A screen for modifiers of RacGAP(84C) gain-of-function in the *Drosophila* eye revealed the LIM kinase Cdi/TESK1 as a downstream effector of Rac1 during spermatogenesis

Karine Raymond*, Evelyne Bergeret, Amélie Avet-Rochex, Ruth Griffin-Shea‡ and Marie-Odile Fauvarque‡

CEA-Grenoble, 17 rue des Martyrs, Département de Réponse et Dynamique Cellulaires, UMR 5092, 38054 Grenoble CEDEX 9, France

*Present address: Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

‡Authors for correspondence (e-mail: mofauvarque@cea.fr; griffinshea@dsvsud.cea.fr)

Accepted 26 January 2004
Journal of Cell Science 117, 2777-2789 Published by The Company of Biologists 2004
doi:10.1242/jcs.01123

Summary

In *Drosophila*, RotundRacGAP/RacGAP(84C) is critical to retinal organisation and spermatogenesis. We show that eye-directed expression of RacGAP(84C) or its GTPase activating protein (GAP) domain induces a dominant rough eye phenotype which we used as a starting point in a gain-of-function screen to identify new partners of RacGAP(84C). Proteins known to function in Ras, Rho and Rac signalling were identified confirming the essential role of RacGAP(84C) in crosstalk between GTPases. Other potential RacGAP(84C) partners identified by the screen are implicated in signal transduction, DNA remodelling, cytoskeletal organisation, membrane trafficking and spermatogenesis. This latter class includes the serine/threonine kinase Center divider (Cdi), which is homologous to the human LIM kinase, Testis specific kinase 1 (TESK1), involved in cytoskeleton control through Cofilin phosphorylation. Eye-directed expression of *cdi* strongly suppressed the phenotypes induced by either RacGAP(84C) gain-of-function or by the dominant negative form of Rac1, Rac1N17. These results are consistent with Cdi being a specific downstream target of Rac1. We showed that *Rac1* and *cdi* are both expressed in *Drosophila* testis and that homozygous *Rac1* mutants exhibit poor fertility that is further reduced by introducing a *cdi* loss-of-function mutation in trans. Thus, results from a misexpression screen in the eye led us to a putative novel Rac1-Cdi-Cofilin pathway, regulated by RacGAP(84C), coordinating *Drosophila* spermatogenesis.

Supplemental data available online

Key words: Serine/threonine kinase, GTPases, Spermatogenesis

Introduction

In mammalian cell systems, the small GTPases of the Rho family, including Rho, Rac and Cdc42, have been shown to control a variety of cellular functions through changes in cytoskeletal organisation and gene transcription (Hall, 1998; Ridley, 1996; Vojtek and Cooper, 1995). Like all GTPases of the Ras superfamily, RhoGTPases act as molecular switches by cycling between active, GTP-bound, and inactive, GDP-bound, states (Nobes and Hall, 1994). GTPase activation requires guanine nucleotide exchange factors (GEFs) inducing the exchange of GDP to GTP, whereas GTPase-activating proteins (GAPs) stimulate GTP hydrolysis, thus down-regulating GTPase-mediated signals (Lamarche and Hall, 1994; Nobes and Hall, 1994). Several lines of evidence suggest that GEFs determine the specificity of GTPase upstream activating signals (Schmidt and Hall, 2002) and that GAPs may serve as effector molecules downstream of Ras-like GTPases (Jullien-Flores et al., 1995; Kozma et al., 1996). In *Drosophila*, the RhoGTPases are specifically required in many developmental events involving actin-mediated cell shape changes and the establishment of cell polarity (Settleman, 2001).

The *Drosophila rotund* locus at cytogenetic position 84C on the third chromosome encodes a RacGAP protein, previously named RotundRacGAP (Agnel et al., 1992), that we now refer to as RacGAP(84C) to distinguish it from the *rotund* (*rn*) transcript included in the same locus (St Pierre et al., 2002). In vitro, RacGAP(84C) enhanced the GTPase activity of Rac1 and Cdc42 but not that of Rho (Raymond et al., 2001). Tissue-specific overexpression experiments demonstrated the same in vivo substrate specificity during eye development and embryonic dorsal closure (Raymond et al., 2001). RacGAP(84C) has 60% similarity with the human male germ cell RacGAP (MgcRacGAP) protein (Toure et al., 1998) although it lacks the N-terminal microtubule-binding domain of MgcRacGAP (Hirose et al., 2001) that is present in the homologous *Drosophila* RacGAP protein, DRacGAP(50C) (Sotillos and Campuzano, 2000). Like *mgcRacGAP*, *RacGAP(84C)* is strongly expressed in the testes, specifically in primary spermatocytes. Analysis of *Drosophila* mutants demonstrated that RacGAP(84C) is necessary for membrane deposition during spermatid elongation. In its absence, testicular cyst growth is extremely limited, while flagellar growth continues independently of cyst elongation, leading to characteristic short testicular cysts with bulbous ends (Bergeret et al., 2001).

We show that expression of RacGAP(84C) or of its GAP

domain dominantly affects eye development. In order to identify new molecular partners of RacGAP(84C), we performed a gain-of-function screen using the EP modular misexpression system devised by P. Rorth, which employs a transposable P-element (EP) containing 14 copies of the UAS element linked to a basal promoter (Rorth et al., 1998). If the EP is inserted 5′ to an endogenous gene (the preferential site for P insertion), directed transcription of that gene can be induced by crossing the EP line to a tissue-specific driver line expressing the yeast transcription factor Gal4 that recognises the UAS sequences (Brand and Perrimon, 1993). The results of our screen strongly support an essential role for RacGAP(84C) in crosstalk between GTPase-dependent signals. EP insertions were identified in several genes encoding regulators of these pathways, as well as in genes coding proteins putatively acting downstream of these GTPases. Of interest is the fact that this eye-directed misexpression screen uncovered a number of proteins with either proven, or potential, function in sperm differentiation. We have demonstrated that *center divider* (*cdi*), encoding a LIM kinase homologous to the human testis specific protein kinase 1 (TESK1), is expressed in *Drosophila* testes where it probably functions as a downstream effector of Rac1 in sperm differentiation.

Materials and Methods

Fly strains and crosses

All flies were maintained on standard medium. X-linked EP lines were obtained from the Bloomington stock center, and autosomal EP insertions were ordered from Exelixis's Fly station (http://flystation.exelixis.com). Transgenic lines UAS-RacGAP(84C), UAS-Zn and UAS-GAP have been described previously (Raymond et al., 2001).

Screen

A recombinant line GMRGal4,UAS-GAP17 (referred to as GG17) inducing a moderate rough eye phenotype was used as the starting point for the screen. A GMRGal4; UAS-GAP35 line (referred to as GG35) inducing a stronger eye phenotype was used to confirm genetic interaction of candidate EP lines. GG17/CyO virgin females were mated to EP males at 20°C (Rorth et al., 1998). F1 progeny were observed with a dissecting microscope for modification of the reference eye phenotype of control GG17/+ flies resulting from crossing GG17 females with w^{1118} males. Lines that modified the original phenotype were retested at both 20°C and 25°C with GG17, GG35 and separately crossed with the GMRGal4 driver. The EP lines presenting a strong eye phenotype with GMRGal4 were discarded whereas those presenting a moderate eye phenotype with GMRGal4, and a strong enhancement or lethality with GG17, were crossed to a GMRGal4; rn^{20}/TM3,Sb strain. The deficiency rn^{20} maps to 84C and deletes the entire *RacGAP(84C)* transcription unit and overlapping genes including *roe* and *rn* (Agnel et al., 1989; St Pierre et al., 2002).

Other genetic tests

Coexpression with the negative forms of either Cdc42, Rac1 or Ras, was achieved by crossing EP lines with GMRGal4,UAS-Cdc42N17/CyO or GMRGal4/CyO; UAS-Rac1N17/TM3,Sb or UAS-RasN17; or GMRGal4/CyO, respectively. Recombinant chromosomes carrying both the cdi^{R47} amorphic mutation (Matthews and Crews, 1999) and either $Rac1^{j11}$ or $Rac2^{\Delta}$ mutations (Hakeda-Suzuki et al., 2002; Ng et al., 2002) were obtained by classic meiotic recombination techniques. The cdi^{R47} mutation was followed genetically (lethality over cdi^{R47}); $Rac1^{j11}$ was followed by the linked marker FRT2A ([w^{+}]); and the deletion $Rac2^{\Delta}$ was detected by genomic PCR amplification using the primers described below.

EP(3)3319 mobilisation

EP excisions were performed by introducing the stable source of transposase in EP lines using standard procedures (Robertson et al., 1988). Loss of EP sequences was detected by Southern analysis using the ^{32}P-random-labelled P[UAS] vector as probe.

Sample preparation and microscopic observation

Optical microscopy

Heads of wild-type and transgenic flies were cut in half in PBS saline buffer and immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours. After subsequent washing in cacodylate buffer, they were postfixed in 1% OsO_4 for 1 hour and then dehydrated and embedded in Epon. Thin sections (2 μm) stained with Toluidine Blue were examined by phase-contrast optical microscopy.

Scanning electron microscopy

Flies were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for 2 hours. After three 30 minutes washes in cacodylate buffer, they were dehydrated in successive baths of 50%, 70%, 95% and 100% ethanol, for 30 minutes each. Samples were dried by the critical point method with liquid CO_2, sputter-coated with gold and observed with a JEOL JSM 840-A scanning electron microscope.

Detection of transcripts

Reverse transcription polymerase chain reactions (RT-PCR)

Fifty testes were dissected from the indicated strains in RNAlater (Ambion, Inc.), and total RNA was isolated with RNAwiz™ (Ambion, Inc.). Contaminating genomic DNA was removed using the DNA-free™ kit (Ambion, Inc.). RT-PCR reactions were performed with 1 μg RNA and 1.25 Units Titanium™ Taq DNA polymerase (Clontech) and 2.5 units AMVRTase (Promega) for 30 cycles of amplification essentially as described previously (Huet et al., 1993) and following manufacturers' instructions. Oligonucleotide primers were chosen to cover genomic regions containing intronic sequence to allow detection of contaminating DNA. *RacGAP(84C)* primers: sense 5′CTTGCCGTGATCTTCGCTC and antisense 5′GGTGAGT-ACTGCTAAGGTTGAC. Expected fragment sizes: 274 bp (cDNA) and 394 bp (genomic DNA). *cdi* primers: sense 5′GATTTTCG-GTCCTCGCATTTGTGAT and antisense 5′CAGTTAGACGGGCA-CGATTTGTTCA. Expected fragment sizes: 498 bp (cDNA) and 5950 bp (genomic DNA). *TMS1d* primers: sense 5′TGCGGCATCAACA-AGTTCTTCATCTCC and antisense 5′ATCCGTGCCCGCCTC-TGTGTCTG. Expected fragment sizes: 426 bp (cDNA) and 780 bp (genomic DNA). *Rac1* primers: sense 5′AGAAGCACCCCCGCAA-TCAGAAATC and antisense 5′GGTTGGGTTGGGTTGGGGG-AATC. Expected fragment size 856 bp (no intronic sequences in the locus). As a control, amplification was also performed in the absence of reverse transcriptase to further ensure that the amplification observed was not the consequence of contaminating DNA (shown for *Rac1*).

Northern blot

Total RNA was extracted from adult flies or dissected testes or ovaries with RNAwiz™. RNAs were resolved on a 1% agarose formaldehyde gel, blotted onto a Hybond-N filter, and hybridised under stringent conditions (Yang et al., 1993). Probes were labelled by random priming in the presence of [^{32}P]CTP. The *cdi* probe was generated by

PCR using the sense primer: 5′TCAGTTGCCCGCCACGTCTATTCC and antisense primer: 5′CACCGCCGCTGTTGCACCGCTACT covering 853 bp of *cdi* coding sequences (exon 6). The filter was dehybridised and rehybridised with an actin cDNA probe as an internal loading control.

Detection of β-galactosidase activity in testes

Isolated testes were fixed in 1% glutaraldehyde in PBS for 15 minutes at room temperature and β-galactosidase activity was detected using standard procedures.

Results

Eye-directed expression of RacGAP(84C) affects eye development

Transgenic rescue experiments in rn^{20} homozygous flies showed that *RacGAP(84C)* function is crucial to internal retinal differentiation and spermatogenesis (Bergeret et al., 2001). In order to identify additional processes in which RacGAP(84C) functions during eye development, we investigated the effect of eye-directed *RacGAP(84C)* expression on internal and external eye morphology. Transgenic UAS-RacGAP(84C) flies were crossed with a GMRGal4 strain that drives the UAS target transgene in all cells posterior to the morphogenetic furrow in the developing imaginal eye disc (Ellis et al., 1993). Ninety-five percent of the progeny die as black pupae. Lethality probably results from leaky expression of RacGAP(84C) in tissues other than the eye disc. Survivor flies exhibit rough and reduced eyes with glossy surfaces and a black patch in the anterior-most part (Fig. 1C). Compared to control flies (Fig. 1A,B), retinal thickness is reduced and a decreased number of misshapen ommatidia are randomly dispersed in a vacuolated matrix (arrows, Fig. 1D). Photoreceptor cells appear fused at the level of their rhabdomeres and mis-oriented (arrowheads, Fig. 1D). The external eye phenotype induced by directed expression of RacGAP(84C) is not modified by either the deficiency H99 covering the three pro-apoptotic genes *reaper*, *hid* and *grim* (Grether et al., 1995; White et al., 1994), or by coexpression of the p35 protein, a suppressor of caspase 8/3-dependent apoptosis (Hay et al., 1994; Riedl et al., 2001; Xu et al., 2001) (not shown). Thus, vacuole formation and decreased cell number observed following eye-directed expression of RacGAP(84C) might result from other cell death mechanisms (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002), or from non-apoptotic processes.

The phenotype induced by eye-directed expression of RacGAP(84C) might result from the dominant inhibition of Rac1 and Cdc42 signalling. Indeed, eye-directed expression of the dominant negative forms Rac1N17 and Cdc42N17 interferes with normal eye patterning (Fanto et al., 2000) and *cdc42* mutant clones in the eye fail to produce adult ommatidia (Genova et al., 2000). When compared to flies expressing RacGAP(84C), the external phenotype and internal organisation was much less affected in flies expressing Rac1N17 or Cdc42N17 (Luo et al., 1994) whether singly or in combination (shown for Rac1N17, Fig. 1E,F). Cdc42 and Rac1 dominant negative forms, working stochiometrically, might not interfere as strongly as RacGAP(84C) which functions catalytically on its substrates. Alternatively, RacGAP(84C) expression may interfere with pathways other than those affected by Rac1N17 or Cdc42N17 transgenes, a finding in accord with previous results demonstrating genetic interaction of RacGAP(84C) with the two proteins Ras and downstream receptor kinase (Drk) from the MAP kinase cascade (Bergeret et al., 2001; Yamamoto, 1994).

Rough eye phenotype induced by directed expression of the GAP domain of RacGAP(84C) provided the starting point for a misexpression screen

The RacGAP(84C) protein contains two major domains: a

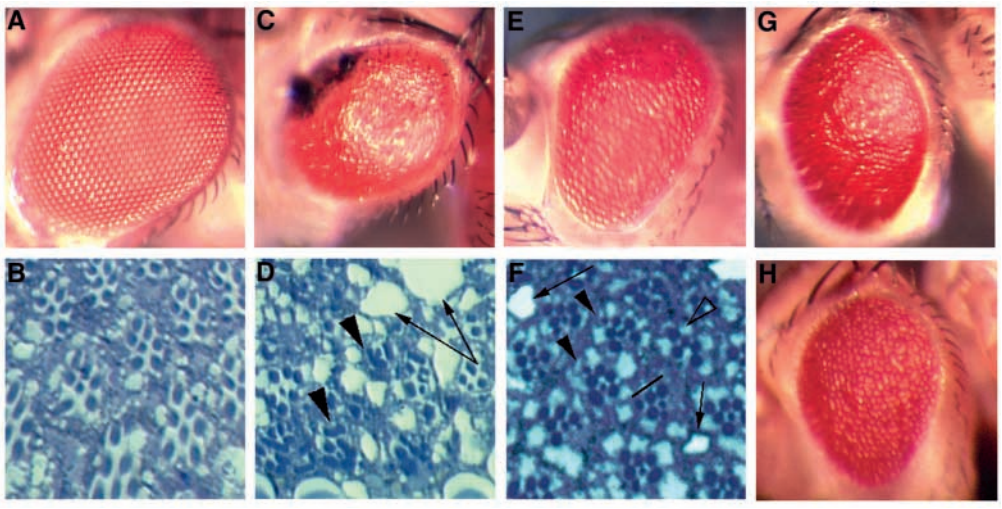

Fig. 1. Eye phenotypes caused by misexpression of the RacGAP(84C) protein or its GAP domain. (A,C,E,G,H) External aspects viewed with a dissecting microscope. (B,D,F) Semi-thin tangential histological sections. (A,B) GMRGal4/UAS-lacZ control flies. (A) Eyes exhibit a regular array of about 800 repeated ommatidia. (B) Normal ommatidial architecture characterised by a trapezoidal array of outer photoreceptor cells R1-6 and central R7 cell, surrounded by accessory cells. (C,D) GMRGal4/UAS-RacGAP(84C). (C) Eye-directed expression of RacGAP(84C) induces rough eyes with black patch in the anterior-most part. (D) RacGAP(84C)-expressing retina shows a reduced number of randomly dispersed ommatidia and vacuolated material (arrows) associated with abnormal numbers of photoreceptor cells and misshapen rhabdomeres (arrowheads). (E,F) GMRGal4/UAS-Rac1N17. (E) Eye-directed expression of Rac1N17 induces rough eyes. (F) Rac1N17-expressing retina shows loss of rhabdomeres (open arrowhead), abnormal photoreceptor cells (black arrowheads), vacuolated material (arrows), and polarity defects (bar). (G) GMRGal4/+; UAS-GAP17/+ eye expressing the isolated GAP domain of RacGAP(84C) (weak strain). (H) Eye-directed expression of RasN17 induces reduced and rough eyes (RasN17/Y; GMRGal4/+).

Table 1. Genetic partners of RacGAP(84C)

Gene/predicted gene	EP line	cytology	GG17	Molecular and functional information	Rac1N17	Cdc42N17	RasN17	rn^{20}
A. Ras, Rac and Rho signalling proteins								
Misexpression of Ras 4 (*MESR4*)	EP(2)386	54C3-7	Su	Suppressor of constitutive Ras-MAPK signalling	No effect	No effect	No effect	NT
A kinase anchor protein (*Akap 200*)	EP(2)2072	29C3-4	Enh	Suppressor of constitutive Ras-MAPK signalling	No effect	No effect	No effect	NT
(*syn.MESR2*)	EP(2)2254		Enh		NT	NT	NT	NT
Drosophila C3G (*DC3-G*)	EP(1)1613*	6D1-2	Enh	RasGEF, activation of Ras-MAPK	NT	NT	NT	No effect
	UAS-DC3G		Enh		Enh	Enh	No effect	NT
CG31012	EP(3)3700	100A6	Enh	SH3 domain	No effect	No effect	No effect	NT
pebble (*pbl*)	EP(3)3415*	66A18	Lethal	RhoGEF, functions in cytokinesis	Lethal	Enh (escapers)	No effect	Lethal
GEF(64C)	EP(3)3035	64B14	Enh	RhoGEF, functions in axonal growth	No effect	No effect	No effect	NT
	EP(3)3322*		Lethal		Lethal	Lethal	No effect	No effect
grapes (*grp*)	EP(2)587	36A10	Enh	Ser/thr kinase, functions in DNA repair	Enh (rough)	Enh (rough)	Enh (glossy)	NT
CG7097	EP(2)2445	56C1-4	Enh	Receptor signalling Ser/thr kinase	No effect	No effect	No effect	NT
SNF/AMP-activated protein S/T kinase gamma subunit	EP(3)648	93C2-6	Enh	AMP-activated Ser/thr kinase, functions as a metabolic sensor in yeast	Enh (escapers)	No effect	No effect	NT
B. Cytoskeletal control and membrane trafficking								
Myo31DF	EP(2)2491	31F3	Enh	Myosin ATPase	Enh	No effect	No effect	NT
Beached1 (*Beach1*)	EP(2)2299*	26A1	Enh	Endocytic pathway, neuronal growth EP may drive a Nter truncated protein (see Table S1)	Enh (escapers)	Enh (escapers)	Enh	Enh
C. DNA remodelling factors								
kismet (*kis*)	EP(2)474	21B2-4	Enh	DNA helicase	No effect	No effect	NT	
	EP(2)563		Enh		No effect	No effect	No effect	
domino (*dom*)	EP(2)2371	57D11	Enh	DNA helicase	No effect	No effect	No effect	
Dodeca-satellite-binding protein 1 (*Dp1*)	EP(2)2422	55D4	Enh	Single strand DNA binding, KH domain	No effect	No effect	No effect	
D. Spermatogenesis								
purity of essence (*poe*)	EP(2)349	28E1	Su	Male sterile, abnormal sperm tail	Su	Su	No effect	
	EP(2)737		Su		Su	Su	No effect	
Target of methylation-induced silencing 1 (*TMS1d*)	EP(3)807	72E5-F1	Enh	Caspase-mediated cell death	No effect	No effect	No effect	
Centre divider (*Cdi*)	EP(3)3319	91E4-F1	Su	Ser/thr kinase TESK1	Su	No effect	No effect	
E. Others								
Traf2	EP(1)325	7D16	Enh	TNF- signalling pathway	No effect	No effect	No effect	
wunen2	EP(2)652	45D	Enh	Phosphatidic acid phosphatase	No effect	No effect	No effect	
CG14959	EP(2)714	63C1	Enh	Chitin binding domain	NT	NT	NT	
	EP(2)3139							
CG5261	EP(2)816	27F1-6	Enh	Dihydrolipoamide-S-acetyl transferase	Enh	Enh	No effect	
CG3624	EP(2)827	58D7	Enh	Immunoglobulin domain				
rhomboid-6 (*rho-6*)	EP(2)2023	33D1	Enh	EGF- MAP kinase signalling	No effect	No effect	No effect	
CG6701	EP(2)2054	50C22	lethal	C2H2 Zn finger domain	Enh	No effect	No effect	
CG8740	EP(2)2233	44E3-4	Enh	No putative conserved domain	Enh	Enh	No effect	
numb	EP(2)2455	30B5	Su	Phosphotyrosine interaction and PH domains	Su	Su	No effect	
couch potato (*cpo*)	EP(3)3395	90C10	Enh	RNA binding motifs	Enh	No effect	No effect	
	EP(3)3608		Enh					
CG2017	EP(3)3503	83C5-6	Su	GTP-binding elongation factor	No effect	No effect	No effect	

Eye directed expression of EP-associated genes with: the GAP domain of RacGAP(84C) (GG17) (column 4), Rac1N17 (column 6), Cdc42N17 (column 7), RasN17 (column 8); or in rn^{20} hemizygous context (last column, A,B).

*EP lines presenting a rough eye in combination with GMRGal4.

NT, not tested.

diacylglycerol-binding, zinc finger domain in the N terminus, and a GAP domain homologous to that of RhoGAP proteins in the C terminus. Previous experiments showed that overexpression of the full-length protein or its GAP domain, in UAS-RacGAP or UAS-GAP embryos respectively, generated similar dorsal closure phenotypes resulting from an inhibition of Rac1 and Cdc42 signalling (Raymond et al., 2001). Among 12 UAS-GAP lines, eight behaved like the strains carrying the full-length protein when crossed with the GMRGal4 driver (not shown) whereas the four remaining strains were viable (70-100%) and had a less severe eye phenotype (Fig. 1G). The differences are presumably due to insertional position effects. Eye-directed expression of the N-terminal part of RacGAP(84C) (UAS-Zn strain) did not induce any eye phenotype indicating that RacGAP(84C) affects eye development and viability through its GAP domain (not shown).

To identify novel signalling components that interact with RacGAP(84C), we performed a gain-of-function screen using the viable recombinant strain GMRGal4,UAS-GAP17 (referred to as GG17). From a set of 1800 EP lines tested, six lines behaved as suppressors and 93 lines, as enhancers. In the latter class, 67 enhancer lines themselves induced abnormal eye shape when crossed with GMRGal4; as such, enhancement of the GG17 eye phenotype might result from either additive or synergistic effects. Of these, four EP lines were retained since other data confirmed the existence of a genetic interaction with RacGAP(84C) (see below and Materials and Methods, indicated with an asterisk in Table 1). The exact position of each EP insertion relative to its target was determined from sequence data supplied from the Berkeley *Drosophila* Genome Project (http://flybase.bio.indiana.edu) so as to assess the likelihood that it drives transcription of a downstream gene (Table S1, http://jcs.biologists.org/supplemental/). Insertions located just upstream of the transcription start, in the 5′UTR, or in the first non-coding intron of a contiguous gene initiate a sense mRNA starting from the EP target promoter which is produced only in the presence of Gal4 (Rorth, 1996). Accordingly, three enhancer EP lines inserted in the opposite orientation were discarded (i.e. EP(2)588 in *kekkon*, EP(2)1195 in *toutatis* and EP(2)2004 in CG7231). To assess the additional possibility that the observed phenotypes may be due to insertional loss-of-function mutation, loss-of-function mutations or deficiencies of each of the candidate genes were systematically tested for their effect on the GG17 eye phenotype (Table S1, http://jcs.biologists.org/supplemental/). No changes were observed except in one case: the deficiency covering *TMS1d*, which dominantly enhanced GG17 (see below).

The final, selected 27 enhancers correspond to 23 loci and the original six selected suppressors, to five independent loci. These RacGAP(84C) partners were subdivided into five main classes according to their putative or proven functions (Table 1): (A) GTPase signalling molecules, (B) proteins implicated in cytoskeleton control or membrane trafficking, (C) DNA remodelling factors, (D) spermatogenesis proteins and, (E) proteins of diverse or unknown function.

A certain number of GG17 modifiers act through dominant inhibition of signalling pathways dependent upon Rac or Cdc42, but not Ras

EP insertions modifying GG17 were subsequently coexpressed in the eye with the dominant negative forms of either Cdc42, Rac1 or Ras (Table 1, column 6-8), which themselves induce reduced and roughened eyes (shown for Rac1N17 and RasN17, Fig. 1E and H, respectively). When coexpression of EP lines with either Rac1N17 or Cdc42N17 induced eye phenotypes similar to those induced by its coexpression with GG17 (although systematically milder with Cdc42N17), one can conclude that interaction with RacGAP(84C) was likely to result from dominant inhibition of Rac (and Cdc42) signalling. Only two lines modify the eye phenotype induced by directed expression of RasN17 (EP(2)587 and EP(2)2299) inducing defects quite different from those observed with GG17 (see below). This suggests that RacGAP(84C) is unlikely to affect Ras activity directly; but rather interacts with Ras regulators or effectors including those found in our screen.

Modifiers of RacGAP(84C) gain-of-function belong to the Ras pathway

Three EP insertions identified in our screen are situated 5′ to genes encoding proteins affecting the Ras-MAPK pathway (Table 1A). MESR4 and MESR2/DAKAP200 are two proteins previously identified as downstream dominant suppressors of constitutive Ras activity (Huang and Rubin, 2000), while the RasGEF DC3G directly enhances Ras activity during fly development (Ishimaru et al., 1999).

MESR4 acted as a suppressor, the expression of which restored the global appearance of the eye associated with normal ommatidial size and packing in GG17/EP(2)386 flies (Fig. 2E,F) compared to GG17/+ flies (Fig. 2C,D). In contrast, the expression of MESR2/DAKAP200 (EP(2)2072, EP(2)2254) enhanced the GG17-induced eye phenotype inducing strong posterior defects and ommatidia sunken below the eye surface (Fig. 2I,J). As expected for downstream antagonists of Ras signalling, neither MESR4 nor MESR2 expression modified the phenotypes induced by eye-directed expression of the dominant negative form RasN17. Their expression also had little or no effect on the eye phenotype induced by expression of Rac1N17 or Cdc42N17. This observation would suggest that MESR interactions with GG17 are independent of dominant inhibition of Rac or Cdc42 signalling.

The EP(1)1613 insertion situated 5′ to the coding sequence of *DC3G* behaved as a strong enhancer of the GG17 phenotype, itself inducing moderate rough eyes (Table 1A). This interaction was confirmed by using three existing UAS-DC3G lines (Ishimaru et al., 1999). While two lines behaved like EP(1)1613, the third line induced no eye phenotype by itself and also strongly enhanced the GG17-induced defects. The detailed phenotype is reminiscent of that induced by coexpressing MESR2 and the GAP domain of RacGAP(84C), with notably the appearance of sunken ommatidia although not of posterior defects (not shown). As expected for a RasGEF, expression of DC3G had no effect on the RasN17-induced eye defects. Indeed, RasN17 is blocked in a negative conformation preventing any effective exchange from GDP to GTP. In contrast, coexpression of DC3G with Rac1N17 or Cdc42N17 resulted in phenotypic enhancement of the reduced and roughened eyes as with GG17, although ommatidia stayed round and did not appear depressed (not shown).

Finally, the same sunken ommatidial phenotype appeared

when the GAP domain of RacGAP(84C) was coexpressed with the predicted gene CG11316 (misexpressed in EP(3)3700) that encodes a putative protein containing SH3 domains (Table 1A, not shown). The common phenotype obtained by coexpression of all three genes with RacGAP(84C) suggest that MESR2/AKAP200, DC3G, the CG11316-encoded protein, and RacGAP(84C) act in a common signalling pathway.

RacGAP(84C) interaction with RhoGEFs

EP insertion in two genes encoding specific activators of Rho, the two RhoGEFs Pbl (EP(3)3415) and GEF(64C) (EP(3)3322) induced rough eyes when expressed alone and pupal lethality in combination with either GG17, Rac1N17 or Cdc42N17 (Table 1A). Furthermore, reducing the dosage of *RacGAP(84C)* in GMRGal4/+; EP3415/rn^{20} flies expressing Pbl also resulted in pupal lethality. Interestingly then, a gain-of-function of *RacGAP(84C)* or its diminution resulted in a common lethal phenotype when in combination with an excess of Pbl. Inversely, the pbl^{5} mutant (Prokopenko et al., 1999) did not modify the phenotype associated with GG17 when in trans. This indicates that RacGAP(84C) would be critical in Pbl-dependent processes whereas in RacGAP(84C)-dependent processes, Pbl lies upstream of RacGAP(84C) or its dosage is not limiting.

Eye-directed expression of *GEF(64C)* in GMRGal4/+; EP(3)3035/+ or in GMRGal4/UAS-GEF(64C) flies (Bashaw et al., 2001) did not induce any rough eye phenotype but enhanced the phenotypic defects of GG17. This suggests that GEF(64C) and RacGAP(84C) might act synergistically in a common pathway. The introduction in trans of either a deficiency of the 64C region (Df104(64C)), or of two loss-of-function mutants ($GEF(64C)^{EMS1}$ and $GEF(64C)^{EMS9}$) (Bashaw et al., 2001) did not affect the GG17 eye phenotype. Thus, it appears that GEF(64C) may lie upstream of RacGAP(84C) or its dosage is not limiting in this pathway.

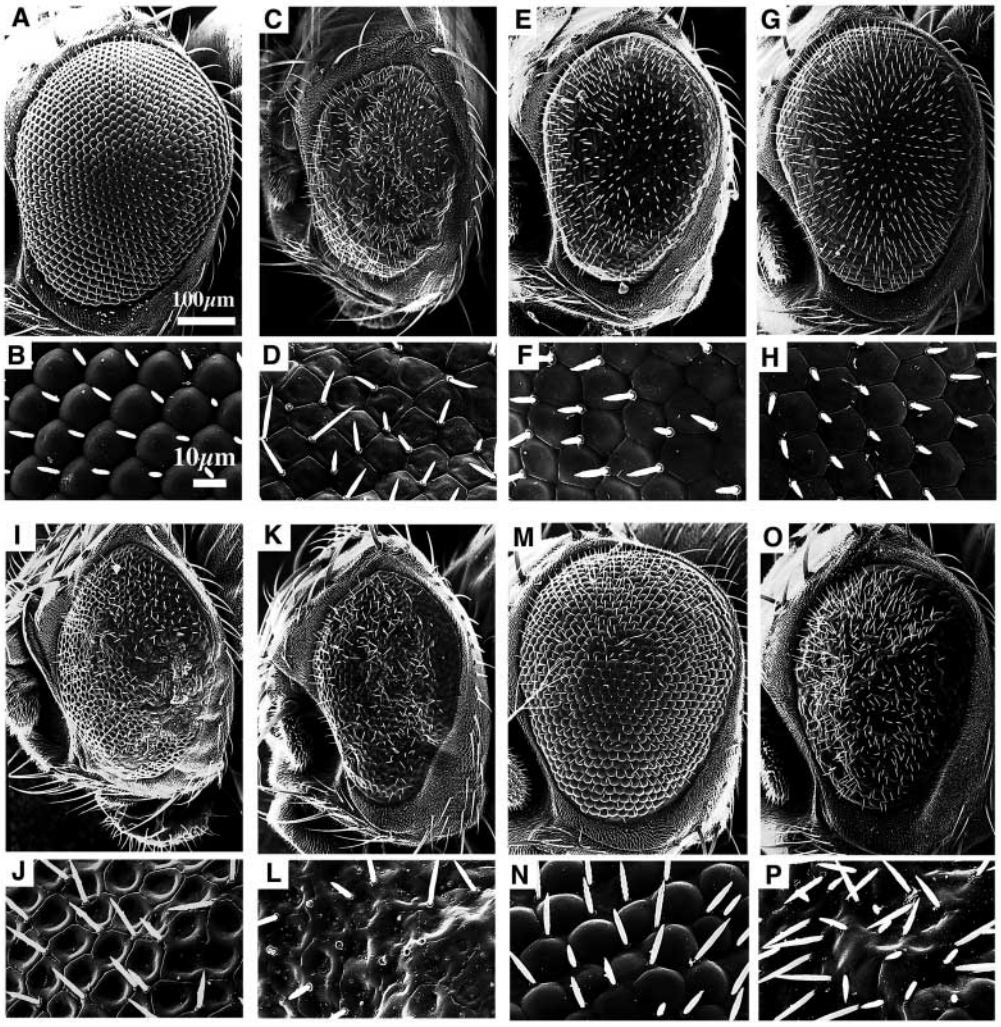

Fig. 2. Modifiers of the reference eye phenotype induced by the GAP domain of RacGAP(84C). Heads from female flies raised at 25°C (A-L) or 20°C (M-P) were viewed by scanning electron microscopy. B,D,F,H,J,L,N,P are higher magnifications of parts of A,C,E,G,I,K,M,O, respectively. (A,B) GMRGal4/UAS-lacZ control flies. (A) The eye surface is convex and of characteristic size and shape. (B) Each ommatidium is uniform and round and there are regular polarised inter-ommatidial bristles. (C,D) GMRGal4,UAS-GAP17/+ flies. (C) Reference rough eye phenotype. (D) Ommatidia are flattened, inter-ommatidial bristles are disoriented, of variable length, and occasionally absent. (E-H) Suppressor lines showing a more wild-type eye in size, shape, roundness and surface texture. Ommatidial architecture is basically restored, and inter-ommatidial bristles are nearly normal in number, length and polarity. (E,F) GMRGal4,UAS-GAP17/EP(2)386 flies misexpressing MESR4. (G,H) GMRGal4,UAS-GAP17/+; EP(3)3319/+ flies misexpressing Cdi/TESK1. (I-P) Enhancer lines. (I,J) GMRGal4,UAS-GAP17/EP(2)2072 flies misexpressing AKAP200. (I) Strong defects are apparent posteriorly corresponding to a black patch when viewed by optical microscopy. (J) Ommatidia are collapsed with distinct borders, except in rare cases of fusion. (K,L) GMRGal4,UAS-GAP17/EP(2)587 flies misexpressing the kinase Grapes. (K) Reduced eye size. (L) Fusion of ommatidial units and loss of bristles. (M,N) GMRGal4/EP(2)2299 flies misexpressing Beach at 20°C exhibit slightly rough eye surface and occasional fusion of ommatidia. (O,P) GMRGal4/EP(2)2299; rn^{20}/+ flies have strongly affected eyes with a rough surface, reduced size (O) and fused ommatidial units (P) (whereas rn^{20}/+ flies appear normal; not shown).

Four serine/threonine kinases, including the LIM kinase Cdi, identify potential effectors of RacGAP(84C)-dependent signalling pathways

GTPase activation either directly controls morphological cell changes or triggers a cascade of downstream serine/threonine kinases that ultimately activates a set of transcription factors. Four serine/threonine kinases were isolated in our screen that may correspond to novel effectors in GTPase-dependent signalling cascades.

The kinase Grapes (Grp) (Table 1A, EP(2)587) is involved in DNA repair following exposure to radiation (Fogarty et al., 1997). *grp* expression strongly enhanced the eye defects when coexpressed with the GAP domain of RacGAP(84C) leading to extensively fused and depressed ommatidia and a significant loss of inter-ommatidial hairs (Fig. 2K,L). Grp induced similar defects when coexpressed with Rac1N17 or Cdc42N17 (noted as 'rough' in Table 1A) and generated other kinds of enhancement when coexpressed with RasN17, leading to a glossy eye surface (not shown). This suggests that Grp mediates multiple signalling pathways whose activation synergises with the inhibition of either Rac, Cdc42 or Ras signalling.

EP(2)2445 (Table 1A) is inserted 5′ to the predicted gene CG7097 that encodes a putative receptor signalling serine/threonine kinase of unknown specificity. CG7097-encoded protein is a good candidate for being a RhoGTPase target as it contains a Citron-homology domain similar to that found in the protein kinase Citron, an effector of Rho-dependent signalling. Its expression did not significantly modify the eye phenotype associated with either Rac1N17, Cdc42N17 or RasN17 expression suggesting that interaction with RacGAP(84C) is not due to dominant inhibition of these GTPases.

A third kinase selected in the screen is homologous to SNF4γ (Yoshida et al., 1999), an AMP-activated serine/threonine kinase that induces derepression of gene expression in response to glucose limitation in yeast (EP(3)648, Table 1A). Its expression enhanced the eye defects induced by Rac1N17 expression as observed in escaper flies, but not that of Cdc42N17 or RasN17, indicating that interaction with RacGAP(84C) gain of function might specifically result from Rac inhibition. By analogy, these results suggest the interesting possibility that Rac signalling may function in a stress response linked to the deregulation of glucose metabolism in the fly.

The last serine/threonine kinase isolated in this screen is encoded by the gene *center divider* (*cdi)* and was classified as a protein putatively involved in spermatogenesis (EP(3)3319, Table 1D and see below). Cdi is the only case in which expression of a serine/threonine kinase suppressed the eye defects induced by expressing the GAP domain of RacGAP(84C) (Fig. 2G,H). Interestingly, EP(3)3319 also behaved as a strong suppressor of the eye phenotype induced by Rac1N17 (Fig. 3C,D), but had no effect on the eye phenotype induced by expressing either Cdc42N17 (Table 1, not shown) or RasN17 (Fig. 3I,J). To confirm that phenotypic suppression by EP(3)3319 is attributable to the directed expression of *cdi*, we generated P-excised lines that did not contain any P[UAS] sequence as detected by Southern analysis. These P-excised lines did not show any suppression effect toward either GG17 or Rac1N17 (not shown). Our results indicate that Cdi would be a specific downstream effector of Rac1, the expression of which is able to rescue the dominant inhibition of Rac activity.

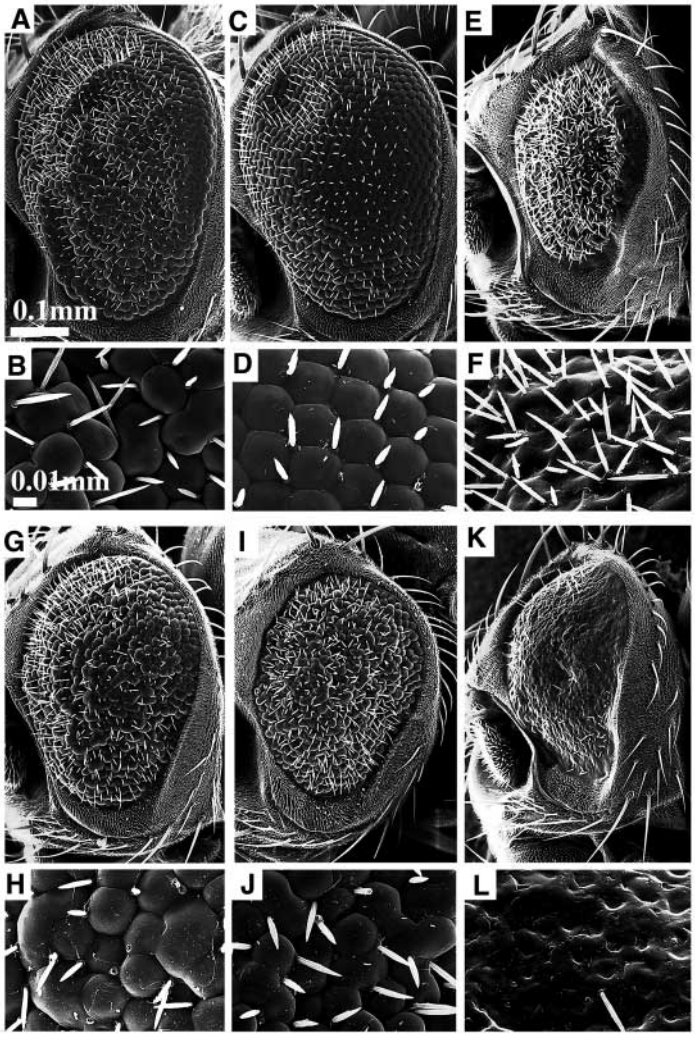

Fig. 3. Specificity of Cdi and Beach towards Rac and Ras signalling. Eyes of flies raised at 20°C viewed by scanning electron microscopy. Coexpression of EP lines with Rac1N17 (A-F) or RasN17 (G-L). (A,B) Control flies expressing only Rac1N17 (GMRGal4/+; UAS-Rac1N17/+) show moderately rough eyes, occasional ommatidial fusion and bristle polarity defects. (C,D) GMRGal4/+; UAS-Rac1N17/EP(3)3319 flies. The LIM kinase Cdi is a strong suppressor of the dominant inhibition of Rac1, restoring normal eye size and ommatidial patterning. (E,F) GMRGal4/EP(2)2299; UAS-Rac1N17/+ flies. Beach is a strong enhancer of the dominant inhibition of Rac. Eye size is further reduced and the ommatidial surface is totally fused with a high density of randomly-oriented bristles. (G,H) UAS-RasN17/Y; GMRGal4/+ control flies expressing RasN17 exhibit rough eyes with frequent fusion between ommatidia. (I,J) UAS-RasN17/Y; GMRGal4/+; EP3319/+ flies expressing Cdi do not exhibit modification of the eye defects induced by RasN17. (K,L) UAS-RasN17/Y; GMRGal4/EP(2)2299 flies expressing Beach exhibit strong enhancement of RasN17, further reducing eye size and leading to completely fused ommatidia and almost complete loss of bristles.

Partners of RacGAP(84C) regulating cytoskeletal and membrane dynamics

RhoGTPases define a major communications network in the regulation of the actin cytoskeleton directing specific cell morphological changes during development. We isolated several EP insertions in genes encoding cytoskeleton-associated proteins but only one, EP(2)2491 in *MyoD31DF*, did not itself induce an eye phenotype with GMRGal4 (Table 1B). *MyoD31DF* encodes a myosin ATPase, the expression of which enhanced the eye phenotype of GG17 and to a lesser extent that of Rac1N17 (not shown) but not that of Cdc42N17 or RasN17. This suggests that Rac, but not Cdc42 or Ras, is required to modulate its function.

Beach, identified by this screen as a functional partner of RacGAP(84C) is of particular interest when considering its role in membrane trafficking-dependent processes, i.e. endocytosis (Cornillon et al., 2002) and axonal growth (Abdelilah-Seyfried et al., 2000; Kraut et al., 2001), two processes requiring coordinated actin polymerisation and membrane dynamics. While expression of *Beach* alone induced a slightly rough eye phenotype when flies were raised at 20°C (Fig. 2M,N), there was a dramatic enhancement when Beach was coexpressed with RacGAP(84C) in GG17 flies (not shown) or when Beach was expressed in a rn^{20}/+ background inducing a similar strong eye phenotype (Fig. 2O,P). This indicates that *RacGAP(84C)* dosage is critical in Beach-dependent processes. Misexpression of Beach also enhanced the defects induced by expression of Rac1N17, Cdc42N17 and RasN17, inducing both common and specific phenotypes with each corresponding transgene: as with the GAP domain of RacGAP(84C), coexpression of Beach with Rac1N17 or Cdc42N17 leads to reduced eyes exhibiting abnormally abundant inter-ommatidial hairs and totally fused ommatidia (Fig. 3E,F) whereas its coexpression with RasN17 induced similarly reduced eyes and fused ommatidia but a total loss of inter-ommatidial hairs (Fig. 3K,L). The major structural component of inter-ommatidial hairs is polymerised actin. Presumably, these results reflect coordinate activity of Rac, Cdc42, Ras and Beach in coupling actin polymerisation and membrane growth.

GTPase nuclear targets encoding DNA remodelling factors

The GTPase-dependent activation of kinase cascades ultimately leads to the phosphorylation and the consequent activation of transcription factors that themselves activate specific genes by direct binding to DNA. Several transcription factors were isolated in the screen but none was conserved as their misexpression alone in the eye systematically induced eye defects. Changes in transcription levels also require proteins involved in DNA remodelling that ensure accessibility of transcription factors to DNA binding sites. The finding of EP-associated genes encoding helicases like *kismet* (*kis*) (EP(2)474 and EP(2)563) or *domino* (*dom*) (EP(2)2371) (Table 1C) supports the idea that GTPase-dependent signals induce transcriptional enhancement of target genes through direct signalling to DNA remodelling factors. Finally, another potential partner of RacGAP(84C) is the putative single-strand DNA-binding protein encoded by Dodeca-satellite-binding protein 1 (Dp1) implicated in DNA replication (Table 1C).

Three partners of RacGAP(84C) are expressed in *Drosophila* testis

We have isolated three genes having either proven or putative function in sperm differentiation, where RacGAP(84C) is known to play an essential role (Table 1D). These are *purity of essence* (*poe*) for which loss-of-function mutations are male sterile (Castrillon et al., 1993; Fabrizio et al., 1998), *TMS1d* and *cdi* for which the respective human orthologues, *TMS1* (Target of methylation-induced silencing) and *TESK1* are expressed in mammalian testes (Grossman et al., 2000; Toshima et al., 1995). Further we show by RT-PCR on isolated testes that *cdi* and *TMS1d* are also expressed in *Drosophila* testis as is *RacGAP(84C)* (Fig. 4A, lanes 1-4). Thus, the corresponding proteins represent probable partners of RacGAP(84C) acting in sperm differentiation.

It has been reported that three alternatively spliced transcripts of lengths 6.8, 6.0 and 5.7 kb are encoded by the *cdi* locus (Matthews and Crews, 1999). We show by northern analysis that there is considerable transcript-specific expression of *cdi* in the germline; i.e. the 6.0 kb form is specific to the testis, and the 6.8 kb form, to the ovary (Fig. 4B). This strongly suggests a specific function of each Cdi isoform in spermatogenesis and oogenesis, respectively. Taking advantage of the cdi^{P242} enhancer trap line containing the *lacZ* reporter gene under the control of *cdi* regulatory sequences (Matthews and Crews, 1999; Nambu et al., 1991), we assayed for β-gal activity in both imaginal eye discs of cdi^{P242} larvae and in testes of cdi^{P242} adult males. Staining was detectable in the larval central nervous system and optic lobes but not in any imaginal structures (not shown). Therefore, the observed interaction in the eye is either the indirect consequence of abnormal neuronal connections or the result of ectopic effects of Cdi on signalling cascades. In contrast, we detected specific expression of *cdi* in both extremities of the testis: in the apical part where cells are actively dividing and in the distal part during terminal differentiation of the spermatids (Fig. 4C).

In the case of *TMS1d*, the enhancer line EP(3)807 is inserted 24 bp upstream of the putative transcription start, and thus, it very probably produces Gal4-dependent *TMS1d* transcripts. However, since *Df(3L)st-e4* including *TMS1d* also behaved as a GG17 enhancer, we cannot exclude that *TMS1d* loss-of-function would also result in the aggravation of the GG17 eye phenotype. In any event, both results indicate the existence of a genetic interaction between the two genes. The EP(3)807 insertion in *TMS1d* had no effect on the eye defects induced by the dominant negative forms of the three GTPases Rac1, Cdc42 or Ras, suggesting that interaction with RacGAP(84C) depends upon other signalling cascades.

Eye-directed expression of Poe, like that of Cdi, resulted in dominant suppression of the eye phenotype induced by expressing Rac1N17 or the GAP domain of RacGAP(84C). Additionally, expression of Poe but not of Cdi, suppressed the rough eye phenotype observed in flies expressing Cdc42N17 (Table 1). In contrast, the eye phenotype induced by RasN17 was not affected by coexpression of either Poe or Cdi. These phenotypic interactions suggest that Poe and Cdi act as downstream antagonists of RacGAP(84C) or that they negatively modulate its function. Given that Cdi is a serine/threonine kinase, it is probably a specific downstream effector of Rac1. We set about to test this hypothesis with respect to spermatogenesis.

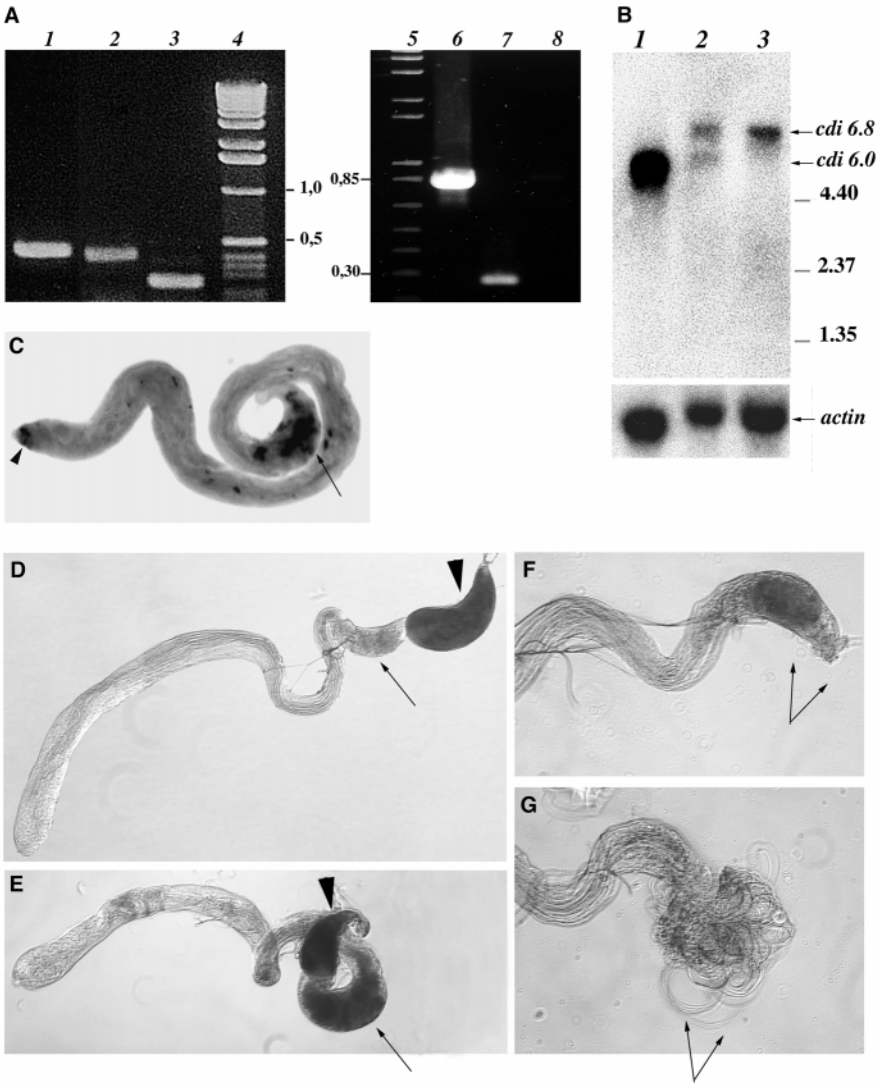

Fig. 4. Partners of RacGAP(84C) encode testis-specific genes. (A) Ethidium bromide-stained agarose gel showing RT-PCR amplified fragments after electrophoretic separation. Lane 1: *cdi* (498 bp); 2: *TMS1d* (426 bp); 3: *RacGAP(84C)* (274 bp); 4: Fragment size marker (Gibco-BRL); 5: Fragment size marker (Invitrogen); 6: *Rac1* (856 bp); 7: *RacGAP(84C)* (274bp); 8: *Rac1* amplification without reverse transcriptase (negative control). (B) Northern analysis. Total RNA from: lane 1, 300 isolated testes; lane 2, 200 adult flies and lane 3, 200 isolated ovaries. Detection of *cdi* transcripts with a ^{32}P-random-labeled probe shows that the 6.0 kb transcript is specific for testes and the 6.8 kb transcript is specific for ovaries. The third form of 5.7 kb described by Matthews and Crews (Matthews and Crews, 1999) was detected only in total adult RNA after longer exposure of northern blot (not shown). (C) Detection of β-gal activity in testis from the enhancer trap line cdi^{P242}. Staining is present in the apical part of the germarium with dividing spermatogonia (arrowhead), and in the distal part where the final stages of differentiation occur (arrow). (D-E) Dissection of testes and seminal vesicles of 5-day-old males. (D) Control w^{1118} males have transparent testes (arrow) and seminal vesicles full of sperm (arrowhead). (E) *w*; $Rac1^{j11}$,*FRT2A*,cdi^{R47}/$Rac1^{j11}$,*FRT2A* males exhibit an accumulation of dense material in the distal region (arrow) and seminal vesicles full of sperm (arrowhead). (F-G) Isolated testis of $Rac1^{j11}$,*FRT2A*,cdi^{R47}/$Rac1^{j11}$,*FRT2A* separated from its seminal vesicle. (F) Before crushing, no sperm are detectable (arrows). (G) After crushing, in addition to uncharacterised material, we observe motile sperm issuing from the distal extremity of the testis (arrows).

Rac1-Cdi interaction is required during *Drosophila* spermatogenesis

Homozygous amorphic $Rac1^{j11}$ mutants are viable and show no abnormal external phenotype (Hakeda-Suzuki et al., 2002). However, we observed that 46% of $Rac1^{j11}$ homozygous males are sterile in individual crosses with wild-type females although the testes appear normal and the seminal vesicle is full of motile sperm when viewed by optical microscopy (not shown). We further show by RT-PCR on isolated testes that *Rac1* is indeed expressed in *Drosophila* testes (Fig. 4A, lanes 5-8) consistent with Rac1 acting during *Drosophila* spermatogenesis.

To assess the contribution of a putative Rac1-Cdi pathway in sperm differentiation, we constructed independent *w*; $Rac1^{j11}$,cdi^{R47}/*TM3,Sb* recombinant lines where cdi^{R47} is a lethal deletion into the *cdi* transcription unit (Matthews and Crews, 1999). Half the genic dosage of *cdi* further reduced fertility of $Rac1^{j11}$ homozygous males: 74% of *w*; $Rac1^{j11}$,cdi^{R47}/$Rac1^{j11}$ are fully sterile in 100 individual crosses. By comparison, both $Rac2^{\Delta}$ homozygous and $Rac2^{\Delta}$,cdi^{R47}/$Rac2^{\Delta}$ males were fully viable and fertile indicating that spermatogenesis specifically requires Rac1 function.

Dissection of *w*; $Rac1^{j11}$,cdi^{R47}/$Rac1^{j11}$ testes revealed an abnormal accumulation of dense material in the distal part of the testis corresponding to the last stages of sperm differentiation where we had previously detected *cdi* expression (Fig. 4C,E arrows). When crushing the isolated testis, we observed motile sperm issuing from the distal extremity (Fig. 4F,G). This indicates that sperm individualisation has been completed without accompanying passage into the seminal vesicle, thereby implying a deregulation of the signals normally regulating their coordination. Our results thus strongly suggest the requirement of a Rac1-Cdi pathway, putatively regulated by RacGAP(84C), during the terminal stages of *Drosophila* spermatogenesis.

Discussion

We devised a gain-of-function screen to identify new genetic partners of RacGAP(84C). The quantity, diversity and apparent hierarchy of action of the candidate proteins that emerged strongly suggest that RacGAP(84C) may contribute to the regulation of multiple and distinct processes including GTPase crosstalk, cytoskeletal dynamics and gene transcription in a

capacity exceedingly more complex than solely as a negative inhibitor of Rac and Cdc42. Moreover, we have shown that the relative ease of a misexpression screen in the fly eye can be used as a valid assay for targeting genes whose main function may be in other tissues, in that we identified several genes potentially involved in sperm differentiation, where RacGAP(84C) function has been previously demonstrated. The finding of candidate proteins with known or putative function in GTPase-dependent pathways and spermatogenesis validates the specificity of the screen.

RacGAP(84C) mediates crosstalk between GTPase regulatory proteins

In response to an external stimulus, several signalling pathways are likely to be activated within the cell that would require active crosstalk in order to ensure coordinated nuclear responses and cell shape changes. RacGAP(84C) interferes with Rac1 and Cdc42 activity during fly development and interacts with the Ras-dependent pathway during retinal and sperm differentiation (Bergeret et al., 2001; Raymond et al., 2001). Our screen identified candidate RacGAP(84C) partners that may ensure this crosstalk including three genes that encode specific regulators of Ras activity.

MESR4 and MESR2 were first isolated as suppressors of a gain-of function of the Ras-MAP kinase pathway (Huang and Rubin, 2000). MESR2/AKAP200 encodes the *Drosophila* A kinase anchor protein, AKAP200, with a molecular mass of 200 kDa. AKAP200 binds the cyclic AMP-dependent protein kinase A (PKA) that has a non-uniform distribution in cells (Li et al., 1999). We previously demonstrated that overexpression of RacGAP(84C) perturbs the sub-cortical localisation of polymerised actin in embryonic cells (Guichard et al., 1997; Raymond et al., 2001), which would presumably prevent the normal localisation of AKAP200 with respect to the cytoskeleton. Enhancement by MESR2/AKAP200 of the eye defects induced by RacGAP(84C) gain-of-function would then be explained in that excess AKAP cannot be correctly targeted to its intracellular position.

MESR4 acts as a suppressor of both a gain-of-function of RacGAP(84C) and Ras (Huang and Rubin, 2000). This can perhaps be explained if a gain-of-function of RacGAP(84C) indirectly induces the activation of the Ras-MAPK pathway which is then inhibited by MESR4 misexpression. The proposed Ras activation by RacGAP(84C) might depend upon its synergistic interaction with the RasGEF DC3G and the putative scaffolding protein encoded by CG11316 that we uncovered in this screen. Indeed, coexpression with the GAP domain of RacGAP(84C) of either DC3G or CG11316 induced the same enhancement of ommatidial defects, i.e. a depressed central region in the ommatidium, although distinct borders remained. In that constitutive localisation of DC3G to the membrane is sufficient to trigger Ras-MAP-kinase activation (Ishimaru et al., 1999), the combined data suggest that CG11316 ensures the physical link between RacGAP(84C) and DC3G localising them to the membrane, thus initiating Ras activation. As the same phenotype is observed when the GAP domain of RacGAP(84C) is expressed in combination with MESR2, one possible target of this pathway may be AKAP200.

Coordinated activation of the RhoGTPases, Rho, Rac and Cdc42 ensures cytoskeletal rearrangement within the cell. We found that expression of the two RhoGEFs, Pbl and GEF(64C) strongly enhanced a gain-of-function of RacGAP(84C). We previously showed that RacGAP(84C) does not interact directly with Rho and that reducing the genic dosage of *RacGAP(84C)* had no effect on the eye defects induced by a gain-of-function of Rho, thus indicating that RacGAP(84C) is unlikely to be a direct downstream effector of Rho (Raymond et al., 2001). Rather, RacGAP(84C) may mediate Rac/Rho crosstalk through direct or indirect interaction with these two RhoGEFs. Both Pbl and GEF(64C) ensure essential functions during *Drosophila* development, in cytokinesis and axonal growth, respectively, suggesting that crosstalk between Rho and RacGAP(84C) would occur during these morphogenetic processes (Bashaw et al., 2001; Prokopenko et al., 1999).

RacGAP(84C) and its partners regulate cytoskeletal dynamics and membrane trafficking in morphogenetic processes requiring rapid and extensive cell elongation

The absence of sperm elongation observed in RacGAP(84C)-deficient males is a consequence of membrane deposition failure attributed to defects in actin polymerisation during the elongation process (Bergeret et al., 2001). Similarly, ommatidial elongation depends on RacGAP(84C) and also requires directed localisation of polymerised actin to guide the precise positioning of new membrane. Mechanistically, attaining wild-type retinal depth presents a problem similar to that encountered during sperm elongation: the polarised deposition in a relatively short time of sufficient amounts of membrane into different cell types so that they may coordinately span distances from 10× to more than 100× their original respective lengths.

Our screen identifies potential partners of RacGAP(84C) implicated in morphogenetic processes requiring coordinated cytoskeletal reorganisation and membrane trafficking. Specifically, the myosin head ATPase, Myo31DF, is a candidate effector of Rac1 that might link actin polymerisation dynamics to localised membrane contraction. In addition, *Beach* encodes a protein directly implicated in membrane trafficking (Cornillon et al., 2002) and cell polarisation during motorneuron pathfinding (Abdelilah-Seyfried et al., 2000; Kraut et al., 2001). Finally, the RhoGEF GEF(64C) is required for axonal growth during the formation of the embryonic central nervous system (Bashaw et al., 2001). Similar constraints may be operating during axonal guidance as during sperm elongation, requiring the polarized polymerisation of actin.

Integration of GTPase-dependent signals by DNA remodelling factors

Activation of GTPase-dependent nuclear pathways ultimately results in changes in the transcriptional level of target genes. One major class of determining factors in this process are DNA remodelling factors such as helicases that remove certain DNA conformational constraints thereby favouring accessibility of the designated region to transcription factors. We found two helicases, Kis and Dom, which acted as enhancers in this misexpression screen for modifiers of RacGAP(84C)-dependent signalling. It had been previously shown that a loss-

of-function mutant of *kis* behaved as a suppressor of the constitutive activation of the Ras pathway (Therrien et al., 2000). Taken together, these observations suggest a general mechanism of transcriptional control whereby transmission of GTPase-related signals would be mediated by specific target factors such as Kis and Dom that constitute an integral part of the nuclear machinery preparing DNA for transcription. Our screen also identified a single-strand binding protein that might facilitate DNA replication. Thus, GTPases may signal to DNA-binding factors thereby functioning as part of a cell division checkpoint signalling apparatus.

An eye screen allowed the isolation of testis-specific genes

Three genes with proven, or putative, function in spermatogenesis were identified as genetic partners of RacGAP(84C): *poe*, *TMS1d* and *cdi* (discussed below). The gene *poe* encodes a calmodulin-binding protein, a ubiquitous intracellular Ca^{2+} receptor. Its misexpression in the eye rescued the defects induced by expressing the GAP domain of RacGAP(84C), thus raising the possibility that Poe provides a link from Ca^{2+} signalling to GTPase-dependent pathways. The *poe* gene product is specifically necessary in terminal spermatid maturation for the individualisation of the spermatids (Fabrizio et al., 1998). Spermatids develop as a syncitium connected by localised cytoplasmic bridges; individualisation is then accomplished by the head-to-tail passage of an actin-based cytoskeletal-membrane complex, investing each spermatid in its own plasma membrane and simultaneously extruding most of the syncitial cytoplasm from between spermatids. When the individualisation complex (IC) reaches the tail end, it detaches and the mature sperm are transferred to the seminal vesicle. The identification of Poe as a partner of RacGAP(84) suggests that RacGAP(84C) may also function late in spermatogenesis in addition to its requirement in spermatid membrane elongation.

TMS1 has been identified in a human cell model system in which aberrant CpG island methylation was induced by ectopic expression of DNA methyl transferase. *TMS1* was also aberrantly methylated and silenced in human breast cancer cells. The human gene encodes a 22 kDa predicted protein containing a COOH-caspase recruitment domain, a protein motif found in apoptotic signalling molecules. Ectopic expression of TMS1 induced apoptosis in human cultured cells and inhibited the survival of human breast cancer cells, suggesting that methylation-mediated silencing of TMS1 confers a survival advantage by allowing cells to escape from apoptosis (Conway et al., 2000). *TMS1* is highly expressed in mouse brain, thymus, kidney, liver and testes (Grossman et al., 2000). The function of TMS1d is not known in *Drosophila*. We showed by RT-PCR that *TMS1d* is expressed in fly testes where it may interact with RacGAP(84C). Its putative interaction with an excess of GAP activity provides a molecular link between GTPase-dependent signals and caspase-mediated cell death.

A Rac1-Cdi-Cofilin pathway modulated by RacGAP(84C) may act to control actin dynamics during spermatogenesis

Directed expression of *cdi* resulted in a strong suppression of the eye defects induced by a gain-of-function of RacGAP(84C). Interestingly, high levels of Cdi also suppressed the eye phenotype induced by Rac1N17, but had no effect on that induced by Cdc42N17 or RasN17. This is consistent with Cdi being a specific downstream effector of Rac1, the expression of which then rescues the dominant inhibition of Rac activity. *cdi* encodes a serine/threonine kinase presenting homology with the LIM kinases, a family of kinases particularly involved in the control of cytoskeleton dynamics triggered by RhoGTPase activation (Arber et al., 1998; Maekawa et al., 1999; Yang et al., 1998). In particular, Cdi shows strong sequence homology with the human gene TESK1 expressed in testis (Toshima et al., 1995). Activation of Cdi stimulates phosphorylation of Cofilin, thereby inhibiting its filamentous actin-cutting function and stabilising actin structures. In cell culture, the murine TESK1 is insensitive to either Rho kinase- or p21 kinase-dependent activation, but rather is activated by direct binding to integrin (Toshima et al., 2001a; Toshima et al., 2001b). We show here that Cdi is most probably a downstream target of Rac1. Recently, TESK1 has been isolated in a two-hybrid screen as a direct partner of the SH3-containing protein human Sprouty 4 (hspry4) (Leeksma et al., 2002). Hspry4 is homologous to the *Drosphila* Sprouty protein that behaves as a universal negative regulator of the Ras/MAP kinase pathway. This raises the interesting possibility that crosstalk between Ras- and Rac-dependent signalling pathways that we previously showed to operate during spermatogenesis (Bergeret et al., 2001) may be mediated through the complex Sprouty-Cdi/TESK1.

We show that *Rac1* is expressed in *Drosophila* testes and that in *Rac1* homozygous mutant males fertility is low. A *cdi* loss-of-function mutant further increases sterility of $Rac1^{j11}$ homozygous males and prevents the normal entry of individualised sperm into the seminal vesicle. This phenotype demonstrates that Rac1-Cdi interaction is specifically required during late spermatogenesis and suggests evolutionary conservation of testis-specific function of a Rac1-Cdi-Cofilin pathway from the fly to man. This putative pathway, modulated by RacGAP(84C), in which Rac1 signalling to the downstream effector Cdi inactivates Cofilin, would thereby regulate the spatial and temporal coordination between sperm individualisation, coiling and entry into the seminal vesicle.

We thank Jean-Claude Platel who contributed to this work as a rotation student, Isabelle Pignot-Paintrand for help with preparation of samples for transmission electron microscopy, Michelle Grasset at the UPMC Service de Microscopie Electronique de l'IFR de Biologie Intégrative, 7 Quai Saint Bernard 75252 Paris cedex 05 for help with scanning electron microscopy; Michel Satre for his support as laboratory supervisor, other members of the lab for stimulating discussion, Marie-Claire Joseph for making *Drosophila* food and maintaining stocks. We thank Dr Luo, Dr Ishimaru, Dr Settleman, Dr Bashaw, Dr Matthew, the EP exelexis fly station, the Szeged and the Bloomington Stock Centers for sending flies. This work was supported by grant 9392 from the Association pour la Recherche contre le Cancer and by a grant and an Allocation Doctorale de Recherche to A.A.R. from the Région Rhône Alpes (Emergence 2002).

References

Abdelilah-Seyfried, S., Chan, Y. M., Zeng, C., Justice, N. J., Younger-Shepherd, S., Sharp, L. E., Barbel, S., Meadows, S. A., Jan, L. Y. and

Jan, Y. N. (2000). A gain-of-function screen for genes that affect the development of the Drosophila adult external sensory organ. *Genetics* **155**, 733-752.

Agnel, M., Kerridge, S., Vola, C. and Griffin-Shea, R. (1989). Two transcripts from the rotund region of Drosophila show similar positional specificities in imaginal disc tissues. *Genes Dev.* **3**, 85-95.

Agnel, M., Roder, L., Vola, C. and Griffin-Shea, R. (1992). A Drosophila rotund transcript expressed during spermatogenesis and imaginal disc morphogenesis encodes a protein which is similar to human Rac GTPase-activating (racGAP) proteins. *Mol. Cell. Biol.* **12**, 5111-5122.

Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O. and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **393**, 805-809.

Bashaw, G. J., Hu, H., Nobes, C. D. and Goodman, C. S. (2001). A novel Dbl family RhoGEF promotes Rho-dependent axon attraction to the central nervous system midline in Drosophila and overcomes Robo repulsion. *J. Cell. Biol.* **155**, 1117-1122.

Bergeret, E., Pignot-Paintrand, I., Guichard, A., Raymond, K., Fauvarque, M.-O., Cazemajor, M. and Griffin-Shea, R. (2001). RotundRacGAP functions with Ras during spermatogenesis and retinal differentiation in *Drosophila melanogaster*. *Mol. Cell. Biol.* **21**, 6280-6291.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Castrillon, D. H., Gonczy, P., Alexander, S., Rawson, R., Eberhart, C. G., Viswanathan, S., DiNardo, S. and Wasserman, S. A. (1993). Toward a molecular genetic analysis of spermatogenesis in Drosophila melanogaster, characterization of male-sterile mutants generated by single P element mutagenesis. *Genetics* **135**, 489-505.

Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S. I. and Abrams, J. M. (2002). The damage-responsive Drosophila gene sickle encodes a novel IAP binding protein similar to but distinct from reaper, grim, and hid. *Curr. Biol.* **12**, 137-140.

Conway, K. E., McConnell, B. B., Bowring, C. E., Donald, C. D., Warren, S. T. and Vertino, P. M. (2000). TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. *Cancer Res.* **60**, 6236-6242.

Cornillon, S., Dubois, A., Bruckert, F., Lefkir, Y., Marchetti, A., Benghezal, M., De Lozanne, A., Letourneur, F. and Cosson, P. (2002). Two members of the beige/CHS (BEACH) family are involved at different stages in the organization of the endocytic pathway in Dictyostelium. *J. Cell Sci.* **115**, 737-744.

Ellis, M. C., O'Neill, E. M. and Rubin, G. M. (1993). Expression of Drosophila glass protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. *Development* **119**, 855-865.

Fabrizio, J. J., Hime, G., Lemmon, S. K. and Bazinet, C. (1998). Genetic dissection of sperm individualization in Drosophila melanogaster. *Development* **125**, 1833-1843.

Fanto, M., Weber, U., Strutt, D. I. and Mlodzik, M. (2000). Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the Drosophila eye. *Curr. Biol.* **10**, 979-988.

Fogarty, P., Campbell, S. D., Abu-Shumays, R., Phalle, B. S., Yu, K. R., Uy, G. L., Goldberg, M. L. and Sullivan, W. (1997). The Drosophila grapes gene is related to checkpoint gene chk1/rad27 and is required for late syncytial division fidelity. *Curr. Biol.* **7**, 418-426.

Genova, J. L., Jong, S., Camp, J. T. and Fehon, R. G. (2000). Functional analysis of Cdc42 in actin filament assembly, epithelial morphogenesis, and cell signaling during Drosophila development. *Dev. Biol.* **221**, 181-194.

Grether, M. E., Abrams, J. M., Agapite, J., White, K. and Steller, H. (1995). The head involution defective gene of Drosophila melanogaster functions in programmed cell death. *Genes Dev.* **9**, 1694-1708.

Grossman, T. R., Luque, J. M. and Nelson, N. (2000). Identification of a ubiquitous family of membrane proteins and their expression in mouse brain. *J. Exp. Biol.* **203**, 447-457.

Guichard, A., Bergeret, E. and Griffin-Shea, R. (1997). Overexpression of RnRacGAP in Drosophila melanogaster deregulates cytoskeletal organisation in cellularising embryos and induces discrete imaginal phenotypes. *Mech. Dev.* **61**, 49-62.

Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L. and Dickson, B. J. (2002). Rac function and regulation during Drosophila development. *Nature* **416**, 438-442.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121-2129.

Hirose, K., Kawashima, T., Iwamoto, I., Nosaka, T. and Kitamura, T. (2001). MgcRacGAP is involved in cytokinesis through associating with mitotic spindle and midbody. *J. Biol. Chem.* **276**, 5821-5828.

Huang, A. M. and Rubin, G. M. (2000). A misexpression screen identifies genes that can modulate RAS1 pathway signaling in Drosophila melanogaster. *Genetics* **156**, 1219-1230.

Huet, F., Ruiz, C. and Richards, G. (1993). Puffs and PCR, the in vivo dynamics of early gene expression during ecdysone responses in Drosophila. *Development* **118**, 613-627.

Ishimaru, S., Williams, R., Clark, E., Hanafusa, H. and Gaul, U. (1999). Activation of the Drosophila C3G leads to cell fate changes and overproliferation during development, mediated by the RAS-MAPK pathway and RAP1. *EMBO J.* **18**, 145-155.

Jullien-Flores, V., Dorseuil, O., Romero, F., Letourneur, F., Saragosti, S., Berger, R., Tavitian, A., Gacon, G. and Camonis, J. H. (1995). Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. *J. Biol. Chem.* **270**, 22473-22477.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1996). The GTPase-activating protein n-chimaerin cooperates with Rac1 and Cdc42Hs to induce the formation of lamellipodia and filopodia. *Mol. Cell. Biol.* **16**, 5069-5080.

Kraut, R., Menon, K. and Zinn, K. (2001). A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in Drosophila. *Curr. Biol.* **11**, 417-430.

Lamarche, N. and Hall, A. (1994). GAPs for rho-related GTPases. *Trends Genet.* **10**, 436-440.

Leeksma, O. C., Van Achterberg, T. A., Tsumura, Y., Toshima, J., Eldering, E., Kroes, W. G., Mellink, C., Spaargaren, M., Mizuno, K., Pannekoek, H. et al. (2002). Human sprouty 4, a new ras antagonist on 5q31, interacts with the dual specificity kinase TESK1. *Eur. J. Biochem.* **269**, 2546-2556.

Li, Z., Rossi, E. A., Hoheisel, J. D., Kalderon, D. and Rubin, C. S. (1999). Generation of a novel A kinase anchor protein and a myristoylated alanine-rich C kinase substrate-like analog from a single gene. *J. Biol. Chem.* **274**, 27191-27200.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases, Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8**, 1787-1802.

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. and Narumiya, S. (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* **285**, 895-898.

Matthews, B. B. and Crews, S. T. (1999). Drosophila center divider gene is expressed in CNS midline cells and encodes a developmentally regulated protein kinase orthologous to human TESK1. *DNA Cell Biol.* **18**, 435-448.

Nambu, J. R., Lewis, J. O., Wharton, K. A., Jr. and Crews, S. T. (1991). The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67**, 1157-1167.

Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., Sun, Y., Dietzl, G., Dickson, B. J. and Luo, L. (2002). Rac GTPases control axon growth, guidance and branching. *Nature* **416**, 442-447.

Nicolai, M., Lasbleiz, C. and Dura, J. M. (2003). Gain-of-function screen identifies a role of the Src64 oncogene in Drosophila mushroom body development. *J. Neurobiol.* **57**, 291-302.

Nobes, C. and Hall, A. (1994). Regulation and function of the Rho subfamily of small GTPases. *Curr. Opin. Genet. Dev.* **4**, 77-81.

Pena-Rangel, M. T., Rodriguez, I. and Riesgo-Escovar, J. R. (2002). A misexpression study examining dorsal thorax formation in Drosophila melanogaster. *Genetics* **160**, 1035-1050.

Prokopenko, S. N., Brumby, A., O'Keefe, L., Prior, L., He, Y., Saint, R. and Bellen, H. J. (1999). A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in Drosophila. *Genes Dev.* **13**, 2301-2314.

Raymond, K., Bergeret, E., Dagher, M. C., Breton, R., Griffin-Shea, R. and Fauvarque, M. O. (2001). The Rac GTPase-activating protein RotundRacGAP interferes with Drac1 and Dcdc42 signalling in Drosophila melanogaster. *J. Biol. Chem.* **276**, 35909-35916.

Ridley, A. J. (1996). Rho, theme and variations. *Curr. Biol.* **6**, 1256-1264.

Riedl, S. J., Renatus, M., Snipas, S. J. and Salvesen, G. S. (2001). Mechanism-based inactivation of caspases by the apoptotic suppressor p35. *Biochemistry* **40**, 13274-13280.

Robertson, H. M., Preston, C. R., Phillis, R. W., Jonhson-Schlitz, D. M., Benz, W. K. and Engels, W. R. (1988). A stable genomic source of *P* element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470.

Rorth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. *Proc. Natl. Acad. Sci. USA* **93**, 12418-12422.

Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G. M., Weigmann, K., Milan, M., Benes, V., Ansorge, W. et al. (1998). Systematic gain-of-function genetics in Drosophila. *Development* **125**, 1049-1057.

Schmidt, A. and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases, turning on the switch. *Genes Dev.* **16**, 1587-1609.

Settleman, J. (2001). Rac 'n Rho, the music that shapes a developing embryo. *Dev. Cell* **1**, 321-331.

Sotillos, S. and Campuzano, S. (2000). DRacGAP, a novel Drosophila gene, inhibits EGFR/Ras signalling in the developing imaginal wing disc. *Development* **127**, 5427-5438.

Srinivasula, S. M., Datta, P., Kobayashi, M., Wu, J. W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y. et al. (2002). Sickle, a novel Drosophila death gene in the reaper/hid/grim region, encodes an IAP-inhibitory protein. *Curr. Biol.* **12**, 125-130.

St Pierre, S. E., Galindo, M. I., Couso, J. P. and Thor, S. (2002). Control of Drosophila imaginal disc development by rotund and roughened eye, differentially expressed transcripts of the same gene encoding functionally distinct zinc finger proteins. *Development* **129**, 1273-1281.

Therrien, M., Morrison, D. K., Wong, A. M. and Rubin, G. M. (2000). A genetic screen for modifiers of a kinase suppressor of Ras-dependent rough eye phenotype in Drosophila. *Genetics* **156**, 1231-1242.

Toshima, J., Ohashi, K., Okano, I., Nunoue, K., Kishioka, M., Kuma, K., Miyata, T., Hirai, M., Baba, T. and Mizuno, K. (1995). Identification and characterization of a novel protein kinase, TESK1, specifically expressed in testicular germ cells. *J. Biol. Chem.* **270**, 31331-31337.

Toshima, J., Toshima, J. Y., Amano, T., Yang, N., Narumiya, S. and Mizuno, K. (2001a). Cofilin phosphorylation by protein kinase testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. *Mol. Biol. Cell.* **12**, 1131-1145.

Toshima, J., Toshima, J. Y., Takeuchi, K., Mori, R. and Mizuno, K. (2001b). Cofilin phosphorylation and actin reorganization activities of testicular protein kinase 2 and its predominant expression in testicular Sertoli cells. *J. Biol. Chem.* **276**, 31449-31458.

Toure, A., Dorseuil, O., Morin, L., Timmons, P., Jegou, B., Reibel, L. and Gacon, G. (1998). MgcRacGAP, a new human GTPase-activating protein for Rac and Cdc42 similar to Drosophila rotundRacGAP gene product, is expressed in male germ cells. *J. Biol. Chem.* **273**, 6019-6023.

Tseng, A. S. and Hariharan, I. K. (2002). An overexpression screen in Drosophila for genes that restrict growth or cell-cycle progression in the developing eye. *Genetics* **162**, 229-243.

Vojtek, A. B. and Cooper, J. A. (1995). Rho family members, activators of MAP kinase cascades. *Cell* **82**, 527-529.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-683.

Wing, J. P., Karres, J. S., Ogdahl, J. L., Zhou, L., Schwartz, L. M. and Nambu, J. R. (2002). Drosophila sickle is a novel grim-reaper cell death activator. *Curr. Biol.* **12**, 131-135.

Xu, G., Cirilli, M., Huang, Y., Rich, R. L., Myszka, D. G. and Wu, H. (2001). Covalent inhibition revealed by the crystal structure of the caspase-8/p35 complex. *Nature* **410**, 494-497.

Yamamoto, D. (1994). Signaling mechanisms in induction of the R7 photoreceptor in the developing Drosophila retina. *BioEssays* **16**, 237-244.

Yang, H., McLeese, J., Weisbart, M., Dionne, J. L., Lemaire, I. and Aubin, R. A. (1993). Simplified high throughput protocol for northern hybridization. *Nucleic Acids Res.* **21**, 3337-3338.

Yang, N., Higuchi, O. and Mizuno, K. (1998). Cytoplasmic localization of LIM-kinase 1 is directed by a short sequence within the PDZ domain. *Exp. Cell. Res.* **241**, 242-252.

Yoshida, E. N., Benkel, B. F., Fong, Y. and Hickey, D. A. (1999). Sequence and phylogenetic analysis of the SNF4/AMPK gamma subunit gene from Drosophila melanogaster. *Genome* **42**, 1077-1087.

Table S1. Insertion position and loss- or gain-of-function analysis of targeted genes

Gene	EP line	Position into targeted gene	Loss-or gain-of function analysis towards GG17
A. Ras, Rac and Rho signalling proteins			
MESR4	EP(2)386	-575 bp from mRNA start	Behaves like UAS-MESR4 (Huang and Rubin, 2000).
AKAP200 /MESR2	EP(2)2072 EP(2)2254	-43 bp from mRNA start -114 bp from mRNA start	Behaves like UAS-MESR2 (Huang and Rubin, 2000).
DC3G	EP(1)1613*	In first intron, -475 bp from AUG start codon	UAS-DC3G lines (Ishimaru, 1999) similarly enhanced GG17 (see text).
CG31012	EP(3)3700	-14 bp from mRNA start	*P{SUP-or-P}CG31012P*$^{[KG05741]}$ has no effect
pbl	EP(3)3415*	5'UTR, -457bp from AUG start codon	Described as gof in (Prokopenko et al., 1999). *pbl*^{5} has no effect (see text).
GEF(64C)	EP(3)3035 EP(3)3322*	In first untranslated exon (+26bp) -469 from mRNA start	UAS-GEF (64C) line (Bashaw, 2001) enhanced GG17. *GEF* mutants have no effect (see text).
grp	EP(2)587	In first intron of transcript CG17161-RA, - 16 kb from AUG start codon	Described as gof (Abdelilah-Seyfried et al., 2000). *grp*$^{[06034]}$ has no effect.
CG7097	EP(2)2445	-44 bp from mRNA start	*Df(2R)P34* (55E-56C) has no effect.
SNF4Aγ	EP(3)648	-40 bp from mRNA start of *SNF4Aγ* transcript CG17299-RH	*P{SUPor-P}SNF4Aγ*$^{P[KG00325]}$ has no effect.
B. Cytoskeletal control and membrane trafficking			
Myo31DF	EP(2)2491	-28 bp from mRNA start	*P{lacZ}l(2)k09116* has no effect
Beach1	EP(2)2299*	In first intron, + 194 bp downstream of AUG start codon: may generate a Nter truncated protein.	Described as gof in (Abdelilah-et al, 2000; Kraut et al., 2001). *Df(2L)cl7* (25E-26A) has no effect.
C. Chromatin remodelling factors			
kis	EP(2)474	In first intron of *kis* CG3696-RA transcript, - 5 kb from CTG start codon	Described as gof in (Kraut et al., 2001; Pena-Rangel et al., 2002). *kis*^{1} has no effect.
	EP(2)563	- 1kb from start of *kis* CG3696-RB transcript	
dom	EP(2)2371	-23 bp from mRNA start	*dom*$^{P[K08108]}$ has no effect.
Dp1	EP(2)2422	In first intron, -1152 kb from AUG start codon	*P{GT1}Dp1*$^{[BG01405b]}$ and *P{GT1}Dp1*$^{[BG02288]}$ have no effect.
D. Spermatogenesis			
poe	EP(2)349 EP(2)737	-18 bp from mRNA start -18 bp from mRNA start	EP737 described as gof in (Kraut et al., 2001). *poe*$^{[O3420]}$ has no effect.
TMS1d	EP(3)807	-24 bp from mRNA start	*Df(3L)st-e4* (72D-73A) enhanced GG17 (see text).
cdi	EP(3)3319	In first non coding exon	Described as gof in (Kraut et al., 2001) *cdi*^{r47} has no effect.
E. Others			
Traf2	EP(1)325	- 257 bp from mRNA start	*Df(1)GE202* (7D12-7E3) has no effect
wun2	EP(2)652	-4,6kb from mRNA start may affect *wun* UAS+/-	May affect *wunen* . *wun*^{EMS4} and *P{lacW}wun*^{K10201} have no effect.
CG14959	EP(2)714 EP(2)3139	Both EPs inserted -7,6 kb from mRNA start	Described as gof (Tseng and Hariharan, 2002; Pena-Rangel et al., 2002). *P{SUP-orP}KG0365* has no effect
CG5261	EP(2)816	-3kb from mRNA start	Described as gof (Abdelilah-Seyfried et al., 2000; Pena-Rangel et al., 2002).
		May affect *chameau (chm)* (3' to *chm*)	*P{GT1}chm*$^{[BG02254]}$ has no effect
CG3624	EP(2)827	- 40 bp from mRNA start	*P{SUPor-P}CG3624*$^{[KG05061]}$ has no effect
rho-6	EP(2)2023	-958 bp from mRNA start	*P{SUPor-P}rho-6*$^{[KG09603]}$ has no effect
CG6701	EP(2)2054	5'UTR, -251 bp from AUG	Described as gof (Tseng and Hariharan, 2002; Pena-Rangel et al., 2002). *P{SUPor-P}CG6701*$^{[KG00917]}$ has no effect
CG8740	EP(2)2233	-36 pb from mRNA (GH05582) start	/
numb	EP(2)2455	- 520 bp from *numb* transcript CG3779-RA	*numb*^{1} has no effect
cpo	EP(3)3395 EP(3)3608	Both in second intron of *cpo* transcript, -2,2 kb from CUC start codon	*cpo*^{01432} has no effect
CG2017	EP(3)3503	5' UTR, - 40 bp from AUG start codon	Described as gof (Kraut et al 2001). *Df(3R)Tpl10,DpDfd[rv1]* has no effect

Note that Gal4-dependent regulation can be functional at considerable distance of up to10 kb (Nicolai et al., 2003).

References

Nicolai, M., Lasbleiz, C. and Dura, J. M. (2003). Gain-of-function screen identifies a role of the Src64 oncogene in Drosophila mushroom body development. *J. Neurobiol.* **57**, 291-302.

Pena-Rangel, M. T., Rodriguez, I. and Riesgo-Escovar, J. R. (2002). A misexpression study examining dorsal thorax formation in Drosophila melanogaster. *Genetics* **160**, 1035-1050.

Tseng, A. S. and Hariharan, I. K. (2002). An overexpression screen in Drosophila for genes that restrict growth or cell-cycle progression in the developing eye. *Genetics* **162**, 229-243.