarity among family members ranges from 84% to 87% and includes a match to the consensus PP1-binding motif R/K-I/V-X-F-S (Fig. 4*b*). Similarly, the percentage similarity among family members for the 38 amino acids encompassing the D-spinophilin SAM domain ranges from 54% to 64%, and includes critical amino acid residues previously shown to be involved in protein:protein interactions. However, we have not been able to identify an actin-binding domain in D-spinophilin corresponding to actin-binding domains found in most of the other members of the family (see Section 4). Finally, a BLAST search of the coding potential of the *Drosophila* genome with the conserved domains of either *D-spinophilin* or other family members indicates that *D-spinophilin* is likely to be the only member of this family in *D. melanogaster*. For example, within the conserved PDZ domain D-spinophilin has 65–75% identity with the other family members from diverse species; however, only 36% identity is seen within this domain for its closest relative in *D. melanogaster*, the *canoe* gene (Fig. 4*b*).

(iv) *D-spinophilin expression increases in response to the late larval ecdysone pulse*

The expression of D-spinophilin was next examined using an affinity-purified polyclonal antibody directed against amino acids in the conserved region (Fig. 4*a*; Section 2). We compared D-spinophilin expression in whole-mount stains of larval tissues isolated before (18 h BPF) and after (2–4 h BPF) the late larval ecdysone pulse (Fig. 5*a–i*). Specificity of the antibody was demonstrated by the greatly reduced fluorescence seen in tissues isolated from larvae homozygous for the *P* element insertion mutation *l(3)06911* (Fig. 5*c*, *f* and *i*). This strain contains a semi-lethal *P* element insertion 2·6 kb downstream of the third exon of *D-spinophilin* (Fig. 1; Fig. 1e). We observed a dramatic increase in fluorescence signal in most tissues following the late larval ecdysone pulse. In a number of these tissues we also observed changes in the localization of

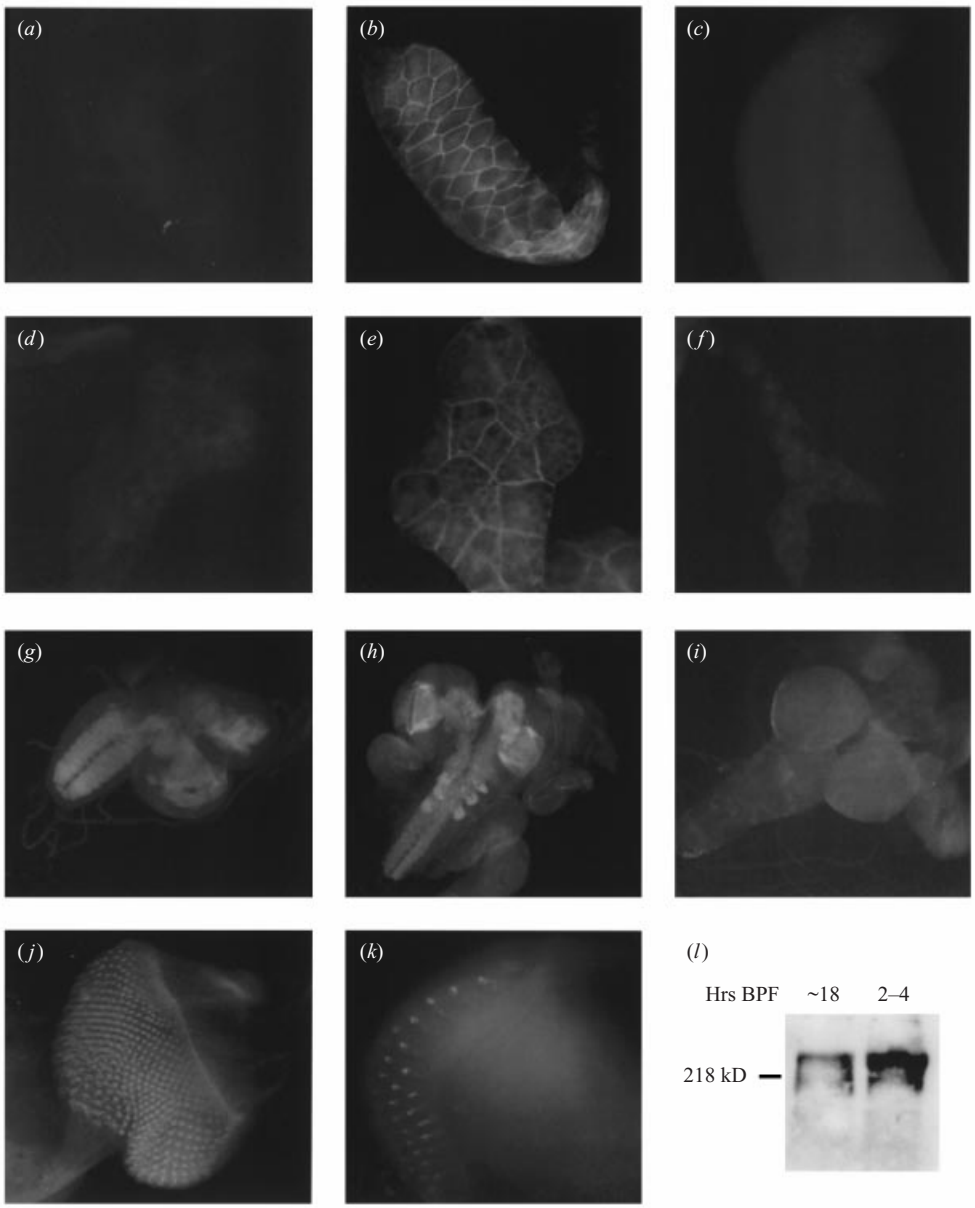

Fig. 5. Expression of D-spinophilin protein. Tissues were isolated from wild-type (Oregon-R) larvae 18 h before puparium formation (18 h BPF: *a*, *d* and *g*) or 2–4 h BPF (*b*, *e* and *h*) and from mutant (*l(3)06911* larvae 2–4 h BPF (*c*, *f* and *i*). Shown are representative staining patterns for salivary gland (*a–c*), fat body (*d–f*) and CNS (*g–i*). Also shown are eye discs from larvae 2–4 h BPF (*j–k*), and a Western blot comparison of D-spinophilin protein before (18 h BPF) and after (2–4 h BPF) the late larval ecdysone pulse.

the protein within particular tissues before (18 h BPF) versus after (2–4 h BPF) the late larval ecdysone pulse. In the salivary gland and the fat body (Fig. 5*a–c* and Fig. 5*d–f* respectively) we saw a dramatic increase in the fluorescence observed at or near cell membranes after the late larval ecdysone pulse (Fig. 5*b*, *e*). In the CNS we observed moderate levels of fluorescence in the neuropil and in the brain, and little or no staining in the eye disc at 18 h BPF (Fig. 5*g* and data not shown). After the ecdysone pulse we observed continued expression in the central neuropil, but also new expression in the thoracic neuromeres and increased expression in the optic lobe and brain (Fig. 5*h*). We also saw, for the first time, a distinctive arrayed pattern of fluorescence in the eye disc associated with the photoreceptor clusters in the developing ommatidia (Fig. 5*j*, *k*). Induced expression is also seen in the imaginal discs, and appears to be both cytoplasmic and membrane associated (data not shown). Little or no expression is seen in eye discs or CNS for tissues isolated from the *l(3)06911* mutant at any time point examined (Fig. 5*i* and data not shown). Consistent with the results observed for whole-mount tissue stains (Fig. 5*a–b*, *d–e*, and data not shown) we also saw an increase in the amount of D-spinophilin protein detected on

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Western blots after the late larval ecdysone pulse (Fig. 5*l*).

4. Discussion

(i) *D-spinophilin corresponds to the 62E early-late puff in the ecdysone genetic regulatory hierarchy*

Previously defined genes corresponding to early-late puffs in the ecdysone genetic regulatory hierarchy encode members of the nuclear receptor superfamily. In contrast, we have presented several lines of evidence indicating that the 62E early-late puff activity is correlated with the transcriptional activity of the *D-spinophilin* gene, encoding a protein that is not likely to act directly as a transcriptional regulator. First, we cytogenetically defined the genomic region likely to contain ecdysone-regulated genes corresponding to the 62E puff by *in situ* hybridization and characterization of deficiencies with proximal breakpoints in the 62E-F region (Fig. 2, Table 1). Second, we identified cDNAs corresponding to three genes in this region and showed that transcription of one of these genes, denoted *D-spinophilin*, is correlated with puff induction, regression and re-induction during the late larval and prepupal stages (Fig. 3*a–c*). Third, we have shown that *D-spinophilin* is regulated by ecdysone in *in vitro* cultured larval tissues (Fig. 3*d*). Last, we have shown that D-spinophilin protein expression is up-regulated in a number of tissues during the late larval ecdysone pulse, including the salivary gland, where the 62E puff is formed at this time (Fig. 5). The *D-spinophilin* gene we have identified appears to be the sole representative in *D. melanogaster* of a gene family found in diverse species, including *C. elegans*, *G. gallus*, *R. norvegicus* and *H. sapiens* (Fig. 4), and is, to our knowledge, the first member of this gene family shown to be regulated by a steroid hormone.

(ii) *D-spinophilin is a member of a family of multi-functional adaptor proteins*

Members of the neurabin/spinophilin protein family contain a number of protein:protein interaction domains, and previous results are consistent with the idea that these proteins act as multi-functional adaptors, tethering target proteins to each other and to the cytoskeleton. This is well-illustrated by the diversity of molecules shown to interact with members of this family. The *spinophilin/neurabin II* gene was originally identified using a yeast two-hybrid screen for neuronal proteins interacting with protein phosphatase 1 (Allen *et al.*, 1997), but was also independently identified as a novel actin-binding protein along with the related neurabin I (Nakanishi *et al.*, 1997; Satoh *et al.*, 1998), as well as in a two-hybrid screen for proteins interacting with the third cytoplasmic loop of the D2 dopamine receptor (Smith *et al.*, 1999). Neurabin I itself was independently identified in yeast two-hybrid screens for proteins interacting with the densely phosphorylated region of $p70^{s6k}$ kinase (Burnett *et al.*, 1998) and for proteins interacting with the trans-Golgi network membrane protein TGN38 (Stephens & Banting, 1999). Both spinophilin and neurabin I were also identified as components of a brain actin-associated PP1 holoenzyme complex (MacMillan *et al.*, 1999). In addition, biochemical analysis of these proteins has shown that they are likely to exist as multimers (Satoh *et al.*, 1999; Macmillan *et al.*, 1999), consistent with the ability of at least some SAM and coiled-coil domains to facilitate self-association of proteins (Kyba & Brock, 1998). Thus, members of the spinophilin/neurabin protein family are capable of interacting with each other as well as with other proteins and with the actin cytoskeleton.

(iii) *D-spinophilin function and F-actin binding*

Both the neurabin I and neurabin II/spinophilin proteins contain an F-actin binding domain near their amino-termini and interact with the actin cytoskeleton. This interaction is likely to be involved in the observed subcellular localization and concentration of these proteins at synapses and cell:cell adhesion sites (Satoh *et al.*, 1998). More generally, these adaptor proteins can provide linkage between the actin cytoskeleton and the membrane-bound proteins; such interactions are known to affect cell adhesion, cell motility and cell shape – all potential targets of ecdysone regulation in both neuronal and non-neuronal tissues. This F-actin binding domain is conspicuously absent from D-spinophilin. However, another gene identified in the 62E puff region, *c62E-11/CG1141* (see Fig. 1), encodes a villin-like, putative actin-binding protein. This gene is transcribed in the opposite direction relative to *D-spinophilin*, but our preliminary results suggest that it, like *D-spinophilin*, is up-regulated in response to the late larval ecdysone pulse, raising the possibility that these genes are co-regulated (D. Garza, unpublished). It is therefore possible that D-spinophilin interacts with actin through association with villin and/or other as yet unidentified actin-binding proteins. Alternatively, D-spinophilin may contain an as yet unrecognized actin-binding domain or carry out its functions independent of actin binding.

(iv) *D-spinophilin is likely to bind and facilitate the function of PP1*

Most members of the spinophilin protein family, including D-spinophilin, contain a conserved protein phosphatase 1 (PP1)-binding domain, and mammalian

spinophilin has been shown to bind to and regulate the activity of PP1 (Allen *et al*., 1997; Hseih-Wilson *et al*., 1999; MacMillan *et al*., 1999; McAvoy *et al*., 1999). These results suggest that regulation of PP1 activity is an important function of spinophilin and that understanding the role of D-spinophilin in regulating PP1 activity is likely to provide critical insights into the role of D-spinophilin in the ecdysone response. PP1 is a serine/threonine phosphatase that is a critical regulator of the function of a number of target proteins controlling many aspects of cellular physiology, including muscle contraction, cell division, gene expression, neurotransmission and glycogen metabolism (Shenolikar, 1994). There are four *PP1* genes in *D. melanogaster*, each defined by their cytological location in the genome (Dombradi *et al*., 1990, 1993). The protein isoforms encoded by three of these genes – *PP1*(*9C*), *PP1*(*87C*) and *PP1*(*96A*) – correspond to the mammalian PP1α/γ proteins that preferentially associate with mammalian spinophilin (Allen *et al*., 1997; Hseih-Wilson *et al*., 1999; MacMillan *et al*., 1999), while the fourth, *PP1*(*13C*), is most similar to mammalian PP1-β. Of the three *Drosophila* genes encoding PP1α/γ-type isoforms, only the *PP1*(*87C*) gene appears to be highly expressed (Dombradi *et al*., 1990, 1993). Mutations in *PP1*(*87C*) have pleiotropic effects on diverse processes including mitosis and position effect variegation (Axton *et al*., 1990; Dombradi *et al*., 1990; Baksa *et al*., 1993). Further, associative olfactory learning and visual conditioning are impaired, indicating that *PP1*(*87C*) is involved in neuronal functions in *Drosophila* (Asztalos *et al*., 1993). These mutations and assays will be particularly useful for genetic tests of the hypothesis that *D-spinophilin* plays a role in regulating PP1 function in *Drosophila*.

There is also evidence that the PDZ domain of neurabin interacts with $p70^{S6k}$ kinase, and cotransfection of neurabin activates kinase activity (Burnett *et al*., 1998). The $p70^{S6k}$ is a multi-functional kinase known to phosphorylate the S6 protein of the 40S ribosomal subunit in response to mitogenic stimuli, causing alterations in the ribosome's affinity for certain abundant mRNAs containing a polypyrimidine tract in their 5′ untranslated regions and a general increase in translation (for a review see Pearson & Thomas, 1995). If the activity of functionally equivalent kinases in *Drosophila* were similarly regulated by D-spinophilin, this would provide a potential link between the ecdysone response and post-transcriptional regulation. Again, the *Drosophila* homologue of the $p70^{S6k}$ kinase gene has been identified (Watson *et al*., 1996) and *S6k* mutations isolated (Montagne *et al*., 1999) that can be used for genetic interaction tests between *D-spinophilin* and *S6k*. We also note that the PP1-binding activity is itself regulated through phosphorylation (McAvoy *et al*., 1999), implicating spinophilin-like proteins as potential adaptor molecules that are involved in the regulation of and are themselves regulated by alternative protein phosphorylation states.

(v) *D-spinophilin, regulation of PP1 function and CNS metamorphosis*

We have shown that D-spinophilin is dynamically expressed in the developing CNS during metamorphosis (Fig. 5*g–i*). The observed D-spinophilin expression pattern in the neuropil, developing thoracic neuromeres and optic lobe of late larval CNS and photoreceptor clusters suggests a number of potential functions for D-spinophilin. Mammalian neurabin I is highly concentrated in the lamellipodia of the growth cone, and neurite outgrowth in hippocampal cultures is disrupted by *neurabin* antisense oligonucleotides, suggesting an important function for family members in developing neurons (Nakanishi *et al*., 1997). Similarly, r-spinophilin/neurabin II regulates spine number and morphology (Allen *et al*., 1997). These results are consistent with a role in development of the nervous system. During metamorphosis, the *Drosophila* CNS undergoes extensive remodelling as part of the ecdysone response (Truman *et al*., 1993), and, given the previously established functions of spinophilin family members, it is likely that ecdysone-regulation of *D-spinophilin* plays an important role in this process. Using molecular and genetic tools available in *Drosophila*, we hope to determine the various functions of this important ecdysone-regulated adaptor protein.

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References

Allen, P. B., Ouimet, C. C. & Greengard, P. (1997). Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proceedings of the National Academy of Sciences of the USA* **94**, 9956–9961.

Andres, A. J., Fletcher, J. C., Karim, F. D. & Thummel, C. S. (1993). Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. *Developmental Biology* **160**, 388–404.

Andres, A. J. & Thummel, C. S. (1995). Methods for quantitative analysis of transcription in larvae and prepupae. *Methods in Cell Biology* **44**, 565–573.

Ashburner, M. (1972). Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. VI. Induction by ecdysone in salivary glands of *D. melanogaster* cultured in vitro. *Chromosoma* **38**, 255–281.

Ashburner, M., Chihara, C., Meltzer, P. & Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symposia on Quantitative Biology* **38**, 655–662.

Ashburner, M. & Richards, G. (1976). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. III. Consequences of ecdysone withdrawal. *Developmental Biology* **54**, 241–255.

Ashburner, M. (1989). Drosophila: *A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

Asztalos, Z., von Wegereer, J., Wustmann, G., Dombradi, V., Gausz, J., Spatz, H. C. & Friedrich, P. (1993). Protein phosphatase 1-deficient mutant *Drosophila* is affected in habituation and associative learning. *Journal of Neuroscience* **13**, 924–930.

Axton, J. M., Dombradi, V., Cohen, P. T. & Glover, D. M. (1990). One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. *Cell* **63**, 33–46.

Baksa, K., Morawietz, H., Dombradi, V., Axton, M., Taubert, H., Szabo, G., Torok, I., Udvardy, A., Gyurkovics, H. & Szoor, B. (1993). Mutations in the protein phosphatase 1 gene at 87B can differentially affect suppression of position-effect variegation and mitosis in *Drosophila melanogaster*. *Genetics* **135**, 117–125.

Bayer, C., Von Kalm, L. & Fristom, J. W. (1996). Gene regulation in imaginal disc and salivary gland during *Drosophila* metamorphosis. In *Cell Biology Series. Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells* (ed. L. I. Gilbert, J. R. Tata & B. G. Atkinson), pp. 321–361. New York: Academic Press.

Bender, M., Imam, F., Talbot, W., Ganetzky, B. & Hogness, D. (1997). *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* **91**, 777–788.

Burnett, P. E., Blackshaw, S., Lai, M. M., Qureshi, I. A., Burnett, A. F., Sabatini, D. M. & Snyder, S. H. (1998). Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton. *Proceedings of the National Academy of Sciences of the USA* **95**, 8351–8356.

Dombradi, B., Axton, J. M., Brewis, N. D., da Cruz e Silva, E. F., Alphey, L. & Cohen, P. T. (1990*a*). *Drosophila* contains three genes that encode distinct isoforms of protein phosphatase 1. *European Journal of Biochemistry* **194**, 739–745.

Dombradi, B., Axton, J. M., Barker, H. M. & Cohen, P. T. (1990*b*). Protein phosphatase 1 activity in *Drosophila* mutants with abnormalities in mitosis and chromosome condensation. *FEBS Letters* **275**, 39–43.

Dombradi, V., Mann, D. J., Saunders, R. D. & Cohen, P. T. (1993). Cloning of the fourth functional gene for protein phosphatase 1 in *Drosophila melanogaster* from its chromosomal location. *European Journal of Biochemistry* **212**, 177–183.

D'Avino, P. P. & Thummel, C. S. (2000). The ecdysone regulatory pathway controls wing morphogenesis and integrin expression during *Drosophila* metamorphosis. *Developmental Biology* **220**, 211–224.

Fristrom, D. & Fristrom, J. W. (1993). The metamorphic development of the adult epidermis. In *The Development of* Drosophila melanogaster (ed. M. Bate & A. Martinez Arias), pp. 843–897. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Garza, D., Ajioka, J. W., Burke, D. T. & Hartl, D. L. (1989). Mapping the *Drosophila* genome with yeast artificial chromosomes. *Science* **246**, 641–646.

Harlow, E. & Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Hsieh-Wilson, L. C., Allen, P. B., Watanabe, T., Nairn, A. C. & Greengard, P. (1999). Characterization of the neuronal targeting protein spinophilin and its interactions with protein phosphatase-1. *Biochemistry* **38**, 4365–4373.

Huet, F., Ruiz, C. & Richards, G. (1995). Sequential gene activation by ecdysone in *Drosophila melanogaster*: the hierarchical equivalence of early and early-late genes. *Development* **121**, 1195–1204.

Karim, F. D. & Thummel, C. S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO Journal* **11**, 4083–4093.

Koelle, M. R., Segraves, W. A. & Hogness, D. S. (1992). DHR3: a *Drosophila* steroid receptor homolog. *Proceedings of the National Academy of Sciences of the USA* **89**, 6167–6171.

Kyba, M. & Brock, H. W. (1998). The SAM domain of polyhomeotic, RAE28, and Scm mediates specific interactions through conserved residues. *Developmental Genetics* **22**, 74–84.

Lam, G. T., Jiang, C. & Thummel, C. S. (1997). Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. *Development* **124**, 1757–1769.

Lam, G., Hall, B. L., Bender, M. & Thummel, C. S. (1999). DHR3 is required for the prepupal–pupal transition and differentiation of adult structures during *Drosophila* development. *Developmental Biology* **212**, 204–216.

Langer-Safer, P., Levine, M. & Ward, D. (1982). Immunological method for mapping genes on *Drosophila* polytene chromosomes. *Proceedings of the National Academy of Sciences of the USA* **79**, 4381–4385.

Lindsley, D. L. & Zimm, G. G. (1992). *The Genome of* Drosophila melanogaster. San Diego: Academic Press.

MacMillan, L. B., Bass, M. A., Cheng, N. Howard, E. F., Tamura, M., Strack, S., Wadzinski, B. E. & Colbran, R. J. (1999). Brain actin-associated protein phophatase 1 holoenzymes containing spinophilin, neurabin, and selected catalytic subunit isoforms. *Journal of Biology Chemistry* **274**, 35845–35854.

McAvoy, T., Allen, P. B., Obaishi, H., Nakanishi, H., Takai, Y., Greengard, P., Nairn, A. C. & Hemmings, H. C. Jr (1999). Regulation of neurabin I interaction with protein phosphatase 1 by phosphorylation. *Biochemistry* **38**, 12943–12949.

Maroni, G. & Stamey, S. C. (1983). Use of blue food to select synchronous, late third-instar larvae. *Drosophila Information Service* **59**, 142–143.

Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C. & Thomas, G. (1999). *Drosophila* S6 kinase: a regulator of cell size. *Science* **285**, 2126–2129.

Nakanishi, H., Obaishi, H., Satoh, A., Wada, M., Mandai, K., Satoh, K., Nishioka, H., Matsuura, Y., Mizoguchi, A. & Takai, Y. (1997). Neurabin: a novel neural tissue-specific actin filament-binding protein involved in neurite formation. *Journal of Cell Biology* **139**, 951–961.

O'Connell, P. & Rosbash, M. (1984). Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Research* **12**, 5495–5513.

Pearson, R. B. & Thomas, G. (1995). Regulation of p70s6k/p85s6k and its role in the cell cycle. *Progression of Cell Cycle Research* **1**, 21–32.

Ponting, C. P., Phillips, C., Davies, K. E. & Blake, D. J. (1997). PDZ domains: targeting signaling molecules to sub-membranous sites. *BioEssays* **19**, 469–479.

Raghavan, S., Williams, I., Aslam, H., Thomas, D., Szoor, B., Morgan, G., Gross, S., Turner, J., Fernandes, J., VijayRaghavan, K. & Alphey, L. (2000). Protein phosphatase 1 beta is required for the maintenance of muscle attachments. *Current Biology* **10**, 269–272.

Richards, G. (1997). The ecdysone regulatory cascades in *Drosophila*. *Advances in Developmental Biology* **5**, 81–135.

Riddiford, L. M. (1993). Hormones and *Drosophila* development. In *The Development of* Drosophila melanogaster (ed. M. Bate & A. Martinez Arias), pp. 899–939. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Robinow, S., Talbot, W. S., Hogness, D. S. & Truman, J. W. (1993). Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. *Development* **119**, 1251–1259.

Russell, S. R., Heimbeck, G., Goddard, C. M., Carpenter, A. T. & Ashburner, M. (1996). The *Drosophila* Eip78C gene is not vital but has a role in regulating chromosome puffs. *Genetics* **144**, 159–170.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Satoh, A., Nakanishi, H., Obaish, H., Wada, M., Takahashi, K., Satoh, K., Hirao, K., Nishioka, H., Hata, Y., Mizoguchi, A. & Takai, Y. (1998). An actin filament-binding protein with one PDZ domain localized at cadherin-based cell-cell adhesion sites. *Journal of Biological Chemistry* **273**, 3470–3475.

Schubiger, M., Wade, A. A., Carney, G. E., Truman, J. W. & Bender, M. (1998). *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development* **125**, 2053–2062.

Shenolikar, S. (1994). Serine/threonin phosphatases: new avenues for cell regulation. *Annual Review of Cell Biology* **10**, 55–86.

Smith, F. D., Oxford, G. S. & Milgram, S. L. (1999). Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *Journal of Biological Chemistry* **274**, 19894–19900.

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S. & Rubin, G. M. (1999). The Berkeley *Drosophila* Genome Project gene disruption project: single P-element insertions mutating 25% of vital *Drosophila* genes. *Genetics* **153**, 135–177.

Stephens, D. J. & Banting, G. (1999). Direct interaction of the trans-Golgi network membrane protein, TGN38, with the F-actin binding protein neurabin. *Journal of Biological Chemistry* **274**, 30080–30086.

Stone, B. L. & Thummel, C. S. (1993). The *Drosophila* 78C early late puff contains E78, an ecdysone-inducible gene that encodes a novel member of the nuclear hormone receptor superfamily. *Cell* **75**, 307–320.

Stowers, R., Russell, S. & Garza, D. (1999). The 82F late puff contains the L82 gene, an essential member of a novel gene family. *Developmental Biology* **213**, 116–130.

Stowers, R., Garza, D., Rascle, A. & Hogness, D. S. (2000). The L63 gene is necessary for the ecdysone-induced 63E late puff and encodes CDK proteins required for *Drosophila* development. *Developmental Biology* **221**, 23–40.

Sun Suh, K., Ting, Y.-T. & Burr, J. G. (1998). An avian cDNA encoding a tyrosine-phosphorylated protein with PDZ, coiled-coil, and SAM domains. *Gene* **219**, 111–123.

Talbot, W. S., Swyryd, E. A. & Hogness, D. S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73**, 1323–1337.

Thummel, C. S. (1996). Files on steroids: *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends in Genetics* **12**, 306–310.

Thummel, C. S. (1997). Dueling orphans: interacting nuclear receptors coordinate *Drosophila* metamorphosis. *Bioessays* **19**, 669–672.

Treisman, J. E., Ito, N. & Rubin, G. M. (1995). Misshapen encodes a protein kinase involved in cell shape control in *Drosophila*. *Gene* **186**, 119–125.

Truman, J. W., Taylor, B. J. & Awad, T. A. (1993). Formation of the adult nervous system. In *The Development of* Drosophila melanogaster (Ed. M. Bate & A. Martinez Arias), pp. 1245–1275. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Wang, M., Champion, L. E., Biessmann, H & Mason, J. M. (1994). Mapping a mutator, mu2, which increases the frequency of terminal deletions in *Drosophila melanogaster*. *Molecular Genetics* **245**, 598–607.

Watson, K. L., Chou, M. M., Benis, J., Gelbart, W. M. & Erikson, R. L. (1996). A *Drosophila* gene structurally and functionally homologous to the mammalian 70-kDa s6 kinase gene. *Proceedings of the National Academy of Sciences of the USA* **93**, 13694–13698.

White, K. P., Hurban, P., Watanabe, T. & Hogness, D. S. (1997). Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science* **276**, 114–117.