Leveraging the withered tail tip phenotype in *C. elegans* to identify proteins that influence membrane properties

Emma Svensk, Jana Biermann, Sofia Hammarsten, Fredrik Magnusson, and Marc Pilon

Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden

**ABSTRACT**

The properties of cellular membranes are critical for most cellular functions and are influenced by several parameters including phospholipid composition, integral and peripheral membrane proteins, and environmental conditions such as temperature. We previously showed that the *C. elegans* *paqr-2* and *iglr-2* mutants have a defect in membrane homeostasis and exhibit several distinct phenotypes, including a characteristic tail tip defect and cold intolerance. In the present study we report that screening for novel mutants with these 2 defects can lead to the identification of genes that are important contributors to membrane properties. In particular we isolated 3 novel alleles of *sma-1*, the *C. elegans* homolog of βH spectrin, and 2 novel alleles of *dpy-23*, which encodes the *C. elegans* homolog of the AP2 μ subunit. We also show that *sma-1* and *dpy-23* act on membrane properties in pathways distinct from that of *paqr-2* and *iglr-2*.

**Introduction**

Membranes are critical for most cellular processes, and their properties, such as composition and fluidity, must be tightly regulated so as to achieve what may be called “membrane homeostasis.”

We have previously shown that *C. elegans* mutants lacking *paqr-2* or *iglr-2* have defects in membrane fluidity accompanied by 3 obvious phenotypes: a characteristic tail tip morphology defect, inability to grow at low temperatures, and inability to grow in the presence of glucose.5-7

The tail tip defect is particularly interesting for several reasons: 1) it is the only *paqr-2* and *iglr-2* mutant phenotype that is readily visible in worms cultivated under standard conditions at 20°C; 2) This phenotype is suppressed by the inclusion of small amounts of nonionic detergent (e.g. 0.05% NP40) in the culture plates; and 3) it affects a distinct and limited number of cells, namely the 4 hypodermal cells of the tail tip, and is very distinct from the tail tip phenotype of the *cdh-3* mutant in which the terminal hypodermal cell exhibits a range of defects including kinked and forked appearances.8 Here, we characterize the *paqr-2* tail tip phenotype in some more detail and, to identify other genes that influence membrane properties, we report the results of a screen for *paqr-2* genocopiers, i.e. novel mutants that exhibit the same phenotypes as the *paqr-2* mutant.

**Results**

**The tail tip phenotype in hermaphrodites and males**

The hermaphrodite tail tip of *paqr-2* mutants is short and withered (Fig. 1A–B). The tail tip in the hermaphrodite is composed of 4 hypodermal cells: the binucleate hyp10 cell makes up the extreme tip of the tail, which is in contact with hyp11 on the dorsal side and hyp9 and hyp8 on the ventral side and anteriorly to hyp10. These cells are born during early embryogenesis and acquire their tapered shapes during the elongation phase of development, which is then maintained throughout the life of the healthy hermaphrodite.9,10 The hermaphrodite tail tip defect in *paqr-2* mutants is post-developmental and manifests itself as the tail tip grows in size during the larval stages; this was confirmed using the adherence junction marker *ajm-1::GFP* to monitor the shape of the tail hypodermal cells during development. Specifically, the hyp8-10 cells are present and properly shaped in early *paqr-2* larvae, and begin to show signs of deformation at the L3...
stage, which becomes increasingly more severe in L4 larvæ and adult worms (Fig. 2).

The male tail is a specialized structure composed of many cells born post-embryonically, and its development involves cell migrations, fusions, differentiation into neurons and 9 pairs of bilateral rays embedded in a cuticular fan, as well as spicules, hooks and other structures used in mating. Development of the male tail is relatively fragile and male tail defects are found in mutants affecting the TGF-β pathway, the Wnt pathway, cuticular glycoproteins and several other pathways. The tail fan and rays are often missing or deformed also in paqr-2 mutant males (Fig. 1C–E).

Screen for paqr-2 genocopiers

We performed a forward genetic screen of 80 000 mutagenized haploid genomes for paqr-2 genocopiers, i.e., mutants that exhibit a tail tip defect and cold sensitivity similar to that of paqr-2 mutants. The term “genocopier” is derived from “genocopy” which is defined as a “genetic mutation that results in a phenotype known to be caused by another mutation or process.” Ten candidate paqr-2 genocopier mutants were selected for further studies because they most resembled paqr-2 for these 2 phenotypes. These were outcrossed 6 times and their genomes sequenced, and outcrossed at least 8 times prior to detailed phenotypic characterization. We previously reported that 5 of the picked mutants were 2 alleles of paqr-2 itself (et35 and et36) and 3 alleles of iglr-2 (et34, et37 and et38), and that these mutants had identical phenotypes in all aspects, including tail tip defect, cold sensitivity, excess saturated fatty acids, glucose sensitivity, impaired regulation of membrane fluidity and suppression by detergents or mutations that promote fatty acid desaturation. The five other mutants picked in the screen correspond to 3 alleles of smo-1 (et39, et40 and et44) and 2 alleles of dpy-23 (et42 and et43) (Fig. 3A–B). These novel smo-1 and dpy-23 alleles are here described in details, with the null paqr-2(tm3410) and iglr-2(et34) alleles used for comparison purposes.
Figure 2. Hermaphrodite tail tip in wild type and paqr-2 mutants during post-embryonic development. The tail hypodermal cells were monitored in wild-type (N2) or paqr-2 mutants carrying the ajm-1::GFP transgene during larval development, and the stage of the worms were verified by scoring the gonad/vulva, as shown in the right-most column. The hyp8-11 cells are indicated in the wild-type L1 larva. Note that the paqr-2 mutant shows no tail tip defect in the L1 and L2 stages, but begins to exhibit deformations in hyp10 at the L3 stage, which worsens and affects also hyp8-11 in later stages (arrows).
Experimental confirmation of the paqr-2 genocopiers

The fact that we isolated multiple independent alleles of each gene is in itself evidence that the correct molecular mutations were identified. Several evidences support the identification of the novel sma-1 alleles: 1) they caused a Sma phenotype; 2) the novel et39, et40 and et44 mutants display a tail tip phenotype similar to that of the reference sma-1(rlu18) and sma-1(e30) alleles (Fig. 4A–B); and 3) the new alleles fail to complement sma-1(rlu18) and sma-1(e30) for both the size and tail tip defects. Similarly, the novel dpy-23 mutant alleles showed growth defects indistinguishable from the reference dpy-23(e840) mutant, and were rescued by a wild-type dpy-23 transgene (Fig. 4C–D). We conclude that the et39, et40 and et44 are undoubtedly novel alleles of sma-1, while et42 and et43 are undoubtedly novel alleles of dpy-23.

The sma-1 and dpy-23 mutants are not complete paqr-2 genocopiers

The newly isolated mutants were evaluated using several assays in order to determine the degree to which they may be true paqr-2 genocopiers. When considering the tail tip defect, it is clear that the paqr-2 and iglr-2 mutants are very similar both in terms of the nature of the defect and its penetrance. The sma-1 alleles also have a tail tip defect but differ from the paqr-2 and iglr-2 mutants in that the penetrance is lower (Fig. 5A), and the shortened tail is smoother (see Fig. 3B). The dpy-23 mutants have a low penetrance of tail tip defect (Fig. 5A), and the defect is more subtle than in paqr-2, iglr-2 or sma-1 mutants. In conclusion, the paqr-2 and iglr-2 mutants have an identical tail tip phenotype that differs from that of the sma-1 and dpy-23 alleles.
Three assays were used to evaluate the temperature sensitivity of the mutants: measuring their length, scoring their developmental stage after cultivating synchronized L1s for 96 hrs at 20°C or 144 hrs at 15°C, and scoring their ability to produce eggs at 15°C, 20°C and 25°C. The results for the growth and developmental staging assays (Fig. 5B–D) show that the paqr-2 and iglr-2 mutants have the clearest cold sensitivity phenotype, while the sma-1 and dpy-23 are less cold sensitive. In terms of fertility (Fig. 5E), the paqr-2 and iglr-2 mutants are all infertile at 15°C and most fertile at 25°C, while the sma-1 and dpy-23 alleles are most fertile at 20°C (with the exception of the sma-1(et44) allele which is slightly more fertile at 25°C than 20°C). In conclusion, the paqr-2 and iglr-2 mutants have identical temperature sensitivity profiles, clearly benefiting from a warmer cultivation temperature (25°C), and differ from the sma-1 and dpy-23 alleles which thrive best at 20°C. All the mutant strains have an abnormally small brood size (Fig. 5F).

Small amounts of nonionic detergent can suppress the tail tip phenotype of the paqr-2 and iglr-2 mutants, but had no beneficial effect on the sma-1 or dpy-23 mutants (Fig. 6A). The paqr-2 and iglr-2 mutants are glucose intolerant, being completely unable to grow in the presence of 20 mM glucose; this was not the case for the sma-1(et39) and the dpy-23(et43) mutants which showed no special sensitivity to glucose (Fig. 6B).

In summary, based on the tail tip phenotype, temperature sensitivity, brood size and suppression by nonionic detergents and glucose sensitivity, we conclude that only iglr-2 is a true paqr-2 genocopier. The sma-1 and dpy-23 alleles are clearly different from each other phenotypically and differ from paqr-2 in most or all of these traits. This is especially true for the 2 dpy-23 alleles where the

Figure 4. Experimental confirmation of the sma-1 and dpy-23 alleles. (A-B) Shows that the novel sma-1 alleles, et39, et40 and et44 have growth and tail tip defects similar to those of the reference alleles ru18 and e30. (C-D) Shows that expression of a wild-type dpy-23 transgene in hypodermal cells can significantly rescue the growth and development defects at 15°C in the dpy-23(e840) reference alleles and in the novel et42 and et43 alleles. **p < 0.01; ***p < 0.001.
tail tip phenotype is subtle and of low penetrance; the 
dpy-23 mutants are also characterized by “jowls,” a swell-
ing of the head at the level of the nerve ring which has 
been described elsewhere.16

Genetic interaction studies show that sma-1 acts in parallel to paqr-2

In spite of several phenotypic differences, the sma-1 and dpy-23 mutants do have tail tip defects and signifi-
cant cold sensitivity, i.e. the very reasons for which they were picked as candidate paqr-2 genocopiers. To 
explore the possibility that sma-1 and dpy-23 may be in the paqr-2 pathway we performed gene interaction 
experiments. A first experiment examined the double 
mutants. The paqr-2(tm3410);dpy-23(et43) double mutant is very sick and difficult to maintain (data not 
shown), suggesting that the 2 genes act in separate 
pathways. This double mutant was not further studied. 
The paqr-2(tm3410);sma-1(et39) double mutant is also 
much more severe than either single mutant, growing 
very poorly at 20°C (Fig. 7A–B). We also examined 
whether the potent paqr-2 suppressor nhr-49(et8) could 
suppress the sma-1 mutant phenotypes. This was not 
the case: the sma-1(et39);nhr-49(et8) double mutant actually had a worse cold adaptation defect than the 
sma-1(et39) single mutant (Fig. 7C), and also showed 
no amelioration of the tail tip defect (Fig. 7D–E). This 
suggests that sma-1 and paqr-2 cause somewhat similar phenotypes but likely act in separate pathways.

Figure 5. sma-1 and dpy-23 are not as good paqr-2 genocopiers as iglr-2. The penetrance of the tail tip defect (A) and severity of the 
cold tolerance defect (B–D) is very similar for the paqr-2 and iglr-2 mutants, but less severe in the sma-1 and dpy-23 mutants. (E) All 
mutants studied are poorly fertile at 15°C, but differ in that the paqr-2 and iglr-2 mutants are most fertile at 25°C, while the sma-1 and 
dpy-23 mutants favor 20°C (except for sma-1(et44) which was slightly more fertile at 25°C). (F) The brood size at 20°C was low in all 
mutants, and especially low for the dpy-23 mutant alleles. ** p < 0.01; *** p < 0.001.
Discussion

By screening 80,000 mutagenized haploid genomes, we isolated 10 candidate *paqr-2* genocopi-ers that had a tail tip morphology defect as well as a defect in their ability to grow at 15°C. Among the mutants were 2 novel alleles of *paqr-2* and 3 alleles of the gene *iglr-2*; these mutants exhibited remarkable similarities in their phenotypes, which suggests that they act together in the same pathway. The other alleles, described in the present study, affected either *sma-1* or *dpy-23* and showed several phenotypic differences compared to each other or to the *paqr-2* and *iglr-2* mutants; *sma-1* and *dpy-23* likely impact the tail tip and cold adaptation via separate pathways. *sma-1* encodes an βH-spectrin homolog important for epithelial cell sheet morphogenesis, a process during which the SMA-1 spectrin network is remodeled under the apical membrane surface and is essential to organize actin filaments. Spectrins are essential components of the membrane cytoskeleton where they interact with phospholipids and provide a structural platform for the stabilization and activation of various types of membrane proteins. *dpy-23* (also known as *apm-2*) encodes the *C. elegans* μ subunit of the clathrin adaptor AP2 that mediates the endocytosis of membrane proteins and functions at the plasma membrane as an interaction hub for transmembrane cargoes (e.g., Wnt), accessory proteins, and clathrin. It is therefore not surprising that the *dpy-23* mutant exhibits several membrane-related phenotypes, including the tail tip defect, “jowls” and perhaps even the dumpy phenotype. Based on these observations and also on the known molecular functions of the implicated genes, we propose a simple model by which at least 3 separate pathways act on the membrane properties to account for the observed phenotypes (Fig. 8). It is interesting that several alleles of *paqr-2*, *iglr-2*, *dpy-23*, and *sma-1* were isolated in our screen: it suggests that short body length may be a common outcome in mutants with membrane property defects. Note however that while all these mutants are shorter than wild-type, this is clearly not sufficient to cause the observed tail tip defect because mutants in dozens of other genes known to cause Sma or Dpy phenotypes were not picked.

The hermaphrodite tail may be particularly sensitive to membrane defects for several reasons. It is a rather large structure with a very narrow and elongated shape, hence far from thermodynamic equilibrium, and composed of only 4 cells. The tail tip is extremely thin over a rather long stretch and any defect in the properties of the cell membranes or the associated membrane cytoskeleton is likely to result in readily visible deformations. An additional Achilles heel for the tail tip is its relative isolation from the rest of the worm: any metabolite or building block, for example fatty acids or even phospholipids, that are turned over and equilibrated throughout the worm are less likely to do so efficiently in the narrowest end of the worm, i.e., the tail tip. Finally, defects in this structure seem to be well tolerated by worms, and indeed is such a subtle defect that it is easily missed; indeed, previous articles on *sma-1* or *dpy-23* did not mention a tail tip defect. We conclude that screening for mutants with tail tip defects may be a useful approach to identify genes important for the regulation of membrane properties, but that this approach is not stringent enough to selectively identify components of the *paqr-2* pathway.

Figure 6. *sma-1* and *dpy-23* mutants are not rescued by detergents and are not glucose sensitive. (A) The inclusion of 0.05% NP40 completely rescues the tail tip defect in the *paqr-2* and *iglr-2* mutants but has no beneficial effect on the *sma-1* or *dpy-23* mutants. (B) The *paqr-2* and *iglr-2* mutants are unable to grow in the presence of glucose, which has no effect on the *sma-1* or *dpy-23* mutants. *p < 0.05; ***p < 0.001.
Materials and methods

C. elegans strains and transgenes

The wild-type reference strain was the *C. elegans* Bristol variety strain, N2. Unless otherwise stated, strains were obtained from the *C. elegans* Genetics Center (CGC; MN, USA), and experiments were performed using the *E. coli* strain OP50 as food source, which was maintained by passaging either on NGM plates or liquid cultures in LB medium.

Figure 7. Genetic interactions between *paqr-2*, *sma-1(et39)* and the *paqr-2* suppressor *nhr-49(et8)*. (A-B) The double mutant *paqr-2*sma-1(et39) is more severe than either single mutant in terms of growth and development at 20°C. (C-D) nhr-49(et8) did not suppress the growth or tail tip defects of the *sma-1(et39)* mutant, with representative tail tip images shown in (E). **p < 0.01; ***p < 0.001.
using standard protocols.\textsuperscript{21} jcs1, which carries an ajm-1::gfp transgene that expresses a AJM-1::GFP fusion protein localized to adherens junctions\textsuperscript{22} was obtained from the C. elegans Genetics Center. Ppdi-2::dpy-23 expressed the wild-type dpy-23 gene under the hypodermal pdi-2 promoter\textsuperscript{20} and was a kind gift from Gian Garriga.

\textit{Mutagenesis and screen for paqr-2 genocopiers}

As previously described,\textsuperscript{7} 2 strategies were used to isolate novel mutants that are phenotypically similar to \textit{paqr-2}(tm3410) among the F2 progeny of EMS-mutagenized worms. In the first strategy, we isolated mutants with a tail tip defect then searched among those for mutants that also had the cold sensitivity phenotype. Approximately 11 000 mutagenized haploid genomes were screened in this way, yielding 9 promising mutants (alleles \textit{et34}, \textit{et35}, \textit{et37}-\textit{et43}). In the second strategy, we isolated mutants with the cold sensitivity phenotype then searched among those for mutants that also had a tail tip defect. Approximately 70 000 mutagenized haploid genomes were screened in this way, yielding 1 mutant (allele \textit{et36}). Each mutant was outcrossed 4-6 times prior to whole-genome sequencing, and a minimum of 8 times prior to careful phenotypic characterization.

\textit{Whole genome sequencing and mutant identification}

The genomes of the novel mutants outcrossed 4 or 6 times were sequenced to a depth of 25-40x and differences between the reference N2 genome and that of the mutants were identified as previously described.\textsuperscript{6} For each novel mutant, one or 2 mutation clusters, i.e. small genomic areas containing several mutations, were identified, which is in accordance to previous reports. These candidate mutations were tested experimentally as described in the text.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Model in which DPY-23 and SMA-1 act in pathways distinct from that of PAQR-2 and IGLR-2. The results presented here show that DPY-23 and SMA-1 are important for tail tip morphology and cold adaptation via pathways that are at least partly parallel to PAQR-2 and IGLR-2. Specifically, DPY-23 encodes an AP2 \( \mu \) subunit important for clathrin-based endocytosis, hence recycling of membrane components or secretory cargo (e.g. WNT) via endosomes, while SMA-1 encodes a \( \beta H \) spectrin that is a component of the membrane cytoskeleton and hence directly influences the biophysical properties of membranes. PAQR-2 and IGLR-2 act together in a separate pathway that regulates membrane properties by influencing the relative abundance of saturated and unsaturated fatty acids in the phospholipids. Note that DPY-23 could play a role in recycling or membrane localization of PAQR-2, but that it must also have functions separate from PAQR-2 given the distinct phenotypes of the mutants.}
\end{figure}
**Fertility assay**

To determine the percentage of fertile worms, the worms were synchronized by bleaching. The following day, 50 L1 worms were placed on a plate (3 plates for each genotype). The plates were cultivated at 15, 20 and 25°C. The worms were scored daily for pregnancy; pregnant adults and dead worms were removed until no worms were left.

**Other methods**

Generation of transgenic animals, self brood size assay, 15°C growth assay, scoring of tail tip phenotype and preparation of glucose plates were performed as previously described.6

**Statistics**

Error bars for worm length measurements show the standard error of the mean, and t-tests were used to identify significant differences between worm lengths. Error bars for the frequency of the tail tip defect show the 95% confidence interval and the significant differences in the tail tip, staging and fertility assays were determined using Z-tests.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

Some strains were provided by the C. elegans Genetics Center, which is funded by the National Institutes of Health - Office of Research Infrastructure Programs (P40 OD010440). This work was funded by the following Swedish agencies: Vetenskaprådet (grant no. 621-2012-2152), Cancerfonden (grant no. 150326), Carl Trygger Stiftelse (grant no. CTS 15:391), and Diabetesfonden (grant no. DIA 2013-033).

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