The P-type ATPase CATP-1 is a novel regulator of *C. elegans* developmental timing that acts independently of its predicted pump function

Anne-Françoise Ruaud and Jean-Louis Bessereau*

During postembryonic stages, metazoans synchronize the development of a large number of cells, tissues and organs by mechanisms that remain largely unknown. In *Caenorhabditis elegans* larvae, an invariant cell lineage is tightly coordinated with four successive molts, thus defining a genetically tractable system to analyze the mechanisms underlying developmental synchronization. Illegitimate activation of nicotinic acetylcholine receptors (nAChRs) by the nicotinic agonist dimethylphenylpiperazinium (DMPP) during the second larval stage (L2) of *C. elegans* causes a lethal heterochronic phenotype. DMPP exposure delays cell division and differentiation without affecting the molt cycle, hence resulting in deadly exposure of a defective cuticle to the surrounding environment. In a screen for DMPP-resistant mutants, we identified *catp-1* as a gene coding for a predicted cation-transporting P-type ATPase expressed in the epidermis. Larval development was specifically slowed down at the L2 stage in *catp-1* mutants compared with wild-type animals and was not further delayed after exposure to DMPP. We demonstrate that CATP-1 interacts with the insulin/IGF and Ras-MAPK pathways to control several postembryonic developmental events. Interestingly, these developmental functions can be fulfilled independently of the predicted cation-transporter activity of CATP-1, as pump-dead engineered variants of CATP-1 can rescue most *catp-1*-mutant defects. These results obtained in vivo provide further evidence for the recently proposed pump-independent scaffolding functions of P-type ATPases in the modulation of intracellular signaling.

**KEY WORDS:** P-type ATPase, Developmental timing, Dauer formation, DAF-2/InsR, Ras-MAPK, *Caenorhabditis elegans*

**INTRODUCTION**

During embryonic and postembryonic stages, metazoans have to synchronize the development of a large number of cells, tissues and organs. As an example, the symmetrical organs of Bilateria usually grow and develop at the same speed. Recent light has been shed on the molecular mechanisms responsible for such regulation, with the finding that retinoic acid is essential in vertebrates to synchronize the somitogenesis clock between the left and right developing mesoderm (reviewed by Brent, 2005). However, the mechanisms responsible for synchronization during most steps of metazoan development are still unknown (Rougvie, 2005).

The nematode *Caenorhabditis elegans* provides a genetically tractable model system to understand how animals synchronize postembryonic developmental events. After hatching, the *C. elegans* larva proceeds to adulthood through a discontinuous development with four larval stages (L1 to L4), each terminated by a molt. This molt cycle is tightly coordinated with postembryonic cellular events. During embryonic and postembryonic stages, metazoans have to synchronize the development of a large number of cells, tissues and organs by mechanisms that remain largely unknown. In *Caenorhabditis elegans* larvae, an invariant cell lineage is tightly coordinated with four successive molts, thus defining a genetically tractable system to analyze the mechanisms underlying developmental synchronization. Illegitimate activation of nicotinic acetylcholine receptors (nAChRs) by the nicotinic agonist dimethylphenylpiperazinium (DMPP) during the second larval stage (L2) of *C. elegans* causes a lethal heterochronic phenotype. DMPP exposure delays cell division and differentiation without affecting the molt cycle, hence resulting in deadly exposure of a defective cuticle to the surrounding environment. In a screen for DMPP-resistant mutants, we identified *catp-1* as a gene coding for a predicted cation-transporting P-type ATPase expressed in the epidermis. Larval development was specifically slowed down at the L2 stage in *catp-1* mutants compared with wild-type animals and was not further delayed after exposure to DMPP. We demonstrate that CATP-1 interacts with the insulin/IGF and Ras-MAPK pathways to control several postembryonic developmental events. Interestingly, these developmental functions can be fulfilled independently of the predicted cation-transporter activity of CATP-1, as pump-dead engineered variants of CATP-1 can rescue most *catp-1*-mutant defects. These results obtained in vivo provide further evidence for the recently proposed pump-independent scaffolding functions of P-type ATPases in the modulation of intracellular signaling.

**KEY WORDS:** P-type ATPase, Developmental timing, Dauer formation, DAF-2/InsR, Ras-MAPK, *Caenorhabditis elegans*
DMPP resistance assay and dauer pheromone
1,1-Dimethyl-4-phenylpiperazinium (DMPP) (Sigma) was dissolved in water and added to 55°C-equilibrated NG agar at a concentration of 0.75 mM, unless noted otherwise, just before plates were poured. Gravid adult worms were allowed to lay eggs for several hours on standard plates. Eggs were then carefully transferred onto DMPP-containing plates and counted. Surviving L4, adults and dauer larvae were scored after 3 days of development (20°C). Dauer pheromone was purified as described (Golden and Riddle, 1984) and added to streptomycin-containing (Sigma, 50 μg/mL) plates when mentioned.

DMPP resistance screen and cap-1 allele identification
N2 worms were mutagenized by germline mobilization of the Drosophila transposon Mosl (Williams et al., 2005). Young-adult F1 worms were transferred onto 0.75 mM DMPP plates and allowed to lay eggs for 1 day. Three days later, plates were screened for healthy living adult animals. In EN17 cap-1(kr17::Mos1), a mutagenic Mos1 insertion localizes at position 14,438,562 of chromosome I by inverse PCR (WormBase web site, http://www.wormbase.org, release WS160, date 07/2006).

Plasmid constructions and PCR amplification
pCatp-1(C. briggsae) was a 2.9 kb C. briggsae cap-1 promoter fragment was PCR amplified from C. briggsae genomic DNA using a Taq/Plu mix and primers 5'-GCCGATACCA-CCTTTACTGTATGCGGAAACC-3' and 5'-GGGAATTCCCTTACA-TTTGAAAGCGGAAACA-3' and inserted downstream of the cap-1 C. briggsae promoter in pAF29.

pAF88 Pcatp-1(C. briggsae)-cap-1(d0409E) was mutagenized using the QuikChange Site-Directed Mutagenesis kit (Stratagene) using primer 5'-CCACCAGTATCTGCGGAGGAAATCGGACCTC-3'. A 1 kb-long region containing the mutagenized site was sequenced and a pool of four independently mutagenized plasmids was injected.

pAF90 Pcatp-1(C. briggsae)-cap-1(R669Q) is a 1637 bp EcoRV-EcoRI fragment from pAF31 was cloned in pBS SK and mutated using primer 5'-GCTCGCAAGACTGCGGACAATGTCGCCACCTC-3'. The mutagenized region was sequenced and cloned back into pAF31.

pAF92 Pdpy-7::cap-1(dsRNA) was a 2.9 kb C. briggsae cap-1 promoter fragment was PCR amplified from C. briggsae genomic DNA using a Taq/Plu mix and primers 5'-GCCGATACCA-CCTTTACTGTATGCGGAAACC-3' and 5'-GGGAATTCCCTTACA-TTTGAAAGCGGAAACA-3' and inserted downstream of the cap-1 C. briggsae promoter in pAF29.

Germline transformation
Transformation was performed by microinjection of plasmid DNA into the gonad (Mello et al., 1991). cap-1(kr17) worms were injected with a DNA mixture containing a C. briggsae genomic fragment and pT96 (sur-5::GFP) (100 ng/μL) or pAF31 (10 ng/μL) and pT96 (90 ng/μL) for rescue experiments. The pump-dead mutant plasmids pAF88 and pAF90 were injected in cap-1(kr17) at 10 ng/μL with pdP115.62 (pMyo-3::GFP) (5 ng/μL) as a co-injection marker and 1 kb+ DNA ladder (INVITROGEN)
(85 ng/µL). For tissue-specific RNAi, pAF92 was injected in ccds4251; sid-1(q2) hermaphrodites at 0.1 ng/µL together with pHU4 (Prab-3::GFP, 20 ng/µL), pPD115.62 (5 ng/µL) and 1 kb+ DNA ladder (75 ng/µL). pAF29 was injected in lin-15(n765ts) at 20 ng/µL with EKL15 (lin-15(+)) (80 ng/µL) as a co-injection marker.

**Light microscopy**

Light microscopy was performed as previously described (Ruaud and Bessereau, 2006).

**RESULTS**

**Mutation of catp-1 confers resistance to the nicotinic agonist DMPP**

Stimulation of nicotinic receptors by the agonist DMPP during the second larval stage of *C. elegans* development causes a lethal heterochronic phenotype by slowing cellular developmental events but not molting. Consequently, animals die at the L2/L3 molt. To identify the molecules required to implement the effect of DMPP, we performed a forward genetic screen for mutants that can develop on the drug. An insertional mutagenesis technique based on the mobilization of the drosophila transposon *Mos1* in the *C. elegans* germ line (Bessereau et al., 2001; Williams et al., 2005) was used to facilitate rapid identification of the mutated genes. Among seven resistant mutants (Ruaud and Bessereau, 2006), we identified one strain containing the mutant allele *kr17* that was strongly resistant to DMPP compared with wild type (Fig. 1A). *kr17* homozygous mutants are healthy and do not display any gross behavioral nor morphological abnormalities by DIC inspection.

To identify the gene carrying the *kr17* mutation, we performed inverse PCR on genomic DNA of the mutant strain and detected a *Mos1* insertion in the predicted open reading frame Y105E8A.12 (Fig. 1B). This gene was named *catp-1* because it codes for a cation-transporting ATPase of the P-type family (see below). The identification of *catp-1* as a novel DMPP resistance gene was confirmed by three sets of experiments. First, the *kr17* mutation genetically mapped to the right end of chromosome I where Y105E8A.12 is located. Second, RNAi against *catp-1* phenocopied the DMPP resistance of *kr17* mutants (data not shown). Third, *catp-1*...
**catp-1 encodes a cation-transporting ATPase of the P-type family**

Based on sequence homology, catp-1 was predicted to encode a P-type ATPase (Fig. 2) (Kuhlbrandt, 2004; Okamura et al., 2003). P-type ATPases form a large family of diverse membrane proteins that actively transport charged substrates such as cations and phospholipids across biological membranes. P-type ATPases possess ten hydrophobic membrane-spanning helices (M1-M10), and highly conserved cytoplasmic domains inserted between helices M2 and M3 and between M4 and M5, an organization found in the P-type ATPase CATP-1 (see Fig. 2B, see Fig. S2 in the supplementary material). They are biochemically characterized by the presence of an acid-stable phosphorylated aspartate residue that forms during the pumping cycle. This phosphorylatable residue is easily identified in CATP-1 at position 409 (Fig. 2C). In catp-1(kr17::Mos1) mutants, the transposon insertion introduces a premature STOP codon before exon 16 (CATP-1b). It encodes a truncated product compared with the vertebrate classes (Fig. 2). Out of these, C09H5.2, C02E7.1 and CATP-1 lack the ouabain-binding site and the motifs correlated with \( \alpha/\beta \) assembly, and show little conservation in the amino acids associated with ion specificity (see Fig. S2 supplementary material) (Okamura et al., 2003). These characteristics may reflect an ancestral form of the Na\(^{+}/K^{+} \)-ATase, and preclude a more precise prediction of CATP-1 transport specificity.

**catp-1 functions in the epidermis**

To get further insights into catp-1 function, we analyzed its tissue expression pattern. GFP was placed under the control of the promoter sequence previously used to express CATP-1 for mutant rescue. GFP was detected in epidermal cells including the head hyp8 to hyp11, and the ventral Pn.p cells. GFP expression was driven by the promoter sequence previously used to express CATP-1 for mutant rescue. Therefore, we used RNA interference to selectively inhibit CATP-1

**CATP-1 has an ATPase-independent activity**

Apart from regulating ion gradients through their pump activity, recent reports suggest that Na\(^{+}/K^{+} \)-ATases might function to modulate intracellular signaling pathways. Specifically, reducing Na\(^{+}/K^{+} \)-ATPase activity alters signaling through the Src, FAK and MAPK pathways by direct protein-protein interactions between the pump and the signaling kinase Src (Tian et al., 2006) (reviewed by Xie and Askari, 2002).
CATP-1 controls *C. elegans* postembryonic developmental timing

**Fig. 2.** CATP-1 encodes a cation-transporting ATPase of the P-type family with an ATPase-independent activity. (A) Phylogenetic tree of *C. elegans* and vertebrate Ca\(^{2+}\), H\(^+/\)K\(^+\)- and Na\(^+/\)K\(^+\)-ATPases determined using the ClustalW analysis on full-length sequences. SCA-1, PMR-1, EAT-6, C01G12.8, CATP-1, C02E7.1 and C09H5.2 are *C. elegans* proteins. (B) Domain structure of human Na\(^+/\)K\(^+\)-P type ATPase \(\alpha_1\) and CATP-1. TM, transmembrane domain (black); P, phosphorylatable P-domain (light gray); N, ATP-binding N-domain (dark gray). (C-E) CATP-1 has an ATPase-independent activity. (C, D) Amino-acid sequence comparison among the predicted H\(^+/\)K\(^+\) (HK) and Na\(^+/\)K\(^+\) (NK) P-type ATPase \(\alpha\) subunits. The amino-acid numbers are according to *C. elegans* CATP-1. Residues identical or similar in more than 50% of the proteins are shaded in black or gray, respectively; residues similar to the identity consensus are also shaded in gray (BOXSHADE 3.21, http://www.ch.embnet.org/software/BOX_form.html). (C) Part 1 of the phosphorylatable P-domain. Arrow indicates the phosphorylatable aspartate characteristic of P-type ATPases. (D) ATP-binding region of the N-domain. Arrow indicates the arginine equivalent to R544 in pig kidney Na\(^+/\)K\(^+\)-ATPase, which is essential for ATP binding (Jacobsen et al., 2002). (E) Survival on 0.75 mM DMPP. The D409E mutation disrupts the obligatory phosphorylation site conserved in all P-type ATPases. Error bars represent s.e.m. (n=3 independent experiments, N=81 individuals, two independent lines). Two pump-dead mutants of CATP-1 partially rescue *catp-1(kr17)* DMPP resistance (*P*<0.05, Mann-Whitney test).
are largely independent from its catalytic function as a pump-dead mutant can rescue the signaling activity of Na⁺/K⁺-ATPase-depleted cells (Liang et al., 2006). To test if CATP-1 might also function independently of its pump activity during C. elegans postembryonic development, we engineered two different mutations that had been demonstrated to abolish the activity of the sodium pump. The D409E mutation removes the conserved aspartate which is phosphorylated during the catalytic cycle (Fig. 2C) (Liang et al., 2006; Ohtsubo et al., 1990). The R669Q mutation disrupts ATP binding and, consequently, the pump activity (Fig. 2D) (Jacobsen et al., 2002). CATP-1 engineered point mutants were expressed in catp-1(kr17). Each of these pump-dead proteins was able to partially rescue DMPP resistance of the mutant animals (Fig. 2E). These results indicate that CATP-1 functions, at least in part, independently of its ATPase function during C. elegans postembryonic development.

**catp-1 modulates C. elegans developmental rate at the second larval stage**

Chronic exposure to DMPP is lethal by slowing development at the second larval stage without affecting the molt timing (Ruaud and Bessereau, 2006). Insensitivity to DMPP was achieved (1) when DMPP was unable to induce a lineage delay, as demonstrated in the *daf-12(0)* mutant, and (2) when development was slowed down at the L2 larval stage, thus enabling resynchronization of molting with a slowed development, as for animals entering a L2 diapause. To identify which of these mechanisms might account for the DMPP resistance of *catp-1(kr17)* mutants, we monitored the molt cycle and L2 development. First, we observed that the duration of the L2 stage was extended in mutants to 145% of wild type (Fig. 3A) and L2 cell divisions were accordingly postponed (Fig. 3B). This developmental delay was specific for the L2 stage, as the duration of the other larval stages was not altered. Second, exposure of *catp-1(kr17)* mutants to DMPP did not cause any further developmental delays (Fig. 3B). Together, these results suggest that *catp-1* functions to tune both the developmental and molting timers during wild-type L2 development.

**catp-1 functions in parallel with UNC-63-containing nAChR and lipophilic hormone signaling to implement DMPP toxicity**

In a previous study, we showed that the nAChR subunit UNC-63 (Culetto et al., 2004) and the nuclear hormone receptor DAF-12 (Antebi et al., 1998; Antebi et al., 2000) are required to implement
DMPP toxicity. UNC-63 might be part of a DMPP receptor, whereas DAF-12 is thought to provide a permissive activity to implement DMPP signaling. These two genes interact in a non-linear pathway (Ruaud and Bessereau, 2006). To further understand the function of catp-1 during the developmental response triggered by exposure to DMPP, we tested genetic interactions between catp-1(kr17::Mos1) and null mutations of unc-63 and daf-12 that affect sensitivity to DMPP. As both catp-1(kr17) and daf-12 null mutants show a strong DMPP resistance (Ruaud and Bessereau, 2006) (Fig. 4), we used a high drug concentration (1 mM) that kills a fraction of catp-1 and daf-12 mutants in order to unmask possible synergistic effects. In these conditions, we found that both double mutants [unc-63(kr13) catp-1(kr17) and catp-1(kr17); daf-12(rh61rh411)] were more resistant than any of the single mutants (Fig. 4), suggesting that catp-1 acts in parallel to both daf-12 and unc-63.

**catp-1 specifically interacts with the daf-2/InsR branch of the dauer pathway to control dauer formation and morphogenesis**

Previously identified DMPP-resistant mutants such as daf-9 and daf-12 do not properly take the decision to enter dauer, a facultative L3 diapause stage chosen under adverse environmental conditions (Antebi et al., 1998; Gerisch et al., 2001; Jia et al., 2002). Does this property also apply to catp-1 mutants? Under replete culture conditions, catp-1(kr17) animals never form dauer larvae and are therefore not dauer formation constitutive (Daf-c). We tested whether catp-1 mutants were dauer defective (Daf-d) using partially purified dauer pheromone (Golden and Riddle, 1984). catp-1(kr17) mutants do not form morphologically typical dauer larvae on high pheromone. Instead, we observed a high proportion of short dauers that had not molted from their L2 cuticle (Fig. 5A). These animals were SDS-resistant, suggesting that occlusion of the buccal cavity typical of dauer morphogenesis had been completed. Interestingly, these abnormal dauer larvae unable to molt are highly reminiscent of worms grown on lophenol, a methylated derivative of cholesterol (Matyash et al., 2004). In these conditions, it is hypothesized that hormones required for molting and for non-dauer development, derived from non-methylated cholesterol, are not synthesized. Our result could implicate CATP-1 in the control of the L2/L3 molt.

The decision to enter dauer diapause is controlled by a complex genetic network. Schematically, signals from the DAF-2/InsR (insulin receptor) and the DAF-7/TGFβ pathways are integrated at the level of DAF-12/NHR via the production of lipopholic hormones (Fig. 5B). To place catp-1 in this network, we performed epistasis experiments between catp-1(kr17) and dauer constitutive mutants of the different pathways. Among all mutant combinations tested, we specifically detected genetic interactions between catp-1 and daf-2 (Fig. 5C). catp-1(kr17) fully suppressed the Daf-c phenotype of daf-2(m50) mutants and caused abnormal dauer morphogenesis of daf-2(m96) and daf-2(e199) mutants in epidermal tissue (Fig. 5D and data not shown); the dauer alae were abnormal and animals did not elongate properly. Because the pharynx was constricted as in normal daf-2 dauers, it appeared squeezed in the head. These results identify catp-1 as a novel allele-specific suppressor of the daf-2/InsR dauer constitutive phenotype and suggest that catp-1 could function downstream or in parallel with daf-2/InsR to control dauer formation and morphogenesis.

**CATP-1 functions independently of DAF-16/FOXO to modulate DAF-2/InsR signaling**

To further investigate the interaction between catp-1 and daf-2/InsR signaling, we examined the DMPP resistance of mutants of daf-2/InsR and its main downstream effector, the transcription factor daf-16/FOXO (Lin et al., 1997; Ogg et al., 1997). daf-2(e1370) mutants are strongly DMPP resistant and this phenotype is fully suppressed by a daf-16(mgDf50) mutant (Fig. 6). By contrast, a daf-16(mgDf50) catp-1(kr17) double mutant is as resistant as catp-1(kr17) alone (Fig. 6), suggesting that catp-1 functions downstream or in parallel of daf-16 to mediate DMPP sensitivity and probably dauer formation. Several genetic differences distinguish CATP-1 and DAF-16: (1) daf-16(0) suppresses constitutive dauer formation and increased life span of all daf-2(0) alleles (Kenyon et al., 1993; Riddle et al., 1998) whereas catp-1(kr17) does not (Fig. 5C and see Fig. S3 in the supplementary material); (2) daf-16(0) suppresses age-1/PE3K whereas catp-1(kr17) does not (Fig. 5C); and (3) daf-16(0) is DMPP sensitive whereas catp-1(kr17) is DMPP resistant (Fig. 6). These data are not in favor of catp-1 acting downstream of daf-16 but rather support a model where catp-1 and daf-16 would act in parallel to differentially modulate signaling through activated DAF-2/InsR.

**CATP-1 modulates Ras-MAPK signaling**

Analysis of dauer formation and aging in *C. elegans* has defined a linear DAF-2/InsR signaling pathway regulating DAF-16/FOXO transcriptional activity. However, two recent studies have unmasked functions of the Ras-MAPK pathway in DAF-2-dependent regulation of development and aging in *C. elegans* (Hopper, 2006; Nanji et al., 2005). An activated Ras mutation, let-60(n1046gf), which affects the GTPase domain of Ras (Han and Sternberg, 1990) and causes an extended life-time of LET-60 in its active GTP-bound conformation (Barbacid, 1987; Beitel et al., 1990; Polakis and McCormick, 1993) was demonstrated to weakly suppress the constitutive dauer formation of some daf-2 mutants. However, the interaction between daf-2(m41) and let-60(n1046gf) had not been analyzed previously. We observed that dauer entry of daf-2(m41) was partially suppressed by let-60(n1046gf), as opposed to daf-2(e1370) which was unaffected.
by the activation of Ras (Fig. 7A). These results further support the role of the Ras pathway in DAF-2/InsR signaling during larval development.

Because catp-1 appeared to play a role in insulin signaling using a branch parallel to daf-16, we speculated that it might function by modulating a DAF-2-dependent Ras-MAPK pathway. To test this hypothesis, we first assayed DMPP resistance of mutants in the Ras-MAPK pathway. A loss-of-function mutant of mek-2, the MAPKK of the ERK-MAPK pathway (Wu et al., 1995), was partially DMPP-resistant (Fig. 7B). We could not test stronger mutants of the pathway because of their rod-like L1 lethality (Sundaram, 2006). The weak viable mutant of mpk-1 (Lackner et al., 1994), which encodes the C. elegans homolog of the ERK-MAPK, was not DMPP resistant. However, this allele has been previously shown to induce weaker phenotypes than the weakly resistant mek-2 allele (Nicholas and Hodgkin, 2004). In addition, a null mutant of the adaptor protein-encoding gene ksr-1 (Kornfeld et al., 1995; Sundaram and Han, 1995) was also resistant to DMPP (Fig. 7B). The very weak DMPP resistance of ksr-1(n2526) could result from redundancy between KSR-1 and a second KSR protein, KSR-2, as previously demonstrated for a subset of MAPK-controlled developmental decisions (Ohmachi et al., 2002). By contrast with the ERK-MAPK pathway, null mutants of the p38 and JNK MAPK pathways (Sakaguchi et al., 2004) were sensitive to DMPP (data not shown). Altogether, these results suggested that the C. elegans ERK-MAPK pathway is required to implement DMPP toxicity.
To test if CATP-1 was interacting with the MAPK pathway, we used the activated Ras mutation. let-60(n1046gf) individuals were DMPP sensitive. The activated Ras partially suppressed catp-1(kr17) DMPP resistance (Fig. 7B), hence suggesting that catp-1 and let-60 might act in the same pathway to implement DMPP toxicity. To test if Ras signaling is indeed involved in DAF-2-dependent sensitivity to DMPP, we introduced the let-60(n1046gf) in catp-2 mutants. Interestingly, activation of Ras only weakly suppresses the DMPP resistance of the daf-2(e1370) mutants whereas it fully suppresses the resistance of daf-2(m41) mutants (Fig. 7B). Altogether, these results suggest that both Ras-dependent and Ras-independent pathways are involved in DAF-2/InsR signaling during development modulation by DMPP-stimulation of AchRs. Moreover, CATP-1 would mostly interact with DAF-2/InsR signaling by modulating a Ras-dependent pathway.

**DISCUSSION**

In a forward genetic screen for *C. elegans* mutants able to develop in the presence of the nicotinic agonist DMPP, we identified catp-1, a gene coding for an epidermal cation-transporting ATPase. catp-1 is involved in the control of postembryonic developmental timing and in the decision to enter the dauer diapause stage. These features likely result from interactions with daf-2/InsR signaling, most probably through the interaction between CATP-1 and the Ras-MAPK pathway. By rescuing catp-1(kr17) mutants with ATPase-dead versions of CATP-1, we could demonstrate that the developmental functions of CATP-1 are largely independent of its putative cation transport function but might rely on scaffolding properties provided by this transmembrane protein, as recently proposed for other members of the P-type ATPase family.

**CATP-1 regulates L2 developmental timing**

We previously demonstrated that illegitimate activation of nAChRs by DMPP during the second larval stage induced a lethal heterochronic phenotype by slowing developmental speed without affecting the molting timer, hence resulting in deadly exposure of a defective cuticle to the surrounding environment at the subsequent molt (Ruaud and Bessereau, 2006). Most probably, the defective cuticle exposed at the L2/L3 molt does not fulfill its diffusion barrier function and animals dissolve rapidly in a way reminiscent of an osmotic shock. Different parameters can be modified by environmental conditions or genetic mutations to cause DMPP resistance. Some mutants, such as daf-12(0), do not slow development in the presence of DMPP and thus do not desynchronize. Alternatively, animals reared on restricted amounts of food or mutants such as eat-6(0f) possess extended intermolt periods, hence enabling the compensation of developmental delay. Finally, some osmotic-stress-resistant mutants like osm-7 (Wheeler and Thomas, 2006) might survive even with a defective cuticle (A. F. Ruaud and J. L. Bessereau, unpublished). At first glance, catp-1 might function in osmoregulation, osmotic stress sensing or response to osmotic stress as CATP-1 is expressed in the epidermis and has a predicted ion transport function. However, we do not favor this hypothesis. First, catp-1 mutants are as sensitive as wild-type animals to high osmolarity on 800 mM sodium acetate, in contrast to osm-7(n1515) (data not shown). Second, catp-1 mutants do not delay their development in the presence of DMPP. Under physiological conditions, catp-1(kr17) animals develop at normal rate during all larval stages, except at the L2 stage which is considerably extended compared with wild type. L2 lengthening could then account for the DMPP insensitivity of catp-1 mutants by occluding the reduction of development speed normally caused by activation of nicotinic receptors. Interestingly, the two daf-2 alleles that tested DMPP resistant are also slow growing; at 20°C, daf-2(m41) and daf-2(e1370) need one additional day to reach adulthood compared with wild type (Gems et al., 1998) (A. F. R. and J. L. B., unpublished). Part of this delay is due to an extended L2 stage, which is likely to be different from L2d as dauer formation was marginal in these conditions (A. F. R. and J. L. B., unpublished). As in catp-1 mutants, L2 lengthening in daf-2 might account for the resistance to DMPP.

**Signal bifurcation downstream of the *C. elegans* DAF-2/InsR**

Signaling downstream of the insulin and IGF receptors has been extensively studied in vertebrates at the cellular and molecular levels. When activated, these receptor tyrosine kinases (RTKs) are able to phosphorylate multiple intracellular substrates, including the insulin receptor substrate proteins (IRS), Shc, Gab-1, Cbl and APS. Upon tyrosine phosphorylation, each of these substrates can recruit a distinct subset of signaling proteins containing Src homology 2 (SH2) domains and initiate different signaling pathways, among which are the Akt/PKB and the MAPK pathways (Lizcano and Alessi, 2002; Saltiel and Pessin, 2002). Each of these pathways plays a separate role in the different cellular effects of insulin and IGF-1. Most of the insulin and IGF receptors transduction machinery described in vertebrates is conserved in *C. elegans*. However, the genetics of dauer formation and aging have defined a linear pathway for insulin signaling, which consists of elements both necessary and sufficient for dauer formation and aging under the control of daf-2. Ultimately, DAF-2/InsR activation leads to the segregation of DAF-16/FOXO in the cytoplasm, preventing its interaction with transcriptional targets. This pathway is required to implement DMPP toxicity as daf-16(0) suppresses daf-2(e1370) DMPP resistance (Fig. 8A).

Despite the functional importance of the DAF-16/FOXO-dependent pathway for DAF-2/InsR signal transduction, increasing evidence substantiates the existence of DAF-16-independent DAF-2/InsR pathways (Gerisch and Antebi, 2004; Yu and Larsen, 2001). Our results, together with two recent
DMPP resistance (*individuals). dauer formation (*not test the interactions between the null alleles of interaction data must be interpreted cautiously because we could not test the interactions between the null alleles of daf-2 and let-60/Ras which are both lethal early during development. However, these results raise the possibility that two signaling branches bifurcate downstream to DAF-2/InsR, one independent of LET-60/Ras and one involving LET-60/Ras. According to this simple model, the LET-60/Ras-dependent pathway would be prominently reduced in daf-2(m41) while both pathways would be depressed in daf-2(e1370) mutants. As suggested by Nanji et al. (Nanji et al., 2005), such differential alteration of the coupling between DAF-2/InsR and the Ras-MAPK pathway might participate in the phenotypic differences observed between daf-2 mutant alleles as DAF-2/InsR probably positively regulates LET-60/Ras activity to control both L2 developmental timing and dauer formation (Fig. 8).

**Dual interaction between catp-1 and a Ras-MAPK-dependent DAF-2/InsR pathway**

Genetic interaction data indicate that CATP-1 participates in two processes regulated by DAF-2/InsR signaling during *C. elegans* larval development: catp-1 positively interacts with daf-2 in the control of L2 developmental timing and negatively interacts in the decision to form dauer larvae. In both cases, CATP-1 function seems to interact with the Ras-MAPK pathway discussed above. Like daf-2 mutants, the DMPP resistance of catp-1 mutants is suppressed by let-60(gf). However, catp-1(kr17) DMPP resistance was independent of DAF-16/FOXO, thus indicating an interaction of CATP-1 with a DAF-16/FOXO-independent DAF-2 pathway (Fig. 8A). Opposite to their interaction in the control of L2 developmental timing, CATP-1 negatively interacts with LET-60/Ras signaling to control entry into the dauer diapause, as both catp-1(kr17) and let-60(gf) suppress constitutive dauer formation in a daf-2(m41) mutant.

Such dual interaction illustrates the complexity of the network that integrates insulin/IGF-1 signaling into developmental decision at the organism level.

One striking feature of the interactions between catp-1, let-60/ras and daf-2 is the high degree of allele specificity, as previously reported for many phenotypic traits of the daf-2 mutants. Despite extensive analysis of multiple daf-2 mutant alleles, the relationship between the molecular lesions of the DAF-2 receptor and mutant phenotypes remains poorly understood. In our study, we observed the strongest interaction with m41, which corresponds to a G383E mutation in the Cys-rich region of the ectodomain (Yu and Larsen, 2001), outside of the ligand-binding interface (McKern et al., 2006). Three other mutations in the ectodomain (Kimura et al., 1997; Scott et al., 2002) show weak (m596) or no (e1368 and m577) genetic interaction with catp-1(kr17). Similarly, mutants of the kinase domain display either weak (e1391) or no (e1370) genetic interaction with catp-1(kr17). If differences in relative levels of disruption of LET-60/Ras and PI3-kinase signaling account for phenotypic differences based on genetic data (Nanji et al., 2005), the cellular and molecular mechanisms at work are unknown. For example, the m41 mutation in the ectodomain might affect the binding of one or a group of the many *C. elegans* insulin-like peptides that preferentially activate the Ras-MAPK pathway. Alternatively, the different mutations might cause subtle changes of the overall receptor activity, and phenotypic differences might arise from differential coupling with intracellular signaling pathways among the cells executing insulin/IGF-1-dependent programs. The multiplicity of the functions of DAF-2 in *C. elegans* and the prominence of cell non-autonomous processes has hampered such analysis so far.

**A scaffolding function of CATP-1 for signal transduction?**

Sequence analysis unambiguously identifies CATP-1 as an α-subunit of the Na⁺/K⁺- and H⁺/K⁺-pump P-type ATPase family. Four additional *C. elegans* genes are predicted to encode closely related proteins, including eat-6 which codes for a bona fide Na⁺/K⁺-ATPase α-subunit required for proper excitability of pharyngeal muscle cells (Davis et al., 1995). The three other predicted genes...
CATP-1 controls *C. elegans* postembryonic developmental timing

**Fig. 8. A genetic model for CATP-1 action in L2 developmental timing and dauer formation.** (A) Control of L2 developmental timing. CATP-1 speeds up both a cell division and a molting timer independently of the UNC-63/nACHr and DAF-12/NHR pathways described in Ruaud and Bessereau (Ruaud and Bessereau, 2006). *daf-2* mutant DMPP resistance probably results from a similar developmental delay and involves both the DAF-16/FOXO and Ras-MAPK pathways. CATP-1 effect on developmental timing likely involves a Ras-MAPK branch of the *daf-2* pathway. Whether *catp-1* directly interferes with *daf-2*NSR, modulates Ras/MAPK activity and/or functions through a third unidentified parallel pathway remains equally possible at this stage. Dashed lines: hypothetical interactions. (B) Dauer formation. In addition to the DAF-16/FOXO and DAF-12/NHR pathways, DAF-2NSR controls dauer formation through a Ras-MAPK pathway. CATP-1 is likely to modulate dauer formation by negatively interacting with this Ras-MAPK branch, but could also directly alter *daf-2*NSR signaling or work through an uncharacterized parallel pathway (see the text for a full discussion).

We thank Ian Johnstone for the gift of a Pdpy-7 plasmid. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). B. Matthieu is thanked for help with confocal microscopy, H. Gendrot for technical help, M.A. Félix, M. Labouesse, I. Katic and V. Robert for critical reading of the manuscript. A.F.R. was supported by a fellowship from the
Ministère de la Recherche et by the Association pour la Recherche contre le Cancer. This work was funded by an AVENIR grant from the Institut National de la Santé et de la Recherche Médicale and by the ACI BDR from the Ministère de la Recherche.

**Note added in proof**

After this manuscript was accepted, Paul et al. reported an in vivo pump-independent function of the Na,K-ATPase for epithelial junction function in *Drosophila* (Paul et al., 2007).

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/5/867?

**References**


CATP-1 controls *C. elegans* postembryonic developmental timing.


