Fly

ISSN: 1933-6934 (Print) 1933-6942 (Online) Journal homepage: https://www.tandfonline.com/loi/kfly20

Mtl interacts with members of Egfr signaling and cell adhesion genes in the Drosophila eye

Verónica Muñoz-Soriano, Yaiza Belacortu, Fabrice C. Durupt, Silvia Muñoz-Descalzo & Nuria Paricio

To cite this article: Verónica Muñoz-Soriano, Yaiza Belacortu, Fabrice C. Durupt, Silvia Muñoz-Descalzo & Nuria Paricio (2011) *Mtl* interacts with members of Egfr signaling and cell adhesion genes in the Drosophila eye, Fly, 5:2, 88-101, DOI: 10.4161/fly.5.2.15457

To link to this article: https://doi.org/10.4161/fly.5.2.15457

Published online: 01 Apr 2011.

Fly 5:2, 88-101; April/May/June 2011; © 2011 Landes Bioscience

Mtl interacts with members of Egfr signaling and cell adhesion genes in the Drosophila eye

Verónica Muñoz-Soriano,[‡] Yaiza Belacortu,[‡] Fabrice C. Durupt, Silvia Muñoz-Descalzo[†] and Nuria Paricio*

Departamento de Genética; Facultad CC Biológicas; Universidad de Valencia; Burjasot, Spain

[†]Current address: Department of Genetics; University of Cambridge; Cambridge, UK

[‡]These authors contributed equally to this work.

Key words: *Mtl*, Rho GTPases, ommatidial rotation, Egfr pathway, cell adhesion, *hbs*

Abbreviations: *Mtl*, *Mig 2-like*; Egfr, epidermal growth factor receptor; LOF, loss-of-function; PCP, planar cell polarity; MF, morphogenetic furrow; ECM, extracellular matrix; JNK, jun N-terminal kinase

Mtl is a member of the Rho family of small GTPases in Drosophila. It was shown that Mtl is involved in planar cell polarity (PCP) establishment, together with other members of the same family like Cdc42, Rac1, Rac2 and RhoA. However, while Rac1, Rac2 and RhoA function downstream of Dsh in Fz/PCP signaling and upstream of a JNK cassette, Mtl and Cdc42 do not. To determine the functional context of Mtl during PCP establishment in the Drosophila eye, we performed a loss-of-function screen to search for dominant modifiers of a *sev>Mtl* rough eye phenotype. In addition, genetic interaction assays with candidate genes were also carried out. Our results show that *Mtl* interacts genetically with members and effectors of Egfr signaling, with components and/or regulators of other signal transduction pathways, and with genes involved in cell adhesion and cytoskeleton organization. One of these genes is *hibris* (*hbs*), which encodes a member of the immunoglobulin superfamily in Drosophila. Phenotypic analyses and genetic interaction assays suggest that it may have a role during PCP establishment, interacting with both Egfr and Fz/PCP signaling during this process. Taken together, our results indicate that Mtl is functionally related to the Egfr pathway regulating ommatidial rotation during PCP establishment in the eye, being a positive regulator of this pathway. Since Egfr signaling is linked to cytoskeletal and cell junctional elements, it is likely that Mtl may be regulating cytoskeleton dynamics and thus cell adhesion during ommatidial rotation in the context of that pathway.

Introduction

Rho GTPases constitute a distinct family within the superfamily of Ras-related small GTPases that control some of the most fundamental processes of cell biology common to all eukaryotes, including morphogenesis, polarity, cell movement and cell division.[1] In Drosophila, six members of this family have been described: Rac1, Rac2, Cdc42, RhoA, RhoL and Mtl, which are involved in multiple developmental processes like dorsal closure, axon guidance, bacterial response, blood cell migration or planar cell polarity (PCP) establishment.[2-15] Mtl is the Drosophila ortholog of the *C. elegans* Mig-2 Rho GTPase and is structurally similar to Rac1, Rac2 and Cdc42.[16,17] Several studies have demonstrated that it behaves functionally like Rac1 and Rac2 because these three GTPases act redundantly in regulating dorsal closure or axon guidance in Drosophila.[5,7,10] However, it seems that they display differential activities in processes like macrophage migration or fly immunity.[2,8] It has been also demonstrated that Mtl, Rac1, Rac2 and Cdc42 may have partially overlapping functions during PCP generation and that they are probably acting in two parallel pathways.[6]

Cell polarization is essential for cellular function, patterning and cytoskeletal organization. In many tissues, epithelial cells are polarized not only along the apical-basal axis but also within the plane of the epithelium, which is referred to as PCP. In the Drosophila eye, PCP is manifest in the precise orientation of ommatidia relative to the dorso-ventral midline, the equator. This ordered pattern is generated during larval life in the eye imaginal disc, where PCP establishment takes place as two different and simultaneous processes: ommatidia adopt opposite chirality in the dorsal and ventral halves of the eye disc while they rotate 90° towards the equator in two 45° steps.[18] Chirality is achieved by the proper differentiation of R3 and R4 photoreceptors, process controlled by the Fz/PCP signaling pathway that is activated upon reception by the Fz transmembrane receptor of a gradient of an unknown ligand (reviewed in ref. 19). The signal is then transduced to the nucleus via Dishevelled (Dsh) through Rho family GTPases, the STE20-like kinase Misshapen (Msn),

*Correspondence to: Nuria Paricio; Email: nuria.paricio@uv.es
Submitted: 01/26/11; Revised: 03/11/11; Accepted: 03/11/11
DOI: 10.4161/fly.5.2.15457

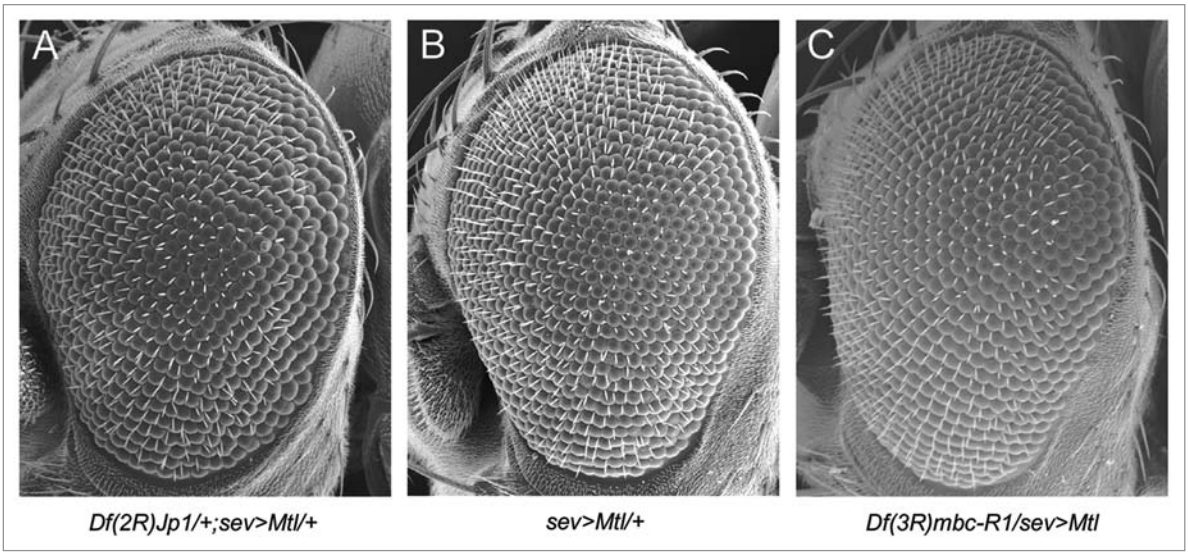

Figure 1. Dose-dependent modification of the *sev>Mtl* rough eye phenotype. Scanning electron micrographs of adult eyes: (A) *Df(2R)Jp1/+; sev>Mtl/+*, (B) *sev>Mtl/+* and (C) *Df(3R)mbc-R1/sev>Mtl/+* eye. Enhancement and suppression of the *sev>Mtl/+* eye phenotype is observed in (A and C), respectively (compare A, C to B).

JNK/p38 kinases and the transcription factors Jun and Fos,[20-23] leading to *Delta* upregulation in R3 and thus activating Notch (N)-signaling in the neighboring R4 precursor.[24,25] Moreover, the transcription factor Pointed (Pnt) is also required in R4 specification probably downstream of Epidermal growth factor receptor (Egfr) signaling.[23] Ommatidial rotation represents the final step in establishing PCP during eye development. The direction of rotation depends on proper R3/R4 cell fate specification since misrotation is a common phenotype observed in loss and gain-of function mutants of PCP genes.[19] In parallel to PCP signaling, which may regulate ommatidial rotation through effects on cytoskeletal elements via the Rho-Kinase Drok,[26] this process is also regulated by the Egfr pathway. It has been shown that both over- and under-activation of Egfr signaling give rise to ommatidial rotation defects, providing evidence for its involvement in that process.[27-29] Moreover, genes functionally related with cytoskeleton reorganization and cell adhesion act as downstream effectors of Egfr signaling, thus linking ommatidial rotation with cell adhesion and cytoskeleton rearrangement processes.[28,30] Other genes required in ommatidial rotation are *nemo* (*nmo*), *scabrous* (*sca*) and *zipper* (*zip*).[31-34] *nmo* encodes a serine/threonine kinase, which is essential to regulate the speed of the process.[31,34,35] Sca, a fibrinogen-related secreted protein,[36] has been implicated in controlling the normal arrest of rotation at 90°.[32] Finally, *zip* encodes the non-muscle Myosin II heavy chain, a protein that regulates the rate of rotation and has been shown to act downstream of Fz/PCP and Egfr signaling.[4,26,28,33]

Several Rho GTPases, such as Rac1, Rac2, RhoA, Cdc42 and Mtl, are involved in PCP establishment.[3,4,6,9] It has been reported that only clones triple mutant for *Rac1*, *Rac2* and *Mtl* showed mild PCP defects, which are enhanced when reducing *Cdc42* function, thus suggesting that these four GTPases may act redundantly during this process.[6] Genetic interaction assays indicated that, while Rac1, Rac2 and RhoA function in Fz/PCP signaling upstream of JNK/p38 kinases,[20-22] Mtl and Cdc42 seem to be acting in a parallel pathway, being both probably connected through Rac1-Mtl functional interaction.[6] Since *Mtl* overexpression in the eye mainly caused ommatidial rotation defects and it interacted genetically with *argos* (encoding the Egfr-inhibitory ligand), we suggested that it could function in the Egfr pathway regulating that aspect of the PCP process.[6]

In order to determine the functional context of Mtl during PCP establishment in the Drosophila eye, we have performed a loss-of-function (LOF) screen and a candidate gene approach looking for dominant modifiers of the *sev>Mtl* rough eye phenotype. Our results show that *Mtl* interacts genetically with members and effectors of Egfr signaling, and suggest that Mtl acts as a positive regulator of the Egfr pathway. Furthermore, we identify and characterize several candidate genes that might be functionally related to Mtl during PCP establishment and have a role in ommatidial rotation. Among them, we find genes encoding proteins involved in cell adhesion and cytoskeleton organization suggesting that *Mtl* could be a general regulator of both processes in the context of Egfr signaling. One of the genes identified is *hibris* (*hbs*), which encodes a member of the immunoglobulin superfamily in Drosophila involved in myoblast fusion and eye morphogenesis.[37-40] Preliminary phenotypic analyses of *hbs* mutants and genetic interaction assays suggest that this gene may have also a role during PCP establishment and ommatidial rotation in the Drosophila eye.

Results

Identification of dominant modifiers of the *sev>Mtl* eye phenotype. Previous studies showed that overexpression of a wild-type form of *Mtl* in the Drosophila eye produces externally rough eyes that in tangential sections display PCP defects. They are mainly misrotations and ommatidial chirality is rarely affected. We

Table 1. Chromosomal regions that genetically interact with *sev>Mtl*

Deficiency	External modification[a]	Cytology	Candidate genes	LOF alleles tested[b]
Df(2L)J39 (1469)	S	31C-D; 32D-E	*hup*	NT
Df(2L)TW161 (167)	S	38A6-B1; 40A4-B1	*dia*	dia^{k07135} (-)
Df(2R)ST1 (1888)	S	42B3-5; 43E15-18	*jing, pk*	$pk^{pk\text{-}sple9}$ (E)
Df(2R)enA (190)	S	47D3-48A5	-	-
Df(2R)en30 (1145)	S	48A3-4; 48C6-8	-	-
Df(2R)CX1 (442)	E	49C1-4; 50C23-D2	*arr, mam, RacGAP50C, E(Egfr) B56, sca, drk*	mam^{8} (-), sca^{BP2} (-), drk^{e0A} (-)
Df(2R)BSC11 (6455)	E	50E6-F1; 51E2-4	*phyl, hbs*	$phyl^{2245}$, UAS-*phyllR* (E), hbs^{361} (E)
Df(2R)Jp1 (3518)	E	51D3-8; 52F5-9	*hbs, Dg, scb, sli*	hbs^{361} (E), scb^{2} (-), sli^{2} (-)
Df(2R)Jp8 (3520)	E	52F5-9; 52F10-53A1	*shark*	$shark^{1}$ (-)
Df(2R)M60E (2471)	S	60E2-3; 60E11-12	-	-
Df(3L)Ar14-8 (439)	S	61C5-8; 62A8	*Rac1, trio, rho*	$Rac1^{J11}$ (E), $trio^{S095914}$, $trio^{6A}$ (S), rho^{ve1}, rho^{AA69} (S)
Df(3L)29A6 (2479)	S	66F5-67B1	-	-
Df(3L)st-f13 (2993)	E	72C1-D1; 73A3-4	*aos, bul, Mbs*	aos^{rlt}, $aos^{\Delta 7}$ (E), Mbs^{3} (-)
Df(3L)VW3 (3000)	S	76A3; 76B2	-	-
Df(3L)XS533 (5126)	S	76B4; 77B	*gig, Gβ76C, trc, fat2*	gig^{109} (-), $G\beta 76C^{1}$ (-), trc^{1} (-)
Df(3R)mbc-R1 (2585)	S	95A5-7; 95D6-11	*mbc, E(rst)C436, E(rst)F84*	mbc^{c1}, $mbc^{D11.2}$ (S)
Df(3R)D605 (823)	S	97E3; 98A5	*Ser, side*	NT

Dose-dependent modifiers of the rough eye phenotype observed in adult *sev Mtl* flies. The deficiency name is followed by the Bloomington stock number in parenthesis. [a]E or S indicate enhancement or suppression of the external *sev>Mtl* phenotype. [b](E), (S) or (-) indicate enhancement, suppression or no modification, respectively, of the internal *sev>Mtl* phenotype by the corresponding mutant allele; NT indicates that no alleles were tested. Quantification of the interactions are shown in **Table 2**. The interactions with $Rac1^{J11}$, $pk^{pk\text{-}sple9}$ and aos^{rlt} alleles were previously reported.[6]

also found that these defects are already evident in eye imaginal discs, and are thus primary defects.[6] In contrast, LOF alleles of *Mtl* do not show any obvious phenotype.[5] In order to identify loci functionally related to *Mtl*, we decided to carry out a dominant modifier screen in a *Mtl*-missexpression background, since LOF alleles are not amenable for genetic screens. First, and to validate the suitability of the *sev>Mtl* phenotype to perform a large-scale genetic screen, we performed a pilot screen for dose-dependent modifiers of the *sev>Mtl* rough eye phenotype by testing 80 deficiencies on the second chromosome, 84 on the third chromosome, and two on the fourth chromosome that remove a total of ~75% of the loci on these chromosomes (see Materials and Methods). Seventeen deficiencies reproducibly modified the *sev>Mtl* rough eye phenotype (~8% of the tested lines; example in **Fig. 1**): five were enhancers and 12 were suppressors (**Table 1**). Next, we looked for candidate genes in the deficiencies that could be potentially responsible for the detected interaction, selecting those involved in PCP establishment or encoding components of signaling pathways or proteins involved either in regulation of GTPase function or in cytoskeleton organization and cell adhesion. Several mutant alleles of the candidate genes were tested for their ability to interact with *Mtl* by analyzing their effect on the internal *sev>Mtl* phenotype (**Tables 1 and 2**). In some cases, there was an inconsistency in the direction of the interaction found when using those alleles. Either the existence of additional unknown *Mtl*-interacting genes mapping within the deficiencies, or the different level of detection of the interactions (external or internal modification of the *sev>Mtl* eye phenotype) could explain these results. Furthermore, mutant alleles of several genes selected as candidates did not modify the *sev>Mtl* phenotype,

Table 2. Quantification of the genetic interactions between *Mtl* and candidate genes from the deficiency screen

Genotype (*sev>Mtl*/+ for all genotypes)	Wild-type ommatidia (% ± sd)	Number of ommatidia
w^{1118} (+/+, control)	74.3 ± 2.4*	1123
$phyl^{2245}$	63.3 ± 6.1*	433
UAS-*phyllR*	49.7 ± 3.7**	456
hbs^{361}	22.6 ± 2.2**	246
$trio^{S095914}$	81.3 ± 3.2*	498
$trio^{6A}$	89.7 ± 3.6**	374
rho^{ve1}	84.8 ± 1.7**	257
rho^{AA69}	87.2 ± 4.8**	413
$aos^{\Delta 7}$	62.6 ± 4.6**	590
mbc^{c1}	84.4 ± 1.6**	498
$mbc^{D11.2}$	81.6 ± 5.0*	426

Percentage of wild-type ommatidia (± standard deviation) of eyes from flies heterozygous for the indicated alleles and containing one copy of *sev>Mtl*. All alleles listed show significant modification of the *sev>Mtl* phenotype (*p < 0.05, **p < 0.005, t-test).

Table 3. P-insertion lines that modify the *sev>Mtl* phenotype

P line	External modification[a]	Cytology	Disrupted gene	Tested alleles[b]	Comments
P{lacW}syt^{k05909}	S	23A6-B1	*dawdle* (*daw*)	*daw*^{DG14702} (-)	Non-BMP TGFβ ligand
P{lacW}mts^{s5286}	S	28D1-2	*microtubule star* (*mts*)	*mts*^{02496} (S)	Catalytic subunit of the PP2A complex
P{lacW}l(2)k13305^{k13305}	S	30F5-6	*Translocation protein 1* (*Trp1*)	*Trp1*^{c03479} (S), *Trp1*$^{EP-663}$ (E)	Protein transporter activity
P{lacW}l(2)35Bb^{k11524a}	S	35B6-7	*moladietz* (*mol*)	*mol*^{e02670}, *mol*$^{35Bb-1}$ (E)	Asymmetric protein localization
P{lacW}l(2)k13805^{k13805}	S	37A1-2	*gluon* (*glu*)?	*glu*$^{88-37}$, *glu*$^{88-44}$ (E)	Sister chromatid segregation, chromatin remodelling
P{lacW}spi^{s3547}	S	37F2	*spitz* (*spi*)	*spi*^{1}, *spi*^{s3547} (S)	Activating ligand of the EGFR pathway
P{lacW}Rs1^{k09514}	S	44C1-2	*Rs1*	*Rs1*^{k09513}, *Rs1*^{k09514} (S)	ATP-dependent RNA helicase activity
P{lacW}Uba1^{s3484}	S	46A1-3	*Ubiquitin activating enzyme 1* (*Uba1*)	*Uba1*^{05642} (E), *Uba1*^{DG24412} (S)	Axon pruning and polarisation
P{lacW}SelD^{k12303}	S	50E4-7	*Glycogen phosphorylase* (*GlyP*)	*GlyP*^{k07918} (-)	Glycogen metabolism
P{lacW}Cdk4^{s4639}	S	53C9	*Cyclin-dependent kinase 4* (*Cdk4*)	*Cdk4*^{05428} (S)	JAK-STAT signaling pathway
P{lacW}mbl^{k07103}	S	54B1-5	*muscleblind* (*mbl*)	*mbl*^{E27}, *mbl*^{E127} (S)	Eye and muscle development
P{lacW}l(2)k08002^{k08002}	S	56F6-9	*18 wheeler (18w)*	*18w*$^{\Delta 7-35}$, *18w*^{k02701} (S)	Toll-like receptor, cell adhesion, Rho-signaling pathway
P{lacW}blw^{k00212}	S	59B1-2	*bellwether (blw)*	*blw*^{1}, *blw*^{DG06809} (S)	Mitochondrial ATP synthase α subunit
P{lacW}sls^{j1D7}	S	62C1-2	*sallimus (sls)*	*sls*^{1} (S)	Myoblast fusion and muscle development
P{lacW}dos^{P115}	S	62E	*daughter of sevenless (dos)*	*dos*^{R31} (-)	Photoreceptor differentiation
R^{PIE-6}*P{lacW}J45 Psn*^{9} *P{lacW}K33*	E	68A1-2, 96F10-11	*Roughened (R)*	*R*^{CD5}, *R*^{67} (E)	Ras family of small GTPases, Integrin activator
P{lacW}RpS12^{s2783}	S	69F5-6	*Ribosomal protein S12* (*RpS12*)	*RpS12*^{EY04767}, *RpS12*^{EY23269} (S)	Structural constituent of the ribosome
P{lacW}l(3)L2100^{L2100}	S	84B2-3	*CG1965*	NA	Transcription factor activity
P{lacW}l(3)L1231^{L1231}	S	88C9-11	*CG7832*	*CG7832*^{EY04982} *CG7832*^{EP3047} (-)	Unknown molecular and biological function
P{lacW}Dad^{j1E4}	S	89E10-11	*Daughters against dpp (Dad)*	*Dad*^{c02089}, UAS-*DadIR* (S)	Dpp antagonist
P{lacW}l(3)j12B4^{j12B4}	S	96C8-9	*CG11859*	*CG11859*^{f03457}, *CG11859*^{j12B4} (S)	Protein kinase activity
P{lacW}l(3)j8B9^{j8B9}	S	99F8-9	*prolyl-4-hydroxylase-αEFB (PH4αEFB)*	*PH4αEFB*^{EY13295}, *PH4αEFB*$^{j8B9Ex13-11}$ (S)	Collagen modification activity
P{lacW}l(3)s1921^{s1921}	S	100E1-2	*CG2245*	NA	Peptidyl-lysine modification to hypusine

All P lines that modify externally the *sev>Mtl* rough eye phenotype are listed. [a]S, E denotes external suppression or enhancement, respectively, of that phenotype. The cytological location of the insertions is shown. The disrupted gene in each P-line has been identified by performing inverse PCR or by analyzing the flanking DNA sequences available. In the *P{lacW}l(3)j12B4*^{j12B4} line the identification was performed by complementation analyses. [b]Mutant alleles of the genes identified have been used to validate the interactions by analyzing the modification of the internal *sev>Mtl* phenotype, (E), (S) or (-) as in **Table 1**; NA, no alleles available. During this analysis, we found that the *P{lacW}l(2)k13805*^{k13805} line does complement the *glu* LOF alleles tested, thus indicating that it is probably not affecting that gene (marked with a ?). Quantifications of the interactions are shown in **Table 4**.

suggesting that other genes included in the deficiencies could be responsible for the interactions. As a result of this candidate gene approach we have identified the following genes: *rhomboid* and *phyllopod*, which encode Egfr signaling components; *trio*, a gene previously related to *Mtl* function in other cellular contexts;[16] *myoblast city* (*mbc*), encoding a GTPase regulator (a

Table 4. Quantification of the genetic interactions between *Mtl* and candidate genes from the P-insertion screen

Genotype (*sev>Mtl*/+ for all genotypes)	Wild-type ommatidia (% ± sd)	Number of ommatidia
w^{1118} (+/+, control)	74.3 ± 2.4	1123
mts^{02496}	84.7 ± 5.7**	708
Trp^{EP-663}	63.2 ± 6.3*	403
Trp^{c03479}	88.7 ± 5.1**	443
mol^{e02670}	66.7 ± 1.7**	461
mol^{35Bb-1}	60.6 ± 6.6*	351
glu^{88-37}	63.0 ± 5.4**	485
glu^{88-44}	63.0 ± 4.3*	290
spi^{1}	84.7 ± 4.1**	573
spi^{s3547}	84.9 ± 2.4**	354
$Rs1^{k09513}$	78.0 ± 4.8*	833
$Rs1^{k09514}$	85.0 ± 5.2**	549
$Uba1^{05642}$	66.9 ±5.2*	518
$Uba1^{DG24412}$	81.8 ± 3.4**	390
$Cdk4^{05428}$	80.7 ± 2.6*	405
mbl^{E27}	80.5 ± 3.4*	308
mbl^{E127}	82.7 ± 3.8**	430
$18w^{\Delta 7-35}$	84.1 ± 5.6*	330
$18w^{k02701}$	81.5 ± 4.7*	364
blw^{1}	80.8 ± 6.9*	580
$blw^{DG06809}$	78.9 ± 2.0**	339
sls^{1}	81.0 ± 4.1*	465
R^{CD5}	60.1 ± 7.4**	623
R^{67}	66.2 ± 1.4**	292
$RpS12^{EY04767}$	79.7 ± 3.1**	573
$RpS12^{EY23269}$	80.2 ± 3.0*	377
Dad^{c02089}	82.2 ± 5.6*	385
UAS-*DadIR*	82.5 ± 2.1**	343
$CG11859^{j12B4}$	77.4 ± 2.0*	336
$CG11859^{f03457}$	85.3 ± 5.8*	422
$PH4\alpha EFB^{EY13295}$	82.2 ± 5.4**	695
$PH4\alpha EFB^{i889Ex13-11}$	82.2 ± 3.5*	286

Percentage of wild-type ommatidia (± standard deviation) of eyes from flies heterozygous for the indicated alleles and containing one copy of *sev>Mtl*. All alleles listed show significant modification of the *sev>Mtl* phenotype (*p < 0.05, **p < 0.005, t-test).

RacGEF); and *hibris* (*hbs*), a gene encoding a protein involved in cell adhesion (**Tables 1 and 2**). Moreover, other candidate genes included in the deficiencies are *prickle*, *argos* and *Rac1* (**Tables 1 and 2**), previously found to interact genetically with *Mtl*.[6]

By using the same experimental approach than in the pilot screen, we tested a total of 672 independent, autosomal P-element lethal insertions (498 on the second and 174 on the third chromosomes, respectively) for interaction with the *sev>Mtl* rough eye phenotype. During the initial round of the screen, we retained a total of 23 lines (~3.4% of the tested lines) for further analysis: 22 suppressors and one enhancer that were re-tested to confirm the repeatability of the interaction (**Table 3**). The genes associated with the insertions were identified by analyzing the genomic DNA sequences flanking the P-element insertion site, either available in Flybase (http://flybase.org/) or determined by inverse PCR (see Material and Methods). For the *P{lacW}l(3)j12B4*^{j12B4} line, complementation analyses with LOF alleles of neighboring loci (*Aats-Gln* and *CG11859*) were performed (data not shown) since no information about the flanking sequences could be obtained. In summary, we found that 19 lines were inserted in or close to known genes and four in uncharacterized genes. Subsequently, we validated the interactions by using additional alleles, finding that most of them showed a significant interaction with *sev>Mtl* phenotype (**Tables 3 and 4**). However, in some cases we found that different mutants of the same gene either enhanced or suppressed the *sev>Mtl* phenotype. We speculate that perhaps the position of the insertions/molecular lesions in those mutant lines determined the expression levels of the corresponding genes and affected the *sev>Mtl* phenotype differently, as suggested in other genetic interaction assays.[41-43] The identified genes in the P-element screen could be grouped into different categories following a functional criterion. Some of them were related to signal transduction pathways (*spitz, Daughters against dpp*), cytoskeleton organization and extracellular matrix (ECM) composition/cell adhesion (*microtubule star, prolyl-4-hydroxylase-α EFB*). We also found interactions with genes involved in different developmental processes like eye and muscle development or dorsal closure (*muscleblind*, *Roughened*) and in cellular metabolism (*Rps12*). Several uncharacterized genes were also identified (*CG11859, CG7832, CG1965* and *CG2245*), but only the genetic interaction with *CG11859*, which encodes an atypical protein kinase, could be confirmed (**Tables 3 and 4**).

Modifiers encoding Egfr pathway components and effectors. It has been reported that the Egfr pathway is involved in ommatidial rotation during PCP establishment in the Drosophila eye.[27-29] This is of particular interest in light of the rotation defects observed in *sev>Mtl* eyes.[6] Consistent with this, some of the genes that dominantly interact with *Mtl* encode components or modulators of the Egfr pathway or proteins functionally related to it during the ommatidial rotation process like *spitz* (*spi*), *argos* (*aos*), *rhomboid* (*rho*), *phyllopod* (*phyl*) or *Roughened* (*R*) (**Tables 2 and 4**). *aos* encodes an Egfr inhibitory ligand,[44] whereas *rho* encodes a serine protease localized in the Golgi where it promotes the cleavage of the Egfr activating ligand Spi.[45] Phyl is a transcriptional target of the Egfr pathway, and its expression is dependent on RTK signaling.[46] *R* encodes a Ras-related Rap1 small GTPase involved in cell adhesion and motility[47,48] that has been recently shown to be critical for differentiation of Egfr-dependent cell types in eyes and wings, and for PCP establishment in these tissues.[49] To confirm these results, we tested for genetic interactions with other members of Egfr signaling. Crosses to LOF alleles of *Egfr* and *sprouty* (*sty*), encoding the sole receptor of the pathway and a negative regulator of Egfr signaling,[50] respectively, and *Ras85D*, *pointed* (*pnt*) and *canoe* (*cno*), which encode members of the Ras/Raf/MAPK cascade and Ras/Cno signaling required downstream of Egfr in ommatidial rotation,[28] also resulted in statistically significant modification of the *sev>Mtl* phenotype

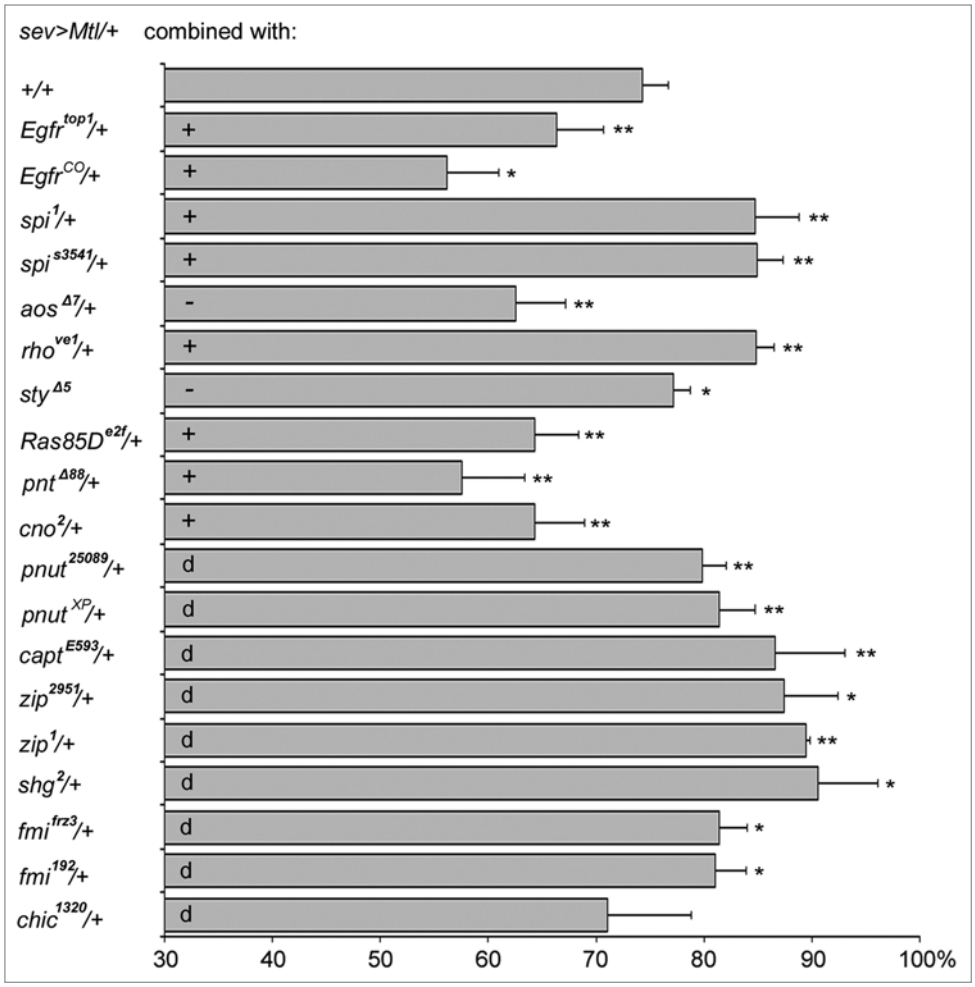

Figure 2. Quantification of genetic interactions with the *sev>Mtl* phenotype. The percentage of correctly oriented ommatidia in the corresponding allelic combination with *sev>Mtl* is shown. +, -, and d in the bars indicate that the corresponding gene is a positive regulator, a negative regulator or a downstream effector of the Egfr pathway during ommatidial rotation,[28] respectively. Data are based on the analysis of 200 to 800 ommatidia from three to ten independent eyes per genotype. Standard deviations calculated across all eyes of a given genotype are indicated by a line in each bar. Asterisks indicate significant interactions (*p < 0.05, **p < 0.005, t-test).

(**Figs. 2 and 3**). Since we have shown that *Mtl* overexpression has dominant negative effects on Mtl function,[6] an obvious conclusion about the role of *Mtl* in Egfr signaling can not be drawn from the observed interactions. Thus, and to more firmly establish the functional link between *Mtl* and Egfr signaling during ommatidial rotation, we performed genetic interaction assays between *Mtl* and *Star* (*S*) by using LOF alleles of both genes. S is a transmembrane protein required for Spi transport from the endoplasmic reticulum to the Golgi apparatus, an essential process for this ligand to be functional,[50] then Egfr signaling is reduced in *S* mutants. *S* is haploinsufficient for eye development, and loss of one gene copy produces mild rough eyes that show loss of photoreceptors but also rotation defects.[28] Two different *S* alleles (S^{485} and S^{23N}) were used in the assays, and for both the $S^{+/-}$ rotation phenotype is enhanced by removing one copy of *Mtl* (**Table 5** and not shown). In addition, it has been reported that expression of an activated Egfr (λ-*top*) under *sep*-GAL4 control produces rough eyes and results in ommatidial rotation and photoreceptor recruitment defects due to gain of Egfr pathway activity.[49] We found that loss of one copy of *Mtl* did not have an effect on the *sep*>λ-*top* photoreceptor phenotype (29.9% ± 5.4 in *sep*>λ-*top*/Mtl^{Δ} vs. 28% ± 6.8 in *sep*>λ-*top*/+eyes), although it significantly suppressed ommatidial rotation defects (16.2 ± 2.0 in *sep*>λ-*top*/Mtl^{Δ} vs. 31.9 ± 6.7 in *sep*>λ-*top*/+eyes; $p < 0.02$, t-test) as well as the external roughness of *sep*>λ-*top* eyes (**Fig. 4**). Thus, with respect to ommatidial rotation, we found that reducing *Mtl* levels dominantly enhanced the effects of Egfr loss (*Star*) and dominantly suppressed the effects of Egfr overactivation (*sep*>λ-*top*). These results suggest that Mtl may cooperate with the Egfr pathway in ommatidial rotation either being a positive regulator or an effector of Egfr signaling during this process, although it could also act in a parallel pathway. To distinguish between these possibilities, we tested whether dpERK (the dually phosphorylated form of MAPK) levels were affected in *sev>Mtl* eye imaginal discs. In wild-type discs, dpERK is most prominent at the morphogenetic furrow (MF). Posterior to the MF, dpERK is expressed at lower levels where it is predominantly cytoplasmic but also transits to the nucleus to phosphorylate target proteins (see ref. 51 and **Fig. 5A**). Stainings of *sev-GAL4/UAS-Egfr* discs were also performed, in which an evident increase of dpERK levels posterior to the MF is observed by overactivation of the pathway in such cells under *sev*-GAL4 control (**Fig. 5B**). Conversely, we found that dpERK levels posterior to the MF were drastically reduced in *sev>Mtl* eye imaginal discs, appearing predominantly cytoplasmic (**Fig. 5C**). Since we previously reported that Mtl function is reduced in *sev>Mtl* flies,[6] genetic interaction assays and dpERK stainings suggest that Mtl functions as a positive regulator of Egfr signaling upstream the MAPK kinase cassette. Consistent with this, we found that LOF alleles of genes encoding positive regulators or transcriptional targets of the pathway, like *Egfr*, *phyl*, *R*, *Ras85D*, *cno* or *pnt*, enhance the *sev>Mtl* phenotype while a LOF allele of *sty,* which encodes a negative regulator of the pathway, suppresses that phenotype (**Tables 2 and 4, Figs. 2 and 3**). In contrast, we found inconsistent results when analyzing LOF alleles of *spi*, *aos* and *rho* (**Tables 2 and 4, Figs. 2 and 3**). Since

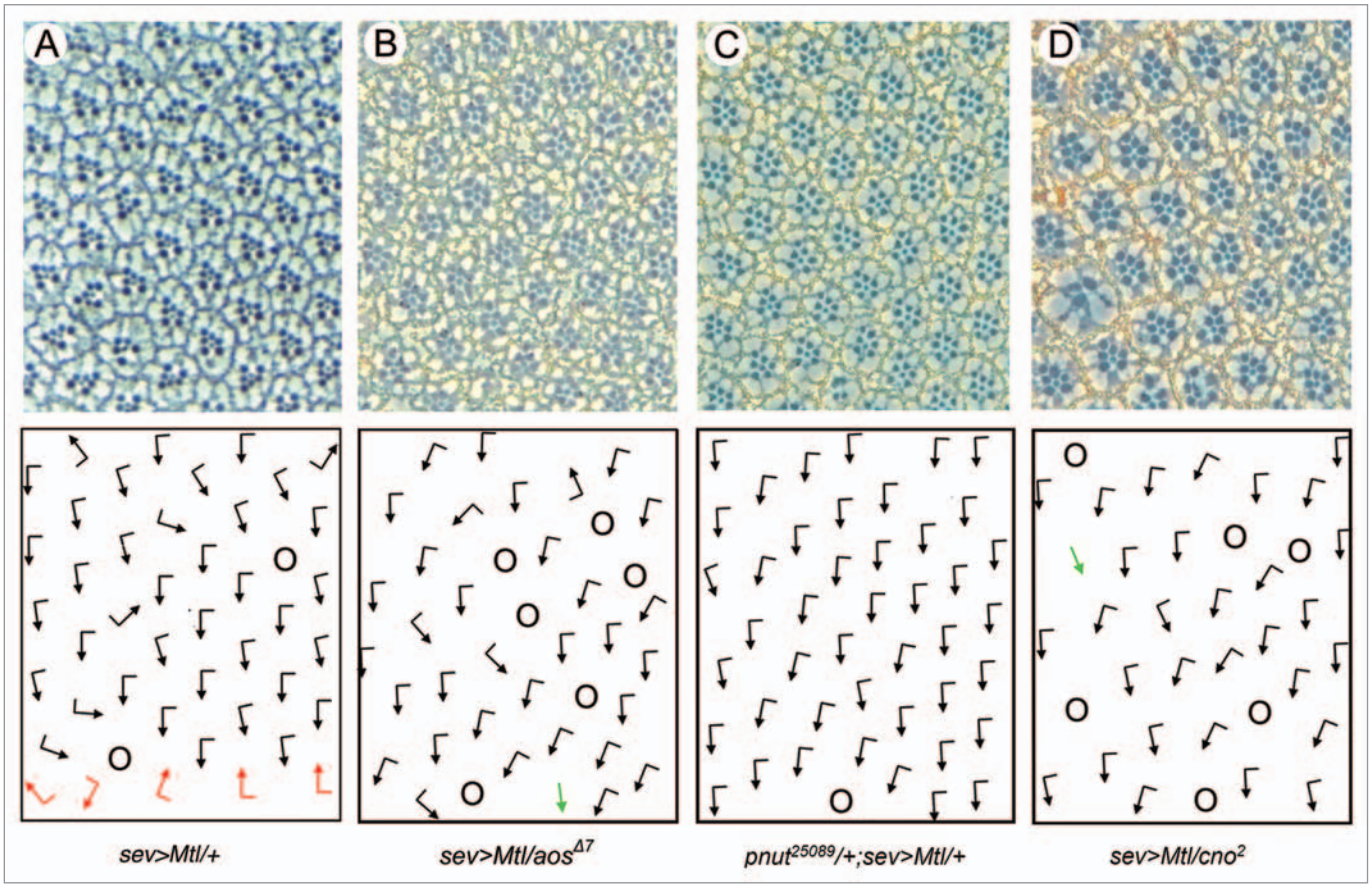

Figure 3. Components and effectors of the Egfr pathway dominantly modify the *sev>Mtl* phenotype. All parts show tangential sections of adult eyes and the corresponding schematic representation: (A) *sev>Mtl/+*, (B) *sev>Mtl/aos*$^{\Delta 7}$, (C) *pnut*^{25089}*/+; sev>Mtl/+* and (D) *sev>Mtl/cno.*[2] For comparison, a tangential section of a wild-type eye is shown in **Figure 6A**. Dorsal and ventral chiral ommatidia are indicated by black and red arrows, respectively. Green arrows without flag represent achiral ommatidia and circles ommatidia with an abnormal number of photoreceptors. Quantifications of the interactions are shown in **Figure 2**. Anterior is to the left and dorsal is up.

Table 5. Star haploinsufficient rotation phenotype is enhanced by *Mtl* and *hbs*

Genotype	% misrotated ommatidia (±sd)
$S^{48.5}$/+	8.9 ± 4.3
$S^{48.5}$/+; Mtl^{Δ}/+	37.2 ± 4.2
$S^{48.5}$/hbs^{361}	21.7 ± 3.7

Percentage of misrotated ommatidia and standard deviation calculated between eyes of the corresponding genotype. The enhancement of the $S^{+/-}$ rotation phenotype is statistically significant in both cases ($p < 0.001$, t-test).

we obtained the same results in the genetic interaction assays by using different LOF alleles of these genes and deficiencies uncovering them (see ref. 6 and **Tables 1, 2 and 4**), this excludes the possibility that secondary mutations on the chromosomes could account for the observed interactions. Then, it is likely that the molecular nature of the *sev>Mtl* mutation could be the cause of such inconsistent results. Similar complex genetic interactions were also reported between *jun/fos* and *menin*, which encodes a regulator of their transcriptional activity.[52] Furthermore, and confirming our results, we found dominant interactions between *Mtl* and genes encoding cytoskeletal elements and cell adhesion components required downstream of Egfr during ommatidial rotation, as well as several genes involved in PCP establishment like *shg* or *zip* (**Fig. 2**). In addition, *Mtl* also interacts with core PCP genes like *flamingo* (*fmi*) (**Fig. 2**) or *prickle* (*pk*),[6] as it has been shown for other Egfr pathway components such as *S*.[28]

Taken together, our results suggest that *Mtl* is functionally related to the Egfr pathway during ommatidial rotation and that this signaling is probably compromised in *sev>Mtl* flies, thus accounting for the rotation defects observed in such mutants. This relationship must be specific for the rotation process since no defects in differentiation of photoreceptors and/or wing veins associated to loss of Egfr signaling are observed in Mtl-overexpressing flies.[6] Alternatively, another explanation could be that, as reported for other Egfr pathway components,[27] rotation is more sensitive than photoreceptor recruitment to the possible perturbation of Egfr signaling caused by Mtl overexpression.

Modifiers involved in cell adhesion and cytoskeleton organization. Our results suggest that Mtl may have a role in ommatidial rotation in the context of Egfr signaling, probably regulating cytoskeleton dynamics and cell adhesion. Indeed, this process requires cellular rearrangements including changes in cell

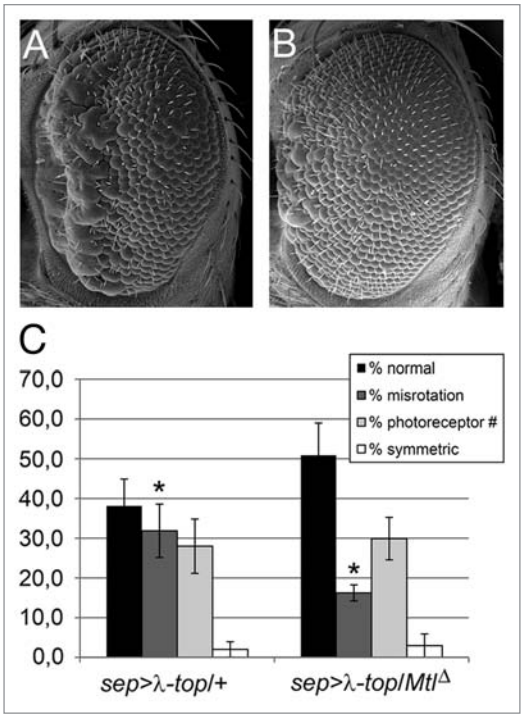

Figure 4. *Mtl* loss-of-function dominantly suppresses eye phenotypes caused by overactivation of Egfr signaling. (A and B) Scanning electron micrographs of adult eyes: (A) *sep*>λ-*top*/+ and (B) *sep*>λ-*top*/*Mtl*$^{\Delta}$. There is a clear suppression of the *sep* > λ-*top* rough eye phenotype when reducing *Mtl* dosage. (C) Quantification of genetic interactions between *Mtl* and the *sep*>λ-*top* phenotype. There is a significant suppression of the percentage of misrotated ommatidia in *sep*>λ-*top*/*Mtl*$^{\Delta}$ compared to *sep*>λ-*top*/+ eyes, while no changes occur in the rest of phenotypic classes (at least 300 ommatidia were scored from four independent eyes). Asterisks indicate statistically significant differences (*p < 0.02, t-test).

contacts/adhesion, cell movements and cytoskeleton reorganization. Consistent with this, some of the genes identified to interact significantly with *Mtl* encode proteins with a direct or indirect role in cytoskeleton reorganization and cell adhesion like *prolyl-4-hydroxylase-α EFB* (*PH4αEFB*), *microtubule star* (*mts*) and *hibris* (*hbs*). *PH4αEFB* encodes a subunit of the prolyl 4-hydroxylase multienzymatic complex that catalyzes the hydroxylation of proline residues in collagens, a major component of the ECM, as well as other secreted proteins.[53] Modifications in the ECM are probably essential for ommatidial rotation. Besides, *mts* encodes the catalytic subunit of the Protein phosphatase 2A (PP2A) complex.[54] Although its exact role in the PCP context remains unknown, it has been shown that PP2A is required both for hair formation and orientation in the wing.[55] To test whether PP2A is required during PCP establishment in the eye, we examined the effect of PP2A loss of function in that tissue. As *mts* null alleles are cell lethal,[56] we overexpressed in the eye a truncated version of the Mts protein that lacks the active site and is unable to interact productively with its regulatory subunits.[55] However, our results were not conclusive since we found that while *sev*-GAL4, UAS-*mts*^{I81HA4a} flies have wild-type eyes, overexpression of the dominant negative form of Mts with *GMR*-GAL4 produced very rough eyes[57] that in tangential sections showed complete disorganization of the retina (data not shown). Since PP2A has a role in the regulation of cell adhesion and cytoskeleton dynamics in human mammary endothelial cells and functions in a protein complex including Rac,[58] a similar situation could be occurring in Drosophila thus explaining the genetic interaction found between *Mtl* and *mts*.

One of the Mtl-interacting genes is *hbs* (**Tables 1 and 2**), which encodes one of the Drosophila Nephrin homologs, a member of the immunoglobulin (Ig) superfamily of transmembrane proteins, involved in myoblast fusion during embryogenesis.[37,39] Hbs is also essential for interommatidial precursor cell (IPC) morphogenesis in the pupal eye together with the Drosophila Neph1 homolog Roughest (Rst), another protein of the Ig superfamily.[38] This process involves cell rearrangements and movements which require changes in adhesive interactions between retinal cells.[59] Both proteins are expressed in complementary cell types and mediate specific interactions between them, by modulating adherens junction dynamics.[38] Furthermore, Hbs also contributes to the establishment of the native configuration of cone cell contacts and shape.[40] Since *hbs* mutants have rough eyes, we decided to determine whether Hbs could have a role during PCP establishment. Tangential sections of eyes from flies homozygous for the hypomorph *hbs*^{361} allele (which contains an amino acid substitution in the extracellular Ig domain of the protein) revealed that 18.6% ± 3.5 ommatidia exhibit PCP defects (mainly misrotations and rare defects in chirality) and 8.2% ± 3.7 show photoreceptor recruitment defects (n = 5, 744 total ommatidia) (**Fig. 6C**). We also analyzed eyes from flies heterozygous for the null *hbs*^{459} allele (in which no Hbs protein is produced due to the presence of a premature stop codon in the *hbs* gene) and the *Df(2R)X28* deficiency (which uncovers the *hbs* genomic region) finding that they showed similar but weaker phenotypes than *hbs*^{361} homozygous eyes (**Fig. 6B**). Previous observations already indicated that *hbs*^{361} embryos displayed stronger phenotypes than *hbs*^{459}/*Df(2R)X28* transheterozygous mutants when analyzing defects in muscle development although they showed wild-type protein expression levels.[37] To confirm our results, we also studied the effect of reducing *hbs* levels specifically in the eye by using *sev-GAL4* and *GMR*-GAL4 to drive expression of an *hbs-IR* transgene. We found that *sev*>*hbs-IR* mutant eyes are externally rough at 29°C, and in tangential sections display mild but consistent ommatidial rotation defects (**Fig. 6D**). *GMR*>*hbs-IR* mutant eyes at 25°C were also rough and displayed stronger phenotypes, similar than the mutant alleles analyzed (data not shown). Taken together, these results suggest that the presence of a mutant form of Hbs (in *hbs*^{361} homozygous animals) has stronger phenotypic effects than reduced levels or absence of the wild-type protein, confirming previous results.[37] Indeed, the eye phenotypes observed in our study are also stronger than those reported for recently generated *hbs* null alleles, which showed very mild rotation defects.[40] To further confirm the role of Hbs in PCP determination, we also analyzed the consequences of *hbs* overexpression in the eye with the *sev*-GAL4 driver. A rough eye phenotype was also obtained (see ref. 39 and data not shown). This phenotype is due to photoreceptor recruitment defects (28.2% ± 6.2), as

previously suggested,[39] but also to PCP defects (13.1% ± 2.0) mainly misrotations (n = 5, 558 ommatidia) (**Fig. 6E**). Since some of the phenotypes observed in *hbs* mutants could be consequence of a later role of this gene during eye development, we performed anti-Armadillo (Arm) antibody stainings of hbs^{361} and *sev*-GAL4/UAS-*hbs* third instar larvae imaginal discs that confirmed that the rotation defects in *hbs* mutant flies result from an early failure in PCP establishment (**Fig. 6F–H**). Finally, to gain further insight into Hbs requirements during ommatidial rotation, we performed preliminary genetic interaction assays designed to identify a possible link between *hbs* and Egfr or Fz/PCP signaling, both required to regulate this process. Our results showed that *hbs* dominantly enhanced the $S^{+/-}$ rotation phenotype caused by reduction of Egfr signaling (**Table 5**), probably indicating that *hbs* cooperates with or is a positive regulator/effector of this signaling pathway during ommatidial rotation. This is consistent with the enhancement of the *sev>Mtl* phenotype obtained when reducing *hbs* function (see **Table 2**). Regarding the possible link between *hbs* and Fz/PCP signaling, we found that reduction of one copy of *hbs* dominantly enhanced the *Dsh* gain-of-function rough eye phenotype of *sE-Dsh* flies (data not shown). However, tangential sections revealed that the hbs^{361} allele caused a clear reduction of the number of symmetric ommatidia but an increase of photoreceptor loss in *sE-Dsh* eyes, thus masking PCP defects in many ommatidia and precluding the analysis (data not shown). Thus, we analyzed whether reduction of *hbs* function was able to dominantly modify the PCP defects displayed by dsh^{1} mutant eyes. We found that the hbs^{361} allele significantly reduced the percentage of ommatidia with PCP defects in such eyes (48.5 ± 6.7 in dsh^{1}, hbs^{361}/+ vs. 58.1 ± 4.1 in dsh^{1} eyes; $p < 0.05$, t-test; n = 6 eyes per genotype). Quantifications of the different classes of ommatidia with PCP defects showed that the acquisition of chirality aspect of the dsh^{1} phenotype was clearly modified by reduction of *hbs* function (as seen in **Fig. 7**). However, when only quantifying rotation defects (in either chiral or achiral ommatidia) we observed that they were also significantly suppressed by the hbs^{361} allele (45.1 ± 3.8 in dsh^{1}, hbs^{361}/+ vs. 54.2 ± 6.7 in dsh^{1} eyes; $p < 0.05$, t-test). Then, our results show that reduction of *hbs* function affects both chirality and rotation in dsh^{1} eyes.

Taken together, our genetic and molecular epistasis data suggest a role of Hbs during PCP establishment in the Drosophila eye, and indicate that it is functionally related to both Fz/PCP and Egfr signaling pathways. Further experiments are being performed to determine its exact role in this process, and to test whether other proteins of the Neph1/Nephrin subfamily, related to Hbs in other functional contexts, could also participate in it.

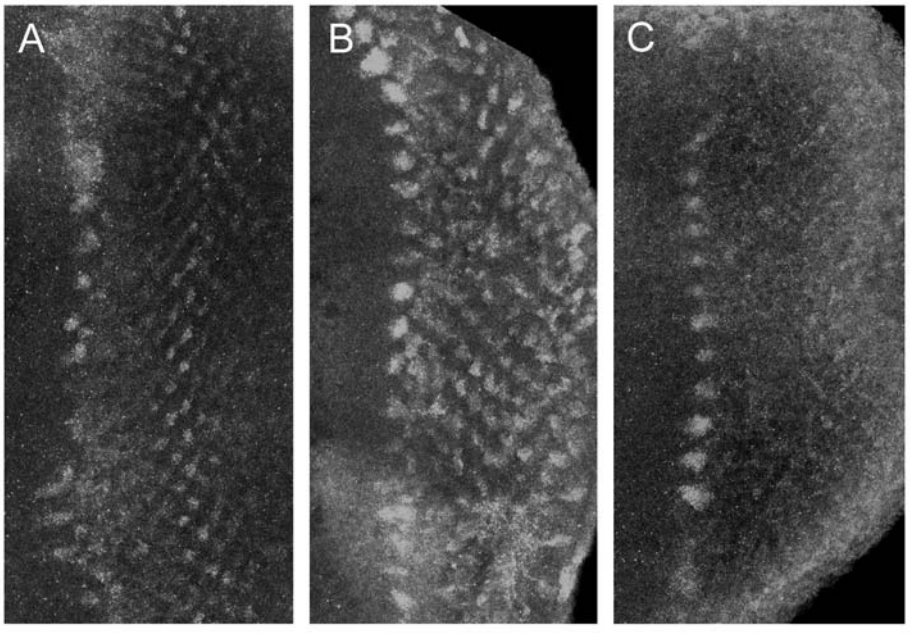

Figure 5. dpERK expression posterior to the MF is reduced in *sev>Mtl* eye imaginal discs. Confocal micrographs of eye discs stained with anti-dpERK from (A) wild-type, (B) *sev*-GAL4, UAS-*Egfr* and (C) *sev>Mtl* larvae. In wild-types discs, nuclear dpERK staining is observed at the MF (arrow) and posterior to it (arrowhead), remaining cytoplasmic in between. In *sev>Mtl* discs, dpERK staining appears unchanged at the MF but it is reduced posterior to the MF. On the contrary, overexpression of Egfr with the *sev*-GAL4 driver produces a ubiquitous nuclear dpERK staining. Anterior is to the left.

Discussion

In this work we have used a genetic approach to identify genes that could be functionally related to *Mtl*, a member of the Rho family of small GTPases in Drosophila. Since *Mtl* LOF mutants are phenotypically wild-type,[5] a dominant modifier screen in a *Mtl*-missexpression background was performed as alternative despite of the clear limitations of this approach. Using the *Mtl*-overexpression rough eye phenotype we screened 166 deficiencies, 672 P-lines and LOF alleles from candidate loci, and identified several genes that exhibit significant dose-sensitive genetic interactions with that phenotype. These genes belong to different functional classes: core PCP genes, components of signaling pathways, regulators of GTPase function, and genes involved in cell adhesion, cytoskeleton dynamics and other developmental processes. From these results, several hypotheses about *Mtl* function can be proposed.

Previous analyses indicated that *Mtl* overexpression in the eye causes very subtle defects in chirality or photoreceptor number and mainly affects ommatidial rotation.[6] These results suggested that transmission of the PCP signal through the eye epithelium is essentially unaffected in *sev>Mtl* eye discs, although the adhesive/cytoskeletal response to PCP could be compromised. Consistent with this, we find that some of the *Mtl*-interacting

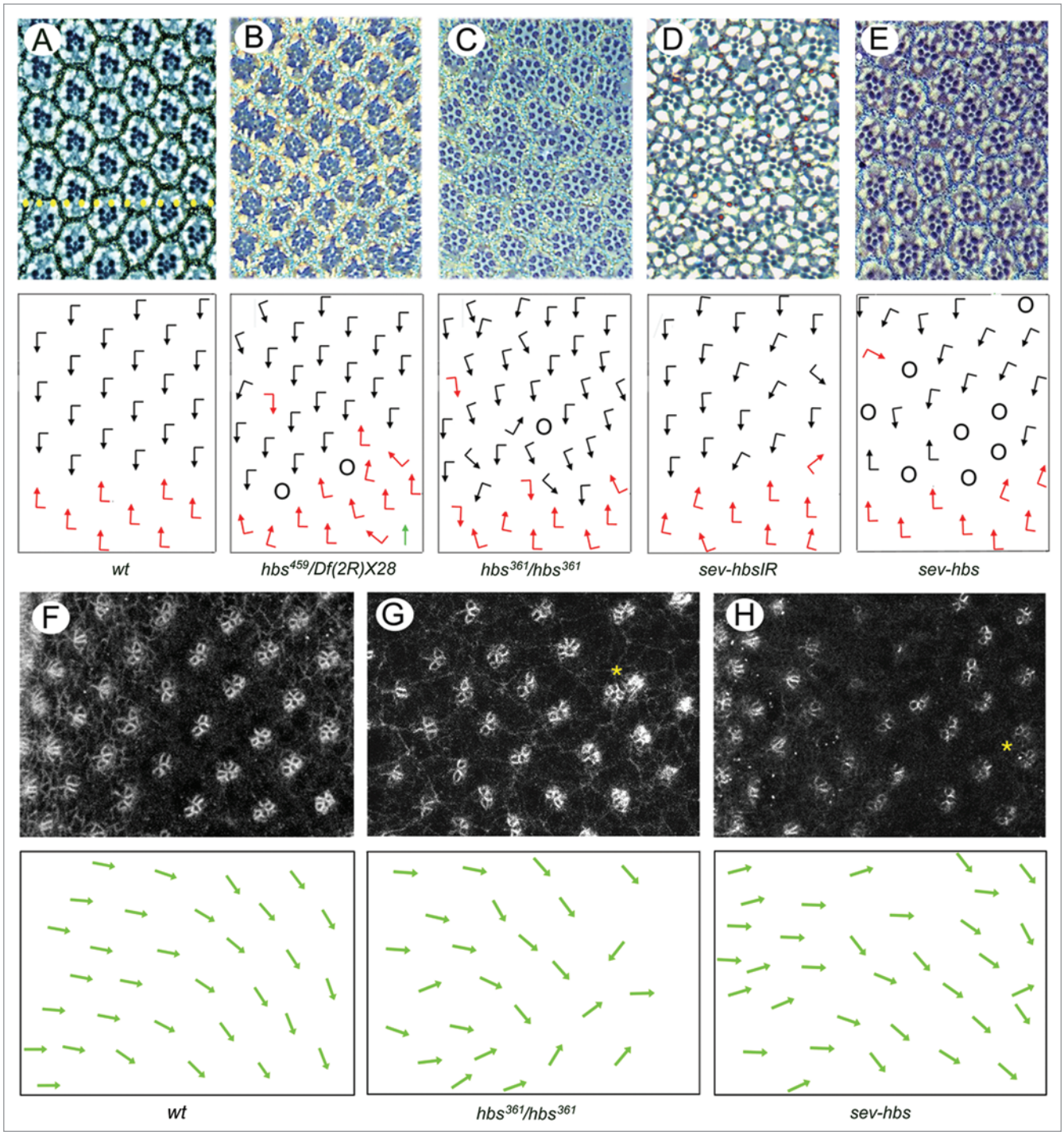

Figure 6. *hbs* mutants show PCP defects in Drosophila adult eyes and eye imaginal discs. (A–D) Tangential sections of adult eyes and the corresponding schematic representations (arrows are drawn as in **Fig. 3**): (A) wild-type, (B) $hbs^{459}/Df(2R)X28$, (C) hbs^{361}, (D) *sev*-GAL4, UAS-*hbsIR* and (E) *sev*-GAL4, UAS-*hbs* eyes. (F–H) Confocal images of eye imaginal discs stained with anti-Arm antibody from (F) wild-type, (G) hbs^{361} homozygous and (H) *sev*-GAL4, UAS-*hbs* larvae. Yellow asterisks indicate ommatidial fusions. In lower parts, green arrows bisect R8 and run through the R3/R4 interface, highlighting the angle of orientation of each ommatidium. Anterior is to the left and dorsal is up.

genes identified in our study encode components and effectors of the Egfr pathway, which has been reported to act downstream of or in parallel to Fz/PCP signaling to control ommatidial rotation.[27-29] Our results suggest that Mtl may function as a positive regulator of the Egfr pathway specifically during the ommatidial rotation process since *Mtl* LOF only affects this process but not photoreceptor differentiation and recruitment. Indeed, reduction of *Mtl* dosage is able to suppress the eye phenotype caused by overexpression of an activated form of Egfr (λ-*top*) and to enhance the eye phenotype caused by reduction of Egfr signaling

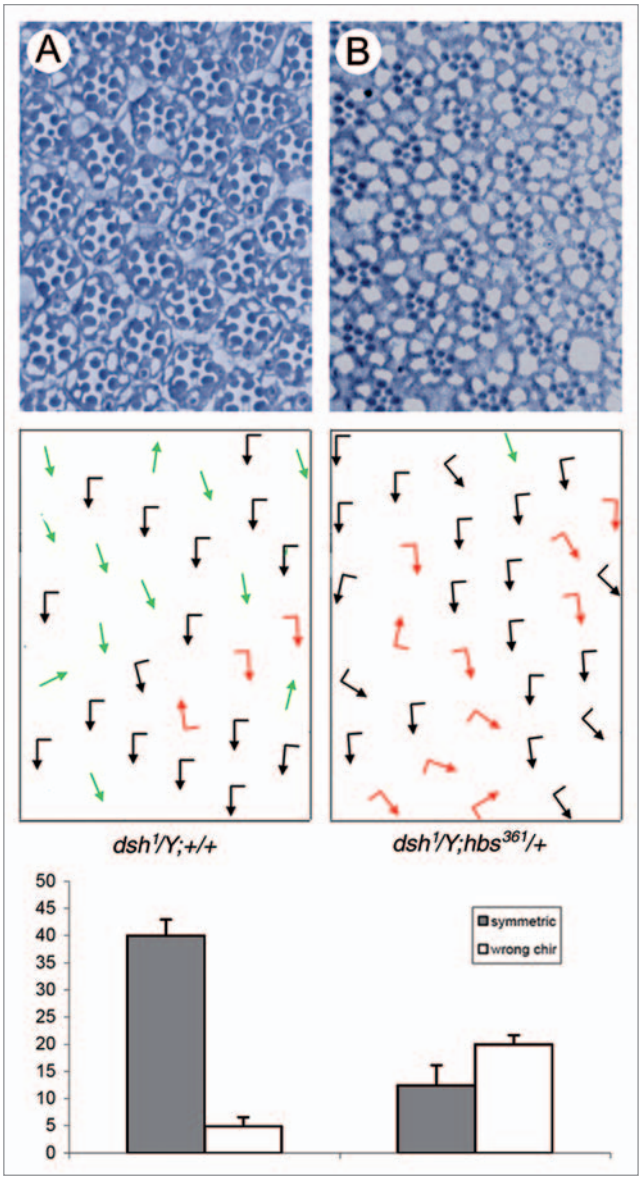

Figure 7. Mutations in *hbs* dominantly modify the *dsh*^{1} phenotype. (A and B) Tangential sections of adult eyes and the corresponding schematic representations (arrows are drawn as in **Fig. 3**): (A) *dsh*^{1}/*Y* and (B) *dsh*^{1}/*Y*; *hbs*^{361}/+. For comparison, a tangential section of a wild-type eye is shown in **Figure 6A**. Anterior is to the left and dorsal is up. (C) Quantification of genetic interactions between *hbs* and *dsh*.^{1} Reducing *hbs* function suppresses symmetrical ommatidia formation and mildly increases the percentage of ommatidia with wrong chirality (at least 400 ommatidia were scored from five independent eyes). All differences are statistically significant (in both cases p < 0.0001, t-test).

in *S*$^{+/-}$ mutants, but specifically affecting the ommatidial rotation defects. Although our genetic data do not reveal a molecular mechanism for the function of Mtl in the Egfr pathway, reduction of dpERK levels posterior to the MF observed in *sev>Mtl* flies, which has a dominant negative effect on *Mtl* function,[6] indicates that Mtl exerts its positive regulation upon the Egfr pathway upstream the MAPK cassette. It is likely that it functions in parallel to other GTPases like Rac1, Ras and Rap1 (see ref. 6 and this study). Indeed, LOF alleles of all of them are able to modify the *sev>Mtl* phenotype in the same direction. This functional parallelism has been extensively found among other Rho GTPases in many different processes.[1] It is interesting to mention that *Mtl* overexpression in the wing causes PCP phenotypes[6] similar than those observed in *Rap1* LOF mutant clones,[49] despite Egfr signaling has not been shown to play a role during PCP establishment in this tissue. These results suggest that both GTPases could have an Egfr-independent role to establish PCP in the wing, and support the idea that they could be functionally related.

The finding of genes that are implicated in cell adhesion and cytoskeleton rearrangements is consistent with a putative role of *Mtl* in regulating such processes during ommatidial rotation. It is evident that actin rearrangements and modification of the adhesive properties of the ommatidial preclusters have to occur during the ommatidial rotation process.[33] Indeed, it has been shown that the classic cadherins E-Cad and N-Cad and the Ig cell adhesion molecules (CAMs) Echinoid (Ed) and Friend of Echinoid (Fred) function in controlling this process.[30,60] Cadherin transmembrane proteins are the core components of adherens junctions (AJs), although CAMs of the Ig superfamily have been also identified as components or modulators of AJs. Both cadherins and Ig-type receptors interact with the actin cytoskeleton through cytoplasmic adaptor proteins and through signaling cascades, and this is essential for effective adhesion and AJ assembly and stability.[59] Interestingly, *Mtl* interacts genetically not only with *shg* (**Fig. 2**), which encodes DE-Cad, but also with *hbs* and *ed* (**Table 2** and data not shown), both encoding CAMs of the Ig superfamily. All these genes seem to have a role during ommatidial rotation/PCP establishment and to interact genetically with members of Egfr and Fz/PCP signaling[30,60,61] (this study). Although previous phenotypic analysis of null *hbs* mutant alleles showed very mild rotation defects and did not reveal a clear role of this gene in the process,[40] our results show that *hbs* mutants display reproducible PCP phenotypes suggesting that this gene could function redundantly with other members of the Ig superfamily during PCP determination, as shown in muscle precursor formation.[62] We have also observed that PCP phenotypes are stronger in hypomorph than in null alleles, as previously reported in references 37 and 40. It is likely that amino acid substitutions in the Ig domain (in the *hbs*^{361} hypomorph allele) may affect binding to its functional partner during this process, since this protein regulates cell adhesion in a heterotypic manner in other contexts, thus causing stronger phenotypes. In addition, we have shown that *hbs* LOF mutants interact genetically with members of Egfr and Fz/PCP signaling, affecting not only ommatidial rotation but also the chirality aspects of PCP. These results suggest that *hbs* could regulate PCP establishment at least partly through control of both pathways or integrating inputs from both, as proposed for other CAMs.[30,60] Further analyses will be required to clarify this issue.

Other genes identified in our study encode GTPase regulators that are probably modulating Mtl function (**Tables 1–4**) like Trio which functions activating Rac GTPases through its GEF domain, and was already shown to activate Mtl in the axon guidance process,[16] or *myoblast city* (*mbc*) that is involved in small GTPase activation during several morphogenetic processes in

which actin rearrangements are implicated, such as dorsal and thorax closure, myogenesis, and neural development.[63,64] It has been recently shown that Mbc functions together with Elmo as a RacGEF to activate Rac1 and Rac2 during eye development and myoblast fusion.[65] It is possible that Mbc could also activate Mtl, thus explaining the genetic interaction, although further experiments will be required to confirm this assumption.

We also found that other *Mtl*-interacting genes encode members or regulators of several signal transduction pathways other than Egfr and Ras signaling (**Table 3**), like Cdk4, a cyclin-dependent kinase 4 involved in cell growth regulation[66] that interacts with the JAK-STAT pathway.[67] Although this interaction remains unclear, it is interesting to mention that JAK-STAT signaling is involved in PCP establishment in the eye.[68] We also find interaction with genes related to the TGF-β pathway like *Dad*, which encodes a Dpp antagonist that acts as its negative feedback regulator.[69] The genetic interactions between *Mtl* and members of other pathways might reflect the existence of a functional cross-talk between them. Finally, genes encoding proteins involved in cellular metabolism and other cellular processes were also identified to interact with *Mtl* (**Tables 3 and 4**).

In summary, our results suggest that Mtl may exert its function by directly interacting with the cytoskeleton, maybe forming complexes with other proteins (as it happens with other Rho GTPases), or by indirectly regulating the expression of genes which encode proteins that in turn regulate cytoskeleton dynamics, or are involved in cell-matrix and cell-cell adhesion or AJs dynamics. Since we have shown that Mtl is functionally related to Egfr signaling during ommatidial rotation, and this pathway is linked to cell adhesion components and cytoskeleton regulators,[28,29] both functions could be exerted by Mtl in the context of that pathway. Further analyses will be required to determine whether some of the genes identified in this study are also involved in the process that will help to clarify the mechanisms driving ommatidial rotation, an example of intercellular motility in the Drosophila eye disc that requires polarized regulation and dynamic remodelling of adhesive contacts between cells, and is reminiscent to collective cellular movements that occur during vertebrate development. Regarding this, it is interesting to mention that Mig-2, the *C. elegans* ortholog of Mtl, has been recently shown to be involved in cell migration and in establishing polarity in migrating cells.[70]

Materials and Methods

Fly strains. The *sev>Mtl* recombinant line was previously described.[6] EP insertion lines *Trp1*^{EP663} and *CG7832*^{EP3047} were obtained from the Szeged Drosophila Stock Center, the *Rs1*^{k09513} allele from the Kyoto Drosophila Gene Research Center, the *Trp1*^{c0347}, *Dad*^{k02089} and *CG11859*^{f03457} stocks from Exelixis Inc., and the *UAS-hbsIR* line from the Vienna Drosophila RNAi Center. All the mutants in genes encoding components of the Egfr pathway were kindly provided by Dr. Mlodzik. Other stocks used were: *Mtl*$^{\Delta}$,[5] *dos*^{R31},[71] *R*^{CD5}, *R*^{67},[48] UAS-*mts*^{181HA4a},[55] *hbs*^{361}, *hbs*^{459}, *Df(2R)X28*, UAS-*hbs*,[37] UAS-*Egfr*, *sep*>λ-*top*,[49] 2x*sev-Dsh*, *GMR*-GAL4 and *sevenless*-GAL4 (a gift from U. Weber). The TRiP lines *y*^{1} *v*^{1}*; P{TRiP.JF03369}attP2* (named as UAS-*phylIR*) and *P{TRiP.JF02133}attP2* (named as UAS-*DadIR*), as well as other mutant lines used in this study were obtained from the Bloomington Drosophila Stock Center. All crosses were performed at 25°C unless otherwise stated.

Dominant modifier screen. Males either from the Deficiency kit or from the P-element collection (both from the Bloomington Drosophila Stock Center) were crossed to virgin female *sev>Mtl* flies at 25°C. Heterozygous *sev>Mtl* flies carrying one copy of the deficiency/P-insertion were examined under a dissecting microscope for enhancement or suppression of the external *sev>Mtl*/+ rough eye phenotype, and compared to *sev>Mtl*/+ flies in order to eliminate the possible modifier effect of the balancer on the eye roughness. All modifiers were tested again to confirm the repeatability of the interaction. As a result of this, we identified several candidate genes that could be functionally related to *Mtl.* To confirm these results, we tested LOF alleles of the candidate genes for interactions with the *sev>Mtl* phenotype by crossing mutant males to virgin female *sev>Mtl* flies at 25°C. In case of modification of the external phenotype, eyes from the F_1 progeny were sectioned and scored for internal modification of the *sev>Mtl*/+ phenotype (**Tables 2 and 4**). The same procedure was followed in the genetic interaction assays performed between *Mtl* and members or effectors of the Egfr pathway and other candidate genes. For each allele tested, at least 250 ommatidia were scored from three to nine independent eyes. The Student's t-test was used to measure the statistical significance of the interactions.

Inverse PCR. In order to identify the genes hit by the P-elements in several insertion lines (*P{lacW}l(2)k13305*^{k13305}, *P{lacW}l(2)35Bb*^{k11524a} and *P{lacW}l(2)k13805*^{k13805}) we performed inverse PCR, since no flanking sequences to the insertions were available. Using a standard protocol, complete genomic DNA was recovered from 30 flies of the corresponding line, and subsequently digested with *Hin*P1. The DNA fragments were then self-ligated overnight and precipitated. In order to amplify only the recircularized fragment containing the *PlacW* element, we performed a PCR using Pry1 (CCT TAG CAT GTC CGT GGG GTT TGA AT) and Pry2 (CTT GCC GAC GGG ACC ACC TTA TGT TAT T) as specific primers for the 3'end of the P element. The PCR product was purified from agarose 2% using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, cat. # AP-GX-250), and sequenced. Each particular DNA sequence was then compared with the Drosophila genome by using a BLAST tool (http://flybase.bio.indiana.edu/blast/). The hit gene was the one considered responsible for the interactions.

Histology and immunohistochemistry. Sections of adult eyes were performed as previously described.[72] Approximately 50 sections were obtained out of each independent eye that were mounted in DPX and observed through the optical microscope in dark field (PH3 63x/1.25). Imaginal disc stainings with anti-Armadillo (1:40, Developmental Studies Hybridoma Bank, cat.# N2 7A1) and anti-dpERK (1:200, Sigma, cat.# 8159) antibodies were performed as previously described.[6] Secondary antibodies coupled to fluorochromes were purchased from Calbiochem (cat.# DC18L). Pictures were taken using a Leica TCS-NT confocal laser-scanning microscope.

Analysis of the adult retina by scanning electronic microscopy. Adult flies were anesthetized and put into fixative solution (paraformaldehyde 1.6%, glutaraldehyde 3.2% in PBS) for two hours. An increasing ethanol series was used to dehydrate the tissue, starting from 25% to 100%, 12 hr per step. The flies were then subjected to critical point drying and sputter-coated. Scanning electron microscopy analysis was performed in a Hitachi S-2500 and Philips XL-30.

Acknowledgements

We are grateful to M. Mlodzik, C.F. Lehner, T. Raabe, U. Gaul, U. Weber, R. Artero, S. Eaton, C. Wilson, J. Curtiss, the Bloomington and Szeged Drosophila Stock Centers, the Kyoto Drosophila Gene Research Center and Exelixis Inc., for flies and reagents. Scanning electron microscopy and confocal microscopy were performed at the SCSIE (Universitat de València). Fabrice C. Durupt, Silvia Muñoz-Descalzo and Yaiza Belacortu were supported by fellowships from Ministerio de Educación y Ciencia, Conselleria de Cultura, Educació i Ciència and Gobierno de La Rioja, respectively. This work has been supported by grants from Ministerio de Educación y Ciencia (BFU2004-00498 and BFU2007-63213) and Generalitat Valenciana (PROMETEO/2010/081 and ACOMP/2010/242) to Nuria Paricio.

References

1. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 2005; 21:247-69.
2. Avet-Rochex A, Perrin J, Bergeret E, Fauvarque MO. Rac2 is a major actor of Drosophila resistance to *Pseudomonas aeruginosa* acting in phagocytic cells. Genes Cells 2007; 12:1193-204.
3. Eaton S, Auvinen P, Luo L, Jan YN, Simons K. CDC42 and Rac1 control different actin-dependent processes in the Drosophila wing disc epithelium. J Cell Biol 1995; 131:151-64.
4. Fanto M, Weber U, Strutt DI, Mlodzik M. Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the Drosophila eye. Curr Biol 2000; 10:979-88.
5. Hakeda-Suzuki S, Ng J, Tzu J, Dietzl G, Sun Y, Harms M, et al. Rac function and regulation during Drosophila development. Nature 2002; 416:438-42.
6. Muñoz-Descalzo S, Gómez-Cabrero A, Mlodzik M, Paricio N. Analysis of the role of the Rac/Cdc42 GTPases during planar cell polarity generation in Drosophila. Int J Dev Biol 2007; 51:379-87.
7. Ng J, Nardine T, Harms M, Tzu J, Goldstein A, Sun Y, et al. Rac GTPases control axon growth, guidance and branching. Nature 2002; 416:442-7.
8. Paladi M, Tepass U. Function of Rho GTPases in embryonic blood cell migration in Drosophila. J Cell Sci 2004; 117:6313-26.
9. Strutt DI, Weber U, Mlodzik M. The role of RhoA in tissue polarity and Frizzled signalling. Nature 1997; 387:292-5.
10. Woolner S, Jacinto A, Martin P. The small GTPase Rac plays multiple roles in epithelial sheet fusion—dynamic studies of Drosophila dorsal closure. Dev Biol 2005; 282:163-73.
11. Sasamura T, Kobayashi T, Kojima S, Qadota H, Ohya Y, Masai I, Hotta Y. Molecular cloning and characterization of Drosophila genes encoding small GTPases of the rab and rho families. Mol Gen Genet 1997; 254:486-94.
12. Murphy AM, Montell DJ. Cell type-specific roles for Cdc42, Rac and RhoL in Drosophila oogenesis. J Cell Biol 1996; 133:617-30.
13. Hariharan IK, Hu KQ, Asha H, Quintanilla A, Ezzell RM, Settleman J. Characterization of rho GTPase family homologues in *Drosophila melanogaster*: overexpressing Rho1 in retinal cells causes a late developmental defect. EMBO J 1995; 14:292-302.
14. Harden N, Loh HY, Chia W, Lim L. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in Drosophila. Development 1995; 121:903-14.
15. Luo L, Liao YJ, Jan LY, Jan YN. Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev 1994; 8:1787-802.
16. Newsome TP, Schmidt S, Dietzl G, Keleman K, Asling B, Debant A, Dickson BJ. Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in Drosophila. Cell 2000; 101:283-94.
17. Zipkin ID, Kindt RM, Kenyon CJ. Role of a new Rho family member in cell migration and axon guidance in *C. elegans*. Cell 1997; 90:883-94.
18. Klein TJ, Mlodzik M. Planar cell polarization: an emerging model points in the right direction. Annu Rev Cell Dev Biol 2005; 21:155-76.
19. Mlodzik M. Planar polarity in the Drosophila eye: a multifaceted view of signaling specificity and cross-talk. EMBO J 1999; 18:6873-9.
20. Boutros M, Paricio N, Strutt DI, Mlodzik M. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell 1998; 94:109-18.
21. Paricio N, Feiguin F, Boutros M, Eaton S, Mlodzik M. The Drosophila STE20-like kinase misshapen is required downstream of the Frizzled receptor in planar polarity signaling. EMBO J 1999; 18:4669-78.
22. Weber U, Paricio N, Mlodzik M. Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. Development 2000; 127:3619-29.
23. Weber U, Pataki C, Mihaly J, Mlodzik M. Combinatorial signaling by the Frizzled/PCP and Egfr pathways during planar cell polarity establishment in the Drosophila eye. Dev Biol 2008; 316:110-23.
24. Cooper MT, Bray SJ. Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. Nature 1999; 397:526-30.
25. Fanto M, Mlodzik M. Asymmetric Notch activation specifies photoreceptors R3 and R4 and planar polarity in the Drosophila eye. Nature 1999; 397:523-6.
26. Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD, Luo L. Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 2001; 105:81-91.
27. Brown KE, Freeman M. Egfr signalling defines a protective function for ommatidial orientation in the Drosophila eye. Development 2003; 130:5401-12.
28. Gaengel K, Mlodzik M. Egfr signaling regulates ommatidial rotation and cell motility in the Drosophila eye via MAPK/Pnt signaling and the Ras effector Canoe/AF6. Development 2003; 130:5413-23.
29. Strutt H, Strutt D. EGF signaling and ommatidial rotation in the Drosophila eye. Curr Biol 2003; 13:1451-7.
30. Mirkovic I, Mlodzik M. Cooperative activities of drosophila DE-cadherin and DN-cadherin regulate the cell motility process of ommatidial rotation. Development 2006; 133:3283-93.
31. Choi KW, Benzer S. Rotation of photoreceptor clusters in the developing Drosophila eye requires the *nemo* gene. Cell 1994; 78:125-36.
32. Chou YH, Chien CT. Scabrous controls ommatidial rotation in the Drosophila compound eye. Dev Cell 2002; 3:839-50.
33. Fiehler RW, Wolff T. Drosophila Myosin II, Zipper, is essential for ommatidial rotation. Dev Biol 2007; 310:348-62.
34. Fiehler RW, Wolff T. Nemo is required in a subset of photoreceptors to regulate the speed of ommatidial rotation. Dev Biol 2008; 313:533-44.
35. Escudero LM, Bischoff M, Freeman M. Myosin II regulates complex cellular arrangement and epithelial architecture in Drosophila. Dev Cell 2007; 13:717-29.
36. Mlodzik M, Baker NE, Rubin GM. Isolation and expression of scabrous, a gene regulating neurogenesis in Drosophila. Genes Dev 1990; 4:1848-61.
37. Artero RD, Castanon I, Baylies MK. The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. Development 2001; 128:4251-64.
38. Bao S, Cagan R. Preferential adhesion mediated by Hibris and Roughest regulates morphogenesis and patterning in the Drosophila eye. Dev Cell 2005; 8:925-35.
39. Dworak HA, Charles MA, Pellerano LB, Sink H. Characterization of Drosophila *hibris*, a gene related to human *nephrin*. Development 2001; 128:4265-76.
40. Grillo-Hill BK, Wolff T. Dynamic cell shapes and contacts in the developing Drosophila retina are regulated by the Ig cell adhesion protein hibris. Dev Dyn 2009; 238:2223-34.
41. Kim YI, Ryu T, Lee J, Heo YS, Ahnn J, Lee SJ, Yoo O. A genetic screen for modifiers of Drosophila caspase Dcp-1 reveals caspase involvement in autophagy and novel caspase-related genes. BMC Cell Biol 11:9.
42. Magwire MM, Yamamoto A, Carbone MA, Roshina NV, Symonenko AV, Pasyukova EG, et al. Quantitative and molecular genetic analyses of mutations increasing Drosophila life span. PLoS Genet 6:1001037.
43. Taylor J, Abramova N, Charlton J, Adler PN. Van Gogh: a new Drosophila tissue polarity gene. Genetics 1998; 150:199-210.
44. Jin MH, Sawamoto K, Ito M, Okano H. The interaction between the Drosophila secreted protein argos and the epidermal growth factor receptor inhibits dimerization of the receptor and binding of secreted spitz to the receptor. Mol Cell Biol 2000; 20:2098-107.
45. Freeman M. The *spitz* gene is required for photoreceptor determination in the Drosophila eye where it interacts with the EGF receptor. Mech Dev 1994; 48:25-33.
46. Dickson BJ, Dominguez M, van der Straten A, Hafen E. Control of Drosophila photoreceptor cell fates by phyllopod, a novel nuclear protein acting downstream of the Raf kinase. Cell 1995; 80:453-62.
47. Asha H, de Ruiter ND, Wang MG, Hariharan IK. The Rap1 GTPase functions as a regulator of morphogenesis in vivo. EMBO J 1999; 18:605-15.

48. Boettner B, Harjes P, Ishimaru S, Heke M, Fan HQ, Qin Y, et al. The AF-6 homolog *canoe* acts as a Rap1 effector during dorsal closure of the Drosophila embryo. Genetics 2003; 165:159-69.
49. O'Keefe DD, Gonzalez-Nino E, Burnett M, Dylla L, Lambeth SM, Licon E, et al. Rap1 maintains adhesion between cells to affect Egfr signaling and planar cell polarity in Drosophila. Dev Biol 2009; 333:143-60.
50. Shilo BZ. Signaling by the Drosophila epidermal growth factor receptor pathway during development. Exp Cell Res 2003; 284:140-9.
51. Doroquez DB, Orr-Weaver TL, Rebay I. Split ends antagonizes the Notch and potentiates the EGFR signaling pathways during Drosophila eye development. Mech Dev 2007; 124:792-806.
52. Cerrato A, Parisi M, Santa Anna S, Missirlis F, Guru S, Agarwal S, et al. Genetic interactions between *Drosophila melanogaster* menin and Jun/Fos. Dev Biol 2006; 298:59-70.
53. Abrams EW, Andrew DJ. Prolyl 4-hydroxylase alpha-related proteins in *Drosophila melanogaster*: tissue-specific embryonic expression of the 99F8-9 cluster. Mech Dev 2002; 112:165-71.
54. Snaith HA, Armstrong CG, Guo Y, Kaiser K, Cohen PT. Deficiency of protein phosphatase 2A uncouples the nuclear and centrosome cycles and prevents attachment of microtubules to the kinetochore in Drosophila *microtubule star* (*mts*) embryos. J Cell Sci 1996; 109:3001-12.
55. Hannus M, Feiguin F, Heisenberg CP, Eaton S. Planar cell polarization requires Widerborst, a B' regulatory subunit of protein phosphatase 2A. Development 2002; 129:3493-503.
56. Wassarman DA, Solomon NM, Chang HC, Karim FD, Therrien M, Rubin GM. Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in Drosophila. Genes Dev 1996; 10:272-8.
57. Vereshchagina N, Ramel MC, Bitoun E, Wilson C. The protein phosphatase PP2A-B' subunit Widerborst is a negative regulator of cytoplasmic activated Akt and lipid metabolism in Drosophila. J Cell Sci 2008; 121:3383-92.
58. Sontag JM, Sontag E. Regulation of cell adhesion by PP2A and SV40 small tumor antigen: an important link to cell transformation. Cell Mol Life Sci 2006; 63:2979-91.
59. Tepass U, Harris KP. Adherens junctions in Drosophila retinal morphogenesis. Trends Cell Biol 2007; 17:26-35.
60. Fetting JL, Spencer SA, Wolff T. The cell adhesion molecules Echinoid and Friend of Echinoid coordinate cell adhesion and cell signaling to regulate the fidelity of ommatidial rotation in the Drosophila eye. Development 2009; 136:3323-33.
61. Seifert JR, Mlodzik M. Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility. Nat Rev Genet 2007; 8:126-38.
62. Shelton C, Kocherlakota KS, Zhuang S, Abmayr SM. The immunoglobulin superfamily member Hbs functions redundantly with Sns in interactions between founder and fusion-competent myoblasts. Development 2009; 136:1159-68.
63. Ishimaru S, Ueda R, Hinohara Y, Ohtani M, Hanafusa H. PVR plays a critical role via JNK activation in thorax closure during Drosophila metamorphosis. EMBO J 2004; 23:3984-94.
64. Nolan KM, Barrett K, Lu Y, Hu KQ, Vincent S, Settleman J. Myoblast city, the Drosophila homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes. Genes Dev 1998; 12:3337-42.
65. Geisbrecht ER, Haralalka S, Swanson SK, Florens L, Washburn MP, Abmayr SM. Drosophila ELMO/CED-12 interacts with Myoblast city to direct myoblast fusion and ommatidial organization. Dev Biol 2008; 314:137-49.
66. Meyer CA, Jacobs HW, Datar SA, Du W, Edgar BA, Lehner CF. Drosophila Cdk4 is required for normal growth and is dispensable for cell cycle progression. EMBO J 2000; 19:4533-42.
67. Chen X, Oh SW, Zheng Z, Chen HW, Shin HH, Hou SX. Cyclin D-Cdk4 and cyclin E-Cdk2 regulate the Jak/STAT signal transduction pathway in Drosophila. Dev Cell 2003; 4:179-90.
68. Zeidler MP, Perrimon N, Strutt DI. Polarity determination in the Drosophila eye: a novel role for unpaired and JAK/STAT signaling. Genes Dev 1999; 13:1342-53.
69. Tsuneizumi K, Nakayama T, Kamoshida Y, Kornberg TB, Christian JL, Tabata T. Daughters against dpp modulates dpp organizing activity in Drosophila wing development. Nature 1997; 389:627-31.
70. Ou G, Vale RD. Molecular signatures of cell migration in *C. elegans* Q neuroblasts. J Cell Biol 2009; 185:77-85.
71. Raabe T, Riesgo-Escovar J, Liu X, Bausenwein BS, Deak P, Maroy P, Hafen E. DOS, a novel pleckstrin homology domain-containing protein required for signal transduction between sevenless and Ras1 in Drosophila. Cell 1996; 85:911-20.
72. Tomlinson A, Ready DF. Neuronal differentiation in Drosophila ommatidium. Dev Biol 1987; 120:366-76.