Overactive EGF signaling suppresses a *C. elegans pnc-1* egg-laying phenotype independent of known signaling mediators.

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**Abstract**

Nicotinamide recycling is critical to the development and function of *Caenorhabditis elegans*. Excess nicotinamide in a *pnc-1* nicotinamidase mutant causes the necrosis of uv1 and OLQ cells and a highly penetrant egg laying defect. An EGF receptor (*let-23*) gain-of-function mutation suppresses the Egl phenotype in *pnc-1* animals. However, gain-of-function mutations in either of the known downstream mediators, *let-60/Ras* or *itr-1*, are not sufficient. Phosphatidylcholine synthesis is neither required nor sufficient, in contrast to its role in the *let-23gf* rescue of uv1 necrosis. The mechanism behind the *let-23gf* suppression of the *pnc-1* Egl phenotype is unknown.
**Figure 1**: A let-23(sa62)gf mutation in *C. elegans* strongly suppresses a pnc-1(pk9605) egg-laying (Egl) phenotype, where adult pnc-1(pk9605) hermaphrodites form “bags of worms” by four days post L4->adult molt. A) A gain-of-function mutation in *let-23* is sufficient to rescue the *pnc-1* Egl phenotype, but gain-of-function mutations in downstream genes *let-60* and/or *itr-1* are not. B) Phosphatidylcholine synthesis is neither required nor sufficient for the *let-23(sa62)gf* rescue of the *pnc-1* Egl phenotype. WT = wild-type *C. elegans* N2 Bristol strain. Error bars are 95% confidence intervals, with proportion and sample size in the data labels. Proportions were analysed by pairwise.prop.test in R with Holm p value adjustment; *** and n/s represent p<0.0001 and non-significant, respectively.

**Description**

NAD$^+$ is an electron carrier and a co-substrate for NAD$^+$-dependent enzymes such as poly(ADP-ribose) polymerases (Bouchard et al. 2003; Sauve 2008). The byproduct of these enzymatic reactions, nicotinamide (NAM), must be salvaged to maintain a readily available NAD$^+$ pool. In *Caenorhabditis elegans* the nicotinamidase PNC-1 acts both cell autonomously and non-cell autonomously to convert NAM into nicotin acid (NA), an NAD$^+$ precursor in this organism (Huang and Hanna-Rose 2006; Vrablik et al. 2009; Crook et al. 2014). Loss of PNC-1 function affects NAD$^+$ pathway metabolites in two ways. It results in an increase in NAM, causing necrosis of OLQ and uv1 cells, and an egg-laying phenotype due to reduced muscle function. It also reduces NAD$^+$ levels, resulting in gonad developmental delay and a male mating defect (Huang and Hanna-Rose 2006; Vrablik et al. 2009; Vrablik et al. 2011; Upadhyay et al. 2016).

LET-23 is the sole *C. elegans* Epidermal Growth Factor (EGF) receptor and is involved in a range of biological and developmental processes, including vulval development and specification of the uv1 cells (Chang et al. 1999; Moghal and Sternberg 2003). A gain-of-function mutation in the extracellular domain, *let-23(sa62)gf*, results in precocious activation of LET-23 independent of its EGF ligand LIN-3 (Katz et al. 1996). Overactivation of LET-23 rescues the uv1 cell necrosis phenotype of *pnc-1* loss-of-function mutants, and this rescue requires phosphatidylcholine synthesis (Huang and Hanna-Rose 2006; Crook et al. 2016; Crook and Hanna-Rose 2020). We noted that the egg-laying phenotype of *pnc-1* was also ameliorated by overactivation of LET-23 and decided to investigate the mechanism.

To study the role of EGF signaling in the prevention of the egg-laying phenotype we placed individual L4 hermaphrodites on Nematode Growth Medium (NGM) agar plates spotted with *Escherichia coli* OP50. Individual animals were observed after two, three, and four days at 20°C and scored as non-Egg laying defective (nonEgl) adults or “bags of worms” (Egl), where larvae hatch in the uterus due to a failure to lay eggs. Proportion nonEgl was calculated as the number nonEgl adults/total number of individuals at day four. All nonEgl adults had laid eggs by day 4. We found that the *pnc-1*(pk9605) loss-of-function allele reduced the proportion of nonEgl adults to 0.21 and that the *let-23(sa62)* gain-of-function (gf) allele in a *pnc-1*(pk9605) background restored that to 0.9 (Fig. 1a). However, gain-of-function mutations in *let-60* or *itr-1*, which mediate signal transduction downstream of *let-23* (Clandinin et al. 1998; Chang et al. 1999), had no effect on the *pnc-1* egg-laying phenotype (Fig. 1a).

Phosphatidylcholine synthesis is required for *let-23(sa62)gf* mediated rescue of uv1 necrosis and exogenous phosphatidylcholine alone is partially sufficient for uv1 survival (Crook et al. 2016). PMT-1 is part of the Sequential Methylation Pathway (SMP) that synthesizes phosphocholine (Brendza et al. 2007), and PCYT-1 turns phosphocholine from the Sequential Methylation and Kennedy pathways into CDP-choline, the precursor of phosphatidylcholine (Kennedy and Weiss 1956). To test if phosphatidylcholine synthesis was required for the *let-23(sa62)gf*-mediated rescue of the *pnc-1* egg-laying phenotype we knocked down *pmt-1* or *pcyt-1* by RNAi. *unc-22* (control), *pmt-1* and *pcyt-1* RNAi bacterial cultures were spotted onto NGM plates containing 50 μg.ml$^{-1}$ ampicillin and 1 mM IPTG, then individual L4 hermaphrodites were added to each plate and scored as above. We found that neither *pmt-1* nor *pcyt-1* were required for rescue in a *let-23(sa62)gf*; *pnc-1*(pk9605) background (Fig. 1b). *pmt-1* or *pcyt-1* RNAi did however reduce uv1 cell survival in nonEgl adults in experiments run concurrently with this project (Crook et al. 2016), suggesting that RNAi knockdown of the target genes was effective. Next, we wanted to see if phosphatidylcholine alone was sufficient for rescue, as it ameliorates the uv1 necrosis phenotype (Crook et al. 2016). We supplemented *pnc-1* animals with 0.3 mM phosphatidylcholine but found no effect on the *pnc-1* egg-laying phenotype (Fig. 1b).

We have shown that overactivation of the *C. elegans* let-23 EGF receptor robustly rescues the *pnc-1* egg-laying phenotype, but that gain-of-function mutations in the known downstream signaling mediators *let-60*/*Ras* and *itr-1* are not sufficient. Phosphatidylcholine synthesis is not required for the *let-23(sa62)gf* rescue of the egg-laying phenotype and phosphatidylcholine supplementation of *pnc-1* had no significant effect at the sample sizes used, in contrast to the role of phosphatidylcholine in *let-23(sa62)gf* rescue of uv1 necrosis. We have clearly demonstrated another role for *let-23* outside that of growth and development. However, the mechanism by which overactive LET-23 rescues egg-laying in *pnc-1* animals is not
clear. LET-23 may act via an as yet unknown pathway that restores uterine or vulval muscle function by either reducing the production of nicotinamide in those tissues or promoting some other compensatory mechanism.

**Reagents**

**Strains:**
N2 Bristol
BL5715 inIs179 (ida-1::gfp) II
HV560 inIs179 (ida-1::gfp) II; pnc-1(pk9605) IV
HV639 inIs179 (ida-1::gfp) II; pnc-1(pk9605) let-60(n1046gf) itr-1(sy290gf) unc-24(e138) IV
HV662 inIs179 (ida-1::gfp) II; pnc-1(pk9605) let-60(n1046gf) IV
HV663 inIs179 (ida-1::gfp) II; pnc-1(pk9605) itr-1(sy290gf) unc-24(e138) IV
HV776 let-23(sa62gf) inIs179(ida-1p::gfp) II; pnc-1(pk9605) IV

The strains used in this study are available from the authors upon request.

We used the following clones from the Ahringer RNAi library: pmt-1 ZK622.3 II-4G04, pcyt-1 F08C6.2 X-3N20, unc-22 ZK617.1 IV-6K06.

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**References**


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