Insulin-like signalling to the maternal germline controls progeny response to osmotic stress

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In 1893 August Weismann proposed that information about the environment could not pass from somatic cells to germ cells1, a hypothesis now known as the Weismann barrier. However, recent studies have indicated that parental exposure to environmental stress can modify progeny physiology2–7 and that parental stress can contribute to progeny disorders8. The mechanisms regulating these phenomena are poorly understood. We report that the nematode Caenorhabditis elegans can protect itself from osmotic stress by entering a state of arrested development and can protect its progeny from osmotic stress by increasing the expression of the glycerol biosynthetic enzyme GPDH-2 in progeny. Both of these protective mechanisms are regulated by insulin-like signalling: insulin-like signalling to the intestine regulates developmental arrest, while insulin-like signalling to the maternal germline regulates glycerol metabolism in progeny. Thus, there is a heritable link between insulin-like signalling to the maternal germline and progeny metabolism and gene expression. We speculate that analogous modulation of insulin-like signalling to the germline is responsible for effects of the maternal environment on human diseases that involve insulin signalling, such as obesity and type-2 diabetes8.

Maternal exposure to a wide variety of environmental stresses alters progeny growth, development and physiology of diverse organisms2–7 and is thought to be a contributing factor to several human pathologies, including obesity and diabetes8. The mechanisms by which the maternal environment can modify progeny biology are poorly understood. Parental exposure of the nematode Caenorhabditis elegans to mild osmotic stress can protect progeny from the effects of strong osmotic stress8. This finding and similar observations of other organisms2 suggest that besides the potentially deleterious effects of maternal environmental stress on progeny, maternal exposure to environmental stress might epigenetically precondition progeny and protect them from similar environmental insults in the future. How maternal exposure to environmental stress can protect progeny from future environmental stress remains largely unknown.

To determine how parental exposure to mild osmotic stress (300 mM NaCl) protects progeny from the effects of strong osmotic stress (500 mM NaCl) we first examined the effects of 500 mM NaCl on C. elegans. Embryos placed at 500 mM NaCl completed embryonic development and hatched but arrested development immediately after hatching (Fig. 1a and Supplementary Fig. 1a). These arrested animals were unable to move, feed or respond to touch (Supplementary Fig. 1a). However, when arrested animals were returned to normal osmotic conditions (50 mM NaCl), they regained mobility and resumed development (Fig. 1b). We made similar observations when NaBr, KCl or sucrose was used to cause osmotic stress (Supplementary Fig. 1b–d). We conclude that young C. elegans larvae can enter a state of immobile arrested development in response to osmotic stress.

C. elegans also arrests development in response to other environmental stresses, such as starvation10,11. These arrests are caused by the loss of insulin-like signalling via the insulin receptor DAF-2 and the consequent activation of the FOXO transcription factor DAF-16 (refs 10–12). We tested whether the activation of DAF-16 is required for developmental arrest in response to osmotic stress. Animals lacking DAF-16 were less likely than the wild type to arrest development in response to osmotic stress (Fig. 1a), whereas animals with increased DAF-16 activation, such as daf-2 mutants13, were more likely to arrest development in response to osmotic stress (Fig. 1a). Genes in other stress response pathways, such as those that regulate the responses of C. elegans to oxidative stress13 or infection14, were not required for developmental arrest in response to osmotic stress (Supplementary Fig. 1e,f). In addition, DAF-2 activity in the intestine was required for development at 300 mM NaCl (Fig. 1c), and exposure to 500 mM NaCl caused the translocation of DAF-16 from the cytoplasm to the nucleus (Fig. 1d). Like the developmental arrest caused by starvation10, the arrest caused by osmotic stress required dense-core vesicle release (Supplementary Fig. 1g); furthermore, we found that it is

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Received 16 November 2016; accepted 10 January 2017; published online 6 February 2017; DOI: 10.1038/ncb3470
dense-core vesicle release from sensory neurons that was required for developmental arrest in response to osmotic stress (Supplementary Fig. 1g), and daf-16 mutants, which are resistant to developmental arrest, showed an increased susceptibility to osmotic stress (Fig. 1b). These results indicate that like starvation, osmotic stress causes developmental arrest by inhibiting insulin-like signalling and that arrested development correlates with enhanced survival.

In contrast to starvation-induced developmental arrest, we found that developmental arrest in response to osmotic stress is regulated by a different insulin-like peptide, INS-3 (Supplementary Fig. 1h and Supplementary Table 1), than arrest in response to starvation (INS-4 and DAF-28; ref. 15) and that animals that arrest development in response to osmotic stress are immobile and unable to respond to touch, unlike arrest in response to starvation in which animals remain mobile10. In addition, we found that a majority (78%) of genes the expression of which reproductively changed in response to osmotic stress were not affected by starvation (Supplementary Fig. 2 and Supplementary Table 2). These results suggest that these two arrest phenotypes are controlled by partially overlapping but distinct pathways.

Frazier and Roth (2009) found that parental exposure of C. elegans to mild osmotic stress protects progeny from the effects of strong osmotic stress and that this protection required DAF-2 activation9. These authors described an apparently anomalous result: daf-2; daf-16 double mutants were significantly better at adaptation to osmotic stress than both wild-type animals and either daf-2 or daf-16 single mutants9. We hypothesized that this anomaly might be at least in part caused by differing effects of maternal insulin-like signalling and progeny insulin-like signalling with respect to progeny response to osmotic stress. Specifically, we suspected that DAF-2 activation in embryos was required for adaption to osmotic stress, consistent with both the earlier observations9 and our findings (Fig. 1a). We also suspected that it was an inhibition rather than an activation of parental insulin-like signalling that resulted in the protection of progeny from osmotic stress, just as the inhibition of larval insulin-like signalling induced by osmotic stress protects larvae from osmotic stress by causing developmental arrest. To test this hypothesis, we crossed wild-type animals with daf-2 and daf-2; daf-16 double-mutant animals in normal osmotic conditions (50 mM NaCl) and assayed the response of their progeny to 500 mM NaCl. Approximately 60% of the progeny from the cross of wild-type males with daf-2 mutant hermaphrodites hatched and developed at 500 mM NaCl (Fig. 2a). By contrast, the reciprocal cross of daf-2 mutant males with wild-type hermaphrodites and the cross of wild-type males with daf-2; daf-16 double-mutant hermaphrodites did not produce any progeny that hatched and developed at 500 mM NaCl (Fig. 2a). These results demonstrate that like parental exposure to mild osmotic stress, reduced maternal insulin-like signalling can protect progeny from the effects of strong osmotic stress. Importantly, these observations also
Figure 2 Insulin-like signalling to the maternal germline regulates progeny response to osmotic stress. (a) Percentage of wild-type (WT), daf-2(e1370) and daf-2(e1370); daf-16(mu86) cross progeny failing to arrest development after 48 h at 500 mM NaCl. Males contained (Pceh-28::4xNLS::mCherry; him-5(e1490); nls349) for the identification of cross progeny. The pie-1 promoter was used to drive germline-specific expression of DAF-2 and the mex-5 promoter was used to drive germline-specific expression of DAF-16. Error bars, s.d. n=7, 3, 6, 3, 3, 3 and 3; see Supplementary Table 6. (b) Percentage of wild-type, gpdh-1(ok1558) and gpdh-2(ok1733) animals failing to arrest development at 500 mM NaCl after 48 h. Error bars, s.d. n=3 experiments of >100 animals. (c) Average fold change of 2 replicates of the 25 most upregulated genes in embryos in response to osmotic stress after 6 h. FPKM, fragments per kilobase of transcript per million mapped reads. (d) Percentage of wild-type, daf-2(e1370) and gpdh-2(ok1733) cross progeny failing to arrest development after 48 h at 500 mM NaCl. Males contained als39 (Punc-47::GFP); him-5(e1490) for the identification of cross progeny. Error bars, s.d. n=3 experiments of >20 animals. The quantified results are presented as mean ± s.d. using ANOVA. *P < 0.05, ***P < 0.001, ****P < 0.0001 were considered significant. NS, not significant. See Statistics Source Data in Supplementary Table 6.

indicate that there is a previously undescribed link between maternal insulin-like signalling and progeny physiology.

Parental exposure to osmotic stress has been hypothesized to protect progeny from the effects of osmotic stress by increasing the deposition of glycerol from mothers into embryos, since embryos from parents exposed to 300 mM NaCl have more glycerol than embryos from animals grown at normal osmotic conditions (50 mM NaCl)⁹ and glycerol is known to be protective against various environmental stresses¹⁶,¹⁷. We confirmed that exposure of parents to 300 mM NaCl resulted in progeny that are resistant to the effects of 500 mM NaCl (Fig. 2b and Supplementary Table 3), and we discovered that the glycerol biosynthetic enzyme GPDH-2 is required for parental exposure to 300 mM NaCl to protect progeny from 500 mM NaCl (Fig. 2b) but does not affect the response to osmotic stress of animals with parents grown at 50 mM NaCl (Supplementary Fig. 3a). These observations are consistent with the hypothesis that an increased level of glycerol is required for adaptation to osmotic stress. However, it remained unclear how these observations relate to our finding that reduced maternal insulin-like signalling can protect progeny from strong osmotic stress (Fig. 2a), since previous studies indicated that embryos from daf-2 mutant hermaphrodites contain the same amount of glycerol as embryos from wild-type animals⁹ and hence that daf-2 mutant mothers do not deposit more glycerol into embryos. We hypothesized that reduced maternal insulin-like signalling protects progeny from the effects of osmotic stress not by increasing deposition of glycerol from mothers into embryos but rather by increasing glycerol production in embryos via GPDH-2. To test this hypothesis, we crossed gpdh-2 mutant males with daf-2; gpdh-2 double-mutant hermaphrodites. GPDH-2 was required for reduced maternal insulin-like signalling to protect progeny from developmental arrest (Fig. 2d). To test whether GPDH-2 functions maternally, we crossed wild-type males with daf-2; gpdh-2 double-mutant hermaphrodites. GPDH-2 was not required in mothers to protect progeny from developmental arrest in
response to osmotic stress (Fig. 2d). We conclude that the inhibition of maternal insulin-like signalling does not result in the increased deposition of glycerol into embryos but rather results in increased glycerol production in embryos. Importantly, these results reveal that there is a heritable link between maternal insulin-like signalling and progeny metabolism.

We hypothesized that the heritable effects of maternal insulin-like signalling on progeny might be mediated by insulin-like signalling to the germline. To test this hypothesis, we expressed rescuing copies of the wild-type daf-2 or daf-16 genes specifically in the germline. Germline-specific expression of either daf-2 or daf-16 was sufficient to rescue the effects of deficient maternal insulin-like signalling on progeny response to osmotic stress (Fig. 2a). In addition, overexpression of daf-2 in the germline blocked the protective effects of parental exposure to 300 mM NaCl on progeny response to 500 mM NaCl (Supplementary Fig. 3b). These data suggest that maternal exposure to 300 mM NaCl inhibits insulin-like signalling to the germline and that this loss of insulin-like signalling to the germline protects progeny from the effects of osmotic stress.

Insulin-like signalling to the C. elegans germline both inhibits DAF-1618 and activates the RAS–ERK pathway19, which includes the Raf protein LIN-45, the Mek protein MEK-2 and the Erk protein MPK-110. We found that partial loss-of-function mutants in lin-45, mek-2 or mpk-1 (null mutants are lethal) did not arrest development at 500 mM NaCl (Fig. 3a and Supplementary Fig. 3c). In addition, treatment of wild-type animals with the MEK inhibitor U0126 (ref. 21) or RNAi knockdown of mek-2 prevented developmental arrest in response to 500 mM NaCl (Supplementary Fig. 3d–f); RAS–ERK signalling functioned maternally to regulate progeny response to osmotic stress (Fig. 3b); GPDH-2 was required for reduced RAS–ERK signalling to protect animals from developmental arrest (Fig. 3a); and maternal exposure to osmotic stress inhibited MPK-1 activation in the germline (Fig. 3c and Supplementary Table 4), possibly by inhibiting the release of insulin-like peptides from sensory neurons (Supplementary Fig. 4a). We conclude that reduced insulin-like signalling from the soma (probably from sensory neurons) to the maternal germline protects progeny from the effects of osmotic stress by both activating DAF-16 and inactivating MPK-1.

Figure 3 Insulin-like signalling to the maternal germline modifies progeny response to osmotic stress by regulating the RAS–ERK-like pathway. (a) Percentage of wild-type and lin-45(n2018), mek-2(ku114), mpk-1(n5639) and lin-45(n2018); gpdh-2(ok1733) animals failing to arrest development at 500 mM NaCl after 48 h. Error bars, s.d. n = 6. 6, 3, 3, and 3 experiments of >100 animals. See Supplementary Table 6. (b) Percentage of wild-type and lin-45(n2018) cross progeny failing to arrest development at 500 mM NaCl after 48 h. Males contained otIs39 (Punc-47::GFP); him-5(e1490) for the identification of cross progeny. Error bars, s.d. n = 3 experiments of >20 animals. (c) Representative germlines dissected from wild-type animals exposed to either 50 mM NaCl or 300 mM NaCl and stained for DNA (DAPI, white) and diphosphorylated MPK-1 (dpMPK-1) (red). Each condition was replicated 16 times. Asterisks, mitotic end of germline. (d) Relative expression of gpdh-2 mRNA in wild-type and lin-45(n2018) embryos at 50 mM NaCl measured by qRT-PCR and normalized to the expression of the histone his-24. Error bars, s.d. n = 3 experiments from pellets of >1,000 embryos. (e) Glycerol-to-glucose ratio in wild-type and lin-45(n2018) mutant embryos at 50 mM NaCl. Error bars, s.d. n = 3 experiments from pellets of >1,000 embryos. The quantified results are presented as mean ± s.d. using ANOVA (a, b) and two-tailed t-test (d, e), *P < 0.05, **P < 0.01, ***P < 0.001 were considered significant. NS, not significant. See Statistics Source Data in Supplementary Table 6.
To further examine how reduced maternal insulin-like signalling via the RAS–ERK pathway modifies progeny physiology we performed RNA-seq of wild-type and lin-45 mutant embryos. We identified a total of 616 genes upregulated more than twofold and 1,310 genes downregulated more than twofold in lin-45 mutant embryos (Supplementary Table 5). Among the 616 upregulated genes was gpdh-2, and we confirmed that gpdh-2 messenger RNA expression is upregulated approximately threefold in lin-45 mutant embryos using quantitative PCR with reverse transcription (qRT–PCR) (Fig. 3d).

To test whether increased GPDH-2 expression results in increased glycerol production, we compared the glycerol-to-glucose ratios in wild-type and lin-45 mutant embryos by mass spectrometry. We observed an 82% increase in the glycerol-to-glucose ratio in lin-45 wild-type and lin-45 mutant embryos compared with that in wild-type embryos (Fig. 3e).

These data are consistent with our hypothesis that reduced insulin-like signalling to the maternal germline protects progeny from the effects of osmotic stress by increasing the expression in embryos of the rate-limiting glycerol biosynthetic enzyme GPDH-2.

We note that previous studies found that adaptation to osmotic stress resulted in up to a 1,000% increase in glycerol in C. elegans⁴, significantly higher than the increase we observed in lin-45 mutants. Our genetic data demonstrate that GPDH-2 is required for reduced RAS–ERK signalling in the maternal germline to protect progeny from developmental arrest (Fig. 3a). However, given the modest increase in glycerol levels in lin-45 mutants it remains possible, and perhaps likely, that one or more of the additional 615 genes upregulated in lin-45 mutants also contribute to the resistance of these animals to developmental arrest in response to osmotic stress.

We tested whether parental exposure to other environmental stresses, such as bacterial infection or starvation, could similarly modulate progeny response to environmental stress via RAS–ERK signalling. Exposure of C. elegans to the opportunistic pathogen Pseudomonas aeruginosa PA14 slowed early larval development (Fig. 4a), parental exposure to PA14 enhanced this slowing of larval development (Fig. 4a), and this heritable slowing of larval development in response to bacterial infection required RAS–ERK signalling (Fig. 4a). However, maternal exposure to PA14 did not protect progeny from developmental arrest at 500 mM NaCl (Supplementary Fig. 4b) but rather resulted in progeny that were more sensitive to arrest in response to osmotic stress (Fig. 4b). In addition, we found that RAS–ERK signalling was

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**Figure 4** RAS–ERK signalling regulates C. elegans response to bacterial infection and starvation. (a) Percentage of animals expressing lin-4::YFP after 24 h of exposure to either Escherichia coli OP50 or Pseudomonas aeruginosa PA14. Error bars, s.d. n=3 experiments of >100 animals. (b) Percentage of animals failing to arrest development after 24 h at 350 mM NaCl. Error bars, s.d. n=3 experiments of >100 animals. (c) Percentage of wild-type, lin-45(n2018) and daf-16(mu86) mutants with a divided M-cell after 7 days without food in S-basal at 20°C. Error bars, s.e.m.

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n=4 experiments of >100 animals. (d) Model for how maternal exposure to osmotic stress inhibits DAF-2 activity in the germline and affects progeny response to osmotic stress. See text for details. Red, embryo; green, germline; purple, intestine. The quantified results are presented as mean ± s.d. (a,b) and s.e.m. (c) using ANOVA (a) and two-tailed t-test (b,c). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were considered significant. NS, not significant. See Statistics Source Data in Supplementary Table 6.
required for L1 arrest in response to starvation (Fig. 4c). Collectively, these results suggest that maternal exposure to environmental stress modifies progeny physiology via RAS–ERK signalling, but that the effects of these stresses on progeny are different for different environmental stresses.

In conclusion, we propose a model in which the inhibition of insulin-like signalling to both the intestine and the germline can enhance C. elegans survival during osmotic stress but in which the effects of inhibition of insulin-like signalling to these two tissues are distinct. Specifically, the loss of insulin-like signalling to the intestine enhances resistance to osmotic stress by promoting developmental arrest, whereas the loss of insulin-like signalling to the maternal germline enhances progeny resistance to osmotic stress by increasing glycerol synthesis in embryos (Fig. 4d). In this model, information about the maternal environment is inherited via germ cells to enhance progeny resistance to future environmental stress.

The salt concentrations at which C. elegans arrests development in response to osmotic stress are approximately those of seawater, 480 mM Na\(^+\) and 559 mM Cl\(^-\) (ref. 22). We speculate that both the state of immobile arrested development and the ability of parents to confer progeny resistance to osmotic stress evolved to enhance organisal survival in response to osmotic stress caused by seawater. The insulin signalling pathway is broadly conserved among metazoa, and we postulate that insulin signalling to the germline plays a role in several human developmental and metabolic abnormalities known to result from abnormal insulin signalling, such as intrauterine growth restriction, obesity and type-2 diabetes, all of which have been linked to maternal environmental stress\(^8,23\). Consistent with this hypothesis, a recent report found that feeding parental mice a high-fat diet causes epigenetic changes to oocytes that result in progeny that are more susceptible to both obesity and diabetes\(^7\). These observations of a maternal high-fat diet modifying progeny physiology via oocytes in mice are similar to our observations of maternal exposure to osmotic stress modifying progeny physiology via oocytes in C. elegans. We propose that modified insulin-like signalling to the mouse germline might be the mechanism underlying these epigenetic changes in mouse oocytes.

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METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of this paper.

ACKNOWLEDGEMENTS

We thank E. J. Hubbard, K. Ashrafi, S. Mitani and the Caenorhabditis Genetic Center, which is funded by the NIH National Center for Research Resources (NCRR), for providing strains; N. An for strain management; and K. Burkhart, S. Luo, A. Doi, N. Paquin and A. Corrionero for helpful discussions. H.R.H. and N.O.B. were supported by NIH grant GM024663 and NSF grant 1122374. T.F. and S.A. were supported by NIH grant GM98200 and ACS grant RSG014-044-DCD. L.R.B., A.K.W. and R.E.W.K. were supported by NIH grant GM117408. H.R.H. is an investigator of the Howard Hughes Medical Institute.

AUTHOR CONTRIBUTIONS

N.O.B., T.F., A.K.W., R.E.W.K., S.A., L.R.B. and H.R.H. designed the experiments and analysed the data. N.O.B., T.F., A.K.W., R.E.W.K. and S.A. performed the experiments. N.O.B. and H.R.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3470

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METHODS

Strains. All C. elegans strains were cultured as described previously\(^1\) and maintained at 20 °C unless noted otherwise. The Bristol strain N2 was the wild-type strain. Mutations used are:

\[\text{LGI: } \\
\text{dpy-15(ok1477); gmr::mCherry; prd-1[+]; nIs349 (Pceh-2::daf-16::GFP::nos-2 3'UTR, unci-119(+))}; \text{ gpdh-2(ok1733)} \]

\[\text{LGK: } \\
\text{ins-18(ok1672); dpy-15(ok1477); gmr::mCherry; prd-1[+]; nIs349 (Pceh-2::daf-16::GFP::nos-2 3'UTR, unci-119(+))}; \text{ gpdh-2(ok1733)} \]

Assay for developmental arrest. Approximately 200 developing eggs from mothers grown at 50 mM NaCl (unless otherwise noted) were collected and placed on standard NGM plates containing 300 mM NaCl at 20 °C for 24 h. Percentage failure to arrest is defined by the percentage of animals that failed to develop past the L1 larval stage. All males contained dpy-15(ok1477); gmr::mCherry; prd-1[+]; nIs349 (Pceh-2::daf-16::GFP::nos-2 3'UTR, unci-119(+)); gpdh-2(ok1733)

Assay for survival after arrest. Approximately 100 developing eggs from mothers grown at 50 mM NaCl were collected and placed on standard NGM plates containing 500 mM NaCl and allowed to recover for 24 h. After 24 h the fraction that regained mobility and resumed development were scored as surviving and the fraction that failed to resume development and did not respond to touch were assumed to be dead.

DAF-16::GFP localization. Confocal microscopy was performed using a Zeiss LSM 800 instrument. The resulting images were prepared using ImageJ software (National Institutes of Health). Image acquisition settings were calibrated to minimize the number of saturated pixels and were kept constant throughout the experiment.

mRNA expression analysis by RNA-seq and qRT–PCR. L4-stage wild-type and lin-45 animals were placed on standard NGM plates containing various concentrations of NaCl at 25 °C for 48 h. After 48 h, animals that remained immobile and were not feeding were scored as arrested. Mobile animals that were feeding were scored as developing. Percentage of animals mobile and feeding (unlike animals normally arrested in response to osmotic stress) but includes L1-stage larvae.

mRNA expression analysis by RNA-seq and qRT–PCR. L4-stage wild-type and lin-45 animals were placed on standard NGM plates containing various concentrations of NaCl at 20 °C for 24 h. After 24 h arrested animals were picked onto plates containing 50 mM NaCl and allowed to recover for 24 h. After 24 h the fraction that regained mobility and resumed development were scored as surviving and the fraction that failed to resume development and did not respond to touch were assumed to be dead.

DAF-16::GFP localization. Confocal microscopy was performed using a Zeiss LSM 800 instrument. The resulting images were prepared using ImageJ software (National Institutes of Health). Image acquisition settings were calibrated to minimize the number of saturated pixels and were kept constant throughout the experiment.

mRNA expression analysis by RNA-seq and qRT–PCR. L4-stage wild-type and lin-45 animals were placed on standard NGM plates containing either 50 mM or 300 mM NaCl at 20 °C for 44 h. Developing eggs from these animals were collected and placed at either 50 mM or 500 mM NaCl for 6 h. After 6 h embryos were collected in M9, and RNA was extracted using TissueRuptor and the RNeasy Mini kit (QIAGEN). Metabolite preparation for quantification. Approximately 100 µl of concentrated embryos were collected by egg preparation and placed on normal NGM agar plates for 3 h to recover. After 3 h, embryos were collected in M9, pelleted, and frozen. Frozen embryos were resuspended in 400 µl PBS and homogenized by douncing.

Pathogen exposure and development assay. Wild-type embryos were placed onto either NGM plates seeded with Escherichia coli OP50 or slow-killing assay plates seeded with P. aeruginosa PA14 and allowed to grow at 25 °C for 72 h. After 72 h embryos were collected from adults and placed on new plates seeded with either OP50 or PA14 and placed at 25 °C.

M-cell division in response to starvation. M-cell division analysis was performed as described previously\(^2\). Briefly, preparations were performed within 5 min of adding levamisole to achieve optimal phosphorylated MPK-1 (dpmpk-1) staining. The dissected germines were then fixed in 3% paraformaldehyde for 10 min, followed by a post-fix in 100% methanol at −20 °C. The fixed germ larval tissues were then processed for immunofluorescence staining as described previously\(^2\). Anti-MAP kinase was used at a dilution of 1:200 (Clone MAPK-YT, Sigma). Secondary antibodies were donkey anti-mouse Alexa Fluor 594 and used at a dilution of 1:400. Each gonad was photographed as a montage, with each image taken as a 0.15 μm section and captured with overlapping cell boundaries at ×63 magnification.

Phenotype analysis of C. elegans mutants. Wild-type animals at the L4 stage were placed on either 50 mM or 300 mM NaCl NGM plates seeded with OP50 at 20 °C for 24 h, and germ lines were extruded. Dissections were performed as described previously\(^2\). Briefly, dissections were performed within 5 min of adding levamisole to achieve optimal phosphorylated MPK-1 (dpmpk-1) staining. The dissected germ lines were then fixed in 3% paraformaldehyde for 10 min, followed by a post-fix in 100% methanol at −20 °C. The fixed germ larval tissues were then processed for immunofluorescence staining as described previously\(^2\). Anti-MAP kinase was used at a dilution of 1:200 (Clone MAPK-YT, Sigma). Secondary antibodies were donkey anti-mouse Alexa Fluor 594 and used at a dilution of 1:400. Each gonad was photographed as a montage, with each image taken as a 0.15 μm section and captured with overlapping cell boundaries at ×63 magnification. Images were taken using a Zeiss Axio Imager upright microscope with AxioVision V4.8.2.0 microimaging software and an Axio MRm camera (Zeiss).

Metabolite preparation for quantification. Approximately 100 µl of concentrated embryos were collected by egg preparation and placed on normal NGM agar plates for 3 h to recover. After 3 h, embryos were collected in M9, pelleted, and frozen. Frozen embryos were resuspended in 400 µl PBS and homogenized by douncing.
Homogenized embryos were centrifuged at 800g for 2 min to remove undounced tissue and 200µl of supernatant was mixed with 800µl of methanol and dried to extract polar metabolites. Dried samples were stored at −80°C.

**Metabolite profiling.** Liquid chromatography and mass spectrometry were performed as described previously.

**Assay for L2-, L3- and L4-stage developmental arrest.** Approximately 50 L2-, L3- or L4-stage animals were placed onto standard NGM Petri plates containing 500 mM NaCl for 24 h. After 24 h animals that were immobile and not developing or responding to touch were moved to plates containing 50 mM NaCl. Percentage developing is defined by the percentage of animals that resumed development and mobility after returning to normal growth conditions.

**MEK inhibitor exposure.** The MEK inhibitor U0126 (U120 Sigma-Aldrich) was resuspended in dimethylsulfoxide and added to standard NGM Petri plates at a final concentration of 100 μM. These plates were then seeded with *Escherichia coli* OP50. Wild-type embryos were placed on plates containing either dimethylsulfoxide alone or dimethylsulfoxide and the MEK inhibitor U0126. Animals were allowed to grow for 72 h at 25°C. After 72 h, embryos from adult animals were collected and placed onto Petri plates containing 500 mM NaCl for 24 h and the fraction of animals mobile and developing was determined.

**mek-2 RNAi exposure.** Wild-type embryos fed *Escherichia coli* HT115 containing either the empty vector L4440 or a mek-2 RNAi vector (Ahringer library—Source Biosciences) were grown at 20°C for 72 h. After 72 h, embryos were collected and placed onto Petri plates containing 500 mM NaCl for 24 h at 25°C and the fraction of animals mobile and developing was determined.

**Assay for daf-2 adaptation to osmotic stress.** L4 animals were placed on standard NGM plates containing 300 mM NaCl at 25°C overnight. Embryos from resulting adult animals were extracted with a razor blade into M9 solution and pipetted onto plates containing 500 mM NaCl. These animals were allowed to develop for 48 h at 20°C. Percentage of L2+ is defined by the percentage of animals that developed past the L1 larval stage.

**Data availability.** RNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession codes GSE91073 and GSE91039. Data supporting the findings of Figs 1b,c,d, 2a,b,d, 3a,b,d,e and 4a,b,c and Supplementary Fig. 1b,c,d,e,f,g, Supplementary Fig. 3b,d,e,f and Supplementary Fig. 4b,c,d are provided in Supplementary Table 6. All other relevant data are available from the authors on request and/or are included with the manuscript.

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Supplementary Figure 1 *C. elegans* arrests development in response to osmotic stress. (a) Representative images of wild-type animals after 72 hrs of exposure to 50 mM or 500 mM NaCl. Experiment replicated 12 times. Scale bar 200 μm (b) Percent of wild-type animals developing past the L1 larval stage after 24 hrs of exposure to 50 mM NaCl and 450 mM KCl. Error bars, s.d. n = 3 experiments of >100 animals (c) Percent of wild-type animals developing past the L1 larval stage after 24 hrs of exposure to 50 mM NaCl and 450 mM NaBr. Error bars, s.d. n = 3 experiments of >100 animals (d) Percent of wild-type animals developing past the L1 larval stage after 24 hrs of exposure to 50 mM NaCl and 900 mM sucrose. Sucrose was added at twice the concentration of salts to compensate for the difference in osmolarity. Error bars, s.d. n = 3 experiments of >100 animals (e) Percent of wild-type, pmk-1(km25), or skn-1(zu67) animals developing past the L1 larval stage after 48 hrs of exposure to 300 mM NaCl. Error bars, s.d. n = 3 experiments of >100 animals (f) Percent of wild-type, pmk-1(km25), or skn-1(zu67) animals failing to arrest development at 500 mM NaCl after 48 hrs. Error bars, s.d. n = 3 experiments of >100 animals (g) Percent of wild-type and unc-31(ft1) animals failing to arrest development after 48 hrs. The osm-6 promoter was used to drive the expression of UNC-31 specifically in sensory neurons. Error bars, s.d. n = 3 experiments of >100 animals (h) Percent of wild-type and ins-3(tm3608) mutant animals failing to arrest development at 400 mM NaCl after 48 hrs. Error bars, s.d. n = 3 experiments of >100 animals (i) Percent of wild-type, ins-3(ok2488); ins-3(ok2488); daf-2(e1370) and rescue animals failing to arrest development at 400 mM NaCl 48 hrs post-hatching. Error bars, s.d. n = 3 experiments of >100 animals. The variation between the wild type in panels (a) and (b) is likely to be a result of variations in evaporation between different batches of Petri plates. The quantified results are presented as mean ± s.d. using two-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were considered significant. n.s., not significant. See Statistics Source Data in Supplemental Table 6.
**Supplementary Figure 2** Comparison of gene expression in response to osmotic stress and starvation. (a) Venn-diagram of genes that exhibit statistically significant changes in gene expression in response to osmotic stress and starvation. (b) Scatter plot of gene expression changes for 1605 genes whose expression is regulated by both osmotic stress and starvation.
Supplementary Figure 3: Insulin-like signalling to the germline regulates developmental arrest in response to osmotic stress. Percent of wild-type and gpdh-1(ok1558); gpdh-2(ok1733) animals developing past the L1 larval stage after 48 hrs. Error bars, s.d. n = 3 experiments of >100 animals (b) Percent of wild-type and daf-2(e1370) animals failing to arrest development at 500 mM NaCl. The pie-1 promoter was used to drive DAF-2 expression specifically in the germline. Germline #1 and germline #2 represent two independently integrated transgenes. Error bars, s.d. n = 3 experiments of >100 animals (c) Percent of wild-type and lin-45(n2018) animals developing past the L1 larval stage after 48 hrs. Error bars s.d. n = 3 experiments of >100 animals (d) Percent of progeny from animals exposed to either DMSO or the MEK inhibitor U0126 dissolved in dimethyl sulfoxide (DMSO) developing past the L1 larval stage after 48 hrs (500 mM NaCl). Error bars s.d. n = 3 experiments of >100 animals (e) Percent of progeny from animals exposed to either L4440 empty vector or mek-2 RNAi that developed past the L1 larval stage after 48 hrs (500 mM NaCl). Error bars s.d. n = 3 experiments of >100 animals. The quantified results are presented as mean ± s.d. using ANOVA (b) and two-tailed t-test (d,e,f). **P < 0.01, ***P < 0.001, were considered significant. n.s., not significant. See Statistics Source Data in Supplemental Table 6.
**Supplementary Figure 4** Dense-core vesicle release from sensory neurons regulates MPK-1 activity in the germline. (a) Representative germlines dissected from wild-type or unc-31(ft1) animals, which exhibit reduced dense-core vesicle release, at 50 mM NaCl and stained for DNA (DAPI, white) and diphosphorylated MPK-1 (dpMPK-1) (red). SN::UNC-31(+) animals that expressed a rescuing copy of unc-31 specifically in sensory neurons using the osm-6 promoter. Experiment replicated 4 times. Scale bar 100 μm. (b) Percent of animals mobile and feeding after 48 hrs at 500 mM NaCl. Error bars s.d. n = 3 experiments of >100 animals. We note that the observation that less wild-type animals adapted to 500 mM NaCl when parents were exposed to 300 mM NaCl than in Fig. 2a is likely due to the fact that in this experiment embryos had to be collected by bleaching to remove any contaminating PA14 and this likely added an extra stressor to the embryos.
Supplementary Table Legends

**Supplementary Table 1** Most insulin peptides do not regulate development in response to osmotic stress. Mutant animals that each lacked one of 35 insulin-like peptides were assayed for the percent of animals that developed after exposure to 300 mM NaCl or 500 mM NaCl. WT at 300 mM NaCl: fewer than 1% of animals arresting in response to 300 mM NaCl. WT at 500 mM NaCl: 0% of animals mobile and feeding at 500 mM NaCl. WT, wild-type. The quantified results are presented as mean ± s.d. using two-tailed t-test ***P < 0.001 was considered significant. n.s., not significant. See Statistics Source Data in Supplemental Table 6.

**Supplementary Table 2** Profile of gene expression after 24 hrs of exposure to 500 mM NaCl and starvation. See README tab for detailed description of analysis.

**Supplementary Table 3** Profile of mRNA expression in wild-type embryos at 50 mM NaCl and 500 mM NaCl.

**Supplementary Table 4** Quantification of diphosphorylated MPK-1 levels in wild-type germlines exposed to either 50 mM or 300 mM NaCl for 24 hrs.

**Supplementary Table 5** Profile of mRNAs upregulated and downregulated >2-fold in *lin-45(n2018)* mutant embryos when compared to wild-type embryos.

**Supplementary Table 6** Statistics source data. Table contains individual values for all bar graphs and for quantification for Fig. 1d.

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