The *Caenorhabditis elegans* homologue of the proto-oncogene *ect-2* positively regulates RAS signalling during vulval development

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Guanine nucleotide exchange factors (GEFs) regulate the activity of small GTP-binding proteins in a variety of biological processes. We have identified a gain-of-function mutation in the *Caenorhabditis elegans* GEF *ect-2*, the homologue of the mammalian *ect2* proto-oncogene that has an essential role during cytokinesis. Here, we report that, in addition to its known function during mitosis, ECT-2 promotes the specification of the primary vulval cell fate by activating RAS/mitogen-activated protein kinase (MAPK) signalling before the end of the S-phase. Epistasis analysis indicates that ECT-2 crosstalks to the canonical RAS/MAPK cascade upstream of the RAS GEF SOS-1 by means of a RHO-1 signalling pathway. Our results raise the possibility that the transforming activity of the mammalian *ect-2* oncogene could be due to hyperactivation of the RAS/MAPK pathway.

Keywords: *Caenorhabditis elegans*; signal transduction; development; RAS; guanine nucleotide exchange factor

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INTRODUCTION

The RHO family of small guanine triphosphate hydrolases includes the RhoA, RhoB, RAC and CDC42 subfamilies of small GTPases that control various cellular processes, such as cell migration, adhesion, axonal guidance, gene expression, cell-cycle progression and cytokinesis (reviewed by Malliri & Collard, 2003; Burridge & Wennerberg, 2004). In most of these cases, RHO proteins transduce signals from transmembrane receptors to proteins controlling actin dynamics. The activity of RHO proteins is tightly regulated. They cycle between an inactive GDP-bound and an active GTP-bound form. GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of RHO to

inactivate RHO signalling, and guanine dissociation inhibitors block the release of GDP to keep the RHO protein in the inactive conformation. Conversely, guanine nucleotide exchange factors (GEFs) catalyse the release of GDP and thereby facilitate GTP binding and RHO activation (reviewed by Schmidt & Hall, 2002). The activity of the GEFs is regulated by signals from various growth factor receptors at the plasma membrane. Aberrant RHO signalling in humans has been implicated in tumour formation and in other diseases (Malliri & Collard, 2003).

The mammalian ECT2 protein and its *Drosophila* orthologue Pebble are members of the Dbl family of GEFs, and they activate a RHO-mediated signalling pathway during cytokinesis (Prokopenko *et al*, 1999; Saito *et al*, 2003). ECT2 is necessary to form an actomyosin contractile ring that separates the plasma membranes of the two daughter cells that are forming. Amino-terminally truncated ECT2 and many other Dbl family GEFs show oncogenic transforming activity in cultured cells (Miki *et al*, 1993; Schmidt & Hall, 2002). However, it has remained unclear as to which signalling pathways the transforming activity of the Dbl family GEFs involves.

We show that the *Caenorhabditis elegans* homologue of the mammalian *ect2* proto-oncogene positively regulates the RAS/mitogen-activated protein kinase (MAPK) signalling pathway during vulval development (reviewed by Sternberg & Han, 1998). In the *C. elegans* hermaphrodite, the anchor cell (AC) in the somatic gonad secretes the epidermal growth factor (EGF) LIN-3 to activate the EGF receptor (EGFR)/RAS/MAPK pathway in the vulval precursor cells (VPCs) and to specify the vulval cell fates. In response to the inductive AC signal, the VPC closest to the AC, P6.p, adopts the primary (1°) vulval fate. As a consequence of adopting the 1° fate, P6.p produces a lateral signal that activates the LIN-12 NOTCH pathway in the neighbouring VPCs P5.p and P7.p (Sternberg & Horvitz, 1989). The lateral LIN-12 NOTCH signal prevents P5.p and P7.p from adopting the 1° fate and instructs the secondary (2°) fate (Ambros, 1999; Berset *et al*, 2001). The three remaining VPCs, P3.p, P4.p and P8.p, that receive lower amounts of inductive signal (Dutt *et al*, 2004) and no lateral signal adopt the tertiary (3°) non-vulval cell fate. We have isolated a gain-of-function mutation in *C. elegans ect-2* (previously named *let-21*) in a genetic screen for new regulators

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of the EGFR/RAS/MAPK pathway. Genetic epistasis analysis indicates that ECT-2 activates a RHO-1 signalling pathway that crosstalks to the RAS/MAPK cascade upstream of the RAS GEF SOS-1 (Chang *et al*, 2000).

RESULTS

An activating mutation in ect-2 causes excess induction

To identify novel regulators of the EGFR/RAS/MAPK signalling pathway in *C. elegans*, we screened for mutations that cause excess vulval induction and a multivulva (Muv) phenotype in a sensitized *gap-1(0)* background. *gap-1* encodes a GTPase-activating protein that inhibits LET-60 RAS signalling, but *gap-1(0)* single mutants develop a wild-type vulva (Fig 2A; Table 1, row 5; Hajnal *et al*, 1997). Out of 30,000 EMS mutagenized haploid genomes, we isolated 27 mutants that show a synthetic Muv phenotype and define at least four complementation groups. The

Table 1 | An *ect-2* gain-of-function mutation activates the epidermal growth factor receptor/RAS/mitogen-activated protein kinase pathway upstream of SOS-1

Row	Genotype	Vulval induction*	Vulval phenotype†	n
1	Wild type	3.0	WT	Many
2	Wild type, gonad ablated	0.0	100% Vul	22
3	<i>ect-2(zh8gf)</i>	3.0	WT	44
4	<i>ect-2(zh8gf)</i> , gonad ablated	0.6	93% Vul	27
5	<i>gap-1(0)</i>	3.0	WT	36
6	<i>ect-2(zh8gf)/+; gap-1(0)</i>	3.02	5% Muv	65
7	<i>ect-2(zh8gf); gap-1(0)</i>	4.6	76% Muv	17
8	<i>[hs::mpk-1]</i>	3.0	WT	40
9	<i>ect-2(zh8gf)/+; [hs::mpk-1]</i>	3.4	25% Muv	57
10	<i>ect-2(zh8gf); [hs::mpk-1]</i>	5.6	94% Muv	35
11	<i>lin-3(rf)</i>	0.9	95% Vul	20
12	<i>ect-2(zh8gf)/+; lin-3(rf)</i>	1.8	87% Vul	47
13	<i>ect-2(zh8gf); lin-3(rf)</i>	2.9	22% Vul	23
14	<i>sem-5(rf)</i>	1.5	76% Vul	21
15	<i>ect-2(zh8gf); sem-5(rf)</i>	2.7	13% Vul	30
16	<i>sos-1(0); let-60(gf)</i>	2.5	32% Vul	31
17	<i>ect-2(zh8gf); sos-1(0); let-60(gf)</i>	2.7	26% Vul, 2% Muv	40
18	<i>let-60(rf)</i>	0.0	100% Vul	33
19	<i>ect-2(zh8gf); let-60(rf)</i>	0.2	94% Vul	33
20	<i>mpk-1(0)</i>	0.0	100% Vul	25
21	<i>ect-2(zh8gf); mpk-1(0)</i>	0.0	100% Vul	93

*Average number of vulval precursor cells per animal that have adopted a 1° or 2° vulval fate scored under Nomarski optics. †Percent Vul, animals with fewer than three induced VPCs; percent Muv, animals with more than three induced VPCs. Alleles used: *ect-2(zh8gf)* II, *cis*-linked with *unc-4(e120)*, *mpk-1(ga117)* III, *cis*-linked with *unc-32(e189)*, *lin-3(e1417)* IV, *cis*-linked with *unc-5(e51)*, *let-60(n1876)* IV, *cis*-linked with *unc-22(s7)*, *gals36[HS-mpk-1(+)]*, *EFα-D-mek(+)*, *unc-30(+)* V, *cis*-linked with *him-5(e1490)*, *sos-1(s1031)*, *cis*-linked with *unc-46(e177)*, *sem-5(n2019)* X, *gap-1(ga133)* X.

zh8 mutation causes a penetrant Muv phenotype in the *gap-1(0)* background but no obvious vulval phenotype as a single mutant (Table 1, rows 3,7). The gene was mapped to the right arm of chromosome II to an 86 kbp interval containing 15 candidate genes (supplementary figure online; for details on cloning, see the supplementary information online). As we observed a weak Muv phenotype in *zh8/+; gap-1(0)* animals (Table 1, row 6), we speculated that *zh8* might represent a weak gain-of-function (*gf*) allele. We therefore tested whether RNA interference against one of the 15 candidate genes might suppress the Muv phenotype of *zh8; gap-1(0)* double mutants. RNA interference against the open reading frame T19E10.1, which encodes the *ect-2* gene (previously named *let-21*; Omachi & Lambie, personal communication), efficiently suppresses the *zh8* Muv phenotype. In another sensitized background created by the elevated expression of the MAPK under control of the heat-shock promoter along with *D-mek-2* (*hs::mpk-1*; Lackner & Kim, 1998), the semi-dominant nature of *ect-2(zh8)* was more apparent (Table 1, rows 8–10). Moreover, *ect-2(zh8)* is a semi-dominant suppressor of the vulvaless (Vul) phenotype caused by a reduction-of-function mutation in the *lin-3 egf* gene (Table 1, rows 11–13).

Sequence analysis of the *ect-2* locus in *zh8gf* mutants identified a C-to-T transition in exon 6 at position +998 relative to the ATG start codon (supplementary figure online). *ect-2* encodes a protein of 924 amino acids containing two breast cancer gene carboxy-terminal (BRCT) domains followed by a Dbl homology (DH) and a Plekstrin homology (PH) domain (Fig 1A). The *zh8gf* mutation substitutes the conserved Glu residue 225 in the second BRCT domain with a Lys. *ect-2* is the closest *C. elegans* homologue of *Drosophila pebble* (Prokopenko *et al*, 1999) and the mammalian *ect2* proto-oncogene (Miki *et al*, 1993). *C. elegans* ECT-2 shows 23% overall sequence identity and 53% similarity with human ECT2. Pebble and ECT2 are members of the Dbl family of GEFs for the Rho family of small GTPases (Schmidt & Hall, 2002). The residues that are necessary for the catalytic activity of DH domains in the α-helices α1 or α9 are all conserved in *C. elegans* ECT-2, which indicates that the protein could function as a GEF (Fig 1B, asterisks; Liu *et al*, 1998).

Thus, an activating mutation in the RHO GEF *ect-2* causes excess vulval induction when combined with mutations that sensitize the EGFR/RAS/MAPK pathway.

Overexpression of ECT-2 causes hyperinduction

The Muv phenotype caused by the *zh8gf* mutation could be due to either an increase in normal ECT-2 function or a newly acquired (neomorphic) activity of ECT-2 that is created through the *zh8gf* mutation. The mutant ECT-2(*zh8gf*) protein might recognize a substrate that the wild-type protein does not accept. To distinguish between these two possibilities, we tested whether overexpression of wild-type ECT-2 is sufficient to cause excess vulval differentiation. For this purpose, transgenic lines carrying multiple copies of a genomic DNA fragment spanning the *ect-2* locus were introduced into a wild-type and *gap-1(0)* background (supplementary figure online). All three independent lines that were tested showed a Muv phenotype in a *gap-1(0)* background, and two transgenic lines even showed a Muv phenotype in a *gap-1(+)* background (Table 2). Thus, increasing the dosage of wild-type ECT-2 causes excess vulval induction, which indicates that the *zh8gf* Muv phenotype is due to an increase in normal ECT-2 activity.

Fig 1 | Molecular analysis of *ect-2*. (A) Domain structure of the ECT-2 protein compared with human ECT2 and *Drosophila* Pebble. The amino-acid change induced by *zh8* and the location of the *n1778* in-frame deletion are shown. PH, Plekstrin homology. (B) Sequence alignment of the Dbl homology (DH) domain of *C. elegans* (*C.e.*) ECT-2, human (*H.s.*) ECT2 and *Drosophila* (*D.m.*) Pebble. The asterisks indicate the conserved residues in the two α -helices ($\alpha 1$ or $\alpha 9$) that are necessary for the nucleotide exchange factor activity (Liu *et al*, 1998).

ECT-2 promotes primary cell fate specification

Drosophila Pebble and mammalian ECT2 have an essential role in cytokinesis during the M-phase (Prokopenko *et al*, 1999; Saito *et al*, 2003). However, the specification of the 1° vulval cell fate in *C. elegans* occurs during the G1-phase of the VPC cell cycle (Ambros, 1999). We therefore tested whether ECT-2 controls cell fate specification before the M-phase, using the *egl-17::gfp* reporter transgene as a molecular marker for the 1° fate (Burdine *et al*, 1998). For this purpose, we examined the *ect-2(e1778)* reduction-of-function mutation, which is caused by an in-frame deletion that removes the first BRCT domain but leaves the remaining open reading frame intact (Fig 1B). This allele has been identified in a screen for mutations that cause meiotic arrest, and shows the same germline phenotype as a null allele but only a weak P-cell migration defect (Omachi & Lambie, personal communication and our own observation; also, see below).

In wild-type L3 larvae, EGL-17::GFP (green fluorescent protein) is expressed in P6.p and its 1° descendants (Burdine *et al*, 1998). In *ect-2(e1778rf)* larvae, we found two- to threefold lower levels of EGL-17::GFP in P6.p when compared with the wild-type controls (Fig 2C,D,G). Conversely, in a *gap-1(0)* or an [*hss::mpk-1*] sensitized background, *ect-2(zh8gf)* causes ectopic EGL-17::GFP expression in other VPCs besides P6.p (Fig 2E,F,H). This ectopic expression of the 1° fate marker is accompanied by morphological changes of the vulva that are characteristic of a 2° to 1° cell fate transformation in P5.p and P7.p descendants (Fig 2A,B; Berset *et al*, 2001).

To test in which phase of the cell cycle ECT-2 promotes 1° cell fate specification, we examined the EGL-17::GFP expression pattern in hydroxyurea-arrested *ect-2(zh8gf)* larvae. Treatment of L2 larvae with hydroxyurea blocks the VPC cell cycle in the S-phase, but it does not prevent 1° fate specification (Ambros, 1999). In arrested *ect-2(zh8gf); gap-1(0)* larvae, the frequency of ectopic EGL-17::GFP expression is enhanced rather than reduced when compared with untreated *ect-2(zh8gf); gap-1(0)* larvae (Fig 2H). This slight enhancement of EGL-17::GFP expression by hydroxyurea treatment could be due to the fact that the VPCs are exposed to the inductive LIN-3 EGF signal for a longer time than in untreated animals and thus accumulate higher levels of MAPK activity. However, it is important to note that in hydroxyurea-treated *ect-2(zh8gf); gap-1(0)* double mutants also, the frequency of ectopic EGL-17::GFP expression is enhanced when compared with hydroxyurea-treated *gap-1(0)* single mutants (Fig 2H). Taken together, these experiments indicate that ECT-2 positively regulates 1° cell fate specification before exit from the S-phase, most probably during the G1-phase.

ECT-2 acts upstream of or at the level of SOS-1 GEF

The removal of the gonadal AC prevents the production of the inductive EGF signal and causes a completely penetrant Vul phenotype (Table 1, row 2; Kimble, 1981). However, in *ect-2(zh8gf)* animals that lack a gonad, partial vulval induction can be observed, which indicates that ECT-2 promotes vulval differentiation even in the absence of an inductive AC signal (Table 1, row 4).

◀ Fig 2 | Vulval morphology and 1° cell fate marker expression in *ect-2* mutants. (A) Wild-type vulval morphology in a *gap-1(0)* L4 larva. (B) An *ect-2(zh8gf); gap-1(0)* double mutant showing a multivulva (Muv) phenotype. Note the detachment and invagination of the P5.p and P7.p descendants, which is characteristic for a 2° to 1° cell fate transformation, and the ectopic differentiation of the P8.p descendants. (C) Expression of the 1° cell fate marker EGL-17::GFP (green fluorescent protein) in a wild-type (WT) and (D) an *ect-2(rf)* L3 larva (Pn.p stage). (E) EGL-17::GFP expression in an *[hs::mpk-1]* and (F) an *[hs::mpk-1]; ect-2(zh8gf)* L3 larva at the Pn.px stage. Note the ectopic EGL-17::GFP expression in the P7.p and P8.p descendants, in (F). (G) Relative EGL-17::GFP fluorescence intensity in P6.p of wild-type and *ect-2(rf)* L3 larvae (see Methods). (H) Percentage of animals at the Pn.px stage showing EGL-17::GFP expression in P5.p and/or P7.p descendants in addition to the P6.p descendants. Where indicated, animals were treated with 40 mM hydroxyurea, as described (Ambros, 1999), and EGL-17::GFP expression in the undivided Pn.p cells was scored 5 h later. Scale bars, 10 μm (B,F).

Table 2 | Overexpression of wild-type *ect-2* causes excess vulval induction

Genotype	Array	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p*	Induction‡	n
Wild type	None	0	0	100	100	100	0	3.0	Many
<i>ect-2(gf); gap-1(0)</i>	None	38	59	100	100	100	62	4.6	17
Wild type	<i>zhEx44.1[ect-2(+)]</i>	9	19	100	100	100	9	3.4	43
<i>gap-1(0)</i>	<i>zhEx44.1[ect-2(+)]</i>	10	17	100	100	100	15	3.4	41
Wild type	<i>zhEx44.2[ect-2(+)]</i>	0	5	100	100	100	0	3.1	22
<i>gap-1(0)</i>	<i>zhEx44.2[ect-2(+)]</i>	7	27	100	100	100	13	3.5	15
Wild type	<i>zhEx44.3[ect-2(+)]</i>	0	0	100	100	100	0	3.0	21
<i>gap-1(0)</i>	<i>zhEx44.3[ect-2(+)]</i>	10	24	100	100	100	20	3.5	51

*Percent VPCs adopting 1° or 2° induced fates. ‡Average number of VPCs per animal that have adopted a 1° or 2° vulval fate scored under Nomarski optics. Alleles used: *ect-2(zh8gf)* II, *gap-1(ga133)* X, *zhEx44.1* through *zhEx44.3* are three independent lines.

To determine at which step ECT-2 interacts with the inductive signalling pathway, we combined *ect-2(zh8gf)* with mutations that reduce or eliminate the activity of the EGFR/RAS/MAPK pathway at different steps. *ect-2(zh8gf)* efficiently suppresses the Vul phenotype caused by *rf* mutations in *lin-3* *egf* and *sem-5*, which encodes a GRB2 adaptor protein that transduces the signal between EGFR and RAS (Table 1, rows 11–15; Clark *et al*, 1992). In contrast, neither a null mutation in the RAS GEF *sos-1* (Chang *et al*, 2000) nor in *mpk-1* (Lackner & Kim, 1998) is suppressed by *ect-2(zh8gf)* (Table 1, rows 16,17,20, 21; to rescue embryonic lethality of *sos-1(0)*, we carried out the epistasis test in a *let-60 ras(gf)* background, as described by Chang *et al*, 2000). Furthermore, the strong *let-60 ras(e1678rf)* mutation that causes a completely penetrant Vul phenotype is only slightly suppressed by *ect-2(zh8gf)* (Table 1, rows 18,19; Beitel *et al*, 1990).

We also tested whether reducing *ect-2* function suppresses the Muv phenotype caused by hyperactivation of the RAS/MAPK pathway. As no VPCs are generated in *ect-2* null mutants owing to an earlier requirement for ECT-2 during the ventral migration of the P cells that generate the VPCs (K. Morita and M. Han, personal communication), we examined the vulva in *ect-2(e1778rf)* mutants. Among the 21 *ect-2(e1778rf)* animals examined, we found two cases in which an abnormal vulva was formed, because one of the proximal VPC predecessors (P5, P6 or P7) had not migrated ventrally. In another three animals, all proximal Pn.p cells were present but some of them adopted a hypodermal 3° fate (that is, they divided once) instead of a 1° or 2° vulval cell fate. Considering only those cells that were present in *ect-2(e1778)* mutants, the average number of induced vulval cells

Table 3 | *ect-2* acts upstream of or in parallel with *let-60 ras*

Row	Genotype	Vulval induction*	Vulval phenotype‡	n
1	Wild type	3.0	WT	Many
2	<i>ect-2(rf)</i>	2.7	19% Vul	21
3	<i>zhEx68[lin-3(+)]</i>	4.4	86% Muv	28
4	<i>ect-2(rf); zhEx68[lin-3(+)]</i>	3.6	44% Muv	27
5	<i>let-60(gf)</i>	3.8	64% Muv	30
6	<i>ect-2(rf/+); let-60(gf)</i>	3.8	73% Muv	26
7	<i>ect-2(rf); let-60(gf)</i>	3.9	81% Muv	26

*Average number of VPCs per animal that have adopted a 1° or 2° vulval fate scored under Nomarski optics. ‡Percent Vul, animals with fewer than three induced VPCs; percent Muv, animals with more than three induced VPCs. Alleles used: *ect-2(e1778)* II, *cis*-linked with *unc-4(e120)* and balanced with *mIn1*, *let-60(n1046gf)* IV, *zhEx68[lin-3(+), sur-5::gfp]*.

per animal is 2.7 (Table 3, row 2). Thus, the Vul phenotype of *ect-2(e1778)* animals is partly caused by defects in vulval fate specification or execution. We then examined the effect of reducing *ect-2* function on transgenic worms overexpressing LIN-3 EGF under the control of its own promoter [*lin-3(+)*] and on animals carrying a gain-of-function mutation in *let-60 ras* (Table 3, rows 3,5). Although both alleles cause a Muv phenotype of similar strength, *ect-2(e1778rf)* partially suppresses [*lin-3(+)*] but has no effect on the *let-60 ras(gf)* Muv phenotype (Table 3, rows 4,6,7). We conclude that ECT-2 positively

Table 4|Expression of dominant-negative rho-1 suppresses the ect-2(gf) Muv phenotype

Row	Genotype	Transgene	Vulval induction*	Vulval phenotype‡	n
1	ect-2(gf); gap-1(0)	None	3.9	55% Muv	22
2	ect-2(gf); gap-1(0)	zhEx167.1[hs::rho-1(dn)]	3.0	4% Muv	26
3	ect-2(gf); gap-1(0)	None	3.8	50% Muv	20
4	ect-2(gf); gap-1(0)	zhEx167.2[hs::rho-1(dn)]	2.8	5% Muv, 15% Vul	19
5	ect-2(gf); gap-1(0)	None	4.2	77% Muv	26
6	ect-2(gf); gap-1(0)	zhEx167.3[hs::rho-1(dn)]	2.9	9% Muv, 6% Vul	32

To induce rho-1(dn) expression, synchronized populations of L2 larvae were heat shocked once for 30 min at 33 °C and scored as L4s. *Average number of VPCs per animal that have adopted a 1° or 2° vulval fate scored under Nomarski optics. In all cases shown, the uninduced VPCs adopted a 3° cell fate (that is, they divided once). ‡Percent Vul, animals with fewer than three induced VPCs; percent Muv, animals with more than three induced VPCs. Alleles used: ect-2(zh8) II, cis-linked with unc-4(e120), gap-1(ga133) X, zhEx167.1[hs::rho-1(dn), sur-5::gfp] through zhEx167.3 are independent lines.

regulates the EGFR/RAS/MAPK pathway at the level of or upstream of SOS-1 GEF.

ECT-2 regulates the RAS pathway through RHO-1

The results presented so far raised the possibility that ECT-2 regulates vulval induction by activating a RHO-1 signalling pathway, which feeds into the RAS/MAPK cascade. To test this model, we expressed a dominant-negative form of rho-1 under control of the heat-shock-inducible promoter (Spencer et al, 2001). This approach allowed us to selectively block RHO-1 signalling at a time point after the P cells had migrated to the ventral midline and the VPCs had been generated. Three independent hs::rho-1(dn) transgenic lines were crossed to let-21(gf); gap-1(0) animals and tested for rescue of the Muv phenotype (Table 4). All three transgenes efficiently suppressed the let-21(gf); gap-1(0) Muv phenotype, causing distal VPCs to adopt the 3° cell fate when expression was induced by a single heat shock during the L2 stage (Table 4). In two lines, we even observed a weak Vul phenotype because proximal VPCs occasionally adopted the 3° rather than a 1° or 2° fate (Table 4, rows 4,6). Non-transgenic siblings that were subjected to the same heat shock as controls showed a slight reduction of the Muv phenotype, which was probably due to the heat shock (Table 4, rows 1,3,5).

Thus, ECT-2 regulates the RAS/MAPK pathway by activating RHO-1 signalling.

DISCUSSION

We have isolated a gain-of-function mutation in the C. elegans ect-2 gene, which encodes the homologue of the mammalian GEF ECT2 (Miki et al, 1993). Like most other Dbl family GEFs, mammalian ECT2 shows transforming activity when truncated at the N terminus, and its transforming potential has been correlated with hyperactivation of the GEF activity (Saito et al, 2003). It was therefore proposed that the N-terminal region of ECT2 inhibits the GEF activity of the DH domain. The second BRCT domain may interact with the catalytic centre of the DH domain and thereby prevent substrate binding (Fig 3A; Kim et al, 2005). On activation, for example by phosphorylation of the DH domain, the protein assumes an open conformation that allows it to bind to its substrates (Schmidt & Hall, 2002). The ect-2(zh8gf) mutation exchanges a conserved, negatively charged Glu with a positively charged Lys residue in the second BRCT domain, which may cause ECT-2 to remain in an open, constitutively active conformation and thus have a similar effect as the N-terminal

Fig 3|Models for ECT-2 activation and its role in activating the EGFR/RAS/MAPK pathway. (A) Model for the autoinhibitory function of the second BRCT domain and the effect of the zh8gf mutation (symbolized by a star). (B) Crosstalk between ECT-2 and the RAS/MAPK pathway. EGF, epidermal growth factor; EGFR, EGF receptor; GEF, guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase.

truncations found in the oncogenic variants of mammalian ECT2 (Fig 3A).

The Drosophila and mammalian ECT-2 homologues have an essential role in activating RHO signalling during the M-phase

(Prokopenko *et al*, 1999; Saito *et al*, 2003). More recently, *Drosophila* Pebble was also shown to be required during interphase for mesodermal cell migrations in the embryo (Schumacher *et al*, 2004; Smallhorn *et al*, 2004). Here, we report that ECT-2 regulates 1° vulval cell fate specification, which involves a RAS/MAPK pathway and takes place during the G1-phase (Ambros, 1999). By epistasis analysis, we show that ECT-2 feeds into the canonical RAS/MAPK pathway upstream of or at the level of the SOS-1 RAS GEF. Moreover, the stimulatory effect of ECT-2(gf) on 1° vulval cell fate specification can be suppressed by a dominant-negative form of RHO-1. As we have not determined the cellular focus of *ect-2* function, it is in principle possible that ECT-2 regulates vulval induction from the tissues surrounding the VPCs. However, in view of the genetic data, a cell-autonomous function of *ect-2* in the VPCs seems more likely. Taken together, these results indicate that ECT-2 activates a RHO-1 signalling pathway that not only controls P-cell migration during early larval development (Spencer *et al*, 2001) but also regulates RAS/MAPK signalling later during vulval induction (Fig 3B).

Both positive and negative crosstalks between the RHO and RAS pathways have been observed in several cell types, but, in many cases, the molecular mechanisms connecting the two pathways are unclear (Malliri & Collard, 2003). Our results raise the intriguing possibility that the transforming activity of the mammalian Dbl family of GEFs is at least in part due to hyperactivation of the RAS/MAPK pathway. Small deletions that cause N-terminal truncations and result in the oncogenic activation of a Dbl family GEF are rather unlikely to occur spontaneously in human tumours. However, point mutations similar to the *zh8gf* mutation that we have isolated in *C. elegans* *ect-2* could be more common.

METHODS

General *Caenorhabditis elegans* methods. *C. elegans* strains were maintained as described (Brenner, 1974) and grown at 20 °C. Wild type refers to *C. elegans* variety Bristol, strain N2. The induction of the VPCs was scored under Nomarski optics and the average number of 1° or 2° induced VPCs per animal was calculated as described (Dutt *et al*, 2004). Animals were mounted on 3% agarose slides in M9 buffer containing 20mM Na₂N₃. Larvae were first inspected using Nomarski optics to identify the position of the Pn.p cells or their descendants, and GFP expression was then scored under fluorescent light illumination using a Leica DM RA microscope equipped with a Hamamatsu Orca ER camera using Openlab 3.1 (Improvision, Coventry, UK) software. For quantification of the fluorescence intensity shown in Fig 2G, images of synchronized early L3 larvae (Pn.p stage) were recorded under standardized illumination and recording conditions with non-saturating exposure settings, and the average fluorescence intensity in the entire P6.p cell was determined using the Openlab 3.1 measurement tool. The same contrast settings were applied to all fluorescent images shown in Fig 2. AC ablations were carried out by removing the precursors of the somatic gonad Z1 and Z4 with a laser microbeam at the L1 stage, as described (Kimble, 1981).

For more details on alleles used, positional cloning, hydroxy-urea treatment and transgenesis, see the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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