

# *Drosophila* Mob Family Proteins Interact with the Related Tricornered (Trc) and Warts (Wts) Kinases

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The function of Tricornered (Trc), the *Drosophila* Ndr (Nuclear Dbf2-related) serine/threonine protein kinase, is required for the normal morphogenesis of a variety of polarized outgrowths including epidermal hairs, bristles, arista laterals, and dendrites. In yeast the Trc homolog Cbk1 needs to bind Mob2 to activate the RAM pathway. In this report, we provide genetic and biochemical data that *Drosophila* Trc also interacts with and is activated by *Drosophila* Dmob proteins. In addition, *Drosophila* Mob proteins appear to interact with the related Warts/Lats kinase, which functions as a tumor suppressor in flies and mammals. Interestingly, the overgrowth tumor phenotype that results from mutations in *Dmob1* (*mats*) was only seen in genetic mosaics and not when the entire animal was mutant. We conclude that unlike in yeast, in *Drosophila* individual Mob proteins interact with multiple kinases and that individual NDR family kinases interact with multiple Mob proteins. We further provide evidence that *Mo25*, the *Drosophila* homolog of the RAM pathway *hym1* gene does not function along with Trc.

## INTRODUCTION

*tricornered* (*trc*) encodes the *Drosophila* Ndr protein kinase (Nuclear Dbf2 related; Geng *et al.*, 2000). The Ndr kinases are members of a subfamily of serine/threonine kinases that includes Sax1 (*Caenorhabditis elegans*), Cbk1 (*Saccharomyces cerevisiae*), Dbf2 (*S. cerevisiae*), Warts/Lats (*Drosophila*), Orb6 (*Schizosaccharomyces pombe*) and Cot-1 (*Neurospora*), which regulate cell growth, cell division and cell morphology (Yarden *et al.*, 1992; Justice *et al.*, 1995; Xu *et al.*, 1995; Verde *et al.*, 1998; Zallen *et al.*, 2000). In *S. cerevisiae* Cbk1 and Dbf2/Dbf20 play central roles in the RAM (regulation of *AceII* activity and cellular morphogenesis) and MEN (mitotic exit network) pathways (Mah *et al.*, 2001; Nelson *et al.*, 2003). Mutants of Cbk1 or other RAM pathway genes including its binding partners Mob2 and Tao3 (Pag1) fail to activate the *AceII* transcription factor in daughter cells and result in rounder than normal cells due to a defect in axial growth of the bud (Colman-Lerner *et al.*, 2001; Du and Novick, 2002; Nelson *et al.*, 2003). Little is known about the *in vivo* function of the two human Ndr genes but extensive study of their biochemical characteristics has been carried out for nearly 10 years (Millward *et al.*, 1995; Stegert *et al.*, 2004).

*trc* is the Sole Ndr Gene in *Drosophila*. The function of *trc* and its partner *furry* (*fry*) is required for the development of epidermal hairs, sensory bristles, arista laterals, and dendrite arborization (*da*) sensory neuron dendrites (Geng *et al.*, 2000; Cong *et al.*, 2001; He and Adler, 2001; Emoto *et al.*, 2004). The morphogenesis of these cell extensions involves the regulated activation of both the actin and microtubule cytoskeletons (Fristrom *et al.*, 1993; Wong and Adler, 1993; Verheyen and Cooley, 1994; Eaton *et al.*, 1996; Hopmann *et al.*, 1996; Turner and Adler, 1998; Tilney *et al.*, 2000; Geng *et al.*, 2000; He and Adler, 2001). Mutations in *trc* and *fry* result in split and multiplied hairs and laterals, split and deformed bristles and dendrites with extra branching and tiling defects (Geng *et al.*, 2000; Cong *et al.*, 2001; Emoto *et al.*, 2004).

The single *warts/large tumor suppressor* (*wts/lats*) gene is the *Drosophila* kinase most closely related to *trc* (45% identical and 65% similar over 418 amino acids). Once again there are two *wts* homologues in mammals. There is no *wts* ortholog in yeast. *wts* was first identified in *Drosophila* as a tumor suppressor (Justice *et al.*, 1995; Xu *et al.*, 1995). Homozygous *wts/lats* mutant cells display defects in morphogenesis (such as deformed bristles and altered cuticle morphology) and extensive overgrowths (Justice *et al.*, 1995; Xu *et al.*, 1995).

Ndr, like many kinases is regulated by phosphorylation. The phosphorylation of the activation segment site Ser-281 and the hydrophobic motif site Thr-444 of Ndr increase Ndr kinase activity *in vitro* (Millward *et al.*, 1999). Ser-281 phosphorylation is thought to be due to autophosphorylation, whereas Thr-444 is targeted by an as yet unidentified upstream kinase (Tamaskovic *et al.*, 2003; Stegert *et al.*, 2004). These sites are also important regulatory sites for Trc function in the *Drosophila* epidermis and nervous system. The mutation of these sites in *trc* to ala resulted in dominant negative proteins (Emoto *et al.*, 2004; He *et al.*, 2005).

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Several Ndr family kinases have been shown to function with members of the Furry protein family, which consists of large conserved proteins that lack informative motifs. The first member of this family to be characterized was the *Drosophila fry* gene (Cong *et al.*, 2001). Both genetic and biochemical experiments have shown that in flies *trc* and *fry* function in a common process, are present together in a complex and that Fry is required for Trc kinase activity (Cong *et al.*, 2001; Emoto *et al.*, 2004; He *et al.*, 2005). Mutations in both result in similar phenotypes in both the epidermis and sensory neurons. In addition, the subcellular localization/accumulation of Trc and Fry is interdependent in pupal wing cells (He *et al.*, 2005). The subcellular localization of Cbk1 and Tao3 in *S. cerevisiae* and Orb6 and Mor2 in *S. pombe* (Du and Novick, 2002; Hirata *et al.*, 2002; Nelson *et al.*, 2003) has also been found to be interdependent, although the relationships differ in these systems (Tao3 and Mor2 are the Fry homologues in these systems).

The Trc and Furry family proteins appear to be conserved both in terms of sequence and function in a wide range of eukaryotes. This suggests that homologues of other members of the RAM pathway in *S. cerevisiae* will also play similar roles in higher eukaryotes. The Mob2 protein of yeast has been shown to bind to Cbk1 and be essential for Cbk1 kinase activity (Weiss *et al.*, 2002). In vivo Mob2 is required along with Cbk1 for both mother/daughter separation after cytokinesis and the maintenance of polarized cell growth (Weiss *et al.*, 2002). Furthermore, Mob2 and Cbk1 show interdependent localization (Nelson *et al.*, 2003). A similar situation exists for the related Dbf2 kinase, which is a component of the mitotic exit network (MEN). Dbf2 binds to Mob1 (which is related to Mob2) and this complex is essential for activity (Mah *et al.*, 2001). Similarly, *S. pombe* Mob2 interacts physically with the Orb6 protein kinase and is required for Orb6 function in the coordination of cell polarity with the cell cycle (Hou *et al.*, 2003). Multicellular organisms possess multiple *mob* genes. Recently, it was shown that a basic sequence within the insert in the catalytic domain of Ndr has an autoinhibitory function and that Human Mob1 may stimulate Ndr activity by releasing the autoinhibitory effect of this sequence (Bichsel *et al.*, 2004; Devroe *et al.*, 2004).

There are 4 *Drosophila* genes related to the yeast *mob* genes. Evidence for a two-hybrid interaction between CG13852 (Dmob1/mats) and Trc was described in a genome scale experiment; however, no evidence for such interactions were seen between Trc and any of the other *Drosophila* Mobs (Giot *et al.*, 2003). Nor was there any indication in that paper that any of the *Drosophila* Mobs function with the related Warts/Lats kinase. In this article we provide evidence that Mob1 interacts with both *trc* and *wts* and that at least one additional member of the *Dmob* gene family CG11711 (*Dmob2*) can interact with *trc* and *wts*. While this paper was in revision Lai *et al.* (2005) reported that CG13852 interacted with and activated Wts. They named CG13852 *mats*, and we will follow their lead and use that name.

## MATERIALS AND METHODS

### Plasmid Constructs

**Mutagenesis.** For the *Dmob2* mutants generation, the complete *Dmob2* EST clone: RE70633 was used as the template. The following oligonucleotides were used to introduce amino acids substitution with the QuickChange Multiplesites Mutagenesis kit (Stratagene, La Jolla, CA): C161W: TGAATGCTAGCGTCACACCGCCTCGCTCTATTG; C179L: CTATGGAATATTAGTGAATTTTGACACAATCCGGTTGCGCTGAC; E151K: CCAGCTGGT-

CCGGACTACAATGAATGGCCAGAGTCACACACC; H261Q: CATGTGATAGCGCAACTGTATGCGGCG; I15V: CCGGCGCAATCGATTAGCCGTGCGATC; N280S: CCGCTTGCACCCATCTAACGCTGACTTTCGCGCACCTC; N65I: CCTTCATGCCTCTGGATCACATTCGCGGACGC; Q167R: CCTCGCTCTATTGAGCAAGTGAATTTGGTCTATGG; T85P: GCTAGTGGGGACCAGGTGGCAGG; Y193H: GACCGGACCCGCAACGAACAGTACCATGGTTCGACGAGAAGG.

All the *Dmob2* constructs were subcloned into pGADT7 to test their interaction with *trcp*GBKT7 using *NdeI* and *EcoRI* restriction enzyme sites. The primers used were RE70633*NdeI*: sense: GGCCTTCCATATGAACTGGGCATTTC and RE70633*EcoRI*-antisense: GTGGGAATTCCTACCCCTGTTCATTTC.

***trc* Constructs Subcloning.** Full-length of *trc* cDNA was subcloned into pG-BKT7 from *NdeI*-*Bgl*III. The following primers were used: *trc*-5'-*NdeI*: GGGAATTCATATGCACCATCACCATC; *trc*-3'-*EcoRI*: CCGGAATTCCTACTCCAAATTTTCGCAC. To get truncated forms of *trc* cDNA, we used the same *trc*-5'-*NdeI* primer and the following 3' primers (each containing *EcoRI* site): *trc*D1: CCGGAATTCCTAGTCTACGATGTGCTCCAGTC; *trc*D2: CCGGAATTCCTAGGATTGTCGAGCAGAATGG; *trc*D3: CCGGAATTCCTAGGCACGTCCGAAGTCCGGAGAG; *trc*D4: CCGGAATTCCTAGCGTCATCATATCACCACCAGG; *trc*D5: CCGGAATTCCTAGGTACACATGTCCCGTG; *trc*D6: CCGGAATTCCTAGGCTCTCGTCTCAGCTG.

The PCR products were subcloned into pGBKT7 from *NdeI*-*EcoRI* sites. To subclone wild-type and mutant forms of *trc* cDNA into 3xFLAGpCMV7.1, *Trc*-5'-*EcoRI*: CCAAGAATTCATGCACCATCACCATCACC; *Trc*-3'-*Bgl*III: GGAAGATCTTCACTCCAAATTTTCGCACCTCG.

***mats* and *wts* Subcloning for Two-hybrid Assays.** The *mats* cDNA clone LD47553 was used as a template and subcloned into pGADT7 with *NdeI* and *EcoRI* after PCR using the following primers: *Mats*5: GGAATTCATATGATGGACTTCTGTTCGGTTC; *Mats*3: CCGGAATTCCTATATCTGCCGCTCATC.

The *wts* cDNA clone SD19495 was used as a template and subcloned into the *NdeI* and *EcoRI* sites of pGBKT7 after PCR using the following primers: *Wts*5: GGAATTCATATGAGCAGCAGCATCATGCATCC; *Wts*3: CCGGAATTCATGATGAGCAGCAGCATCAG.

***Dmob2* and *Dmo25* Constructs Subcloning.** Dominant negative *Dmob2* and *Dmo25* constructs were produced by cloning N- or C-terminally truncated subfragments generated by PCR into pUAST. EST clones, GH01659 and GH07469, (Research Genetics, Huntsville, AL) were used as full-length cDNA templates. The primers and restriction sites used are as follows: for truncations of *Dmob2* (5'-*EcoRI* and 3'-*Bgl*III): *Dmob2*-N-5'-*EcoRI*: CCGGAATTCATGAACTGGGCCATTTC; *Dmob2*-N-3'-*Bgl*III: GGAAGATCTCTACGCCGCATACAGATGC; *Dmob2*-C-5'-*EcoRI*: CCGGAATTCATGGTGATAGCGCATC; *Dmob2*-C-3'-*Bgl*III: CGTGGAGATCTCTACCCCTGTTCATTC. For truncations of *Dmo25* (5'-*EcoRI* and 3'-*Bgl*III): *Dmo25*-N-5'-*EcoRI*: CCGGAATTCATGCCACTGTTCG; *Dmo25*-N-3'-*Bgl*III: GGAAGATCTCTACTCAGCTAGCCGGAAG.

**Tagging of *Dmob2* (CG11711).** *Dmob2* (CG11711) was tagged as its C-terminus with CFP or 8xHA by a PCR using both EST clone, GH07469, which expressed the long form of *Dmob2* (CG11711), and CFP and 8xHA complete sequence, which was amplified from the ECFPpCMV5 (Clontech, Palo Alto, CA) and 8xHAPag1-N (Provided by Novick lab) as templates. For *Dmob2* tagged with 8xHA, GH07469 was modified by insertion of *NdeI* and *Hind*III sites before the stop codon and 5'-*XhoI* and 3'-*XbaI* sites at both ends. The primers used are as follows: GH07469-C-8x HA (*NdeI*-*Hind*III)-*XbaI*-CTAGTCTAGACTAAAGCTTGAATTCATATGTCGCGTGGTGGTTCG; GH07469-C-8x HA (*NdeI*-*Hind*III)-*XhoI*-CGATTAATTTCTTCTCGAGATGGGCAAG-GCCCGTC.

The PCR product was subcloned into pBS vector. Simultaneously, *NdeI* and *Hind*III restriction sites were added at both ends of the 8xHA sequence amplified from 8xHAPag1 template and subcloned into GH07469pBS. The resulting *XhoI*-GH07469-8xHA-*XbaI* was then cut from pBS and subcloned into pUAST using *XhoI*-*XbaI* sites.

For *Dmob2* tagged with CFP, PCR products of GH07469 with 5' sequence of ECFP at 3' end and ECFP with 3' sequence at 5' end were made to be templates to get the final C-terminal tagged *Dmob2*. 5' primer of GH07469 and 3' primer of ECFP also contained a *XhoI* site and a *XbaI* site separately for subcloning into pUAST. Primers used are as follows: GH07469-C-ECFP: 5'-GATTGGCTTCTCGAGATGGCAAG; GH07469-C-ECFP: 3'-CTGTCTCAGTCCGCTGGTGGTTC; ECFPpCMV5-C: 5'-GACCACCACCGCAGTGAGCAAG; ECFPpCMV5-C: 3'-GACCCGCGCTCAGTTGGAATTC.

### Genetics

Flies were grown on standard media at 25°C. Mutant stocks were either obtained from the stock center at Indiana University, generated in Charlottesville or generously supplied by Zhi-Chun Lai (Lai *et al.*, 2005) or Peter Bryant (Justice *et al.*, 1995). Genetic interaction tests were done by individually crossing male *ap* (or *ptc*)-Gal4/+; UAS-*trc*<sup>T453A</sup> flies to virgin female flies that

carried mutations or deficiencies. Clones were generated by FLP/FRT technology (Xu and Rubin, 1993). Deficiency stocks used in the interaction and mapping experiments included the following: *Df(3L)vin5*, *Df(3L)vin4*, *Df(3L)vin2*, *Df(3L)vin66*, *Df(3L)lxd6*, *Df(3L)vin6*, *Df(3L)vin7*, *Df(3L)ED4470*, *Df(3L)ED4475* and *Df(3L)BK9* (for *Dmob2*), *Df(2R)42* and *Df(2R)ED1552* (for *CG3403*), *Df(2L)J2* and *Df(2L)Exel6026* (for *CG4946*), *Df(3R)lh* and *Df(3R)Exel6191* (for *CG13852*). The *warts (wts)/large tumor suppressor (lats)* gene is known by both names. In line with FlyBase usage, we use *warts*.

### Immunohistochemistry

Immunostaining was done using standard techniques as described previously (He *et al.*, 2005). Monoclonal anti-FLAG antibody was purchased from Sigma (St. Louis, MO). Anti-GFP polyclonal antibody was purchased from Molecular Probes (Eugene, OR). Secondary antibodies and labeled phalloidin were purchased from Molecular Probes. The anti-Fry antibody has been described previously (He *et al.*, 2005).

### Cell Culture and Coimmunoprecipitation

Immunoprecipitations used S2 cells and were done as described previously (Emoto *et al.*, 2004; He *et al.*, 2005).

### Microscopy and Photography

Confocal microscopy was done using either a Nikon Laser Scanning confocal microscope (Melville, NY) or an Atto CARV spinning disk confocal attached to a Nikon microscope. Bright field images of wings were obtained using a Spot digital camera (National Diagnostics, Manville, NJ) on a Zeiss Axioskop microscope (Thornwood, NY). Adobe Photoshop (San Jose, CA) was used to compose figures.

### Scoring of Mutant Wings

Wings were mounted in Euparal (Asco Labs, Manchester, England) and examined under bright-field microscopy using approached described previously (Wong and Adler, 1993). Because the wing is not effected by the mutants to a uniform level throughout the whole region, the same area within the C region ( $5 \times 20$  cells) was chosen for scoring the number of hair per wing cell for *ap-GAL4*- or *ptc-GAL4*-driven UAS-mutants.

### Scoring of Denticle Phenotypes

We counted the number of split denticles in segments 3–7 of second instar larvae. We used second instar larvae for quantifying the phenotypes because some genotypes of interest yielded very few third instar larvae. The number of denticles varies in different segments, so we only counted larvae in which we could count all of the relevant segments. We found only small differences (nonsignificant) in the number of denticles between most of the relevant genotypes. The one exception was *trc<sup>P</sup> mats<sup>PB</sup>*, which contained ~15% fewer denticles per segment. To correct for the effect of this on the number of split denticles we did the following normalization. We determined the average number of denticles in abdominal segment 6 for each genotype and normalized the number of split denticles for each by dividing the number observed by the ratio of the number of denticles in the relevant genotype to Oregon R. Strong *wts* mutants died before the second instar, so we could not compare denticle phenotype to that of the other mutants.

### Yeast Two-hybrid Assay

For the yeast two-hybrid screen, we used *trc* wild-type full-length cDNA and dominant negative form (*trc<sup>T453A</sup>*) as a bait subcloned into the Matchmaker GAL4 Two-Hybrid System 3 vector pGBKT7 (Clontech). This was used to screen a cDNA library made from RNA of 0–21h old *Drosophila* embryos (Clontech). For the analysis of interaction between defined protein partners, yeast strain AH109 was cotransformed with two-hybrid plasmids (pGBKT7 carrying a DBD fusion and a *TRP1* marker, and pGADT7 carrying an AD fusion and a *LEU2* marker). We followed the directions provided by Clontech. In these experiments we scored three reporter genes (*ADE2*, *HIS3*, and *MEL1/lacZ*), assessing both growth on dropout medium (SD/-Ade/-His/-Leu/-Trp) and the  $\alpha$ -galactosidase/ $\beta$ -galactosidase activity. A positive test for all was required for us to score two proteins as interacting.

### Real Time PCR

We used real time RT-PCR to quantify the amount of Mo25 mRNA. RNA was isolated from either Oregon-R or Dmo25<sup>P</sup>/Df using the Trizol reagent. Ten- to 20-s instar larvae were ground in a 1.5-ml microfuge tube after freezing in liquid nitrogen. Trizol reagent was added and mixed, and linear acrylamide was added followed by a chloroform extraction and isopropanol precipitation. Before reverse transcriptase the sample was treated with DNase I. We used Oligo dT priming for RT using AMV reverse transcriptase (Roche, Indianapolis, IN). The real time PCR was carried out in a Cepheid Smart Cycler system SC-1600 (Sunnyvale, CA). For Dmo25 we used the following primers: Dmo25-rp: forward: ATGCCACTGTTCGGGAAGTC; Dmo25-rp: reverse: GCAGCTTCAAGCTCTGACGC.

We used rp49 as a control for RNA content. For rp49 amplification we used the following primers: rp49: forward: AAGATCGTGAAGCGCCACCAA; rp49: reverse: CTGTTGTCGGATACCCCTGGGGCTT.

## RESULTS

### Four mob Genes Are Found in *Drosophila*

BLAST searches identified 4 *Drosophila* genes that shared similarity to the yeast *mob1* and *mob2* genes: *CG13852 (mats or Dmob1)*, *CG11711 (Dmob2)*, *CG4946 (Dmob3)*, and *CG3403 (Dmob4)*. The BLAST analysis and additional clustering analyses did not allow us to identify one or two of the fly genes as being clear orthologues of either yeast *mob1* or yeast *mob2*. *CG13852* and *CG4946* are the two most closely related to both yeast genes, and *CG3403* is the most distantly related. Gene chip analysis of pupal wing RNA showed all four of the fly *mob* genes are expressed in the pupal wing before, during, and after hair morphogenesis (Adler, unpublished results).

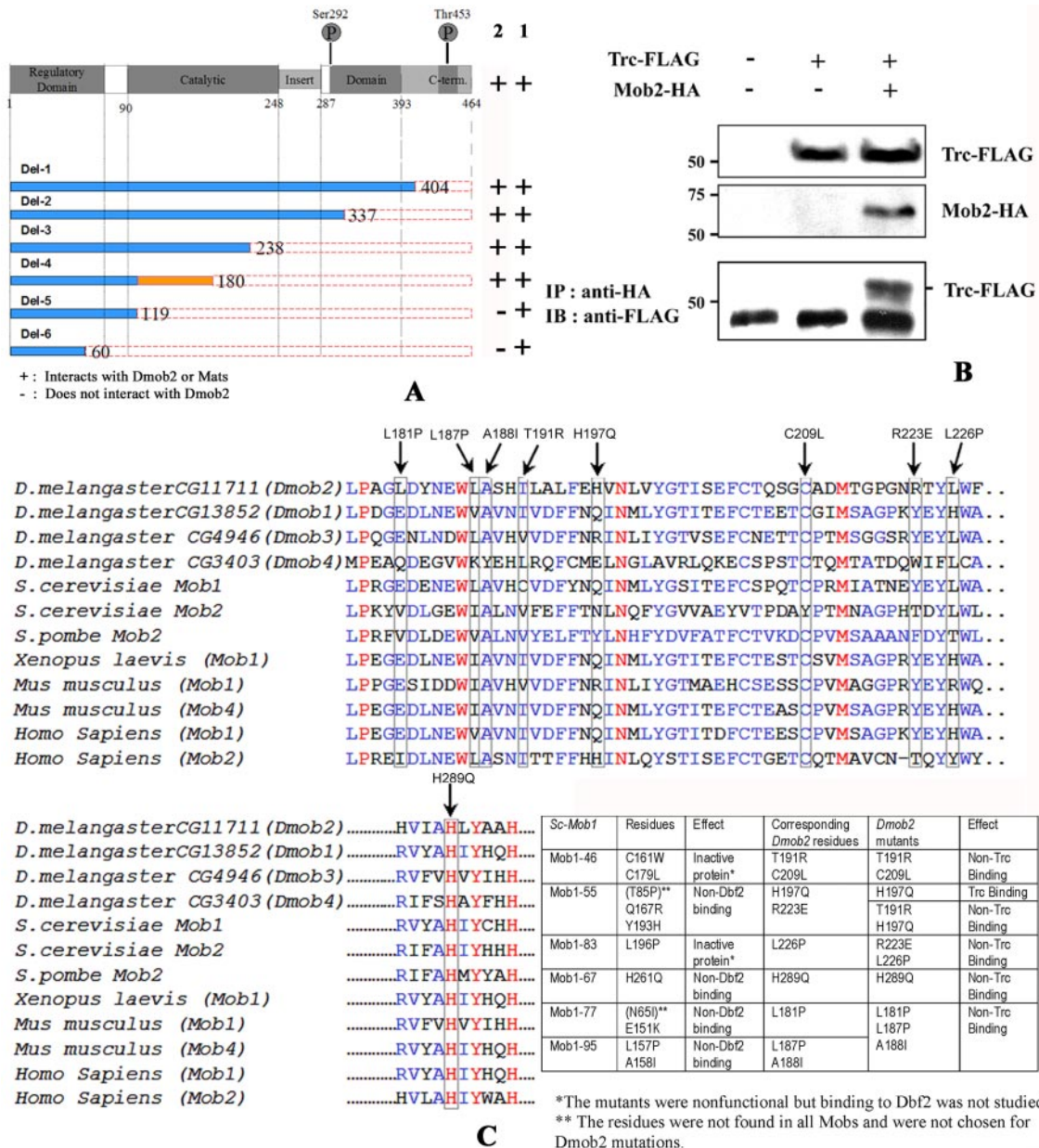
### Evidence of Physical Interaction between Trc and Dmob2

The *S. cerevisiae* Cbk1 and Mob2 proteins are known to interact physically as do the *S. pombe* homologues (Orb6 and Mob2; Weiss *et al.*, 2002; Hou *et al.*, 2003). In addition the related Dbf2 and Mob1 proteins of *S. cerevisiae* also interact physically (Komarnitsky *et al.*, 1998). Thus, we expected that Trc and at least one of the *Drosophila* Mobs would interact physically. Indeed, Trc and Dmob1 (*CG13582*) were identified as interacting proteins in a genome scale two-hybrid screen (Giot *et al.*, 2003). None of the other Dmobs were reported as being able to interact with Trc, and no Dmob was reported as interacting with Wts in that study (Giot *et al.*, 2003), although a recent article demonstrated an interaction between Wts and Mats (*Dmob1*; Lai *et al.*, 2005).

We performed a yeast two-hybrid screen of a *Drosophila* cDNA library using full-length *trc* cDNA as "bait." Most of the clear positive clones recovered contained fusions of segments of Dmob2 fused to the GAL4 activation domain. We did not recover clones of any of the other Dmobs. Perhaps they were not present in the library screened. To determine whether Mats and Trc interacted in the yeast two-hybrid system, we obtained a cDNA clone for *mats* from the BDGP collection and subcloned it into pGADT7. We used this plasmid and confirmed that Trc and Mats interacted in the yeast-two-hybrid system. Thus, Trc appears to be able to interact with at least two different Mob family proteins in *Drosophila*. We similarly tested and confirmed that Wts was able to interact with both Mats and Dmob2 in the two hybrid system. Thus, we did not see evidence of specificity in the *Drosophila* NDR/Mob family interactions.

To determine what portion of the Trc protein interacted with Dmobs, we generated a set of plasmids that contained *trc* C-terminal truncations and assayed them for an interaction with Dmob2 and Mats using the two-hybrid system (Figure 1A). We obtained similar but not identical results with these two mob family members. All of the Trc deleted proteins interacted strongly with Mats and the larger Trc proteins interacted strongly with Dmob2. However, Trc proteins that only contained amino acids 1–60 or 1–119 interacted quite weakly with Dmob2 (slow growth on his<sup>-</sup> ad<sup>-</sup> plates and no blue color in the presence of Xgal or X- $\alpha$ Gal). The data for the binding of human Ndr1 to hMob1 indicates that important residues are found in the amino terminal region of Ndr1 (Bichsel *et al.*, 2004). In hNdr, Tyr-31, Arg-41, Thr-74, and Arg-78 were found to be absolutely required for interaction, whereas the Lys-24, Arg-44, and Leu-79 mutants displayed reduced interaction (Bichsel *et al.*, 2004). The cor-



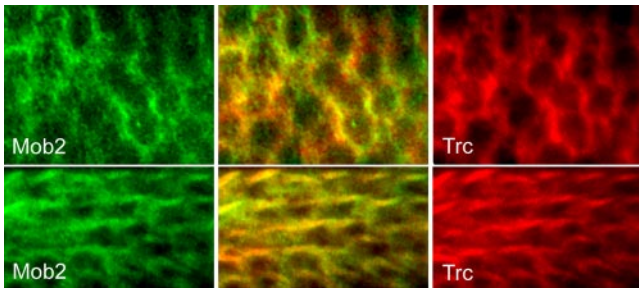


**Figure 1.** Trc physically interacts with Dmob2. (A) A diagram of the Trc protein showing important landmarks and the set of truncated proteins tested for an interaction with Dmob2 in the yeast two-hybrid system. The truncated genes were subcloned in pGBKT7 vector and tested for interactions with Dmob2 fused to the GAL4 transcriptional activation domain. The results are shown as “+” (positive) or “-” (negative) using the stringent criteria described in *Materials and Methods*. The amino acids from 119 to 180 of Trc are important for a strong Trc-Dmob2-interaction as the complementation was quite weak. These experiments showed that neither an intact nor functional Trc kinase domain was essential for the interaction with Dmob2. (B) Trc and Dmob2 coimmunoprecipitate in an S2 cell lysate. Cells were transfected with either UAS-Flag-Trc and/or Dmob2-HA. The top panels show the results from Western blot analysis of the lysate. The bottom panel shows that immunoprecipitation with an anti-HA antibody brought down Flag-Trc protein. The lower band is likely IgG and serves as a loading control. (C) Dmob2 point mutations block its interaction with Trc. Sequence alignment shows the conservation of the Mob family proteins in yeast, frog, fly, mice, and human. The red colored residues are sites that are absolutely conserved, and the blue sites conserved in a majority of the proteins. Arrows indicate the residues mutated and tested. The results from our experiments and the related results from *S. cerevisiae mob1* are shown in the table.

responding residues in Trc are Tyr35, Arg45, Thr78, Arg81, Lys28, Arg48, and Leu82. Hence our data argue that for Mats binding to Trc requires only a subset of the residues needed in human Ndr1, whereas Dmob2 binding is enhanced by additional residues. The significance of these differences is not clear, but each of these examples is consistent with the amino terminal region of the NDR kinase

family members being essential for the interaction with Mob family members.

Because the protein kinase domain of Trc extends from residue 90–393, these results indicate that the kinase domain does not have to be intact for Trc to interact with Dmob2 or Mats. We further found mutations in the conserved regulatory phosphorylation sites, S292A+T453A did not interfere



**Figure 2.** Trc colocalizes with Dmob2 to the cell periphery in wing cells and wing hairs. Confocal images of *ptc*-GAL4; UAS-Flag-*trc*<sup>WT</sup>/UAS-*Dmob2*-CFP pupal wings are shown. Trc (anti-Flag) is shown in red and Dmob2 (anti-CFP) is shown in green. Merged images are also present. The top panels are of a wing before wing hair morphogenesis (~26 h apf) and the bottom panels of a wing during hair morphogenesis (34 h apf). Note that in the top panels the proteins preferentially accumulated together at the cell periphery and later in development they both accumulated in the hairs.

with the interaction. Thus it appears clear that the kinase activity of Trc is not important for its ability to bind to Dmob2. Our results were similar to those seen between kinase inactive Dbf2 and Mob1 (Komarnitsky *et al.*, 1998).

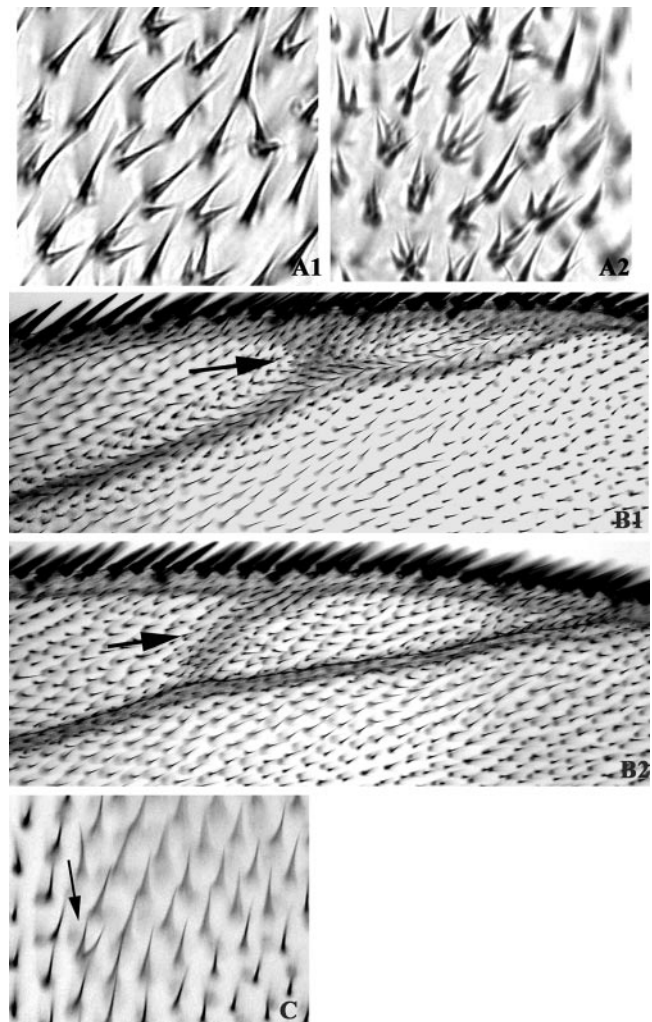
#### Mutations in Dmob2 Interrupt the Interaction with Trc

In *S. cerevisiae* Mob1 a number of sites have been identified as being important for the binding of Mob1 and Cbk1 (Luca and Winey, 1998). To test whether these sites are functionally conserved within the Dmob family, we aligned the sequence of the four *Drosophila* Mobs with yeast, human, and frog to identify the Dmob2 amino acids that corresponded to the important sites in yeast Mob1 for interaction with Dbf2. We generated similar mutants in Dmob2 (RE70633) to confirm the conservation of the Mob-Ndr interaction (Figure 1C). Most of these mutations in Dmob2 disrupted the binding to Trc (Figure 1C), consistent with the conclusion that the mechanism of interaction has been conserved. Most of the residues noted above are also conserved in Mats, Dmob3, and Dmob4, consistent with all Mob family members interacting with Ndr family members in the same manner.

#### Trc and Mob Interact In Vivo

To assess whether these proteins were capable of associating in vivo in *Drosophila* cells, immunoprecipitation experiments were carried out in *Drosophila* S2 cells expressing both Trc and Dmob2. We found Trc in anti-Dmob2-8x HA, but not in control, immunoprecipitates (Figure 1B), consistent with these two proteins interacting in vivo.

As an additional test of these proteins interacting in vivo we examined the subcellular localization of Trc and Dmob2 protein in wing cells. Trc distribution was examined with an anti-FLAG monoclonal antibody (Sigma) using UAS-*trc*<sup>WT</sup> and UAS-*trc*<sup>DN</sup> transgenes driven by *ptc*-GAL4. The proteins encoded by these transgenes carry an amino terminal FLAG epitope. We found that the FLAG staining pattern of overexpressed Trc was the same as the endogenous Trc detected by anti-Trc antibody staining (He *et al.*, 2005). We localized Dmob2 in a similar way using a CFP tag because an anti-Dmob2 antibody was not available. Confocal microscopy demonstrated that before hair formation Trc was cytoplasmic and concentrated at the cell periphery (Figure 2). During hair outgrowth Trc accumulated in the hair, as is the case for the endogenous Trc (He *et al.*, 2005). Both before and after



**Figure 3.** *trc* dominant negative phenotype is enhanced by a mutation in *mats* and the overexpression of a truncated Dmob2 protein caused a very weak *trc*-like phenotype. (A) Multiple hair cell phenotype of *ap*-GAL4/+; UAS-*trc*<sup>T453A</sup>/+ (A1) and *ap*-GAL4/+; UAS-UAS-*trc*<sup>T453A</sup>/*mats*<sup>PB</sup> (A2). Note the dominant enhancement in flies heterozygous for *mats*. (B) An extra vein phenotype caused by *ap*-GAL4-driven UAS-*trc*<sup>K122A</sup> (arrow) (B1) and *ap*-GAL4-driven UAS-*Dmob2*-C (arrow) (B2). (C) Overexpression of a truncated form of *Dmob2* (*ap*-gGAL4-UAS-*Dmob2*-C) induced a very weak *trc*-like dominant negative phenotype (arrow).

hair initiation Dmob-2 was localized similarly to Trc (Figure 2). The subcellular localization of these proteins was similar in flies that carried a single transgene or both UAS-*trc* and UAS-*Dmob2* transgenes (unpublished data). We concluded that Trc and Mob proteins can interact in vivo in *Drosophila* cells.

#### *mats1* Is Required for Both Trc and Wts/Lts Functions In Vivo

As shown previously the directed expression of a dominant negative Trc protein provides a sensitized system for identifying interacting genes (Figure 3A, 1 and 2; He *et al.*, 2005). Deficiencies for each of the fly *mob* genes enhanced the wing hair phenotype that resulted from driving expression of UAS-*trc*<sup>T453A</sup> using either *ap*-GAL4 or *ptc*-gal4 (Figure 3 and Table 1). The strongest enhancement was seen with deficient



**Table 1.** Genetic interactions of four *Dmobs* with a *trc* dominant negative mutant

Genotype	Number of wing hairs/cell		
	Interaction <sup>a</sup>	Average	Std Error
exp 1			
ap-GAL4/+; UAS- <i>trc</i> <sup>T453A2/+</sup>	—	1.8	0.06
ap-GAL4/+; UAS- <i>trc</i> <sup>T453A2</sup> /Df( <i>Dmob1</i> )	Enhancement	3.5	0.08
ap-GAL4/+; UAS- <i>trc</i> <sup>T453A2</sup> /D <i>mob1</i> <sup>P</sup>	Enhancement	3.68	0.08
ap-GAL4/+; UAS- <i>trc</i> <sup>T453A2</sup> /Df( <i>Dmob2</i> )	Enhancement	3.49	0.08
ap-GAL4/Df( <i>Dmob3</i> ); UAS- <i>trc</i> <sup>T453A2/+</sup>	Enhancement	3.42	0.08
ap-GAL4/Df( <i>Dmob4</i> ); UAS- <i>trc</i> <sup>T453A2/+</sup>	Enhancement	3.09	0.08
exp2			
ap-GAL4/UAS- <i>trc</i> <sup>T453A1</sup>	—	1.98	0.07
ap-GAL4/UAS- <i>trc</i> <sup>T453A1</sup> ; Df( <i>Dmob2</i> )/+	Enhancement	3.94	0.09
ap-GAL4/UAS- <i>trc</i> <sup>T453A1</sup> ; UAS- <i>Dmob2-C</i> /+	Enhancement	2.35	0.09
exp 3			
ptc-Gal4/UAS- <i>trc</i> <sup>T453A1/+</sup>	—	1.8	0.11
ptc-Gal4/UAS- <i>trc</i> <sup>T453A1</sup> /UAS- <i>mats</i>	Suppression	1.46	0.06
exp 4			
ptc-Gal4/+; UAS- <i>trc</i> <sup>T453A2/+</sup>	—	1.46	0.05
ptc-Gal4/+; UAS- <i>trc</i> <sup>T453A2</sup> /UAS- <i>mob2C</i>	Enhancement	1.82	0.07
ptc-Gal4/UAS- <i>mob2C</i> ; UAS- <i>trc</i> <sup>T453A2</sup> /UAS- <i>mob2C</i>	Enhancement	2.01	0.05
exp 5			
ptc-Gal4/+; UAS- <i>trc</i> <sup>T453A2/+</sup>	—	1.56	0.05
ptc-Gal4/+; UAS- <i>trc</i> <sup>T453A2</sup> / <i>mats</i> <sup>PB</sup>	Enhancement	1.98	0.06
ptc-Gal4/+; UAS- <i>trc</i> <sup>T453A2</sup> / <i>mats</i> <sup>PBBrv</sup>	—	1.51	0.08
ptc-Gal4/+; UAS- <i>trc</i> <sup>T453A2</sup> / <i>mats</i> <sup>e235</sup>	Enhancement	2.02	0.03
ptc-Gal4/+; UAS- <i>trc</i> <sup>T453A2</sup> +/+ <i>wts</i> <sup>3-17</sup>	Enhancement	1.82	0.1

Genetic interactions were scored as the effect of loss of one wild-type copy of the genes or transgene co-overexpression on the dominant multiple hair cell phenotype from *ap-GAL4* or *ptc-Gal4*-induced overexpression of *Trc*<sup>T453A2</sup> or *Trc*<sup>T453A1</sup>, which are independent lines of UAS-*trc*<sup>T453A</sup>. Because the phenotype is sensitive to both genetic background and environmental effects, results are always compared with a control done at the same time.

<sup>a</sup> Interaction: statistical significance was determined by Student's *t* test ( $p < 0.001$ ). The only exception to this was the enhancement by *wts*, which was significant at  $p < 0.05$ .

cies for *mats* and *Dmob2* (Table 1). These results suggested the possibility that all 4 *Dmobs* can redundantly interact with *Trc*, although it is possible that the interactions could be indirect or due to other genes in deleted regions. It is worth noting that such interactions are not common. When we screened the *Drosophila* deficiency collection for enhancement or suppression of *ap-GAL4* UAS-*trc*<sup>T453A</sup>, <10% of the Dfs showed an interaction (unpublished data).

To confirm that the interaction between *trc* and *Df* (*mats*) was due to the reduction in *mats* dose, we utilized two independent alleles. One was the null allele described by Lai *et al.* (2005) (*mats*<sup>e235</sup>), which is deleted for almost the entire coding region, and the other was a lethal PiggyBac insertion allele of *mats* [PBac{RB}CG13852<sup>e03077</sup>] (we subsequently refer to this allele as *mats*<sup>PB</sup>). Because this later allele had not been well characterized, we first determined that the insertion was lethal over a deficiency for the region (*Df*(3R)*Exel6191*) and it failed to complement the recessive lethality of *mats*<sup>e235</sup> consistent with the lethality being due to the PB insertion. We were able to revert this mutation using a source of PiggyBac transposase (Thibault *et al.*, 2004). We found that both *mats* alleles dominantly enhanced the *trc* dominant negative phenotype (Table 1) and this enhancement was lost in the PB revertant. We also found that overexpression of *mats* from a UAS-*mats* transgene (Lai *et al.*, 2005; driven by *ptc-Gal4*) partially suppressed the multiple hair cell phenotype that resulted from driving expression of *Trc*-DN using *ptc-Gal4* (Table 1). These dose responses argue that *Mats* activates *Trc*. Interestingly, we found that heterozygosity for a *wts* mutation also enhanced the *Trc* dom-

inant negative phenotype, although somewhat less strongly (Table 1).

We also obtained evidence for *mats* functioning with *trc* and *fry* using simple loss of function mutations. Wild-type flies or flies heterozygous for either *trc*, *fry*, or *mats* appeared normal and only rarely (on fewer than 5% of wings) was even a single multiple hair cell seen. Flies that were heterozygous for two of these genes showed a slightly higher frequency of wings with one or a couple of multiple hair cells (often ~10%) but the increase was not routinely significant (Table 2). However, almost half of the wings from flies that were heterozygous for all three genes (e.g., *fry*<sup>2</sup> *trc*<sup>1</sup>+/+ + *mats*<sup>PB</sup>) showed a weak multiple hair cell phenotype (Table 2), a significant increase. This genetic interaction is further support for the hypothesis that *trc*, *fry*, and *mats* function together in regulating wing hair development. In this assay we did not see an equivalent interaction with *wts*<sup>3-17</sup> (Table 2).

Previous studies established that *trc* also had a larval denticle phenotype (Geng *et al.*, 2000). In *trc* mutants the overall pattern of denticles was partly disorganized and many denticles were split (Table 3). Split denticles are infrequent in wild-type larvae (Figure 4A1 and Table 3). The denticle pattern of *mats*<sup>PB</sup>/*mats*<sup>PB</sup> homozygous larvae was also disorganized and contained many split denticles (Figure 4A2 and Table 3). The number of split denticles was similar in *mats*<sup>PB</sup>/*Df* larvae, suggesting that for this phenotype *mats*<sup>PB</sup> is a strong, near phenotypic null allele. The *mats*<sup>e235</sup> also showed a similar denticle phenotype (unpublished data). The phenotype of *mats*<sup>PB</sup> homozygotes was

**Table 2.** Interaction between loss of function alleles at *fry*, *trc*, and *mats*

Genotype	No. of wings	Fraction with an mhc phenotype	Different from Ore-R
Ore R	36	0	ND
<i>trc</i> <sup>1</sup> /+	33	0.03	ND
<i>trc</i> <sup>P</sup> /+	20	0	ND
<i>fry</i> <sup>1</sup> /+	20	0	ND
<i>fry</i> <sup>2</sup> /+	20	0	ND
<i>mats</i> <sup>PB</sup> /+	20	0	ND
<i>mats</i> <sup>e235</sup> /+	20	0	ND
<i>wts</i> <sup>3-17</sup> /+	20	0	ND
<i>fry</i> <sup>2</sup> <i>trc</i> <sup>1</sup> /+ +	24	0.12	ND
<i>fry</i> <sup>1</sup> +/+ <i>mats</i> <sup>PB</sup>	23	0.09	ND
<i>trc</i> <sup>P</sup> +/+ <i>mats</i> <sup>PB</sup>	20	0.05	ND
<i>mat</i> <sup>PB</sup> +/+ <i>wts</i> <sup>3-17</sup>	34	0.03	ND
<i>mats</i> <sup>PB</sup> +/+ <i>wts</i> <sup>P</sup>	16	0.06	ND
<i>fry</i> <sup>2</sup> <i>trc</i> <sup>1</sup> +/+ + <i>mats</i> <sup>e235</sup>	25	0.48	Different <sup>a</sup>
<i>fry</i> <sup>1</sup> <i>trc</i> <sup>P</sup> +/+ + <i>mats</i> <sup>PB</sup>	20	0.4	Different <sup>a</sup>
<i>fry</i> <sup>2</sup> <i>trc</i> <sup>1</sup> +/+ + <i>mats</i> <sup>PB</sup>	22	0.41	Different <sup>a</sup>
<i>fry</i> <sup>2</sup> + <i>trc</i> <sup>1</sup> +/+ Df-mob2 + Df-mats	10	0.5	Different <sup>a</sup>
<i>fry</i> <sup>2</sup> <i>trc</i> <sup>1</sup> +/+ + <i>wts</i> <sup>3-17</sup>	30	0	ND

A z test was used to compare the fraction showing a multiple hair cell phenotype with that of the control Ore-R wings.

mhc, multiple hair cells. ND, no difference at  $p < 0.05$ .

<sup>a</sup> Different at  $p < 0.001$ .

slightly less severe than that of in *trc*<sup>P</sup>/*trc*<sup>P</sup> larvae (Figure 4A3 and Table 3). Notably, *trc*<sup>P</sup> *mats*<sup>PB</sup>/*trc*<sup>P</sup> *mats*<sup>PB</sup> double mutant larvae did not have a more severe phenotype than the single mutants (Table 3). This lack of additivity argues that *trc* and *mats* function in a common pathway during denticle development.

Both *mats* alleles are larval lethals with death typically in the second or early third instar. To examine the phenotype of *mats* in wing cells, we generated mosaics using FLP/FRT. *mats* clones on the wing, leg, thorax, and head displayed two types of phenotype. The most notable was indistinguishable from those produced by clones of *wts* (Justice *et al.*, 1995; Xu *et al.*, 1995), suggesting that *mats* also functions with *wts* as was recently shown by Lai *et al.* (2005). On the wing small clones produced bulges that could be seen at low magnification with a stereomicroscope. In mounted wings individual cell outlines were visible in the cuticle and the cells appeared to have a bulging apical surface (Figure 4B, 1 and 2). The hairs were located on an elevated pedestal, a phenotype that was indistinguishable from those seen in *wts* clones (Figure 4B, 2 and 3). The hairs were often broader than normal. Particularly in other body regions clones were abnormally pigmented (either darker or lighter than normal; Figure 4D) and there were outgrowths of clone tissue (Figure 4F). In highly abnormal wing clones (Figure 4C1) we

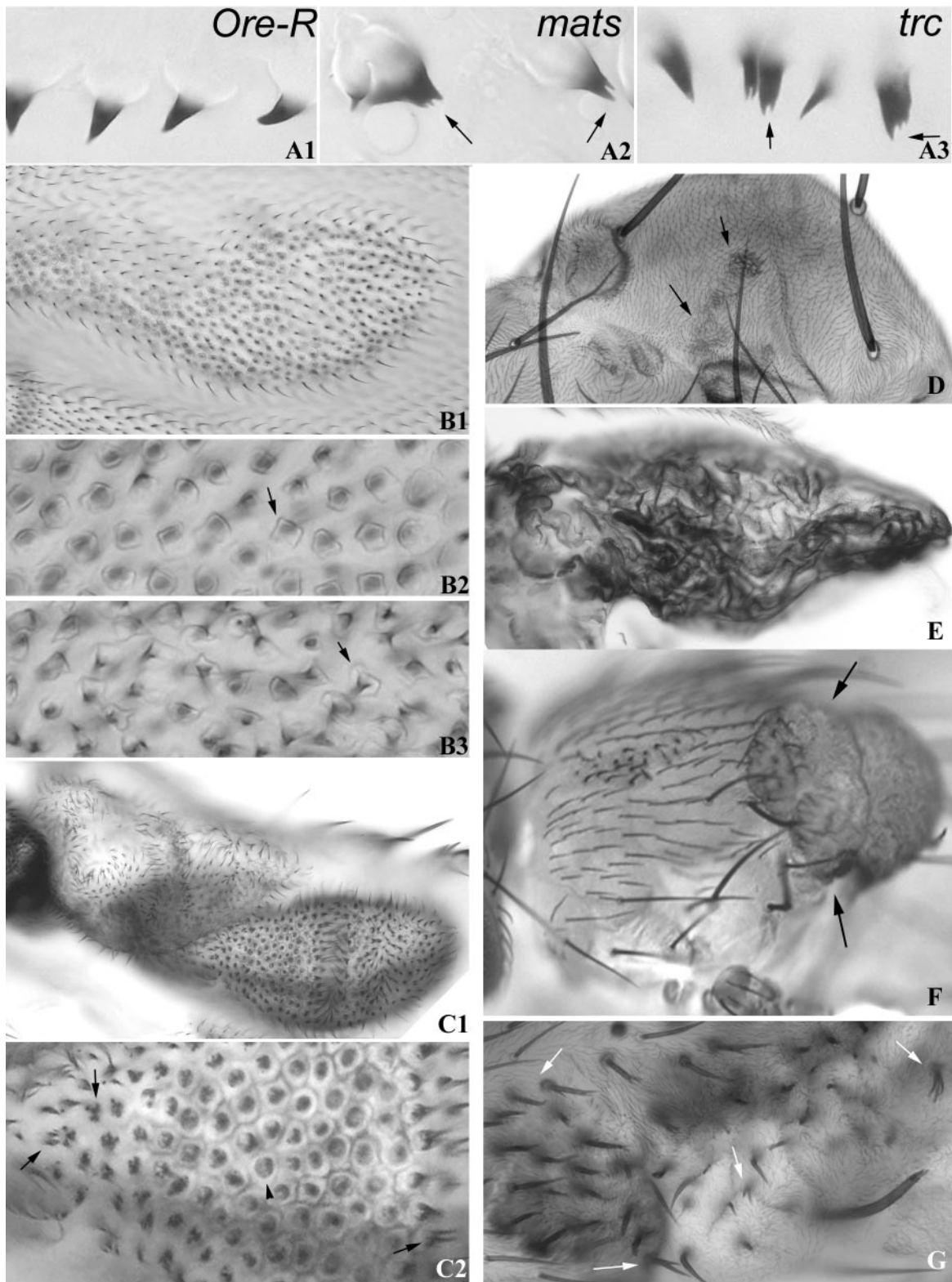
often saw evidence of clustered and split hairs that were typical of *trc* mutant clones (Figure 4C2). Some multiple hair cells were seen in very abnormal *wts* clones but this phenotype appeared less severe (e.g., number of hairs per cell) than that seen with *mats* or *trc*. These observations suggest that *mats* functions with both Trc and Wts.

We also examined *mats*<sup>PB</sup> and *mats*<sup>e235</sup> clones in pupal wings. Mutant *mats* cells were able to outcompete their neighbors and ended up comprising most of the wing when clones were induced early (Figure 5). As was expected from the morphology of clones in adult wings the pupal clones produced bulges in the wing and individual cells also often appeared bulged. Clone cells stained more brightly for F-actin (Figure 5). This was true both in developing hairs and in the general apical cortex. This phenotype was clear-cut enough that we could use it as a convenient marker of *mats* mutant cells. These phenotypes were seen with both *mats* alleles tested. A similar, increase in actin staining was seen in *wts* clones (unpublished data). A similar, but perhaps less severe increase in staining, is seen in *trc* clones (He *et al.*, 2005). In some, but not all *mats* clones large numbers of multiple hair cells could be seen (Figure 5). At later stages we could see a circular pedestal of actin staining surrounding the base of the hair in mutant cells but not in surrounding wild-type cells (Figure 5). At still later stages the wild-

**Table 3.** *mats* and *trc* function together during denticle development

Genotype	Mean number of split denticles/segment (2nd instar larvae)	SEM	p vs. <i>trc</i>	p vs. <i>mob</i>
Ore R	0.2	0.12	<0.000001	<0.000001
<i>trc</i> <sup>P</sup>	38.6	2.25	NR	0.0026
<i>mats</i> <sup>P</sup>	27.8	2.73	0.0026	NR
<i>trc</i> <sup>P</sup> <i>mats</i> <sup>P</sup>	33.5	1.87	0.10	0.091
<i>mats</i> <sup>PB</sup> /Df(3) <i>mats</i>	30.9	2.33	0.048	0.22

NR, not relevant. We scored second instar larvae because for several of the genotypes third instar larvae are rare.



**Figure 4.** Cuticular phenotypes of *Dmob1*. (A1–A3) Larval denticle phenotype of *Dmob1* is similar to that of *trc*. (A1) Oregon R; (A2) *mats<sup>PB</sup>/mats<sup>PB</sup>* (A3) *trc<sup>P</sup>/trc<sup>P</sup>*. Note the split denticles (arrows) in both *mats<sup>PB</sup>/mats<sup>PB</sup>* and *trc<sup>P</sup>/trc<sup>P</sup>*. (B1–B3) Bulging wing cell phenotype of *mats<sup>PB</sup>* is similar to that of *wts*. Unmarked mutant clones were induced using *flp/FRT*. (B1) A moderate-sized *mats<sup>PB</sup>/mats<sup>PB</sup>* clone results in a dimpling of the wing surface. (B2) A blow up of a small region from B1 showing the cuticle defect in *Dmob1* clones. The hair is located on a pedestal like structure (arrows). (B3) Shown is a high magnification image of part of a *wts<sup>3-17</sup>*-induced clone on the wing. Note the presence of pedestal like structures that are very similar to those seen in *mats* clones. (C1–C2) *mats<sup>PB</sup>* clones on the wing sometimes resulted in discrete outgrowths. One is shown in C1. Many cells in such outgrowths display strong multiple hair cells. A blow up of C1 is shown in C2.



type cells also had a circular pedestal of actin staining, suggesting that the mutant cells might be developmentally more advanced. Consistent with this possibility, in many clones hair initiation and outgrowth appeared to be advanced in *mats* mutant cells compared with neighbors (Figure 5). This is also the case for *wts* clones (unpublished data), but it is the opposite of *trc* clones, in which hair development is often delayed (He *et al.*, 2005). Cells in *mats* clones had a smaller cross section so that the array of hairs appeared denser (Figure 5), which is also the opposite of what is seen in *trc* clones, in which there is an increase in cross-sectional area (He *et al.*, 2005). Once again the phenotype of the *wts* clone cells resembles that seen for *mats* cells (unpublished data). Thus, for several wing phenotypes *mats* mutant cells resembled *wts* and not *trc* cells. Indeed, the *mats* phenotype was the opposite of *trc* for both cell area and the timing of hair morphogenesis.

We previously found that the accumulation of Fry in wing cells is subject to feedback control that is dependent on Trc activity. Hence, in a *trc* mutant we find increased Fry accumulation (He *et al.*, 2005). Several of the observations described above suggested the hypothesis that *mats* functioned along with *trc* and was important for Trc activation. From this we predicted that Fry accumulation would also be elevated in *Dmob1* clones. We found increased levels of Fry immunostaining in *Dmob1* clone cells, which was consistent with our hypothesis (Figure 5). This was also seen in *wts* mutant clones (unpublished data), although the increase appeared less dramatic.

Tumorous overgrowth phenotypes are a consequence of mutations in a number of *Drosophila* genes. In several cases, such as *lethal giant discs* overgrown imaginal discs are found in late third instar larvae (Buratovich and Bryant, 1997). To determine whether that was also the case for *mats*, we examined *mats/Df* mutant larvae. These larvae grow slowly and after 5 d of growth, when wild-type larvae begin to pupate, *mats/Df* larvae are the size of early third instar larvae. These larvae routinely die without growing substantially larger. When we dissected 5.5–5-d-old *mats* larvae, we did not see any evidence of tumors or overgrowth of imaginal or other tissues. Rather, the imaginal discs were approximately the size of those seen in 4-d larvae (Figure 6). However, the *mats* homozygous discs did not appear normal, as they were abnormally shaped and more folded than normal discs of this size.

In a number of experiments involving *mats* or *wts* we observed what appeared to be spontaneous tumors or clones. This was seen most often in flies that also carried reduced doses of *Dmob3* and *Dmob4*. We think that these overgrowths were due to spontaneous mitotic recombination, because when the flies were also mutant for *trc* or *fry*, we also saw evidence of *trc* or *fry* clones. The *trc* and *fry* clones were seen less frequently. This could be due to these

genes being located more proximally on the chromosome than *mats* or *wts*, but it might also be due to the competitive advantage of *mats* and *wts* clones, resulting in these clones being larger and easier to detect. The basis for these clones is unclear but suggests genomic instability in *mats* and/or *wts* mutants.

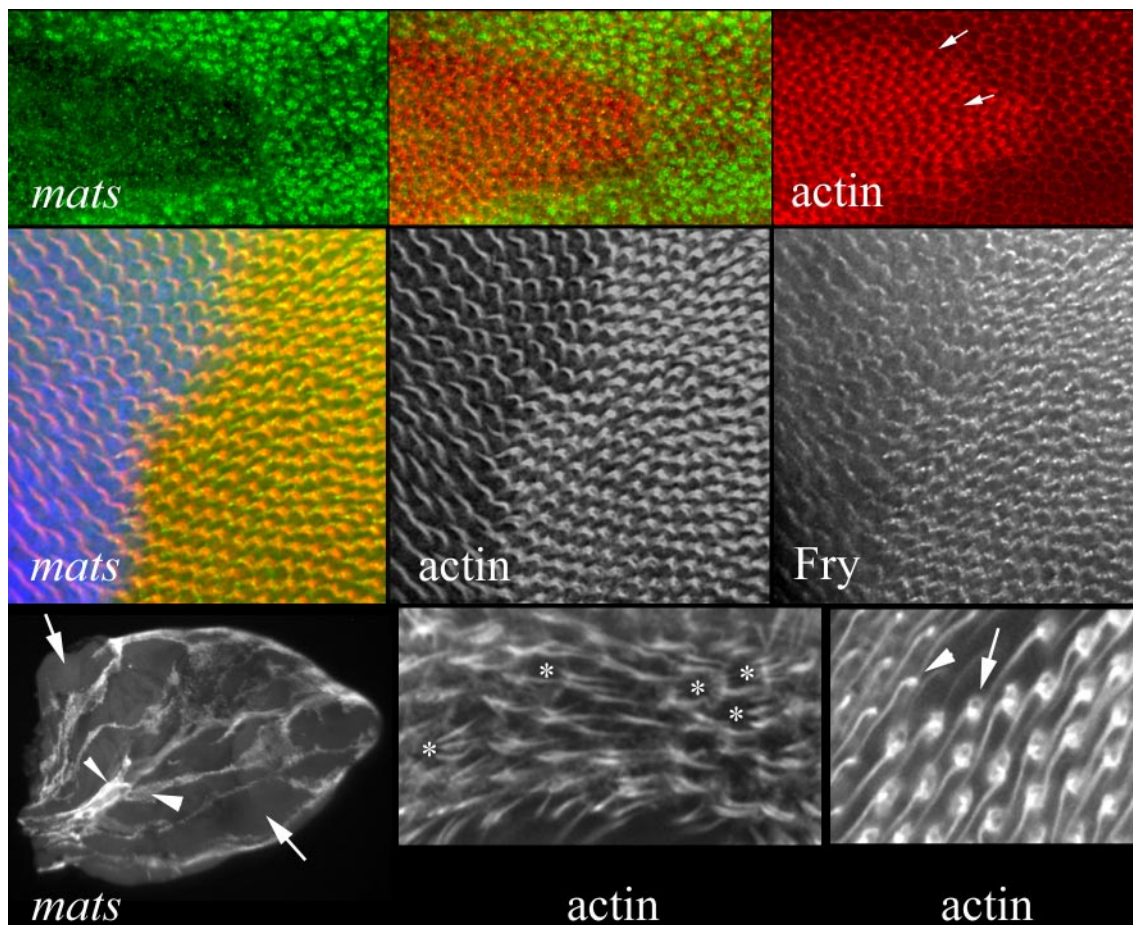
#### *Dmob2* Interacts with *trc*

We tested 10 deficiencies from the 68C region and further mapped the enhancing region to a small interval (68C11–13) that contained *CG11711* (*Dmob2*). Deficiencies from this region were able to similarly enhance the phenotypes that resulted from the directed expression of other dominant negative Trc proteins (unpublished data). The genome project annotation of *Dmob2* suggests it is a complicated gene that encodes at least four variant mRNAs from exons that span >40 kb. These mRNAs encode four proteins with a common c-terminal segment but with different amino terminal regions. There are P insertions in a large intron of *Dmob2*, but these do not inactivate the gene to produce a mutant phenotype (unpublished data). Attempts to use imprecise excision to produce a deletion that would ensure that no *Dmob2* protein could be made were not successful, because we only obtained small deletions that would eliminate one isoform. These did not produce a mutant phenotype. As an alternative approach we generated transgenic flies that carried UAS constructs that encoded either a tagged full-length *Dmob2* (GH07469) protein or partial proteins aa 1–157 (*Dmob2-N*) and aa 148–354 (*Dmob2-C*) that might act as dominant negative proteins. The directed expression of the wild-type *Dmob2* and *Dmob2-N* proteins by *ap-GAL4* did not cause any notable visible phenotype. The interpretation of these results is limited by the fact that we were only expressing one of 4 *CG11711* isoforms. On the other hand, overexpression of the common *Dmob2-C* protein segment resulted in a weak *trc*-like multiple hair cell phenotype (Figure 3C) and it also enhanced the dominant negative *trc* wing hair phenotype in a dose-sensitive way (Table 1), consistent with *Dmob2-C* being a dominant negative and the normal function of *Dmob2* being to activate Trc. In addition, overexpression of *Dmob2-C* caused an extra vein phenotype. This phenotype was enhanced by increasing the number of *UAS-mob2c* transgenes and it was also enhanced by heterozygosity for a deletion for *CG11711* (unpublished data). Thus, *Dmob2-C* acts as a dominant negative for this phenotype. A similar, but weaker vein phenotype was also seen in when *trc<sup>DN</sup>* was overexpressed (Figure 3B, 1 and 2).

#### *Dmo25*, the *Drosophila Hym1* Homolog Does Not Function with *trc*

The yeast *hym1* gene functions upstream of Cbk1 and is needed for RAM pathway function (Dorland *et al.*, 2000; Nelson *et al.*, 2003). There is a single homolog of *hym1* in *Drosophila*, the intronless *Dmo25* gene. *Df(3L)81k19*, which uncovers *Dmo25*, did not enhance or suppress the wing hair phenotype obtained from expressing *UAS-trc<sup>DN</sup>* using *ptc-Gal4*. A P insertion mutation in *Dmo25* [(PZ)Mo25<sup>00274</sup>] was obtained from the Bloomington Stock Center. This 17.3-kb insertion is into the 5' untranslated region of the *Dmo25* mRNA. We confirmed that the insertion was lethal over *Df(3L)81k19* and that the lethal mutation could be induced to revert in the presence of a source of P transposase. Thus, the P insertion in *Dmo25<sup>00274</sup>* is responsible for the recessive lethal mutation. For simplicity we refer to *Dmo25<sup>00274</sup>* as *Dmo25<sup>P</sup>*. Homozygous (or hemizygous) *Dmo25<sup>P</sup>* mutant animals died as larvae without any obvious morphological defect. There was no difference in the lethal stage between

**Figure 4 (cont).** Arrows point to cells with strong multiple hair cell phenotypes that are reminiscent of *trc*. Cells in the center are elevated (due to the bulge) and here we see the cell outlines (arrowhead) and a darkened center (pedestal and base of the hair). (D) Shown is part of the thorax of a fly showing small *mats<sup>PB</sup>* clones. The arrows point to clones that are darkened. (E) A grossly distorted wing due to the presence of a large number of *mats<sup>PB</sup>* clones. This fly was *vg-GAL4 UAS-flp/+; FRT82/FRT82 mats<sup>PB</sup>*. (F) Shown is a fly with a large *mats* "tumor" clone (arrows) on the thorax. (G) Shown is the thorax from a fly that was *vg-GAL4 UAS-flp/±; FRT82/FRT82 mats<sup>PB</sup>* and contained large regions of *mats<sup>PB</sup>* clones. Arrows point to split bristles. Similar defects are seen in *trc* mutants.



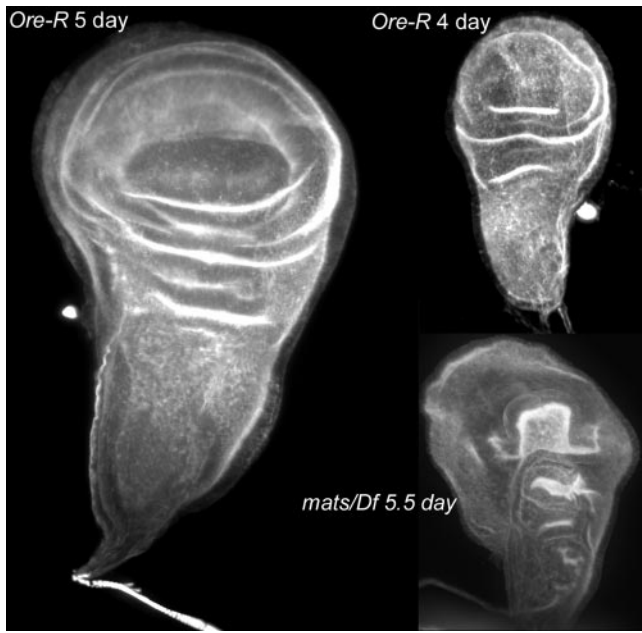
**Figure 5.** *mats* clones in pupal wings. The top panels show a *mats*<sup>PB</sup> clone marked by a loss of the Nmyc epitope in a pupal wing. The left panel shows immunostaining for myc (green). The right panel shows F-actin staining (red; Alexa 568 phalloidin). Note that the clone cells stain more brightly than their wild-type neighbors. The arrows point to two hairs. Note that hairs form in the vicinity of the distal most vertex and point distally. Thus, *mats* function is not required for the function of the *frizzled* pathway in planar polarity. Also note that the mutant cells appear to be advanced in hair morphogenesis compared with their wild-type neighbors. The middle panels also show a *mats*<sup>PB</sup> clone marked by a loss of the Nmyc epitope in a pupal wing. The middle panel in this row shows F-actin staining and the panel on the left anti-Fry antibody staining. The panel on the right is a merge (actin in red, Fry in green and myc in blue). Note once again the increase in actin staining in *mats* mutant cells. Note also that there is an increase in Fry accumulation in the mutant cells. This is similar to what is seen in *trc* mutant clones (He *et al.*, 2005) and is consistent with Dmob1 activating Trc. On the left of the bottom row is a low magnification image of a *vg gal4 UAS-flp; FRT 82 mats<sup>PB</sup>/FRT 82 hs-Nmyc* pupal wing stained with anti-myc antibody. The arrows point to *mats* clone cells that lack *myc* expression. The large arrowhead points to a region with intermediate Myc staining. These should be the heterozygous genotype (no recombination). The small arrowhead points to a region with bright Myc staining. This is the twin spot that has two copies of the *myc*-expressing transgene. Note how the *mats* mutant cells have outcompeted the wild-type cells and now comprise most of the wing. If the *mob* mutation did not alter growth, then the region of *mats* clones should be the same as that of the two-dose *myc* twin spots. The middle panel in the lower row shows an example of prominent multiple hair cells (F-actin staining) formed by *mats* clone cells. This was common only in clones that showed the “outgrowth” phenotype. The right panel on the bottom row shows the boundary between *mats* and wild-type cells late in hair development stained for F-actin. The arrow points to a Dmob1 cell and its prominent “pedestal.” The arrowhead points to a neighboring wild-type cell.

homozygous *Dmo25<sup>P</sup>* and *Dmo25<sup>P</sup>/Df* animals arguing that the P insertion is a strong phenotypic null allele. We compared the amount of *Dmo25* mRNA in wild-type and *Dmo25<sup>P</sup>/Df* second instar larvae using real time RT-PCR. The relative abundance of *Dmo25* mRNA (i.e., compared with the mRNA for the *rp49* ribosomal protein) in the mutant was <1% of that seen for Oregon R. Because there is no intron in *Dmo25*, there was no way to distinguish between mRNA and contaminating DNA in these samples. Further, maternally derived *Dmo25* mRNA could also be contributing to our amplification. Hence the <1% estimate of *Dmo25* mRNA in the mutant is likely to be an underestimate. The

molecular and phenotypic tests indicate that *Dmo25<sup>P</sup>* is a strong if not null allele.

The denticle pattern in the *Dmo25<sup>P</sup>* and *Dmo25<sup>P</sup>/Df* mutants was normal and we did not see a large number of split denticles as we did in *trc* and *Dmob1*. To assess the possible function of *Dmo25* for wing hair and bristle development, we used FLP/FRT-mediated recombination to generate clones of mutant cells. We did not see any *trc*-like multiple hair cells or split bristles, although several phenotypes were seen. When clones were located close to or overlapping wing veins there was a “swelling” of the veins (Figure 7, A and D). Clones some distance from a normal vein did not show any

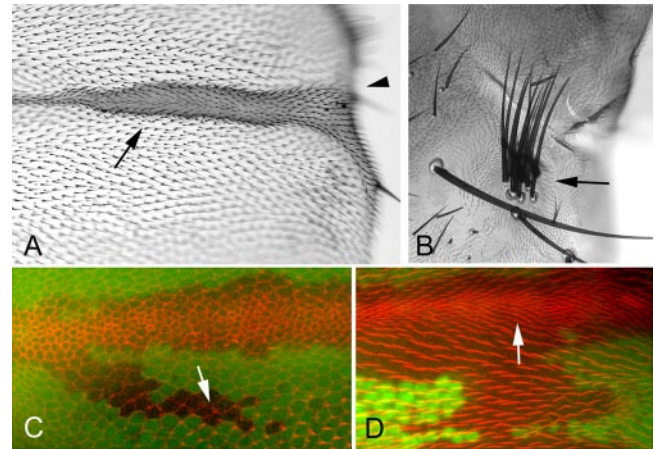




**Figure 6.** Imaginal wing discs in *mats* mutants are smaller than normal. Shown are maximum projections of confocal stacks of wing imaginal discs stained with Alexa 568 phalloidin. All micrographs are at the same magnification. Note how much smaller the 5.5-d *mats/Df* disk is compared with the 5-d Ore-R disk.

phenotype and differentiated normal looking hairs (Figure 7, C and D). We did not see any evidence for a delay in hair initiation (Figure 7C), as is often the case for cells in *trc* mutant clones (He *et al.*, 2005). When we examined adult wings bearing clones, there was often a loss of wing margin (wing notching), suggesting cell death in this region (Figure 7A). In adult flies bearing unmarked clones we also saw examples of “multiplied bristles” (Figure 7B). These were not split, but rather appeared to be due to extra bristles. All three of these phenotypes are reminiscent of mutant phenotypes associated with defects in Notch signal transduction, suggesting that *Dmo25* might function in this pathway. To further test this possibility, we examined the ability of the *Dmo25<sup>P</sup>* allele and a *Df* for the region to interact genetically with Notch pathway mutations. In one set of crosses, we found that a reduction in *Dmo25* dose enhanced the weak wing notching phenotype seen in male flies that carried the weak *N<sup>no1</sup>* allele. The fraction of the wing margin lost increased from 0.31 in *N<sup>no1</sup>* wings to 0.47 ( $p = 0.14$ ) in *N<sup>no1</sup>; Dmo25<sup>P</sup>/+* and to 0.59 ( $p = 2 \times 10^{-6}$ ) in *N<sup>no1</sup>; Df(3L)81k19/+*. Similarly we found that a reduction in *Dmo25* dose enhanced the expanded wing vein phenotype of flies that carried a single copy of the weak *DI<sup>P05151</sup>* allele. The ratio of the vein area to length for Vein 2 distal to the distal cross vein increased from 16.2–21.7 ( $p = 0.00016$ ) for *Dmo25<sup>P</sup>+/+ DI<sup>P05151</sup>* and 20.2 ( $p = 0.0002$ ) for *Df(3L)81k19/+DI<sup>P05151</sup>*. These data are consistent with the possibility that *Dmo25* functions in the Notch pathway, but more extensive experiments will be needed to get definitive evidence for this.

We also generated flies that carried UAS-*Dmo25-N*, a C-terminal half truncated form of *Dmo25* (aa 1–173). Driving expression of this transgene by *ap-GAL4* resulted in swollen wing veins that were similar to the phenotype seen in *Dmo25<sup>P</sup>* mutant clones (unpublished data). This suggests that the C-terminal half of *Dmo25* is essential for the protein's function.



**Figure 7.** *Dmo25*, the *Drosophila* Homolog of Yeast *Hym1*, does not function along with *Trc*. (A) The induction of unmarked *Dmo25<sup>P</sup>* homozygous clones results in thickened wing veins (arrow) and loss of the wing margin (arrowhead). Phenotypes similar to these are often seen in Notch pathway gene mutations. (B) Flies with *Dmo25<sup>P</sup>* clones often showed a multiple bristle phenotype. An example of an unmarked notum clone is shown (arrow). In control experiments (i.e., when *Dmo25<sup>P</sup>* were not induced) such multiplied bristles were not seen. (C) A confocal image of a 32-h pupal wing stained with Phalloidin (red) and anti-GFP (green) antibody. The wing contains a large *Dmo25<sup>P</sup>* clone marked by the loss of GFP. Note that some cells in the region are just starting to elaborate hairs. The genotype with regard to *Dmo25* does not appear to influence the timing of hair initiation. The arrow points to a newly formed hair inside the clone. (D) A confocal image of a 36-h pupal wing stained with phalloidin (red) and anti-GFP (green) antibody. The wing contains a large *Dmo25<sup>P</sup>* clone marked by the loss of GFP. Within the clone the vein region was enlarged, whereas wing hair development is not altered.

## DISCUSSION

### *Trc*, *Dmob*, and *Fry* Function in a Common Signaling Pathway

Previous genetic data pointed out the importance of *trc* and *fry* for the morphogenesis of polarized cellular extensions (Geng *et al.*, 2000; Cong *et al.*, 2001; He and Adler, 2001; Emoto *et al.*, 2004). Based on homology to the RAM, MEN, and SIN pathways (Mah *et al.*, 2001; Hou *et al.*, 2003; Nelson *et al.*, 2003), it seemed likely that one or more of the *Drosophila mob* genes would function along with *trc* and *fry*. We found evidence supporting this hypothesis but the results were complicated by both pleiotropy and redundancy. This was illustrated most clearly in our experiments with *mats*. Mutations in *mats* displayed phenotypes that were typical of both *trc* (split denticles and multiple hair cells) and of *wts/lats* (tumors, bulged cells, advanced hair differentiation). We also found that *Mats* could interact with both *Trc* and *Wts* using the yeast two-hybrid system. These observations stand in contrast to the situation in yeast, in which individual *mob* genes show specificity for individual *Ndr* family members. Further evidence for redundancy came from the gene dosage interactions seen between *trc* and the other *Dmob*s.

Evidence for a direct physical interaction has been reported for *Ndr* and *Mob* family members from yeast, flies and mammals (Colman-Lerner *et al.*, 2001; Mah *et al.*, 2001; Weiss *et al.*, 2002; Hou *et al.*, 2003; Bichsel *et al.*, 2004; Lai *et al.*, 2005). Previous yeast two-hybrid experiments showed evidence for a physical interaction between *Trc* and *Mats*





tions between *Dmo25* and *N* and *DI*. The mammalian Mo25 protein has been shown to be present in a complex with the Lkb tumor suppressor and the Strad $\alpha$  adapter protein (Boudeau *et al.*, 2003). There is no evidence for homologues of Lkb or Strad functioning with Trc or in N signaling. The *Drosophila lkb* homolog has been shown to function in oocyte polarization, but little is known about its function in the epidermis. *fray* is the fly gene most closely related to *strad*. *fray* has been principally studied with respect to its role in nerve development (Leiserson *et al.*, 2000). In mammalian cells the Lkb, Strad, Mo25 complex activated the AMP-activated protein kinase (Baas *et al.*, 2004). The Mamk homolog in *Drosophila* is CG3051 and little is known about the in vivo function of this gene. No deficiency that removed this gene showed an interaction with Trc (unpublished data). It will be interesting to determine whether Mo25 functions with Lkb and Strad in *Drosophila* and if Mamk is a downstream target.

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