Drosophila homologs of transcriptional mediator complex subunits are required for adult cell and segment identity specification

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The origins of specificity in gene expression are a central concern in understanding developmental control. Mediator protein complexes regulate transcriptional initiation, acting as modular adaptors linking specific transcription factors to core RNA polymerase II. Here, we identified the *Drosophila* homologs of 23 human mediator genes and mutations of two, *dTRAP240* and of *dTRAP80* (the putative fly homolog of yeast *SRB4*). Clonal analysis indicates a general role for *dTRAP80* necessary for cell viability. The *dTRAP240* gene is also essential, but cells lacking its function are viable and proliferate normally. Clones reveal localized developmental activities including a sex comb cell identity function. This contrasts with the ubiquitous nuclear accumulation of dTRAP240 protein in imaginal discs. Synergistic genetic interactions support shared developmental cell and segment identity functions of dTRAP240 and dTRAP80, potentially within a common complex. Further, they identify the homeotic *Sex combs reduced* product, required for the same cell/tissue identities, as a functional partner of these mediator proteins.

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Transcription initiation is a central and highly regulated step in eukaryotic gene expression that unites core RNA polymerase II (Pol II) with numerous other factors. These include Pol II-associated general transcription factors (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, and TFIIH) and sequence-specific regulators bound to gene enhancers (for review, see Hampsey and Reinberg 1999; Malik and Roeder 2000). Work of the last 10 years has brought to light a new class of transcription factor complex, the mediator (MED). The first known mediator components, encoded by the yeast *SRB/MED* genes, were identified by dominant mutations suppressing the conditional lethality caused by a C-terminal domain (CTD) mutation of the Pol II large subunit (Thompson et al. 1993). The biochemically purified Srb proteins interact physically with core RNA Pol II in the form of large protein complexes (Thompson et al. 1993; Kornberg 1999).

More recently, mediator complexes have likewise been identified in mammalian cells where, as in budding

yeast, they associate with the core Pol II to form a giant holoenzyme (Asturias et al. 1999). The mammalian MED complexes capable of stimulating basal transcription initiation in vitro contain ~20 subunits, including at least five proteins homologous to yeast Srb/MED proteins (Boyer et al. 1999; Gu et al. 1999; Ito et al. 1999; Naar et al. 1999; Rachez et al. 1999; Ryu et al. 1999). Several related complex forms that mediate transcription in vitro have been isolated through their physical contact with a spectrum of mammalian transcription factors. These include nuclear receptors for thyroid hormone (TRAP complex; Ito et al. 1999) and vitamin D3 (DRIP complex; Rachez et al. 1999), VP16, the p65 subunit of NF- κ B, SREBP-1a (ARC; Naar et al. 1998, 1999), Sp1 (CRSP; Ryu et al. 1999), E1A (Boyer et al. 1999), and p53 (Ito et al. 1999). Alternative protocols have yielded related mammalian complexes (human or mouse mediator; Chao et al. 1996; Jiang et al. 1998; SMCC, Ito et al. 1999) or subcomplexes (negative regulator of activated transcription, NAT, associated with human Srb10/Cdk8; Sun et al. 1998). These related complexes are viewed as versatile interfaces that link specific transcription factors and the general Pol II machinery in a complex equilibrium (Lee et al. 1998). The MED complexes are most often considered transcriptional coactivators, and this property has been used as the basis of their biochemical

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purification. Importantly, however, these complexes are not dedicated activators, and some forms have also been described as corepressors (Song and Carlson 1998; Sun et al. 1998).

A systematic examination of gene expression in yeast *srb* mutants has revealed distinct families of target genes of varying size and composition for different *Srb* subunits (Holstege et al. 1998). This functional diversity is likely to reflect a corresponding diversity of physically interacting regulatory transcription factors. MED complexes appear to integrate regulatory information from multiple transcription factors and relay that information to the core Pol II (for reviews, see Parvin and Young 1998; Hampsey and Reinberg 1999; Kornberg 1999; Malik and Roeder 2000). In support of this view, recent work demonstrates that human ARC complex can interact with two transcription factors and parlay this input into a synergistic transcriptional response (Naar et al. 1998, 1999). In metazoans, the dynamic developmental process requires fine control of the gene expression program, presumably involving their MED complexes. However, for the moment little is yet known of their *in vivo* functions in development. The first known mutation of a metazoan MED subunit, in the *sur2* locus of the nematode *Caenorhabditis elegans*, was isolated as a suppressor of activated Ras in vulval development (Singh and Han 1995). A MED complex has been identified in *C. elegans* and suggested to participate in regulation of developmental target genes (Kwon et al. 1999). Recently, gene inactivations have been described for two mouse subunits, *Srb7* (Tudor et al. 1999) and TRAP220 (Ito et al. 2000). Murine *Srb7* corresponds to a core MED subunit required for yeast cell viability and is apparently required for cell viability in the mouse embryo as well. The inactivation of TRAP220, a subunit implicated in ligand-dependent binding to thyroid hormone receptor, reveals diverse developmental defects in a variety of tissues.

A better understanding of the *in vivo* physiological roles of the MED complexes in metazoan development requires the continued genetic analysis of mutations of their subunits in model organisms. Toward this end, we have identified 23 *Drosophila* homologs of human MED subunits in the recently completed genomic sequence. We have cloned two of these and identified loss-of-function mutations for each. Both mutations are recessive lethal, revealing the functions of the genes as essential. However, the two show marked differences, as one is required for cell viability and the other is required for the integrity of the organism. These mutations have allowed us to identify discrete roles for MED protein functions in the control of adult segment and cell identities. Synergistic genetic interactions between the two putative MED subunits indicate a shared developmental function, potentially within a *Drosophila* complex. Their synergistic interactions with the homeotic *Sex combs reduced* (*Scr*) locus further implicate the SCR homeodomain protein as a functional partner whose activity is modulated by MED protein activity during development.

Results

Identification of 23 *Drosophila* homologs of human mediator proteins and cloning of *pap*/dTRAP240

A new *Drosophila* gene, *poils aux pattes* (*pap*; cytological locus 78A1-3), was identified in a *P*-element screen for dominant genetic modifiers of cell identity functions of the homeotic loci *Scr* (*Hox-A5/B5*) and *proboscipedia* (*pb*; *Hox-A2/B2*; Pattatucci et al. 1991; Cribbs et al. 1995). The *Scr* selector gene confers prothoracic identity, while *pb* alone induces maxillary identity. Together, the *Scr* and *pb* selectors show a combinatorial behavior leading to specification of the adult labial palps (mouthparts). Low-level ectopic expression of PB protein from an *hsp70-pb* mini-gene, the HSPB element, induces several dose-sensitive cell-identity phenotypes (Cribbs et al. 1995; Boubé et al. 1997) that were used to screen for second-site dominant modifier mutations. Of 5000 new autosomal *P* insertions tested, only one, in the *pap* locus, showed dose-sensitive enhancement of the distal sex comb (described below) induced by the HSPB element. Starting from the *P*-element molecular tag, a 50-kb pair interval encompassing the *pap* gene was cloned. Analysis of genomic and complementary DNA (cDNA) sequences indicates a transcription unit spanning at least 22 kb and generating a ~10-kb mRNA (Fig. 1a). The exonic *P* insertion resides upstream of the first in-frame ATG of an open reading frame (ORF) of 2618 amino acids (see Fig. 1a). Full reversal of lethality by mobilizing the *P* element, and the rescue of lethality by a ubiquitin-cDNA construct (not shown), confirmed that this ORF corresponds to *pap*. The ORF encodes the unique *Drosophila* counterpart of TRAP240/ARC250, recently identified as a subunit of the human thyroid hormone receptor-associated protein (TRAP) or activator-recruited cofactor (ARC) protein complexes (Ito et al. 1999; Naar et al. 1999). The *Drosophila* PAP protein shows 27% overall identity (40% similarity) with human TRAP240 and 27% identity (39% similarity) with its *C. elegans* counterpart. This conservation extends across the proteins but is highest in the N- and C-terminal regions (Fig. 1b). We therefore consider *pap* as the presumptive fly homolog of TRAP240.

The recent availability of the *Drosophila* genomic sequence (Adams et al. 2000) and the collection of corresponding cDNAs through the Berkeley *Drosophila* Genome Project (BDGP; Rubin et al. 2000) allowed us to identify a single putative *Drosophila* homolog for each of the 23 known human TRAP/ARC subunits (Fig. 2a; Materials and Methods). For simplicity, we will refer to these proteins and their genes in the text as TRAPs when more than one name exists for the same entity (Malik and Roeder 2000). While the existence of a biochemical entity remains to be demonstrated, the observed structural conservation of such a large number of MED genes provides clear circumstantial evidence for the existence of a fly mediator complex (dMED) similar to the purified complexes from worms (Kwon et al. 1999) and mammals (above).

Figure 1. *pap* encodes the fly homolog of the human transcriptional mediator subunit TRAP240. (a) Molecular organization of the *pap/dTRAP240* region (cytological position 78A1-3, chromosome 3L). The position of the recessive lethal *P-lacW* element insertion allele *pap*¹ is shown with the map of this region. (E) *EcoRI*; (B) *BamHI*; (S) *SalI*. The *pap*⁵³ allele deletes the entire coding portion of exon 1 (amino acids 1–23). The transcription unit is shown with boxes representing exons; ATG is the presumptive initiator codon, filled boxes indicate protein coding region, and broken lines indicate introns (the precise site of transcription initiation is not known). (b) Protein sequence comparisons of TRAP240 homologs from *Drosophila* (d), human (h), and *Caenorhabditis elegans* (c). Because of the length of the proteins, only the most conserved regions (the N and C termini) are shown. Identical amino acids are shaded in black and conservative changes in gray. The Genbank accession numbers for the fly and human proteins are AF227214 and AF117754, respectively. The putative *C. elegans* homolog is derived from EMBL accession number Q93442, with aligned sequence beginning at a conserved methionine.

Figure 2. (Legend on following page)

pap and dTRAP80 encode putative mediator subunits required for development

Among the new *dMED* genes identified was *dTRAP80* at cytological position 90F1-2 on chromosome 3R (Fig. 2b). It encodes a predicted dTRAP80 protein of 642 amino acids exhibiting 40% identity (59% similarity) to its human counterpart. The majority of putative *Drosophila MED* genes, including *pap*, appear to lack a homolog in the complete *Saccharomyces cerevisiae* genome sequence. *S. cerevisiae* *SRB4* encodes a core component of the Srb mediator complex required for the expression of virtually all yeast genes (Holstege et al. 1998). The gene was identified by dominant mutations that directly suppress a Pol II CTD mutation (Thompson et al. 1993). For the dTRAP80 protein, low but potentially significant overall structural conservation (16% identity, 48% similarity) was detected with Srb4 proteins from the MED complexes of the yeast *S. cerevisiae* and *Schizosaccharomyces pombe* (Thompson et al. 1993; Spahr et al. 2000). The overall identity between these two yeast Srb4 proteins is only 25% (60% similarity), with conservation most pronounced in a region with predicted α -helical character between amino acids 214–313 of *S. cerevisiae* *Srb4* (40% identity, 72% similarity; see Fig. 2c). Both primary sequence and predicted helical character are conserved within this interval in metazoan TRAP80 moieties, attaining 34% identity between human TRAP80 and *S. pombe* SRB4. The corresponding sequences appear unique in the budding yeast and *Drosophila* genomes, arguing against a novel reiterated domain. Thus, despite the low level of overall identity, these observations are good evidence for homology of the metazoan TRAP80 genes with yeast *SRB4*.

One lethal *P*-insertion mutation from the BDGP collection (Spradling et al. 1999) is situated within the *dTRAP80* coding sequence. This insertion, *dTRAP80*¹, is located downstream of the apparent initiator ATG within the same exon (Fig. 2b). The cloning and the identification of mutations in these two putative *Drosophila MED* genes allowed us to initiate an in vivo assessment of their physiological roles in normal development. *dTRAP80*¹ mutants die as second-instar larvae with no obvious cuticular defect. The initial *pap*¹ *P*-element insertion and most derived imprecise excisions (including the molecular null allele *pap*⁵³; see Fig. 1a) are recessive embryonic lethals. *pap*[−] embryos appear normal apart

from discrete cuticular defects of the embryonic mouthparts. Thus, both functions are essential for viability, and *pap* is detectably required for normal embryonic development. Ubiquitous accumulation of *pap* and *dTRAP80* mRNA was observed by in situ hybridization in embryos of all stages and in larval imaginal discs (not shown). The presence of mRNA in early embryos further suggests that a maternal contribution partially compensates for the absence of zygotic expression for both *pap* and *dTRAP80*.

dTRAP80 loss of function is cell lethal

The prototypical Srb4 protein is required for transcription of nearly all Pol II-dependent promoters in yeast (Holstege et al. 1998). If *dTRAP80* encodes the functional homolog of Srb4, it is predicted to participate in all aspects of mediator function, and the *dTRAP80*[−] condition should be cell lethal. The survival of *dTRAP80*[−] embryos to second-instar larvae (above) suggested that maternally contributed *dTRAP80* mRNA suffices for embryonic survival. To test the consequences of removing *dTRAP80* while minimizing the complication of maternal contribution, we employed mitotic recombination. From heterozygous mother cells, twin clones of daughter cells homozygous for each of the two chromosome arms were induced, one carrying *dTRAP80*¹ (or *dTRAP80*⁺) and the other its wild-type homolog (plus the associated cuticular markers *Stubble* [*Sb*] and *ebony* [*e*]). The *dTRAP80*^{+/+} or *dTRAP80*^{−/−} cells of interest were identified by their bristle shape (*Sb*⁺). Where *dTRAP80*⁺ yielded 150 clones, none were observed with *dTRAP80*^{−/−} for an equivalent sample size. We conclude that *dTRAP80* is required for cell viability in the adult epidermis. This result provides independent support for a general cellular role of *dTRAP80* consistent with the sequence-based interpretation that it encodes a fly Srb4.

pap encodes a ubiquitous nuclear protein with localized developmental functions

To examine the distribution and cellular localization of PAP protein, a polyclonal antiserum was generated against its N-terminal region, then enriched by affinity chromatography with the same PAP protein fragment

Figure 2. Identification and genomic localization of *Drosophila melanogaster* homologs to known human *MED* genes. These include *dTRAP80*, an apparent metazoan homolog of yeast *SRB4*. (a) Cytological positions of 21 predicted *dMED* loci are shown. These genes have been annotated by the Genome Annotation Database of *Drosophila* (GadFly; <http://www.fruitfly.org/annot/>) and are listed in Materials and Methods. Homology searches identified *dTRAP80* as the apparent metazoan homolog of yeast *SRB4* (see c). (b) Genomic organization of the *dTRAP80* locus (cytological interval 90F1-2 of chromosome 3R) is shown. The *dTRAP80* region map, (E) *EcoRI*; (N) *NotI*; (K) *KpnI*; (Bg) *BglIII*, indicates the position of the recessive lethal *dTRAP80*¹ *P*-element insertion, previously referred to as l(3)s2956 (Spradling et al. 1999). The exon–intron organization is shown beneath with filled boxes for protein coding region and broken lines indicating introns. (c) Aligned protein sequences of TRAP80/Srb4 homologs from *D. melanogaster* (d), human (h), *Schizosaccharomyces pombe* (sp), and *Saccharomyces cerevisiae* (sc). Genbank accession numbers are AF244916, AF117657, CAB10081, and L12026, respectively. A *Caenorhabditis elegans* TRAP80 homolog was also detected (not shown; Genbank accession number CAB76740). Identical amino acids are shaded in black, and conservative changes (Taylor 1986) in gray. Positions of aligned amino acids predicted to adopt helical (H) or extended (E) secondary conformations (<http://www.ibcp.fr/predict.html>) are indicated above the alignment.

(Materials and Methods). The specificity of the antisera was confirmed by immunostaining of embryos and of imaginal discs overexpressing PAP in the posterior compartment of each segment (engrailed-GAL4/UAS-pap) or of discs harboring mitotic clones of *pap*⁵³ cells (Materials and Methods). In overexpression experiments, strong staining was limited to posterior cells, whereas in clones of *pap*⁵³ cells, the signal was no longer detected (not shown). Immunostaining experiments with this specific anti-PAP serum show that *pap* mRNA is translated throughout the imaginal tissues, as predicted by the mRNA distributions. PAP protein accumulation is predominantly nuclear (Fig. 3a,b,d,e), in agreement with a role in the general transcription machinery.

To examine functional requirements for the essential *pap* gene in adult development, we generated mitotic clones of mutant cells. In marked contrast to *dTRAP80* (above), clones were obtained showing that normal *pap* function is not required for cell viability. Clones induced during larval development (Fig. 3) led to distinct consequences in different tissues. First, adult *Drosophila melanogaster* males normally align a single row of specialized bristles, the sex comb teeth, on the first tarsal segment of the prothoracic (T1) leg. These sex comb teeth

are not found elsewhere. In contrast, clones of *pap* mutant cells situated in the distal second tarsal segment differentiated as ectopic sex comb teeth (Fig. 3c). Normal *pap* function thus opposes sex comb cell fate in this position. This role appears cell autonomous, as all observed ectopic sex comb teeth were mutant for *pap* (see Fig. 3c). In contrast, clones within the normal sex comb or bordering it did not affect the number of cells adopting this fate (Fig. 3c). Second, clones localized elsewhere in the T1 leg, or at any position in the T2 and T3 legs, were without effect (not shown). Third, clones in the maxillary palps are associated with malformations. Finally, large clones in the wing blade, the notum, or the antennae led to apparently normal pattern. Therefore in contrast with ubiquitous accumulation of PAP in epidermal cells, *pap* function is required for normal development in only a subset of those cells. Taken together, these data strongly suggest that developmental *pap* activity may be regulated according to the tissue and cell, being required for some identities but dispensable for others.

Previous biochemical characterization identified human TRAP240 as a component of the ubiquitous MED complexes and, hence, of the general transcription machinery. As such, *pap*/*dTRAP240* mutations might have

Figure 3. Expression and activity of the *pap*/*dTRAP240* locus. (a,b,d,e) Localization of PAP in imaginal discs from third-instar larvae using a monospecific rabbit polyclonal serum. Shown are a prothoracic leg imaginal disc (a) with enlargement (b), and a wing imaginal disc (d) with enlargement (e). PAP protein is expressed in most or all imaginal cells (a,d) and accumulates preferentially in the nucleus (b,e). Mutant clones at the distal extremity of the second tarsal segment in the T1 legs (c) exhibited an ectopic distal sex comb (dsc). Ectopic sex comb teeth (small circles) are yellow (*pap*⁻). A maximum of three ectopic teeth were observed in any *pap*⁻ clone. Within the normally placed sex comb (sc) at the distal end of the first tarsal segment of this sample, three mutant cells (marked with circles) are interspersed with *pap*⁺ cells. This sex comb is of normal size. Hence, the *pap*⁻ condition affected neither sex comb cell differentiation nor rotation for alignment. (f) Twin spot clonal analysis of *pap* function in imaginal disc development. Adjacent (twin) clones of *pap*^{-/-} (no GFP; black arrow) and *pap*^{+/+} cells (two copies of ubiquitin-GFP; white arrow) are identified by expression of the cell-autonomous GFP marker in a third-instar larval wing imaginal disc. Note the similar sizes and distributions of the wild-type and mutant cells.

adverse consequences for cell physiology and growth. Consequently, the discrete changes in cell identity observed could reflect a spurious fragilization of the cell rather than a concrete developmental role for *pap* in cell specification. To address this concern, clonal analysis was performed (Materials and Methods and above) using a green fluorescent protein (GFP) cell marker to examine the proliferation and distribution of mutant cells relative to wild type. Clones of cells homozygous for *pap*⁵³ and the reciprocal wild-type product were induced in first- or second-instar larvae, then compared in imaginal discs of late third-instar larvae. An example is shown in Figure 3f, where twin clones are observed in a wing imaginal disc. Adjacent clones of mutant cells (black) are as large as their wild-type counterparts (white) and similarly distributed relative to the axes of the imaginal disc. Similar results were obtained in other imaginal tissues, including the labial and T1 leg discs (examples in Fig. 4 below). These results reinforce the conclusion that the distal sex comb cell identity transformation observed in *pap* mutant clones reflects a normal, localized function of this gene in this position and not an indirect consequence of an impaired general transcription machinery function.

pap and dTRAP80 interact synergistically with the homeotic Sex combs reduced locus in cell and segment identity specification

The ectopic distal sex comb induced by *pap* clones is a readily visible cell identity marker that reflects normal *pap* function. Ectopic distal sex comb teeth are induced in appropriately positioned cells lacking any *pap* function (above). This phenotype is also observed at low frequency with certain *Scr* and *pb* gain-of-function alleles (*Scr*^{ScxP} and *hsp70-pb* [HSPB] mutations; Pattatucci et al. 1991; Cribbs et al. 1995). This effect of the *Scr*^{ScxP} allele is enhanced in *pap* heterozygotes (Table 1). Functions of the homeodomain transcription factor SCR specify prothoracic identity, including sex comb cell fate. The in-

duction of distal sex combs by HSPB also depends on *Scr* activity, as it is no longer detected in *Scr* heterozygotes (see Table 1). Ectopic sex comb differentiation is enhanced in HSPB/*pap*⁵³ heterozygotes, but this effect is abolished in *Scr* heterozygotes (Table 1). These observations of dose-sensitive interactions indicate a synergistic functional link between *Scr* and *pap* in this cell identity specification. *pap* and *dTRAP80* both encode the sole detected fly homologs to human proteins identified by their presence in the MED complex. If the enhancement of the distal sex comb phenotype in *pap* heterozygotes is caused by limiting mediator function, double heterozygotes with *dTRAP80* should aggravate this condition. We therefore tested whether *dTRAP80* acts together with *pap* in this cell identity decision. The loss-of-function allele *dTRAP80*¹ is fully recessive, as for *pap*⁵³. In contrast, 10% of *pap*⁵³ +/+ *dTRAP80*¹ males possess an ectopic distal sex comb tooth, revealing a cooperative function in these cells (Table 1). Synergistic enhancement of the ectopic sex comb caused by *Scr*^{ScxP} is likewise observed in double heterozygotes (Table 1). These functional data indicate a shared function of PAP and dTRAP80, again suggesting the existence of a *Drosophila* mediator complex. They further suggest that at least one common function of PAP and dTRAP80 acts to antagonize *Scr* activity in distal sex comb differentiation.

Apart from the prothorax, normal *Scr* activity is also required for development of the adult labial palps, where it acts in a combinatorial fashion with the homeotic *pb* gene (Cribbs et al. 1995; Percival-Smith et al. 1997). This led us to examine the effects of *pap* and *dTRAP80* mutations on adult mouthparts formation. The wild-type labium is typified by the presence of pseudotracheal rows used for drinking and the absence of a segmental appendage. The hypomorphic *pb*⁴/*pb*⁵ genotype leads to a transformation of distal labium to antennal arista (Fig. 4a), with a concomitant reduction of the pseudotracheae. In this sensitized context, changes in relative *Scr* activity can be readily detected. Reduced *pap* or *dTRAP80* activity in heterozygotes enhances the labial-to-leg transformation, as seen by the appearance of leg-specific cell types: sex comb teeth in males, bracted bristles, and terminal claws (Table 2). In *pap dTRAP80*, double heterozygotes leg structures often entirely replace labial pseudotracheae (Fig. 4c). As in the leg, this effect of *pap* and *dTRAP80* mutants on labial development is synergistic (see Table 2). These data provide further support for a shared role of PAP and dTRAP80 proteins opposed, in this case, to the leg-forming activity of *Scr* in the labial tissue.

pap and the homeotic selector locus *Scr* act in parallel

The observed effects of *pap* and *dTRAP80* mutations in the T1 legs and labium may most simply be rationalized as consequences of increased *Scr* activity. This could result from augmented regulatory activity of SCR protein toward *Scr* target genes. Alternatively, it might reflect higher *Scr* gene expression with greater quantities of SCR protein. To distinguish between these two possibili-

Table 1. *Scr* and dMED in distal sex comb (DSC) cell identity

Genotype	Percentage legs with DSC	Mean DSC teeth per leg	Percentage of legs with three or more DSC teeth
<i>pap</i> ⁵³ /+	0 (n = 200)	0	0
<i>Scr</i> ^{ScxP}	3 (n = 182)	0.04	0
<i>Scr</i> ^{ScxP} / <i>pap</i> ⁵³	15 (n = 102)	0.15	0
HSPB	6 (n = 344)	0.06	0
HSPB/ <i>Scr</i> ⁴	0 (n = 252)	0	0
HSPB/ <i>pap</i> ⁵³	54 (n = 206)	0.54	1
HSPB/ <i>pap</i> ⁵³ <i>Scr</i> ⁴	0 (n = 171)	0	0
<i>dTRAP80</i> ¹ /+	0 (n = 200)	0	0
<i>pap</i> ⁵³ <i>dTRAP80</i> ¹ /++	10 (n = 372)	0.1	0
<i>Scr</i> ^{ScxP} / <i>dTRAP80</i> ¹	9 (n = 140)	0.1	0
<i>Scr</i> ^{ScxP} / <i>pap</i> ⁵³ <i>dTRAP80</i> ¹	34 (n = 150)	0.4	10

Figure 4. *pap* and *dTRAP80* functions contribute to segmental identity specification. Labial palp identity is determined by the joint action of *Scr* and *pb*. (a,c) Loss-of-function mutations of *pap/dTRAP240* and *dTRAP80* affect labial palp development, favoring T1 leg development. (a) The mutant combination *pb⁴/pb⁵* (where *pb⁵* is a protein-null allele and *pb⁴* is a hypomorph leading to a C-terminally truncated PB protein) transforms distal labial palps to antennal arista (arista), whereas the proximal labium retains normal pseudotracheal rows (pst). (c) The *pap¹* and *dTRAP80¹* mutations are recessive in this tissue but enhance the *pb⁴/pb⁵* mutant phenotype. Shown are mouthparts of a *pb⁴/pap¹ pb⁵ dTRAP80¹* adult female. Morphology is altered compared to a; pseudotracheae are replaced by apparent leg structures, and claws replace the arista. (b,d) *Scr* acts with *pb* in the labium to promote labial (or antennal/labial) identity but transforms the labium to distal T1 leg in the absence of *pb*. Accumulation of SCR protein was examined in imaginal labial disc cells of *pb⁴/pb⁵* mutants, giving rise to mixed antennal/labial identities (b) or *pb⁴/pap¹ pb⁵ dTRAP80¹* mutants that give predominantly T1 leg tissue (d). Nuclear SCR protein was detected throughout the discs in both cases, as it was for wild type. SCR accumulation is not increased and, indeed, may be somewhat reduced (cf. b and d), in the discs giving rise to T1 legs. (e–h) *Scr* expression examined in clones of *pap⁵³* homozygous cells. (e) Shown is a labial imaginal disc harboring a twin spot clone of *+/+* (white, vertical arrow) or of *pap⁵³/pap⁵³* cells (black, diagonal arrow). (f) Expression of the SCR homeodomain protein is seen in the same labial disc. The clone is outlined and indicated by the diagonal arrow. SCR accumulation is unaltered in the mutant cells. (g) This T1 leg imaginal disc harbors a prominent clone of *pap⁵³/pap⁵³* cells (black, limit indicated by arrow). (h) SCR protein accumulation in the same T1 disc. The clone is outlined and its border indicated by the arrow. SCR accumulation is unchanged in the *pap⁵³* cells.

ties, we examined SCR homeoprotein accumulation by indirect immunofluorescence in *pb⁴/pb⁵* labial imaginal discs that give rise to mixed labial/antennal or *pb⁴/pap¹ pb⁵ dTRAP80¹*, yielding T1 leg identities. Nuclear SCR protein does not detectably increase with the transition to T1 leg identity (Fig. 4, cf. b and d). These data indicate that PAP and dTRAP80, acting in parallel or downstream of *Scr*, negatively modulate SCR protein function in labial tissue.

The above experiments were performed in heterozygotes for *pap* and *dTRAP80*. In the sensitized genetic context employed, slight but functionally important changes in SCR accumulation could potentially pass undetected in this test. We therefore tested the molecular epistatic relations between *pap* and *Scr* in homozygous mutant cells to determine whether *Scr* gene expression depends on normal *pap* function and vice versa. Both *Scr* and *pap* are normally expressed throughout the labial and T1 leg imaginal discs, and both confer detectable phenotypes there as described above. Mitotic clones of homozygous *pap^{-/-}* or *Scr^{-/-}* cells were induced in first-

and second-instar larvae and identified in mature third-instar imaginal discs by the cell autonomous GFP marker and by the accumulation of SCR or PAP proteins examined in these cells of known genotype. Representative results for SCR protein accumulation are shown for *pap^{-/-}* clones in the labial (Fig. 4e,f) and T1 leg (Fig. 4g,h) imaginal discs. No change in SCR accumulation was detected in *pap^{-/-}* cells compared with neighboring wild-

Table 2. dMED mutations and adult labial segment development

Genotype	Sex comb teeth (%)	Claws (%)
<i>pb⁴/pb⁵</i>	6	0
<i>pb⁴/pb⁵ pap¹</i>	18	41
<i>pb⁴/pb⁵ dTRAP80¹</i>	1	1
<i>pb⁴/pb⁵ pap¹ dTRAP80¹</i>	53	62

Note. Counts ($n > 100$ in all cases) refer to half labia (derived from a single imaginal disc).

type cells in either tissue. These results obtained in homozygous *pap*⁻ cells confirm that *Scr* gene expression, as measured by accumulation of the nuclear homeodomain protein, is not detectably affected by altered *pap* function in these tissues. Conversely, PAP protein accumulation was unchanged in *Scr*^{-/-} cells (not shown), indicating that *pap* transcription is likewise independent of *Scr* function in these tissues. These reciprocal experiments, coupled with the results in heterozygotes described above, provide molecular evidence that the *pap* and *dTRAP80* loci act in parallel with homeotic *Scr* function in distal sex comb and labial identity specification.

Discussion

The MED complexes are believed to serve as adapting interfaces between regulatory proteins bound to specific DNA sequences and the RNA Pol II that executes this input. Overall complex architecture and physical interactions with RNA Pol II appear relatively unchanged between yeast and mammals (Asturias et al. 1999). In this work, we found a single fly homolog for each of 23 known human MED proteins. The presence of so many detectable fly homologs conforms with the observed conservation of overall complex structure between yeast and mammals and clearly supports the existence of a fly complex. The synergistic interactions detected in tests of double heterozygous mutant combinations for *pap* and *dTRAP80* loss-of-function mutations revealed a shared developmental function. The most direct interpretation incorporating these two complementary lines of evidence is that these proteins act in vivo within the shared confines of a fly mediator complex, similar to those detected in worms and mammals.

Whether *pap* and *dTRAP80* act within a mediator complex remains unproven, and elucidating this point must await the biochemical demonstration of a fly complex containing both proteins. Still, the presence of so many recognizable homologs clearly suggests the existence of a metazoan complex, leading us to consider here the developmental and evolutionary implications of this working hypothesis.

The metazoan complex is likely to act as a motor force for evolving genetic programs: overall complex architecture might reflect constraints imposed by interaction with RNA Pol II, while even limited modifications of subunit sequences could alter contacts with specific transcription factors that amplify the modification. Speciation and morphological divergence reflect changes in gene expression programs. Clarifying how these MED protein functions modulate transcription should shed light on the constraints limiting or favoring evolutionary change. One of the two *Drosophila* genes isolated here, *dTRAP80*, encodes the apparent fly homolog of the prototypical yeast complex member Srb4. In contrast, *pap/dTRAP240* does not possess a detectable yeast equivalent. One intriguing possibility is that the *pap* gene may reflect a metazoan-specific MED protein, conceivably replacing a yeast protein of analogous function. The overall number of protein components of yeast and mammals

appears similar, and some subunits of the yeast Srb complex are present in human MED as well. However, others such as *pap* are detectable in nematodes, flies, and mammals but apparently absent in yeast. It is not yet clear whether truly species-specific MED subunits exist. Still, the emergence or exchange of additional metazoan-specific subunits, as well as mixing and matching among various modular subcomplexes such as those detected in yeast (Lee and Kim 1998; Lee et al. 1999), may have been an important element contributing to the diversification of genetic programs in complex organisms.

Ample evidence indicates that yeast Srb complex subunits have diversified functions (Holstege et al. 1998). Some subunits such as Srb4 are required for transcription of nearly all yeast genes, whereas others are specific for discrete, largely nonoverlapping gene subsets. Information concerning the in vivo physiological roles of the metazoan MED complexes and their subunits remains sparse. A knock-out mutation of the ubiquitously expressed mouse *Srb7* gene suggests a requirement for cell viability (Tudor et al. 1999). Similarly, our clonal analysis indicates a requirement of *dTRAP80* for cell viability in *Drosophila*, in agreement with a general role in transcription. In marked contrast, disruption of murine *TRAP220* shows that this gene is essential for normal development, but lethality is associated with a complex variety of defects in several tissues and is not cell lethal (Ito et al. 2000). Similarly, the analysis of *pap* activity suggests a much more specific and restricted role for this gene. No effect on larval cell proliferation was detected, and the tissue-specific consequences of *pap* mutations for adult cells range from no effect to cell or segment identity transformations.

The large size of known complexes, and the functional diversity observed for different yeast Srb subunits (Holstege et al. 1998), suggest their aptitude to receive and integrate multiple cellular inputs. A previous study of the *C. elegans sur-2* gene focused on its role as a genetic suppressor of a Ras/MAP kinase pathway in vulval development, indicating a functional connection between cell signaling and mediator function (Singh and Han 1995). The human TRAP and DRIP complexes were identified by their biochemical interactions with thyroid and vitamin D₃ nuclear hormone receptors, and a different subunit, human TRAP220, mediates ligand-specific complex binding to several nuclear receptors (Yuan et al. 1998). The heightened affinity of this specific ligand-bound complex and its enhancement of in vitro transcription, in contrast to the nuclear receptor-TRAP220 couple, seems likely to represent a recurrent theme for MED utilization in the dynamic expression of genetic programs during development.

This diversity underlines the need for detailed in vivo elucidation of the roles of different mediator proteins. Are all MED protein functions effected within the framework of a single complex, or can these proteins also participate in other biochemical roles? The clonal analysis for *pap* indicates a marked discordance between spatial gene expression and gene activity. These observations may represent evidence for positional information inter-

puted through a mediator complex, which is in accord with a previous proposal that MED complexes may act as a sort of molecular control panel (Hampsey and Reinberg 1999). It will be important to better understand the rules of *in vivo* mediator protein function, notably in transcriptional initiation where potential control points include complex assembly, nuclear localization of subunits or complexes, and posttranslational modifications such as phosphorylation by protein kinases (Singh and Han 1995; Jiang et al. 1998; Boyer et al. 1999) as elements permitting the rapid integration of highly specific spatial information from the cellular environment.

Most descriptions ascribe a coactivator role to MED function. Interestingly, though, observations *in vitro* have also identified specific subcomplexes that serve as transcriptional corepressors (Song and Carlson 1998; Sun et al. 1998; Balciunas et al. 1999). Our genetic tests identify *pap* as a functional antagonist of *Scr*, whereas tests of molecular epistasis indicate that these genes act in parallel. The observed antagonism with *Scr* could result indirectly, reflecting the complex circuitry of the genetic program. However, dose-sensitive synergistic functional interactions such as those detected here may also indicate molecular interactions—which are direct or nearly so—through the intermediary of an as yet unidentified homeotic cofactor. *Scr* is expressed and required throughout the T1 and labial tissues. However, this requirement is not uniform: SCR-expressing cells give rise to the diverse cell types composing an adult segment. The antagonistic effect of *pap* on *Scr* function described here is position-specific within the T1 leg, apparently limited to cells yielding the distal sex comb. These apparently paradoxical observations might reflect a crucial role of the MED complex in integrating diverse inputs. In this manner, the localized effect that appears as a negative regulation of *Scr* might reflect rather the de-repression of a target gene coregulated by *Scr* with other specific factors. A MED function contributing to a combinatorial output that integrates homeotic input with those of other specific transcription factors would be a coherent role for segmental differentiation.

In a study of the roles of Srb subunits in the yeast gene expression program, extensive functional diversity was observed among the subunits (Holstege et al. 1998). In the present initial analysis of two MED genes in *Drosophila*, functional overlap but also clear differences were noted between *dTRAP80* and *pap*. The identification of the *dMED* genes should now facilitate the identification of their mutations toward the detailed *in vivo* characterization necessary for comprehending how the diversity of individual subunit functions can be integrated in a working machine with an important developmental role. The convergence of data from studies of related complexes in yeast, mammals, *C. elegans*, and now *Drosophila* should soon afford a clearer picture of how mediators mediate transcriptional regulation throughout development and evolution.

Materials and methods

Fly techniques

Standard *Drosophila* culture media and culture methods were employed.

Isolation of *pap* mutations

The HSPB transgenic element induces ectopic distal sex combs at low frequency. Among 5000 new autosomal insertions generated by mobilization of an X-linked P-lacW element (<http://flybase.bio.indiana.edu/>), the *pap*¹ allele (for *poils aux pattes*, “hairy legs”) enhanced distal sex comb frequency in combination with the HSPB:4d line. Phenotypic revertants and imprecise excisions were generated by remobilizing the *pap*¹ insertion element with $\Delta 2$ -3 transposase from a stable $\Delta 2$ -3 chromosomal insertion (<http://flybase.bio.indiana.edu/>).

In situ hybridization

Antisense probes were generated by *in vitro* transcription from linearized plasmids, using T3 or T7 RNA polymerases in the presence of digoxigenin-UTP (Boehringer Mannheim). Probe specificity was verified by hybridization to embryos or larvae with localized overexpression of the corresponding mRNA (engrailed-GAL4/UAS-*pap* or UAS-TRAP80).

Identification of the dTRAP80¹ allele

This allele, previously referred to as l(3)s2956 (Spradling et al. 1999), was identified by adjacent sequences located within *dTRAP80* coding sequences.

Clonal analysis

Mitotic recombination experiments were performed using the FLP/FRT recombination system (Xu and Rubin 1993). To examine *pap* function, we employed the strong or null *pap*⁵³ allele that deletes the putative initiator ATG. For adult effects, *y w* hsFLP; *mwh jv pap*⁵³ FRT-2A/TM3, *Sb* females were crossed with *y w*; Dp(1,3)sc⁸⁴ *y*⁺ M(3)i⁵⁵ FRT-2A/TM3, *Sb* males. FLP recombinase was induced by heat shock in first- or second-instar larvae (1 h, 37°C). *Sb*⁺ adults were examined for *pap*[−] *Minute*⁺ clones, using the associated cell-autonomous markers *y*, *mwh*, and *jv*. For twin spot analysis, *pap*[−] clones were induced and examined in imaginal discs using the cell autonomous GFP marker expressed from the *ubiquitin-63E* (UB) promoter (Lee et al. 1988). For these experiments, *y w* hsFLP; *mwh jv pap*⁵³ FRT-2A/TM6B, *Hu Tb* females were crossed with *w*; UB-GFP FRT-2A males. For analysis of *Scr* mutant clones, we employed a chromosome carrying the FRT-82B insertion and the null *Scr*¹ allele (kindly provided by A. Percival-Smith) and a chromosome carrying FRT-82B with UB-GFP (3R; obtained from the Indiana University *Drosophila* Stock Center). Clones were induced by heat shock (1 h, 37°C) during first or second instar, and discs prepared from late L3 larvae. Fixation was for 20 min, 22°C with 4% paraformaldehyde. GFP expression was analyzed by confocal microscopy.

DNA techniques

Standard techniques were employed for cloning, blotting, and sequence analyses.

Cloning and structure of *pap*

A 9.5-kb *EcoRI* genomic DNA fragment 3' to the *pap*¹ insertion site cloned by plasmid rescue was used to initiate the isolation

of 50 kb of genomic DNA encompassing *pap* and to probe cDNA libraries. The intron–exon structure of *pap/dTRAP240* was determined by comparison of genomic and cDNA sequences. For transgenic rescue, a *pap* minigene was constructed containing the ubiquitin promoter and a fusion of *pap* cDNA (the 5' exons; see Fig. 1a) and genomic (3' exons) sequences, and transgenic lines were established following microinjection according to standard methods (Cribbs et al. 1995). GenBank database searches used the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Protein sequence alignments were performed using Clustal W software. Details are available on request.

Structure of dTRAP80

The intron–exon structure of *dTRAP80* was determined by comparison of genomic and cDNA (clone SD10038 from BDGP) sequences. Genbank data base searches and protein sequence alignments were as above.

Localization of dMED homologs

Listed below are the identified *Drosophila* genes corresponding to known human mediator proteins (see Fig. 2a), followed by their GadFly identifiers: *dARC105* (CG4184); *dTRAP26/TRFP* (CG18267); *dARC32* (CG13867); *dTRAP95* (CG5465); *dTRAP150B/SUR2* (CG3695); *dTRAP170/RGR1* (CG12031); *dTRAP36* (CG8609); *dTRAP100* (CG7999); *dTRAP56/CDK8* (CG10572); *dTRAP15/NUT2* (CG5057); *dTRAP230* (CG8491); *pap/dTRAP240* (CG9936); *dTRAP220* (CG7162); *dTRAP18/SOH1* (CG1057); *dTRAP37* (CG1245); *dTRAP32/MED6* (CG9473); *dTRAP34/MED7* (CG6529); *dTRAP33/cycC/SRB11* (CG7281); *dTRAP80/SRB4* (CG7957); *dARC42* (CG4703); *dARC92* (CG12254). *dARC70* (CG1793) is situated on the small chromosome IV (not shown), and *dTRAP19/SRB7* (CG17397) could not yet be mapped.

Generation of anti-PAP antiserum

Recombinant hexahistidine-Pap fusion protein including the first 366 amino acid residues of Pap protein was used to immunize rabbits (Eurogentec SA). Recombinant GST–Pap fusion protein blotted to a nitrocellulose filter permitted affinity purification of anti-Pap antisera. Immunostainings employed commercially available reagents. Details are available on request.

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***Drosophila* homologs of transcriptional mediator complex subunits are required for adult cell and segment identity specification**

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