

# Analysis of the role of the Rac/Cdc42 GTPases during planar cell polarity generation in *Drosophila*

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**ABSTRACT** Initial genetic studies in *Drosophila* suggested that several members of the Rho subfamily (RhoA, Rac1 and Cdc42) are involved in planar cell polarity (PCP) establishment. However, analyses of *Rac1*, *Rac2* and *Mtl* loss-of-function (LOF) mutants have argued against their role in this process. Here, we investigate in detail the role of the Rho GTPases *Mtl*, *Cdc42*, *Rac1* and *Rac2* in PCP generation. These functional analyses were performed by overexpressing *Mtl* in eyes and wings, by performing genetic interaction assays and by using a combination of triple and quadruple mutant LOF clones. We found that *Mtl* overexpression caused PCP phenotypes and that it interacted genetically with other Rho GTPases, such as *Rac1* and *Cdc42* as well as with several PCP genes, such as *stbm*, *pk* and *aos*. However, *Mtl* was not found to interact with *Rac2*, *RhoA* and other members of the Fz/PCP pathway. Triple mutant clones of *Rac1*, *Rac2* and *Mtl* were found to exhibit mild PCP defects which were enhanced by reduction of *Cdc42* function with a hypomorphic *Cdc42* allele. Taken together, these and previous results suggest that Rho GTPases may have partially overlapping functions during PCP generation. Alternatively, it is also possible that the mild PCP phenotypes observed could indicate that they are required at low levels in that process. However, since not all of them function upstream of a JNK cassette, we propose that they may act in at least two parallel pathways.

**KEY WORDS:** *Mtl*, *Rac1*, *Rac2*, *Cdc42*, planar cell polarity, redundancy, *Drosophila*

## Introduction

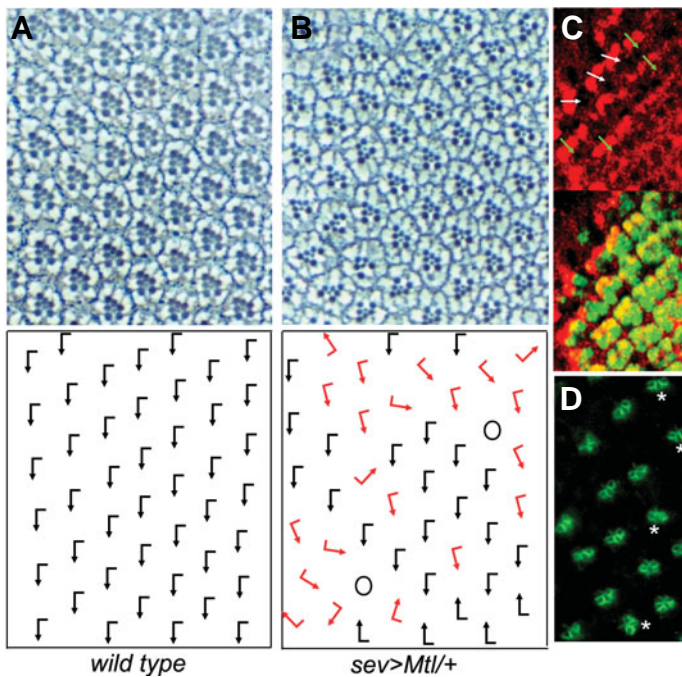
Small GTPases act as signal transducers by switching between inactive GDP-bound and active GTP-bound forms and have been implicated in multiple processes during the development of multicellular organisms (Van Aelst and D'Schouza-Schorey, 1997). In *Drosophila* several members of the Rho subfamily of small GTPases have been identified: *Rac1*, *Rac2*, *Cdc42*, *RhoA*, *RhoL* and *Mtl* (Luo *et al.*, 1994; Harden *et al.*, 1995; Hariharan *et al.*, 1995; Murphy and Montell, 1996; Sasamura *et al.*, 1997; Strutt *et al.*, 1997, Newsome *et al.*, 2000; Hakeda-Suzuki *et al.*, 2002). Expression of constitutively activated and dominant-negative isoforms of these proteins and analysis of loss-of-function mutants have shed light on their physiological roles. They have been implicated in actin cytoskeleton reorganization (Genova *et al.*, 2000), myogenesis, axonal outgrowth and guidance (Luo *et al.*, 1994; Kaufmann *et al.*, 1998; Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002; Fan *et al.*, 2003), gastrulation (Barrett *et al.*, 1997), oogenesis (Murphy and Montell, 1996), embryonic segmentation (Magie *et al.*, 1999) and cell migration (reviewed by

Montell, 1999; Paladi and Tepass, 2004). They also participate in embryonic dorsal closure (Hakeda-Suzuki *et al.*, 2002; Woolner *et al.*, 2005) and epithelial planar cell polarity establishment (Eaton *et al.*, 1995; 1996; Strutt *et al.*, 1997; Fanto *et al.*, 2000; this paper).

In many organs, epithelial cells are polarized not only along the apical-basolateral axis but also within the plane of the epithelium. The acquisition of this planar cell polarity (PCP) is essential for specialized cellular functions (Klein and Mlodzik, 2005). PCP establishment has emerged as a good model to study the role of Rho family small GTPases. In *Drosophila*, two members of this family, *RhoA* and *Rac1*, have been implicated in this process (Eaton *et al.*, 1995; Strutt *et al.*, 1997; Fanto *et al.*, 2000). PCP phenotypes are characterized by the misorientation of cells within the epithelial plane and have been most extensively studied in the context of *Drosophila* eye and wing development. In the eye, PCP is reflected in the mirror-symmetric arrangement of the ommatidia

*Abbreviations used in this paper:* JNK, Jun N-terminal kinase; LOF, loss-of-function; PCP, planar cell polarity.

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**Fig. 1. Overexpression of wild-type Mtl causes planar cell polarity (PCP) defects in the eye.** (A,B) Tangential sections (upper panels) of adult eyes and the corresponding schematic representation (lower panels) with arrows reflecting ommatidial polarity. Anterior is to the left, dorsal is up. Black arrows represent correct ommatidial orientation; red arrows, misrotated ommatidia; circles mark unscorable ommatidia due to missing or malformed photoreceptors. (A) Wild-type eye (dorsal area). (B) *sev-GAL4/UAS-Mtl* eye. (C) Anti-Spalt staining in third instar larval eye imaginal discs of *sev-GAL4/UAS-Mtl* flies (upper panel). The lower panel shows overlay of Spalt (red) and anti-Elav (green). White arrows in the red channel mark misoriented ommatidial preclusters and green arrows correctly oriented clusters. (D) Anti-Arm staining in third instar larval eye imaginal discs of *sev-GAL4/UAS-Mtl* flies. The asterisks show some misoriented ommatidial preclusters.

relative to the dorso-ventral midline, the equator. This pattern is established early in development, when ommatidial preclusters in the dorsal and ventral halves of the disc rotate 90° in opposite directions. At the same time, they lose their symmetry and opposite chiral forms are established in each half of the eye disc (Mlodzik, 1999, 2002). In the wing, PCP is evident in the uniform pattern formed by distally oriented hairs that cover the dorsal and ventral surfaces. Each wing cell orients itself along the proximal to distal axis and generates a single actin hair pointing distally (Mlodzik, 2002; Eaton, 2003). Mutations in genes that regulate PCP result in the loss of mirror-image symmetry in the eye, due to failure of ommatidia to acquire the correct chirality and/or to rotate properly. In the wing, PCP defects are manifest in abnormal hair orientation and number of wing hairs per cell.

Genetic and molecular studies have demonstrated that PCP establishment depends on the activity of the Fz/PCP signaling pathway that regulates changes in both cytoskeletal organization and transcription through the JNK pathway (reviewed in Mlodzik, 1999; 2002). In addition, a conserved group of genes is involved in PCP generation by the formation of multiprotein complexes that are asymmetrically distributed between R3 and R4 (Jenny *et al.*, 2003; Das *et al.*, 2004) and between the proximal and distal wing

cell membranes (reviewed in Klein and Mlodzik, 2005). In the eye, Fz/PCP signaling, together with the Notch pathway, is responsible for R3/R4 fate induction and thus for the establishment of ommatidial chirality (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Besides, it has been shown that the ommatidial rotation, depends on the Egfr pathway (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003). Moreover, downstream of Egfr there is a requirement for the Ras/MAPK cascade and Canoe, an adherens-junction-associated protein (Young *et al.*, 1993), that provides a link from Egfr to cytoskeletal elements (Gaengel and Mlodzik, 2003). Recently, it has been also shown that Egfr signaling regulates Cadherin activity in this context (Mirkovik and Mlodzik, 2006).

Several studies have suggested that the small Rho family GTPases are involved in PCP establishment. *RhoA* loss-of-function mutants display PCP phenotypes in eyes and wings and they dominantly suppress the gain-of-function phenotypes of *fz* and *dsh* (Strutt *et al.*, 1997). Conversely, *Cdc42* mutants were not found to interact with *sev-Fz* or *sev-Dsh* (Boutros *et al.*, 1998), although overexpression of dominant-negative forms of the GTPase in the wing affects actin polymerization during wing hair formation resulting in loss or stunting of hairs (Eaton *et al.*, 1995; 1996), but also producing occasional multiple wing hairs (Baron *et al.*, 2000). The role of *Rac* in PCP establishment was also addressed using dominant-negative and activated isoforms of *Rac1*, which produce PCP phenotypes in the eye (Fanto *et al.*, 2000). In addition, deficiencies uncovering either *Rac1* or *Rac2* dominantly suppress *sevE-Dsh* (Boutros *et al.*, 1998). However, analyses of loss-of-function mutants in *Rac1*, *Rac2* and *Mtl* did not reveal clear PCP defects in eyes or wings (Hakeda-Suzuki *et al.*, 2002). *Mtl* is the *Drosophila* homolog of the *C. elegans* MIG-2 GTPase (Zipkin *et al.*, 1997; Newsome *et al.*, 2000). Genetic studies of null and gain-of-function mutations of *mig-2* in *C. elegans* have shown that this GTPase is required for cell migration and axon guidance and that it functions redundantly with other Rho family GTPases in many cells (Zipkin *et al.*, 1997). Similarly, *Mtl* is functionally related to *Rac1* and *Rac2* in *Drosophila*, because these GTPases act redundantly in regulating

TABLE 1

**QUANTIFICATION OF GENETIC INTERACTIONS WITH THE *SEV>MTL* PHENOTYPE**

| Genotype                          | Wild-type ommatidia (in % $\pm$ sd) |
|-----------------------------------|-------------------------------------|
| <i>sev&gt;Mtl</i> ; +/+ (Control) | 75.3 ( $\pm$ 1.5)                   |
| <i>Mtl</i> <sup>1</sup> /+        | 38.1 ( $\pm$ 5.1)*                  |
| <i>msn</i> <sup>102</sup> /+      | 75.8 ( $\pm$ 3.3)                   |
| <i>hep</i> <sup>R75</sup> /+      | 72.9 ( $\pm$ 3.8)                   |
| <i>bsk</i> <sup>2</sup> /+        | 78.7 ( $\pm$ 2.9)                   |
| <i>jun</i> <sup>2</sup> /+        | 80.2 ( $\pm$ 3.1)                   |
| <i>RhoA</i> <sup>22R</sup> /+     | 74.5 ( $\pm$ 6.1)                   |
| <i>Cdc42</i> <sup>3</sup> /+      | 92.8 ( $\pm$ 2.3)*                  |
| <i>Cdc42</i> <sup>4</sup> /+      | 66.4 ( $\pm$ 4.8)*                  |
| <i>Rac2</i> <sup>3</sup> /+       | 78.1 ( $\pm$ 4.3)                   |
| <i>Rac1</i> <sup>J11</sup> /+     | 63.9 ( $\pm$ 0.3)*                  |
| <i>aos</i> <sup>6</sup> /+        | 47.7 ( $\pm$ 0.6)*                  |
| <i>stbm</i> <sup>X</sup> /+       | 62.7 ( $\pm$ 2.1)*                  |
| <i>pkp</i> <sup>ik-spleg</sup> /+ | 61.4 ( $\pm$ 3.3)*                  |

Percentage of wild-type ommatidia ( $\pm$  standard deviation) of the analyzed eyes of flies heterozygous for the indicated alleles and containing one copy of *sev>Mtl*. The quantifications of allelic combinations are based on scoring of 3 to 7 independent eyes per genotype. The asterisks indicate significant interactions ( $p \leq 0.05$ ,  $t$ -test).

dorsal closure and axon growth and guidance (Hakeda-Suzuki *et al.*, 2002, Ng *et al.*, 2002).

In order to analyze more precisely the requirement of the Rho GTPases Mtl, Rac1, Rac2 and Cdc42 in PCP establishment, we used several strategies. We show that overexpression of wild-type Mtl in eyes and wings gives rise to PCP defects. Moreover, flies hemizygous for a weak hypomorphic *Cdc42* mutant allele also show mild PCP defects in both tissues. In contrast to previous reports (Hakeda-Suzuki *et al.*, 2002), we find that eye clones triple mutant for *Rac1*, *Rac2* and *Mtl* display mild PCP defects, which are further enhanced when reducing the function of *Cdc42* with a hypomorphic mutant allele. Taken together, our data suggest that all four Rho GTPases may have a redundant role during PCP generation. Alternatively, it is also possible that the mild PCP phenotypes observed could indicate that they are required at low levels in that process. However, since we find that Mtl and Cdc42 do not act in the canonical Fz pathway, conversely to Rac1, Rac2 and RhoA, we propose that not all the Rho GTPases act upstream of the JNK module and that there are at least two parallel Rho GTPase family functions.

## Results

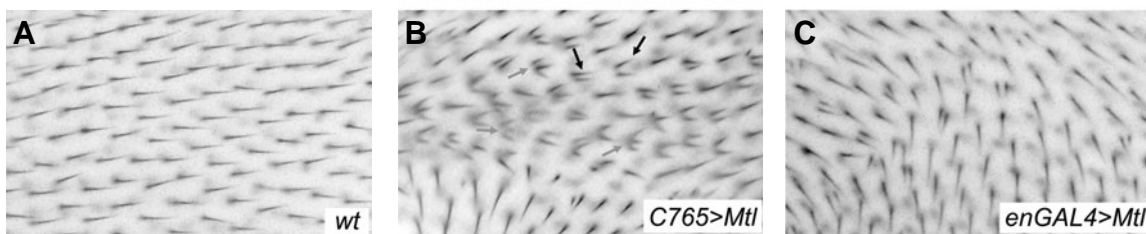
### Overexpression of wild-type Mtl causes PCP phenotypes in eyes and wings

Functional studies of Rac1, Rac2 and Mtl in *Drosophila* have shown that they have overlapping functions in the control of epithelial morphogenesis, myoblast fusion, and axon growth and guidance (Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002). The same study indicates that animals homozygous for a deletion removing the entire *Mtl* open reading frame are fully viable, and suggests that these three GTPases are not required for PCP establishment (Hakeda-Suzuki *et al.*, 2002). However, we have established transgenic flies carrying wild-type *Mtl* under the control of the UAS element (UAS-*Mtl*<sup>WT</sup> lines), and found that overexpression of wild-type Mtl in eyes and wings using several drivers produce PCP defects. *sev*-GAL4 drives the expression of Mtl in the developing eye disc, in the R3/R4 pair, which is critical for establishment of correct polarity. The eyes of the resulting flies (*sev*>*Mtl*<sup>WT</sup>, hereafter referred as *sev*>*Mtl*) are externally rough and reveal typical PCP defects in tangential sections (Fig. 1B). Mtl overexpression resulted in the misorientation of several ommatidia and, at lower frequency, ommatidia with an abnormal complement of photoreceptors. Strikingly, the chirality of the ommatidia was rarely affected and the PCP defects were largely reflected by misrotation (Fig. 1B). As

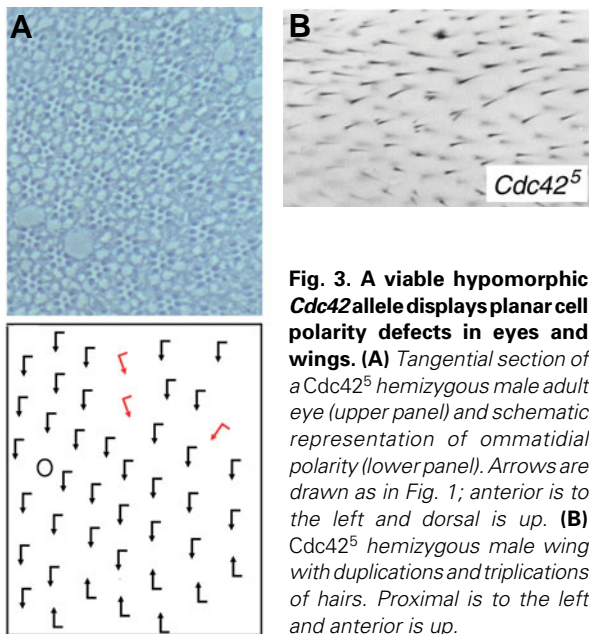
can be seen in Table 1, in *sev*>*Mtl* eyes 75.3% of the ommatidia display wild type orientation and the remaining ommatidia show polarity defects (21%), mainly misrotation, as well as defects in photoreceptor differentiation (3.7%). To establish whether the polarity/rotation defects observed with *sev*>*Mtl* arise early in development and are thus primary defects, we analyzed polarity generation in *sev*>*Mtl* third instar larval eye imaginal discs (when tissue polarity is first apparent and PCP genes are required). *sev*>*Mtl* discs were stained with anti-Spalt (as marker for R3/R4 precursors) and anti-Elav (expressed in all photoreceptors). Our results indicate that ommatidial polarity/rotation is affected in *sev*>*Mtl* eye discs, since the R3/R4 pairs are often incorrectly oriented with respect to their neighbors (Fig. 1C). The misorientation of the photoreceptor clusters is also evident in an anti-Arm staining of *sev*>*Mtl* eye discs (Fig. 1D). We also examined the effect of Mtl overexpression in the wing, using the GAL4 lines C765 and *en*-GAL4. In wild-type wings, each cell produces a single, distally oriented hair (Fig. 2A). Overexpression of Mtl in the whole wing driven by C765 gave rise exclusively to typical PCP defects and many cells exhibited a multiple wing hair phenotype, producing double or even triple hairs. Moreover, in several areas the wing hairs were not pointing distally, but were misoriented forming waves and whorls (Fig. 2B and data not shown). The same phenotypes were found in the posterior part of adult wings from *en*-GAL4, UAS-*Mtl*<sup>WT</sup> flies (Fig. 2C). Strikingly, these defects are reminiscent of those of the core PCP genes like *fz*, *stbm* (also known as *vang*) or *pk* (Vinson *et al.*, 1989; Taylor *et al.*, 1998; Gubb *et al.*, 1999).

To test whether the PCP eye phenotype observed in *sev*>*Mtl*<sup>WT</sup> flies is due to excessive Mtl signaling, we tested the effect of reducing the dosage of *Mtl* on such phenotype, using the *Mtl*<sup>A</sup> null allele (Hakeda-Suzuki *et al.*, 2002). Surprisingly, we found that the *sev*>*Mtl*<sup>WT</sup> phenotype is enhanced in an *Mtl* mutant background (Table 1), thus suggesting that this phenotype is not caused by an excessive Mtl signaling but may actually be due to dominant-negative effects. It is possible that the overexpressed Mtl protein may accumulate in an inactive form that could interfere with endogenous Mtl function by directly sequestering it. Alternatively the overexpressed protein could be sequestering or inactivating limiting components or effectors of that GTPase, thus reducing the ability of the cells to signal productively. A similar situation was found when overexpressing the wild type form of Presenilin at very high levels (Ye and Fortini, 1999).

Taken together, our results suggest that *Mtl* has a role in PCP generation in eyes and wings, but it probably functions redundantly



**Fig. 2. Overexpression of wild-type Mtl causes PCP defects in the wing.** All panels show high magnification areas of wings, distal is to the right. (A) Wild-type wing. Note the regular arrangement of hairs, all pointing distally. (B) C765/+; UAS-Mtl/+ wing at 25°C. (C) *en*-GAL4/+; UAS-Mtl/+ wing at 18°C (this cross is lethal when incubated at 25°C). Overexpression of the wild type form of Mtl in the wing causes typical PCP defects, e.g. duplications and triplications of hairs (black and gray arrows, respectively) and misorientation of hairs. Note many hairs pointing perpendicular to the proximal-distal axis in (B, C).



**Fig. 3. A viable hypomorphic *Cdc42* allele displays planar cell polarity defects in eyes and wings.** (A) Tangential section of a *Cdc42*<sup>5</sup> hemizygous male adult eye (upper panel) and schematic representation of ommatidial polarity (lower panel). Arrows are drawn as in Fig. 1; anterior is to the left and dorsal is up. (B) *Cdc42*<sup>5</sup> hemizygous male wing with duplications and triplications of hairs. Proximal is to the left and anterior is up.

in this process. Since overexpression of wild-type, activated or dominant-negative isoforms of other Rho GTPases like RhoA and Rac1 (Fanto *et al.*, 2000) also results in PCP defects, they could account for the proposed redundancy of function of Mtl during PCP generation.

#### ***Mtl* does not act in the PCP canonical pathway but interacts genetically with *Rac1* and *Cdc42***

Since our results suggested that *Mtl* is involved in polarity generation in eyes and wings, we wanted to place it more specifically in the PCP context. To do this we used the *sev>Mtl* phenotype to test for genetic interactions with mutations in PCP components and the other GTPases. The results obtained in these experiments are shown in Table 1. Our results indicate that there is no significant genetic interaction between *sev>Mtl* and the components of the JNK cascade like *msn*, *hep*, *bsk* or *jun*, suggesting that *Mtl* does not function upstream of the JNK module. The same result was obtained when reducing the gene dosage of the small GTPase *RhoA*. Since *Mtl* is closely related to *Rac1* and *Rac2*, and functionally behaves like both GTPases (Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002), we also tested for genetic interactions with loss-of-function mutations in both genes (*Rac1*<sup>V12</sup> and *Rac2*<sup>1</sup>, respectively). We found that *Rac1*, but not *Rac2*, interacts genetically with the *sev>Mtl* phenotype. These

results indicate that *Mtl* might function with *Rac1* in the PCP process. Therefore, we wanted to determine whether *Mtl* and *Rac1* act in a hierarchy in the PCP context testing the opposite interaction. We crossed the *sev-Rac1*<sup>V12</sup> transgene (a constitutively active isoform of *Rac1*), that produces polarity defects and also interferes with photoreceptor differentiation (Fanto *et al.*, 2000), to the *Mtl* null allele and found that there is a significant suppression of the *sev-Rac1*<sup>V12</sup> phenotype (Table 2), reflected in an increase of the number of correctly oriented wild-type ommatidia. Hence, these results support the idea that *Mtl* functions cooperatively with *Rac1*, and that they may have a redundant role during PCP establishment.

In addition we have found that *Mtl* genetically interacts with *Cdc42*. Two lethal alleles were tested for genetic interactions with the *sev>Mtl* phenotype, *Cdc42*<sup>3</sup> and *Cdc42*<sup>4</sup> (Genova *et al.*, 2000). The *Cdc42*<sup>3</sup> allele has been used in previous studies which demonstrated that *Cdc42* is not involved in Fz signaling (Boutros *et al.*, 1998), and does not interact with *Rac1* (Fanto *et al.*, 2000). We found that both *Cdc42* mutant alleles dominantly interact with the *sev>Mtl* phenotype (Table 1), indicating that *Cdc42* might be functionally related to *Mtl*, and suggesting a possible role of *Cdc42* in PCP generation (see below). However, while *Cdc42*<sup>3</sup> suppressed the *sev>Mtl* phenotype, the *Cdc42*<sup>4</sup> allele enhanced it. *Cdc42*<sup>3</sup> is a lethal allele in which the conserved Gly residue at position 114 of the protein is replaced by Asp, probably inactivating the *Cdc42* protein (Genova *et al.*, 2000). It is interesting to mention that a mutant allele affecting the same region of the *S. cerevisiae* *Cdc42* protein was reported to be more than simply a null allele, having a dominant negative effect (Ziman *et al.*, 1991). The *Cdc42*<sup>4</sup> mutant contains a nucleotide substitution in an splice acceptor site (Genova *et al.*, 2000), probably producing an incomplete protein and thus reducing *Cdc42* function. The results obtained with this allele, which is supposed to be a true loss-of-function allele, suggest that *Cdc42* and *Mtl* may act cooperatively during PCP generation (see below).

#### ***Mtl* interacts genetically with other genes involved in polarity generation**

We also tested for genetic interactions between *Mtl* and other genes involved in PCP establishment like *strabismus* (*stbm*, Wolff and Rubin, 1998) and *prickle* (*pk*, Gubb *et al.*, 1999). It has been reported that *Stbm* and *Pk* are restricted to the R4 precursor cell to properly modulate Fz signaling (Jenny *et al.*, 2003; Rawls and Wolff, 2003). *Pk* and *Stbm* interact physically, leading to the assembly of a *Stbm/Pk* containing signaling complex that is thought to negatively regulate Fz/Dsh activity and membrane localization (Jenny *et al.*, 2003). Both genes dominantly enhance the *sev>Mtl* phenotype (Table 1). One of these genes, *stbm*, was also found to interact genetically with *Rac1* (Fanto *et al.*, 2000). Moreover, we also see a dominant enhancement of this phenotype with the rotation-specific allele of the *Egfr*-inhibitory ligand *argos* (*aos*<sup>rt</sup>, Gaengel and Mlodzik, 2003). It has been recently reported that *Egfr* signaling regulates ommatidial rotation through two Ras-effector pathways, the Ras/Raf/MAPK cascade and Ras/Cno signaling (Gaengel and Mlodzik, 2003). Moreover, it regulates Cadherin activity in this context (Mirkovic and Mlodzik, 2006). This interaction and the fact that overexpression of *Mtl* in the eye mainly causes misrotations, suggests an involvement of *Mtl* in regulating this aspect of the PCP process.

TABLE 2

#### **A NULL *MTL* MUTANT ALLELE SUPPRESSES THE *SEV-RAC1*<sup>V12</sup> PHENOTYPE**

| Genotype  | Wild-type ommatidia (in % ± sd) | Number of ommatidia scored |
|---|---------------------------------|----------------------------|
| <i>sev-Rac1</i> <sup>V12</sup> ; +/+ (Control)              | 34.1 (±4.5)                     | 406                        |
| <i>sev-Rac1</i> <sup>V12</sup> ; <i>Mtl</i> <sup>Δ</sup> /+ | 62.3 (±3.3)                     | 389                        |

The quantifications of allelic combinations are based on scoring of 3 independent eyes per genotype. The percentage shown in this table is the average number of wild-type ommatidia, with the standard deviation calculated across all eyes of a given genotype scored. The suppression is statistically significant ( $p \leq 0.01$ ,  $t$ -test).

TABLE 3

**PERCENTAGE OF ABNORMAL OMMATIDIA IN EYE CLONES MUTANT FOR THE INDICATED ALLELIC COMBINATIONS**

| Genotype  | Abnormal ommatidia (in % $\pm$ sd) | Number of clones analyzed | Number of ommatidia scored |
|---|------------------------------------|---------------------------|----------------------------|
| <i>Cdc42<sup>5</sup></i>  | 2.9 ( $\pm$ 3.8)                   | 6                         | 422                        |
| <i>Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup> #1</i>                 | 4.6 ( $\pm$ 3.1)                   | 7                         | 486                        |
| <i>Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup> #2</i>                 | 5.5 ( $\pm$ 2.2)                   | 19                        | 1252                       |
| <i>Cdc42<sup>5</sup>, Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup></i> | 14.2 ( $\pm$ 3.5)                  | 12                        | 941                        |

*Cdc42<sup>5</sup>* and *Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup> #1* quantifications correspond to control clones generated separately. *Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup> #2* and *Cdc42<sup>5</sup>, Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup>* quantifications correspond to the results obtained for two populations of different clones obtained from the same cross. The difference in the frequency of abnormal ommatidia between *Cdc42<sup>5</sup>, Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup>* and *Rac1<sup>J11</sup>, Rac2<sup>A</sup>, Mtl<sup>A</sup> #1 or #2* is statistically significant ( $p \leq 0.001$ , *t*-test). In all cases about 1% of ommatidia show problems in photoreceptor differentiation.

***Cdc42<sup>5</sup>* mutant flies exhibit mild PCP defects in eyes and wings**

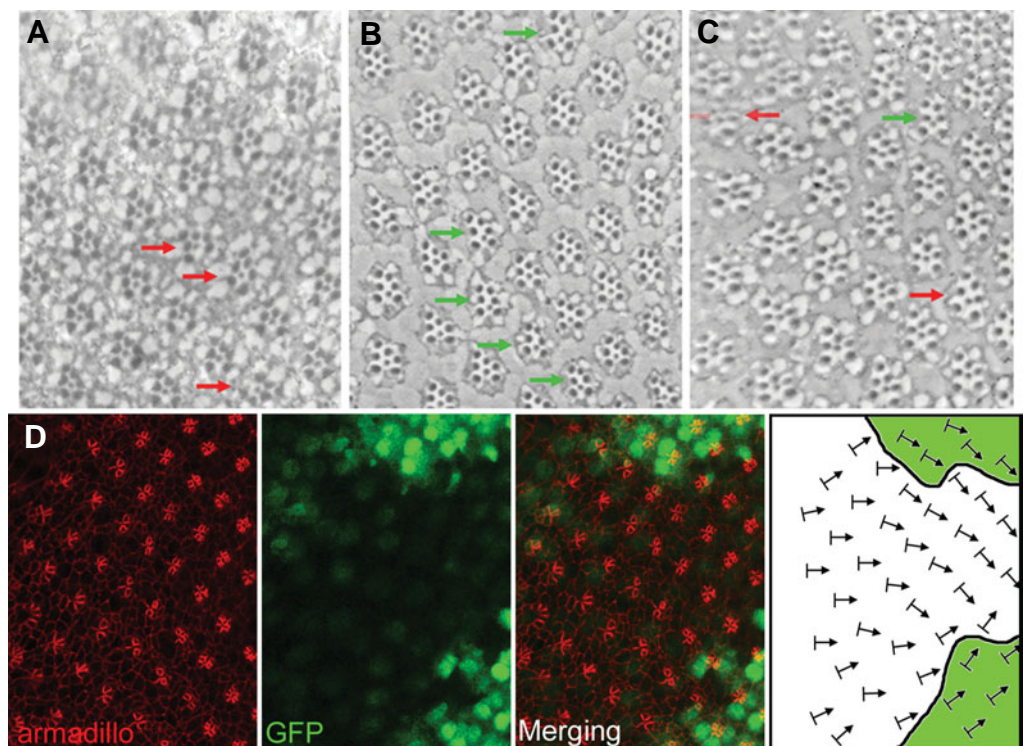
The role of *Cdc42* in PCP establishment in the wing has been previously assessed by ectopic expression of different dominant negative isoforms (Eaton *et al.*, 1995; 1996; Baron *et al.*, 2000). These studies suggested that *Cdc42* is necessary for the formation of polarized actin structures, since overexpression of *Cdc42<sup>F89</sup>* or *Cdc42<sup>L89</sup>* caused abolishment of both actin polymerization and hair outgrowth, resulting in wings with no hair or with stunted hairs (Eaton *et al.*, 1995; 1996). In addition, expression of *Cdc42<sup>N17</sup>* produced a multiple wing hair phenotype (Baron *et al.*, 2000). In the eye, it has been demonstrated that flies heterozygous for weak and strong *Cdc42* mutant alleles have mild rough eyes, although no defects in ommatidial orientation were reported (Boutros *et al.*, 1998; Genova *et al.*, 2000).

To test more definitively whether *Cdc42* plays a role in PCP establishment, we analyzed the effect of reducing *Cdc42* function in eyes and wings. As clones of the null allele *Cdc42<sup>3</sup>* and *Cdc42<sup>4</sup>* do not survive (Genova *et al.*, 2000; data not shown), we analyzed tangential sections of eyes from *Cdc42<sup>5</sup>* (a hypomorphic allele) hemizygous males. These show PCP defects, although at very low frequency (2.9%; Table 3), reflected by the presence of misrotated and achiral ommatidia (Fig. 3A and data not shown). Similar results have been found in females in homozygous mitotic eye clones for the *Cdc42<sup>5</sup>* allele (Table 3). Moreover, analyses of wings from *Cdc42<sup>5</sup>* individuals also revealed mild PCP defects, with cells exhibiting a multiple wing hair phenotype, producing double or

triple hairs (Fig. 3B). These data suggest that *Cdc42* has a role in PCP generation in eyes and wings, and could account for the redundancy of function proposed for the Rac1 and Rac2 in this process. In support of this model, a study has demonstrated that *Cdc42* acts redundantly with Rac1 and Rac2 during embryonic blood cell migration (Paladi and Tepass, 2004).

***Eye clones mutant for different combinations of Mtl, Rac1, Rac2 and Cdc42 alleles display polarity phenotypes***

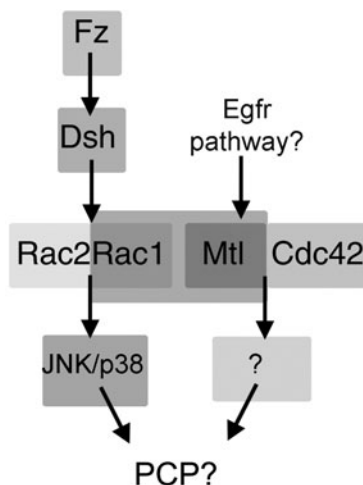
To further investigate the role of *Mtl*, *Rac1*, *Rac2* and *Cdc42* in PCP establishment, we generated eye clones mutant for different allelic combinations of all four genes. We used null alleles for *Rac1*, *Rac2* and *Mtl* (*Rac1<sup>J11</sup>*, *Rac2<sup>A</sup>* and *Mtl<sup>A</sup>*, respectively) and the hypomorphic *Cdc42<sup>5</sup>* allele. Phenotypic analyses of eye clones double mutant for either *Rac1* and *Mtl* or *Rac1* and *Rac2* revealed no PCP defects (data not shown). Besides, clones mutant for either *Mtl* and *Cdc42* or *Rac1*, *Rac2* and *Cdc42* showed a low frequency of PCP defects (data not shown), comparable to the results obtained in *Cdc42<sup>5</sup>* mutants or mutant clones. Next we generated eye clones triply mutant for *Rac1*, *Rac2* and *Mtl*. Although previous analyses of such clones suggested that there is no requirement of these GTPases during PCP generation (Hakeda-Suzuki *et al.*, 2002), we detected reproducible ommatidial polarity defects (Fig. 4A). Analyses of such clones revealed that, although many ommatidia display correct polarity, achiral or misrotated triply mutant ommatidia are reproducibly detected



**Fig. 4. Eye clones which are quadruple mutant for *Rac1*, *Rac2*, *Mtl* and *Cdc42* show planar cell polarity defects.** (A) Homozygous mutant clone for *Rac1<sup>J11</sup>*, *Rac2<sup>A</sup>* and *Mtl<sup>A</sup>*. (B,C) Clones homozygous mutant for *Cdc42<sup>5</sup>*, *Rac1<sup>J11</sup>*, *Rac2<sup>A</sup>* and *Mtl<sup>A</sup>*. Red arrows point to misrotated ommatidia, green arrows point to achiral ommatidia. (D) Anti-Arm staining (red) in third instar larval eye imaginal disc with a *Cdc42<sup>5</sup>*, *Rac1<sup>J11</sup>*, *Rac2<sup>A</sup>* and *Mtl<sup>A</sup>* homozygous mutant clone (marked by the absence of GFP fluorescence). The last panel is a schematic representation of the ommatidial rotation angles in the clone.

(4.6% of abnormal ommatidia, see *Rac1<sup>111</sup>, Rac2<sup>Δ</sup>, Mtl #1* in Table 3). The low penetrance of this phenotype may indicate that the *Rac* genes play a minor role during PCP generation. However, it can also suggest that their function is largely redundant in this process. Since we have found that *Cdc42* also has a role during PCP generation, we asked whether the PCP defects found in eye clones triply mutant for the three GTPases *Rac1*, *Rac2* and *Mtl* could be modified by reducing the function of *Cdc42*. We thus generated clones that were quadruple mutant for all four GTPases (see Material and Methods). Strikingly, these quadruple mutant clones display PCP defects at higher frequency. Analysis of tangential sections of such clones revealed typical PCP defects, like symmetrical and misrotated ommatidia (Fig. 4B,C), with a frequency of close to 15% (Table 3, see *Cdc42<sup>Δ</sup>, Rac1<sup>111</sup>, Rac2<sup>Δ</sup>, Mtl<sup>Δ</sup>*). Control clones triply mutant for the three *Rac* genes (originating from the same cross) showed defects at 5.5% frequency, comparable to the 4.6% obtained in the original triply mutant clones (Table 3, compare *Rac1<sup>111</sup>, Rac2<sup>Δ</sup>, Mtl<sup>Δ</sup>#2* to *Rac1<sup>111</sup>, Rac2<sup>Δ</sup>, Mtl<sup>Δ</sup>#1*). This indicates that the reduction of *Cdc42* function is causing the increased PCP defects and that these are not due to genetic background variation in the clones. Armadillo stainings of quadruple mutant eye disc clones confirmed rotation abnormalities and revealed no loss of accessory ommatidial cells that could lead to problems in local cell stacking (Fig. 4D). To support our results in the eye, we have also generated unmarked quadruple mutant clones in adult wings. In these wings we occasionally observed PCP phenotypes, like duplications of wing hairs and groups of hairs that were not pointing distally (data not shown).

Taken together, these results could indicate that the *Rac/Cdc42* GTPases may have overlapping functions during PCP establishment in the *Drosophila* eye. Then, the low penetrance of the mutant phenotype in the quadruple mutant clones, which is



**Fig. 5. GTPases of the Rho subfamily function in parallel pathways.**

A simplified schematic view of the Fz/PCP pathway and the relative position of several members of the Rho subfamily of GTPases are shown. As was previously reported, *Rac1* and *Rac2* lead to transcriptional activation through the JNK/p38s MAPK cascade. In addition, *Mtl* might function in the *Egfr* pathway. However, the specific effectors of *Mtl/Cdc42* are currently unknown.

comparable to hypomorphic alleles of PCP genes, would be consistent with *Cdc42<sup>Δ</sup>* being a hypomorphic allele. However, another explanation for the mild PCP phenotypes observed in the clones could be that these proteins have a minor role during the PCP process. As mentioned above, the closely related RhoA GTPase plays a non-redundant role in PCP establishment (Strutt *et al.*, 1997). One possibility could be that the *Rac/Cdc42* GTPases cooperate with RhoA during PCP generation. Then, a reduction of *RhoA* function could also modify the PCP defects obtained by loss of function of the *Rac* genes. To test this, we generated clones quadruple mutant for the *Rac1*, *Rac2*, *Mtl* and *RhoA* genes (using the strong hypomorphic allele *RhoA<sup>41</sup>*; Strutt *et al.*, 1997). However, these quadruple mutant clones were not informative as we found mainly photoreceptor loss in such clones, obscuring a potential to score for PCP defects (data not shown). Since the proposed redundancy among the Rho GTPases during PCP generation has not been demonstrated, we can not rule out the possibility that these proteins are required at low levels during this process.

## Discussion

In this report we have analyzed in detail the role of *Mtl*, *Rac1*, *Rac2* and *Cdc42*, four GTPases of the Rho subfamily in *Drosophila*. First, we show that overexpression of different *Mtl* isoforms in eyes and wings produces classical PCP phenotypes, as previously reported for other members of the family, thus suggesting that *Mtl* also has a role during PCP generation in these tissues. Moreover, genetic interaction assays indicate that *Mtl* is functionally related to *Rac1* and *Cdc42*, but it does not function in the JNK pathway. Since previous results showed that *Rac1* interacts genetically with *Rac2* and *RhoA* (Fanto *et al.*, 2000), we conclude that *Rac1*, *Rac2* and *RhoA* could act redundantly in Fz/PCP signaling, and *Mtl* could be acting together with *Cdc42*, both aspects being connected through *Rac1* and *Mtl*. Regarding this, we also show that flies hemizygous for a hypomorphic *Cdc42* allele, as well as mitotic eye clones for the same allele, display typical PCP defects, thus suggesting that *Cdc42* may also function in PCP generation. In such a scenario, and in contrast to previously published results (Hakeda-Suzuki *et al.*, 2002), we also demonstrate that mitotic eye clones triply mutant for the *Rac* GTPases (*Mtl*, *Rac1* and *Rac2*) show polarity defects, albeit at relatively low frequency. Strikingly, the frequency of the defects is increased in the triple null *Rac* mutant background by reducing *Cdc42* function with a *Cdc42* hypomorphic allele. Taken together, all these results suggest that the *Rac/Cdc42* GTPases may have a role during PCP generation but probably function redundantly in this process, since only the removal of the four GTPases causes PCP defects. An explanation for the mild PCP phenotypes observed in the quadruple mutant clones, which is comparable to hypomorphic alleles of PCP genes, would be the fact that *Cdc42<sup>Δ</sup>* is a hypomorphic allele. In such a scenario, *RhoA*, another small GTPase of the Rho subfamily, has a well established and non-redundant role in this process (Strutt *et al.*, 1997), and it interacts genetically with *Rac1* (Fanto *et al.*, 2000). Since there is a high degree of homology among all these proteins, one possibility could be that the *Rac/Cdc42* GTPases cooperate with *RhoA* during PCP generation. However, since we could not demonstrate the proposed redundancy of function between all these GTPases, an alternative explanation could be that the *Rac/Cdc42* GTPases are required at low levels during PCP establishment.

Besides this, the results obtained in the genetic interaction assays indicate that not all the GTPases of the Rho subfamily function upstream the JNK module and that they act in parallel pathways. Our results and previous reports suggest that although Rac1 and Rac2 function downstream of Dsh in the Fz/PCP pathway through JNK/p38 kinases (Boutros *et al.*, 1998; Paricio *et al.*, 1999; Weber *et al.*, 2000), Mtl and Cdc42 might receive a different activating input, as they do not show genetic interactions with gain-of-function *Fz* and *Dsh* phenotypes, and Mtl does not interact with JNK components (Boutros *et al.*, 1998; this paper). It is interesting to mention that the rotation-specific phenotype obtained by Mtl overexpression, together with the fact that this GTPase genetically interacts with members of the Egfr pathway and cell adhesion components related to it (data not shown), could indicate that Mtl function in the Egfr pathway regulating ommatidial rotation during the final steps of PCP establishment (this paper; F. Durupt, S.M.-D. and N.P., in preparation). Taken together, all these observations suggest that the requirement of the four GTPases might be subdivided into pairs: Mtl could share a function with Cdc42 (this is supported by data from mammalian tissue culture experiments, where Cdc42 and mammalian Mtl appear to have the same function; A. Hall, personal communication) and Rac1 with Rac2, and both pairs would be connected through the shared functional Rac1-Mtl interaction (Fig. 5). However, whether the GTPase pairs (Mtl-Cdc42, Rac1-Rac2, and Rac1-Mtl) act in parallel or in a hierarchy remains unclear. Although we did not include RhoA in our model, this GTPase functions downstream of Dsh in the Fz/PCP pathway (Strutt *et al.*, 1997) and upstream of the JNK cassette, and interacts genetically with Rac1 (Fanto *et al.*, 2000). Thus, it will function together with the Rac1-Rac2 pair.

In summary, our results are similar to the data obtained from the study of vertebrate gastrulation. Habas *et al.* (2003) have reported that RhoA and Rac have independent parallel roles during the convergent extension process in vertebrate gastrulation downstream of Fz-Dsh signaling, and that only Rac is able to activate JNK. This is consistent with our data (and previous publications on *Drosophila* PCP generation; Eaton *et al.*, 1996; Fanto *et al.*, 2000). In addition, our data suggest that *Cdc42* could function redundantly with the *Rac* genes. Supporting this, *Cdc42* has also a reported role in convergent extension/vertebrate gastrulation (Choi and Han, 2002). Whether and how this is linked to Fz-PCP signaling remains unclear. Although our results do not provide clear evidence of the redundant function of the GTPases of the Rho subfamily during PCP generation, we can conclude that the situation in vertebrates and *Drosophila* is similar: not all the GTPases act upstream of a JNK cassette, and there are probably (at least) two parallel Rho GTPase family functions.

## Materials and Methods

### Generation of flies expressing Mtl transgenes

To generate the UAS-*Mtl*/wild type construct, the complete *Mtl*cDNA was cloned into the pUAST *Drosophila* transformation vector (Brand and Perrimon, 1993). Transgenic flies were generated by standard P-element-mediated transformation (Spradling and Rubin, 1982).

### Fly strains and genetic interactions

Flies were grown on standard media at 25°C (unless stated otherwise). GAL4 stocks used were: *sev*-GAL4 K25 for the third chromosome (gift from Konrad Basler), *en*-GAL4 and *C765*-GAL4. Mutant stocks used

were: *msn*<sup>102</sup> (Treisman *et al.*, 1997), *RhoA*<sup>ZR</sup>, *RhoA*<sup>AY</sup> (Strutt *et al.*, 1997), *hep*<sup>R75</sup> (Glise *et al.*, 1995), *bsk*<sup>2</sup> (Riesgo-Escovar *et al.*, 1996), *jur*<sup>2</sup> (Kockel *et al.*, 1997), *stbm*<sup>X</sup> (N. Paricio, unpublished), *pk<sup>pk-sple</sup>9* (Gubb *et al.*, 1999), *aos*<sup>4</sup> (Gaengel and Mlodzik, 2004), *Rac*<sup>1/11</sup>, *Rac*<sup>2</sup>, *Mtl*<sup>A</sup> (Hakeda-Suzuki *et al.*, 2002), *Cdc42*<sup>3</sup>, *Cdc42*<sup>4</sup> and *Cdc42*<sup>5</sup> (Genova *et al.*, 2000).

We also used the *sevenless* enhancer driven construct *sev*-*Rac*<sup>V12</sup> for interactions with *Mtl* alleles (Fanto *et al.*, 2000). A *sev*/*Mtl* line was generated by recombination of *sev*-GAL4 and UAS-*Mtl* chromosomes. Genetic interactions with that line were performed at 25°C. The flies analyzed were heterozygous for *sev*/*Mtl* and the mutation of interest. *w*<sup>1118</sup> was used as a negative control.

### Generation of mitotic eye clones

Eye clones were generated with the FRT/FLP recombination system (Golic and Linquist, 1989) using *ey*-FLP lines. To generate clones quadruple mutant for *Rac1*, *Rac2*, *Mtl* and *Cdc42* we set up two independent crosses. First, *Cdc42*<sup>5</sup>/FRT19A females were crossed to *Rac1*<sup>1/11</sup> *Rac2*<sup>3</sup>, *Mtl*<sup>A</sup>/FRT2A/TM6 (Hakeda-Suzuki *et al.*, 2002) males. Simultaneously, we crossed FRT19A/FM6; *ey*-FLP females to *ey*-FLP/Y; *sp* CyO; 2x *Ubi*-GFP, FRT2A/TM6, *Ubx* males. From both, we selected the non-balanced progeny and crossed non-balanced males from the first cross to non-balanced females from the second cross. The eye clones are marked by the absence of pigment in adults and by the absence of GFP fluorescence in discs. Adult eye clones were analyzed only in the female offspring, in which 50% will be quadruple mutant for *Rac1*, *Rac2*, *Mtl* and *Cdc42* and 50% will be triply mutant for *Rac1*, *Rac2* and *Mtl*. Among the clones analyzed, we could distinguish two different populations, based on the frequency of the PCP defects they contained. One population exhibited a similar frequency of defects than the control triple mutant clones generated and corresponds to clones mutant only for the *Rac* genes. The second population showed PCP defects at higher frequency than the controls and corresponds to the quadruple mutant clones. The differences in frequency of defects between both populations are statistically significant.

### Histology and immunohistochemistry

Sections of adult eyes were performed as previously described (Tomlinson and Ready, 1987). Wings were dissected from adult flies in SH solution and mounted in Faure medium. Imaginal disc stainings were done in 0.1 M phosphate buffer, 0.2% Triton X-100 and 10% normal goat serum. Primary antibodies used were mouse anti-Elav and mouse anti-Armadillo (both from the Developmental Studies Hybridoma Bank), rabbit anti-Spalt (gift from Rosa Barrio) and rabbit anti-β-gal (polyclonal from Cappel). Secondary antibodies coupled to fluorochromes were purchased from Calbiochem. Pictures were taken using a Leica TCS-NT confocal laser-scanning microscope.

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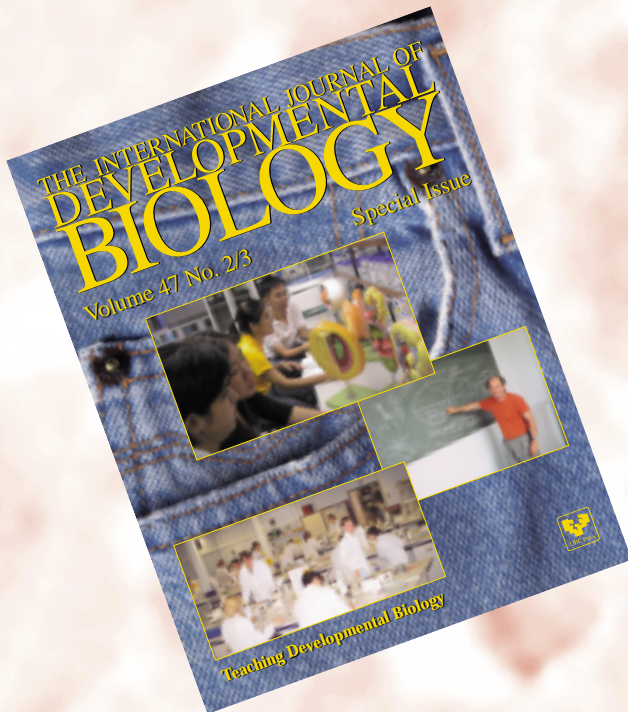
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*Int. J. Dev. Biol.* (1993) 37: 539-546

# Teaching Developmental Biology

Edited by George M. Malacinski and Susan T. Duhon



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