Physiological and molecular mechanisms of salt and water homeostasis in the nematode *Caenorhabditis elegans*

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Submitted 28 February 2013; accepted in final form 25 May 2013


Intracellular salt and water homeostasis is essential for all cellular life. Extracellular salt and water homeostasis is also important for multicellular organisms. Many fundamental mechanisms of compensation for osmotic perturbations are well defined and conserved. Alternatively, molecular mechanisms of detecting salt and water imbalances and regulating compensatory responses are generally poorly defined for animals. Throughout the last century, researchers studying vertebrates and vertebrate cells made critical contributions to our understanding of osmoregulation, especially mechanisms of salt and water transport and organic osmolyte accumulation. Researchers have more recently started using invertebrate model organisms with defined genomes and well-established methods of genetic manipulation to begin defining the genes and integrated regulatory networks that respond to osmotic stress. The nematode *Caenorhabditis elegans* is well suited to these studies. Here, I introduce osmoregulatory mechanisms in this model, discuss experimental advantages and limitations, and review important findings. Key discoveries include defining genetic mechanisms of osmolarity sensing in neurons, identifying protein damage as a sensor and principle determinant of hypertonic stress resistance, and identification of a putative sensor for hypertonic stress associated with the extracellular matrix. Many of these processes and pathways are conserved and, therefore, provide new insights into salt and water homeostasis in other animals, including mammals.

osmoregulation; cell volume; ion; organic osmolyte; protein homeostasis; model organism

**SALT AND WATER HOMEOSTASIS** is a fundamental requirement for metazoan life. Ion and water transport mechanisms that compensate for changes in composition and volume of intracellular and extracellular compartments have been generally well defined using vertebrate cell and in vivo models (38). Alternatively, the upstream molecular mechanisms that animal cells use to sense deviations in salt and water balance and regulate compensatory responses are still poorly characterized (20). Studies in brewer’s yeast demonstrate that osmotic signal detection and transduction within a single eukaryotic cell can be highly complex with numerous components, acting in parallel pathways, that often cross-talk with other processes (40, 55). Osmotic signal detection and transduction are likely to be even more complex in metazoans, which require homeostasis of both the extracellular and intracellular compartments and integration of responses between cells. Genetics is an extremely powerful approach for identifying and characterizing genes and proteins that function in complex biological processes but has been underutilized in the field of osmosensing and signal transduction in metazoans. Invertebrate models, such as *Caenorhabditis elegans* and *Drosophila*, are ideally suited to genetic analysis, and there is a long history of fundamental discoveries in cell biology, animal development, and behavior coming from studies on these species.

A series of studies over the last decade have begun to characterize osmoregulation in *C. elegans* and exploit its experimental advantages to provide novel insights into the molecular nature of extracellular and intracellular osmosensors, signal transduction, cellular hypertonic stress damage, and mechanisms of protection. Considerable progress has been made since mechanisms of osmosensing and signal transduction were last reviewed in *C. elegans* (19). Here, advantages and limitations of *C. elegans* as a model for salt and water homeostasis are summarized, basic aspects of osmoregulation and avoidance are introduced, recent findings are reviewed, and opportunities for future investigation are proposed. Given the fundamental requirement for salt and water homeostasis, pathways and principles discovered in *C. elegans* are likely to be conserved among metazoans.

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Experimental advantages and limitations of *C. elegans*

*C. elegans* is one of the most powerful model organisms for defining the molecular bases of cellular and physiological processes in animals (8, 112, 120). This free-living nematode has several complex and integrated organ systems, but is small (~1 mm) and comprises fewer than 1,000 somatic cells making it a relatively simple model of metazoan physiology. Experimental advantages include a short life cycle (3–4 days), optical clarity at all developmental stages, large numbers of offspring, sexual reproduction, and inexpensive laboratory culture (97). Isolation and maintenance of homozygous strains is straightforward because the vast majority of individuals are hermaphrodites that reproduce by self-fertilization. Males can also be generated at a low frequency to exchange mutations or transgenes between strains.

A 50-yr history of *C. elegans* as an experimental model has yielded a fully sequenced and well-annotated genome, a detailed anatomical and ultrastructural atlas, an invariant fate map for all somatic cells, thousands of mutant and transgenic strains, and a wealth of information on genetics, development, physiology, and behavior. Genomic sequence and biological information for *C. elegans* are assembled in open online databases (119), and mutant worm strains and clones spanning the genome are available through public resources at nominal cost (111). Whole genome DNA microarray chips or RNA sequencing can be used to monitor global gene expression (60, 78, 96, 104). Creation of transgenic worms is relatively easy, rapid, and inexpensive requiring either injection of transgenes into a hermaphrodite’s gonad or bombardment with DNA-coated microparticles (9, 44, 76, 93). Single-copy gene insertions at defined chromosome locations can be generated using transposable elements (28–29).

The culture and reproductive characteristics of *C. elegans* make it one of the most powerful animal models for forward genetics (46). Random mutagenesis and screening in *C. elegans* has been used to identify hundreds of genes that control complex biological processes and to define relationships between protein structure and function. The genome of *C. elegans* contains a similar number of genes as the genome of mammals (~20,000), but it is far more compact at 100 Mb vs. 2.5 Gb for mice (110). A small genome combined with dropping costs of next-generation DNA sequencing has revolutionized the way that mutations are identified following a genetic screen. Sequencing and bioinformatic mapping of alleles have also been generated at a low frequency to exchange mutations or transgenes between strains.

**Review**

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**Osmoregulatory Organ Systems**

A comprehensive description of *C. elegans* anatomy and organ function is available elsewhere (3). Here, the few organ systems with known or suspected osmoregulatory functions are described briefly. Not surprisingly, the “skin”, or hypodermis, and intestine have been shown to play important roles in osmoregulation. The hypodermis underlies a thick extracellular cuticle composed largely of collagen (Fig. 1). Genes encoding signaling proteins and collagens that play roles in regulating volume and responses to high osmolarity are expressed in the hypodermis (see below). In adult worms, 20 epithelial cells make up the intestine, which functions in digestion, nutrient absorption, energy storage, and detoxification. Genes responsible for organic osmolyte synthesis are induced in the intestine during acclimation to high osmolarity (61).

The entire excretory system of *C. elegans* is composed of three cells, the excretory cell, the duct cell, and the pore cell (88). The excretory cell is a remarkably complex and highly specialized “H”-shaped epithelial cell that forms two parallel canals running almost the entire length of the animal (Fig. 1, A–D). Its nucleus and cell body are in the head adjacent to the base of the pharynx (Fig. 1D). From the nucleus, the cell bifurcates laterally into two main branches that each bifurcate again into long anterior and posterior processes (Fig. 1, A and B). In a transmission electron micrograph cross section, it can be seen that one side of the canal borders the hypodermis and...
the other side borders the extracellular fluid of the pseudocel- 
lom (Fig. 1C). A “lumen” is formed in the center of the canal 
that is highly invaginated with canaliculi (Fig. 1C) (10, 88).
The excretory cell expresses numerous channels and transport-
ers, including Cl⁻/H⁺ exchangers, H⁺-ATPase, anion exchangers, and aquaporins (42, 86–87, 91, 103); the 
fluorescence in Fig. 1B is from a green fluorescent protein 
(GFP) transgene that is driven by the promoter for clh-4, a 
ClC-type chloride channel homolog (86). The lumen of the 
excretory cell is connected to the outside environment through 
short duct and pore cells, which emerge through the cuticle 
near the base of the pharynx (Fig. 1D).

Laser ablation of any one of the three cells making up the 
excretory system causes worms to fill with fluid and die, 
suggesting a role in water homeostasis (89). Detailed measure-
ments of excretory cell function have not been made because of 
technical obstacles caused by the small size of C. elegans.

Methods such as using ion-selective, self-referencing probes 
could make it possible to measure fluxes of specific ions (95).
If so, then the excretory system would become a simple and 
genetically tractable model in which to study signaling path-
ways that regulate salt and water transport.

Although transport function is largely unexplored, the 
excretory cell has been an important model for understanding 
mechanisms of unicellular tubule formation. Several mutant 
strains with aberrant excretory canal morphology have been 
isolated and used to investigate tubule formation during normal 
development (e.g., 1–2, 10–11, 32, 37); these studies have 
identified cytoskeletal, signaling, and transport proteins as 
being essential for formation or maintenance of the canals. 
Recent studies have demonstrated that the excretory canals are 
extended when worms are shifted between high and low 
osmolarity (52, 56), implying that osmotic pressure and water 
flux may play important roles in remodeling the excretory
system to match needs dictated by environmental osmolarity (102).

**TRP Channels and Behavioral Avoidance of High Osmolarity**

The natural ecology and habitat of *C. elegans* are poorly defined, but it is clear that they feed, grow, and reproduce in nutrient- and microorganism-rich organic material such asrotting fruit (54). When a food source is consumed or overcrowded, they enter a diapause state called a dauer larva and disperse by crawling through soil or by attaching to larger motile invertebrates. The salt and water content of their natural microhabitats likely fluctuate tremendously. The initial response of a worm when encountering a steep rise in environmental osmolarity is to avoid it by reversing direction, a behavior called “osmotic avoidance” (22). *C. elegans* is attracted to low concentrations of many chemicals associated with food, including sugars and salts, but will avoid these same solutes if they are concentrated enough to generate high osmotic pressures (22). *C. elegans* detects high osmolarity with a pair of amphids, which are sensory structures that contact the environment through pores in the cuticle on both sides of the mouth at the far anterior end of the worm (6). The anatomy and function of amphids are covered in detail elsewhere (3, 6, 43). Briefly, each of the paired anterior amphid pores is filled with the ciliated endings of sensory neuron dendrites (Figs. 2, A–C). The dendrites and cell bodies of six pairs of amphid sensory neurons can be visualized by incubating live worms with the lipophilic fluorescent dye, DiI, which enters through the aphid pore (Fig. 2A). Amphid neuron dendrites connect to cell bodies near the base of the pharynx, and axons extend out from the cell body and synapse with the central nervous system (Fig. 2, A–C).

Laser ablation studies identified a specific pair of sensory neurons termed ASH as being required for osmotic avoidance (Fig. 2, A and B) (7). If wild-type worms are placed on the surface of an agar plate in the middle of a ring of high osmolarity, they will not cross the ring for several minutes until the osmolyte diffuses into the agar. This simple assay was used to screen large populations of randomly mutagenized worms to identify numerous “osm” mutants that are defective for the avoidance behavior (22). One class of osm mutations disrupted amphid neuron development. Another class of osm mutations was found to cause constitutive accumulation of organic osmolytes to high levels (118) (e.g., osm-7, osm-8, and osm-J; see below in Extracellular signaling controls organic osmolytes and osmosensitive gene expression), suggesting that a steep osmotic gradient between environment and worm is required to initiate avoidance. Another class of osm mutations mapped to genes that are thought to be directly involved in osmosensing and signal transduction in ASH neurons (e.g., osm-9, ocr-2, and osm-10).

Strains with mutations in osm-9 and ocr-2 have defects in behavioral avoidance of hypertonicity, noxious chemicals, and mechanical touch of the nose (6). OSM-9 was the first member of the transient receptor potential-vanilloid (TRPV) subfamily of cation channels to be identified (21); OCR-2 is also a TRPV subfamily member (116). TRP channels are a large group of transmembrane proteins that function in sensory signal transduction, epithelial transport, and Ca2+ signaling (90). Transgenic GFP reporter analysis indicates that OSM-9 and OCR-2 are both expressed in the cilia of a set of amphid neurons, including the ASH (21). Dimerization is often required to generate functional TRP channels. Loss of either OSM-9 or OCR-2 prevents the other protein from localizing and functioning properly in amphids, suggesting that they function as a heterodimer and/or in a larger complex with additional proteins (Fig. 2D) (116).

Neither OSM-9 nor OCR-2 has been functionally characterized by electrophysiology, and, therefore, the molecular mechanism of osmosensing is not completely known. However, mechanistic insights were gained from complementation studies with a mammalian TRPV channel. TRPV4, a mammalian volume-sensitive cation channel (66), complemented loss of
osm-9 when expressed in C. elegans ASH neurons (67, 69). TRPV4 is expressed in circumventricular organs of the mammalian central nervous system, which function analogously to ASH neurons to detect noxious stimuli, including high osmolality (68). Transgenic expression of a mutant form of TRPV4 that does not function as a channel failed to complement loss of osm-9, suggesting that cation conductance is required to initiate the avoidance behavior in C. elegans (69). Interestingly, osm-9 and ocr-2 are also required for withdrawal from light touches to the nose, and TRPV4 also complements loss osm-9 in this mechanoreceptor-mediated behavior (69). Consequently, it has been hypothesized that mechanical and osmotic signals may directly activate cation flux through a TRPV channel composed of OSM-9 and OCR-2, likely by distortion of the lipid bilayer or the underlying cytoskeleton. In vivo measurements of worms expressing a genetically encoded Ca\(^{2+}\)-sensitive GFP have confirmed large intracellular Ca\(^{2+}\) transients during stimulation of sensory modalities that require osm-9 (36).

Other genes have also been implicated in osmotic avoidance. A Go protein named ODR-3 is expressed in ASH neurons and is required for all behaviors that require OSM-9 and OCR-2 (Fig. 2D) (98). Genetic and biochemical studies have also demonstrated a role for long-chain polyunsaturated fatty acids upstream from Ca\(^{2+}\) transients in ASH neurons (47). Interestingly, a novel gene named osm-10 was found to be required for osmotic avoidance but not other ASH sensory modalities, suggesting that it could play a role in determining specificity (35).

Volume Recovery and WNK/GCK VI Signaling

Nematodes have an outer cuticle that is predominantly composed of a complex weave of collagen proteins. The nematode cuticle is an effective barrier for many toxins and composed of a complex weave of collagen proteins. The nematode species indicate that the internal osmolarity of nematodes is maintained slightly higher than the external environment (30). If the external osmolarity is manipulated, internal osmolarity changes so that an internal turgor pressure is maintained (30).

In the laboratory, C. elegans is typically cultured on the surface of agar Petri dishes with a NaCl concentration of 51 mM; other ionic and organic solutes make up a minority of the total osmolarity (111). Direct transfer from high to low osmolarity causes swelling and temporary paralysis presumably because of elevated turgor pressure (42, 52). After ~10–15 min, swelling subsides and motility is regained by most individuals. Occasionally, some adult worms actually burst with the intestine and gonads protruding through the vulva, an opening in the cuticle that is used to lay eggs (52). Aquaporins and the excretory system have been suggested to play a role in volume decrease (42, 52), but the mechanism of volume recovery from swelling remains largely uncharacterized.

Remarkably, over 90% of individual C. elegans can survive direct transfer from 51 up to 400 mM NaCl (17–19, 107). Approximate timing of physiological changes that occur following direct transfer to extreme hypertonicity is shown in Fig. 3. After 20–30 min, whole-animal shrinkage is dramatic with a loss of up to half of total body volume, loss of turgor pressure, a rigid posture, and complete loss of motility (18–19, 107). Two to four hours after transfer, body volume and motility recover (Fig. 3) and the life cycle resumes, although with a reduced number of offspring and slower development (61).

The mechanism for recovery from volume shrinkage is poorly characterized, but one signaling pathway has been shown to play an important role. WNK (with no lysine) and GCK-VI (germlinal center kinase, subfamily seven) protein kinases form a signaling cascade that regulates numerous ion transport processes in mammalian cells, including cation-chloride cotransport in response to volume changes (20, 23, 72, 77). This kinase pathway is conserved in C. elegans (18, 37). Silencing of the single worm WNK (wnk-1) or the single worm GCK-VI (gck-3) was shown to reduce volume recovery and long-term survival of hypertonicity (18). By targeting RNAi to specific tissues, it was shown that gck-3 likely functions in the hypodermis and intestine to mediate volume recovery and survival of hypertonicity (18). A model was proposed in which WNK-1 and GCK-3 regulate ion-absorptive mechanisms in these tissues (Fig. 1F), which are in direct contact with the environment. The only known direct phosphorylation substrate for GCK-3 is CLH-3, a volume-sensitive Cl\(^{-}\) channel that is inhibited by GCK-3 (24, 27, 82). It is not known whether CLH-3 functions in volume recovery.

These C. elegans studies were the first to directly demonstrate that a GCK-VI family member is important to whole-animal osmotic homeostasis (18). More recently, genetic manipulations in mice have demonstrated a role for the mammalian GCK-VI kinases SAPK and OSR1 in regulating blood

![Fig. 3. Time courses for C. elegans responses to hypertonic stress. Whole body shrinkage, inhibition of protein synthesis, and induction of gpdh-1 mRNA occur within 30 min after transfer to hypertonic agar. Damage leading to polyglutamine aggregation also occurs in less than 30 min, but full aggregation takes several hours. Recovery of volume typically occurs within 4–6 h. Lastly, accumulation of glycerol reaches an elevated steady state after about 1 day of exposure.](image-url)
pressure (70, 94). Recent studies of *C. elegans* gck-3 mutants have broadened GCK-VI function to include multiple developmental processes, including epithelial tube morphogenesis and spermatogenesis (37, 58). Further work in *C. elegans* will undoubtedly provide more insights into functions and molecular substrates of this conserved volume-sensitive signaling cascade.

Despite the simplicity with which volume recovery can be induced and observed in *C. elegans*, there is still very little known about the underlying mechanisms that this animal uses to achieve dramatic volume recovery. It remains a highly tractable, but underutilized, model for dissecting genetic pathways that control ion and water transport in animals.

**Extracellular Signaling Controls Organic Osmolytes and Osmosensitive Gene Expression**

If *C. elegans* remains in a high osmolarity environment for several hours, it will synthesize and accumulate high levels of the organic osmolyte glycerol, a small polyol (19, 59). The functions of osmolytes like glycerol have long been assumed to include balancing high environmental osmolarity and stabilizing protein structure as a chemical chaperone (53, 121). Glycerol synthesis is essential for long-term growth and reproduction of *C. elegans* on agar plates containing elevated NaCl (61). Transcriptional induction of the gene encoding GPDH-1 (glycerol-3-phosphate dehydrogenase-1) is at least partially responsible for glycerol synthesis during hypertonic stress. GPDH-1 catalyzes the rate-limiting step of glycerol synthesis. The *gpdh-1* gene is expressed at low levels in worms on standard agar but is induced several-fold within the first hour of exposure to nonlethal high osmolarity (59, 61–62) (Fig. 3). Induction of *gpdh-1* is specific to high osmolarity and does not occur during heat shock, oxidative stress, or endoplasmic reticulum stress (61).

The sensors and signal transduction pathways that respond to high osmolarity and activate osmosensitive gene expression in animal cells are poorly defined. The robust and specific induction *gpdh-1* in *C. elegans* is a powerful system for identifying genetic components of these pathways. Using a transgenic strain that expresses GFP under control of the *gpdh-1* promoter and a feeding RNAi library, ~16,000 genes were screened for regulators of *gpdh-1* (61). The 122 genes found to regulate *gpdh-1* are predicted to function in diverse processes, including extracellular matrix, signaling, metabolism, protein trafficking, transcription, and protein homeostasis, suggesting that osmosensitive gene expression is under complex regulation. Five pathways identified by this genetic screen are discussed below and summarized in Fig. 4.

Four of the extracellular matrix genes identified in the RNAi screen encode collagens, *dpy-7*, *dpy-8*, *dpy-9*, and *dpy-10*. Mutations in these genes were confirmed to cause constitutive accumulation of glycerol under standard culture conditions (61). Over 170 distinct collagens are thought to comprise the extracellular cuticle of *C. elegans*, which functions as a flexible barrier and exoskeleton (92). Mutations in *dpy-7*, *dpy-8*, *dpy-9*, and *dpy-10* also cause a shortened body morphology known as the dumpy phenotype (*Dpy*) (92). Mutations in ~10% of cuticle collagens are known to cause Dpy (75), but only some of these mutations also cause glycerol accumulation (118). Specificity may reside in repeated, circumferential indentations in the outer surface of the cuticle called annular furrows (Fig. 1, E and F). Mutations in five *dpy* collagens were shown to disrupt furrow formation, and at least three of these (*dpy-7*, *dpy-8*, and *dpy-10*) are known to also activate glycerol accumulation (61, 75, 118). *Dpy-7* and *Dpy-10* proteins have been localized to annular furrows (Fig. 1F) (75). During larval development, nematodes synthesize a new cuticle before each of four molts (45). During cuticle synthesis, circumferential bundles of actin filaments form in the hypodermis underneath each furrow and are hypothesized to function as anchoring points for furrow formation (75, 92). Taken together, these data suggest that a yet to be identified osmotic sensor complex is formed in or between the hypodermis and cuticle, possibly associated with annular furrows. Disruption of annular furrows by collagen mutations presumably mimics changes to the sensor that occur with high environmental osmolarity and activates downstream signaling mechanisms.

The RNAi screen also identified 10 genes that are predicted to encode secreted proteins as negative regulators of *gpdh-1* expression (61). Mutant strains for three of these genes (*osr-1*, *osm-7*, and *osm-11*) have been characterized, and all three were confirmed to cause constitutive accumulation of glycerol (107, 118). A later study characterized another gene encoding a predicted secreted protein, *osm-8*, as also functioning to suppress *gpdh-1* expression and glycerol accumulation (100). Mutant strains for all four of these genes, *osr-1*, *osm-7*, *osm-8*, and *osm-11*, are also defective for osmotic avoidance, a phe-
notype presumably caused by elevated systemic glycerol that reduces ASH neuron shrinkage. All four of these genes are expressed in the hypodermis, although in some cases not exclusively (57, 100, 107), suggesting that they could function with, or downstream from, the cuticle (Fig. 1C). Whole genome cDNA microarray studies have confirmed that, in addition to gpdh-1, mutations in osr-1, osm-7, osm-8, and osm-11 constitutively activate many genes that are responsive to high osmolarity, indicating that they mimic signaling changes that normally occur with high osmolarity (99).

A recent study characterized the role of osm-8 in control of osmosensitive gene expression (100). OSM-8 contains a domain with similarity to human mucin 2 (Muc2). Muc2 is a large, heavily glycosylated protein that is secreted on the mucosal surface of many epithelial cells, where it forms a protective and lubricating gel (34). Expression was driven in specific tissues to determine the site of OSM-8 function. Importantly, expression of osm-8 from the hypodermis, but not other tissues, was sufficient to repress gpdh-1 expression in an osm-8 mutant strain, suggesting that OSM-8 functions by being secreted into the extracellular space facing the cuticle (100) (Fig. 1F). Similar to many cuticle collagen, expression of osm-8 was shown to oscillate during larval development and then to be permanently downregulated after the final molt (100). Furthermore, using a heat-shock inducible promoter to temporally control induction, the authors demonstrated that expression of osm-8 before the final molt, but not during adulthood, was sufficient to regulate gpdh-1. Unlike dpy-7, dpy-8, and dpy-10 mutations, the osm-8 mutation does not cause any obvious changes in the structure of the cuticle or body morphology that can be observed with light microscopy (100). Taken together, these data suggest that OSM-8 could be critical for establishing an extracellular osmotic sensor before, or during, the final larval molt. Further work is clearly needed to understand the nature of this putative extracellular osmotic sensor and to determine whether OSM-8 interacts with cuticle collagens.

To identify downstream components of the osm-8 pathway, an RNAi screen for gene inactivations that suppress expression of a gpdh-1 GFP reporter in an osm-8 mutant genetic background was performed (100). Loss of ptr-23 (patched-related protein 23), a member of a protein family that functions as a transmembrane receptor for secreted Hedgehog ligands, was found to strongly suppress expression of gpdh-1 and other osmotically induced genes (100). These findings place ptr-23 downstream from osm-8 (Fig. 4), but it is not known whether, or how, the proteins they encode interact. Importantly, ptr-23 is not required for glycerol accumulation or gpdh-1 induction during exposure to high osmolarity, suggesting the presence of parallel and redundant pathways (100) (Fig. 4).

OSR-1 shares no obvious similarity with proteins outside of the nematode phylum. Similar to osm-8, osr-1 expression from the hypodermis, but not other cells, is sufficient to rescue osmotic phenotypes, suggesting a role in the cuticle or as a systemic signal (107) (Fig. 1F). Mitogen-activated protein kinases signal organic osmolyte accumulation in yeast and mammals (12, 39). Genetic epistasis analysis suggests that calmodulin-dependent and p38 MAPK cascades may function downstream from osr-1 to mediate osmotic stress resistance (Fig. 4) (107). However, the molecular and biochemical interactions between OSR-1 and these kinases have not been defined (107). Additionally, the p38 MAPK pathway is not required for osmotic stress resistance in an osm-7 mutant background (118), indicating that its role in osmotic stress responses may be limited to a specific branch of signaling (Fig. 4).

The functions of osm-7 and osm-11 in osmotic stress responses are poorly defined, but some insight can be drawn from their recently defined roles in development and behavior (57, 106). These genes encode proteins with similarity to ligands for transmembrane Notch receptors, which are internalized and regulate nuclear gene expression after binding to ligands (57, 106). Loss of osm-11 disrupts development of the vulva, the opening of the uterus, and OSM-11 was shown to be a secreted protein that can interact with the C. elegans Notch receptor LIN-12, which regulates specific tissue differentiation events (57). Relative to standard agar, growth of worms on agar containing 400 mM NaCl decreases immunoreactivity for OSM-11 in hypodermal cells, suggesting that decreased expression may occur during exposure to high osmolarity, which would mimic loss-of-function (106). Further work is needed to define signaling downstream from OSM-7 and OSM-11. However, these studies raise the intriguing possibility that Notch signaling may coordinate adaptive physiological responses to osmotic stress (Fig. 4), which has not previously been implicated in any species (57).

A major remaining question is the identity of the inducible transcription factor that transactivates gpdh-1 and other osmosensitive genes (99). In many mammalian cells, the rel-type transcription factor NFAT5/TonEBP accumulates in the nucleus in response to high osmolarity and orchestrates adaptive gene expression (81). C. elegans does not have a close homolog of this or other rel-type factors. Bioinformatic analysis determined that GATA transcription binding sites are enriched in the regulatory sequences of osmosensitive genes (99). Furthermore, putative GATA binding sites and two GATA factors, elt-2 and elt-3, were shown to be required for activation of the gpdh-1 promoter by high osmolarity and osm-7 and osm-11 mutations. While these findings establish the requirement for these two GATA factors, it is still unclear what role they serve in osmosensitive gene expression. Importantly, elt-2 and elt-3 appear to be generally required for expression of numerous genes in the intestine and hypodermis, respectively (31, 51, 73–74). Therefore, it is unclear whether either factor is induced by osmotic stress like NFAT5/TonEBP or whether they simply serve as tissue specifying cofactors for a yet to be identified osmotically inducible factor. If GATA factors are not osmotically induced, then further genetic suppressor screens and biochemical pull-down studies with osmosensitive promoters may help identify the osmotically inducible transactivation factor in C. elegans. Identification of this factor is an important step toward a comprehensive understanding of how the multiple signaling components represented in Fig. 4 are integrated to control osmosensitive gene expression.

Remarkably, almost all of the progress on defining C. elegans osmotic sensors and signal transduction started with a single genome-wide RNAi screen published seven years ago (61). Numerous components have been identified, laying the foundation for a comprehensive understanding of extracellular osmotic sensing and signal transduction (Fig. 4). Future work should focus on the biochemical nature of the putative extracellular osmotic sensor(s), understanding the interactions between the numerous upstream and downstream signaling components, and identification of the inducible transcription factor. These studies will doubt-
edly yield further insights into fundamental aspects of osmosensing and signal transduction in animals.

Protein Damage as a Sensor and Principle Determinant of Hypertonic Stress Resistance

Interestingly, the genome-wide RNAi screen introduced in the previous section found “protein homeostasis” as the largest functional class of genes regulating gpdh-1 (54 out of 122) (61). This class includes genes that function in multiple steps of RNA and protein metabolism, and their silencing is predicted to cause an increase in damaged or denatured proteins in the cell. A model based on these results was proposed in which hypertonicity leads to an increase in the pool of damaged proteins, which, in turn, acts as a signal for expression of gpdh-1 (61) (Fig. 4). Importantly, damage to cellular proteins by other types of stressors, including heat, did not activate gpdh-1 (61), suggesting that hypertonicity causes a specific type of signal-inducing damage that the cell can discriminate. Specificity may be linked to modulation of synthesis and/or processing of new proteins because silencing of many genes involved in RNA metabolism, translation, and folding of new proteins activated gpdh-1 (61). This study identified cellular damage as a novel sensor for osmotic stress responses and served as the foundation for a series of studies aimed at defining the type of damage caused by hypertonicity and the signaling pathways that activate gene expression in response to damage (see below).

After the screen for regulators of gpdh-1, a separate genomewide RNAi screen was performed to identify genes that are required for survival of acute hypertonic stress (17). Silencing of 40 genes was found to strongly sensitize C. elegans to hypertonic stress. Half of these genes are predicted to function in protein sorting, transport, or degradation. Over 25 years ago, in vitro studies demonstrated that high concentrations of inorganic ions or artificial crowding agents can destabilize protein secondary structure and decrease enzyme activity (108). More recent in vitro studies demonstrated that macromolecular crowding can cause nonnative protein-protein interactions that can promote aggregation (26, 80, 84, 122). However, the fate of proteins during hypertonic stress is largely unknown for intact animal cells, which have complex mixtures of macromolecules, are divided into distinct compartments, and maintain dynamic and active mechanisms of protein turnover and quality control. A strain of worm expressing a fluorescent reporter protein containing an aggregation-prone 35 glutamine repeat was used to demonstrate that cell shrinkage promotes protein aggregation in vivo (17). Transfer of worms to high osmolarity agar plates containing the impermeable solutes NaCl or sorbitol (made with equal total osmotic pressures) caused similar levels of whole-animal shrinkage and aggregation. Alternatively, transfer to agar with high concentrations of the more permeable protein denaturant urea did not, suggesting that conditions associated with cell shrinkage, such as macromolecular crowding, cause aggregation (17) (Fig. 5). A more recent study demonstrated that damage leading to polyglutamine reporter aggregation occurs in as little as 10 min of hypertonicity and that aggregation is not reversible once initiated (13). Hypertonic stress was also shown to disrupt the function of thermally sensitive mutant proteins and cause aggregation of numerous endogenous proteins in vivo (13).

Finally, it was recently shown that cell shrinkage also induces aggregation of fluorescent polyglutamine reporters in mammalian cells demonstrating that aggregation is a general outcome of cell shrinkage that is not specific to C. elegans (83).

An earlier study revealed that preexposure of C. elegans to mild levels of hypertonicity increases resistance to subsequent exposures to extreme levels (59). This acclimation response also decreases hypertonicity-induced aggregation of the polyglutamine reporter, implying that acclimation could be the result of enhanced protein homeostasis (17). A series of experiments were conducted to investigate mechanisms of acclimation (14). A simple mechanism would be glycerol accumulation raising internal osmolarity and preventing shrinkage. Acclimated worms do shrink less than nonacclimated worms when transferred to equivalent high osmolarity agar (14). Glycerol could also function as a chemical chaperone to promote native protein conformations (53). However, several observations indicate that other mechanisms of acclimation are equally, or more, important. For example, acclimation prevents hypertonicity-induced aggregation of the polyglutamine reporter and native proteins better than an osm-11 mutation, even though acclimated worms have less glycerol and shrink more than osm-11 mutants (14). Acclimation also strongly reduced native protein aggregation, even when GPDH function was eliminated by mutations in gpdh genes (14).

Protein homeostasis is maintained by the dynamic interaction of RNA metabolism together with protein synthesis, fold-
ing, assembly, trafficking, disassembly, and degradation. During heat shock, essentially, all cells increase molecular chaperone activity by inducing expression of heat-shock proteins (HSPs) (117). Many HSP genes are also induced by other types of stress (117). However, C. elegans does not activate canonical HSPs during hypertonic stress (99). Alternatively, it was reported that acclimation to hypertonicity strongly and constitutively decreases synthesis of new proteins without increasing the rate of protein degradation (14). Genetic and pharmacological inhibition of protein synthesis also reduced protein aggregation. Therefore, C. elegans appears to decrease the rate of protein synthesis during acclimation to mild hypertonicity. This is thought to reduce protein aggregation and improve resistance during hypertonicity by reducing the input of new proteins that must be managed by the protein homeostasis network (Fig. 5).

Mechanisms used to regulate protein synthesis during hypertonic stress and the downstream signals that link protein homeostasis to osmosensitive gene expression were investigated in a recent study (62). The authors first confirmed that silencing of multiple genes previously found to repress a gpdh-1 reporter (61) reduce protein synthesis and increase expression of endogenous gpdh-1 mRNA (62). They also demonstrated that protein synthesis is reduced nearly 50%, and gpdh-1 mRNA is induced >50-fold, within an hour of transfer to hypertonic agar (Fig. 3), implying the involvement of rapid signaling events. During nutrient deprivation and some other types of stress, yeast and mammalian cells modulate RNA translation by phosphorylation of eukaryotic translation initiation factors (eIFs) via general control nonderepressible two (GCN2) kinase (85). In C. elegans, inhibition of protein synthesis and roughly half of gpdh-1 induction following transfer to hypertonic agar were shown to require the ortholog gcn-2 (62). Interestingly, while investigating downstream signaling mechanisms in an RNAi screen for genes required for gpdh-1 expression, the authors recovered wnk-1, the protein kinase previously found to be required for volume recovery (18). Induction of gpdh-1, but not eIF phosphorylation, requires wnk-1 and gck-3, suggesting that the WNK/GCK-VI cascade may function to activate osmosensitive gene expression downstream from changes in protein synthesis (62). Importantly, these findings are the first to link regulation of ion transport by the conserved WNK/GCK-VI cascade with protein synthesis and osmosensitive gene expression in any species (Fig. 5); future work is needed to determine whether this new function is conserved in vertebrates.

Perspectives and Future Questions

Over the last 50 plus years, C. elegans has been developed into an extremely powerful model for understanding molecular mechanisms of metazoan cell biology, development, and physiology. Although this nematode has multiple osmoregulatory organs and is highly tolerant of extreme and rapid shifts in environmental osmolarity, it has only recently been used as a model for understanding mechanisms of salt and water homeostasis and their regulation. Genetic screens and detailed molecular and biochemical studies conducted in the last seven years have identified numerous genes and pathways involved in detecting osmotic imbalances, surviving hypertonic stress, and transducing signals to activate compensatory responses.

How relevant are these discoveries in C. elegans to other animals? As discussed above, many of the genes identified in C. elegans, and the processes in which they function, are conserved in vertebrates, including humans (Table 1). For example, genetic analysis of osmotic avoidance and functional analysis of OSM-9 helped determine that mammalian TRPV4 functions in detection of osmolarity. WNK1 and PASK/OSR1, the mammalian homologs of WNK-1 and GCK-3, function together to regulate volume-sensitive ion transporters, renal salt transporters, and blood pressure (41), indicating that the function of this signaling cascade in salt and water homeostasis was highly conserved. It remains to be seen whether WNK1 and PASK/OSR1 also play a role in regulating osmotic responsive gene expression as was recently shown in C. elegans (62).

Protein confirmation is determined by fundamental chemical and biophysical principles. Therefore, it is not surprising that hypertonicity was recently found to promote protein aggregation in cultured mammalian cells similar to that first reported in C. elegans (83). Further work is needed to determine what role

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Biochemical Function</th>
<th>Osmotic Function</th>
<th>Homologs</th>
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<td>serine/threonine protein kinase</td>
<td>volume recovery and regulation of gpdh-1 expression</td>
<td>STK39 (PASK), OXSR1 (OSR1)</td>
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<tr>
<td>WNK-1</td>
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<td>kinase for elongation factor</td>
<td>inhibition of protein synthesis and regulation of gpdh-1</td>
<td>EIF2AK4 (GCN2)</td>
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</table>

C. elegans protein and sequence names are those used by WormBase.org. Homologs are human proteins with the best scores via BLAST protein searches. Human proteins are given as listed by the HUGO Gene Nomenclature Committee; other common names are given in parentheses.
hypertonicity plays in homeostasis of endogenous proteins of intact vertebrates, particularly in tissues like the renal medulla that are regularly exposed to high osmotic pressures and concentrations of the denaturant urea. Regulation of protein synthesis by phosphorylation of translation initiation factors is highly conserved in eukaryotes (85). It remains to be seen what role regulation of protein synthesis plays in protection of mammalian cells from hypertonicity and if the pathways identified in C. elegans can be therapeutically targeted to modulate protein confirmation diseases. Although the mechanism remains largely unknown, genetic data do suggest the presence of an extracellular osmotic sensor in C. elegans. Further work is needed to confirm this putative sensor and to determine whether a homologous or analogous mechanism is present in vertebrates. Important remaining questions include the following:

What is the mechanism of fluid transport in the excretory cell and how is it regulated?

What mechanisms do C. elegans use for volume recovery and how are they regulated?

What are the factors responsible for transactivation of osmotic responsive genes and how are they coordinated by the multiple signaling pathways identified to date?

How conserved is the role of protein translation inhibition in protection from hypertonic stress?

Are specific mRNAs selectively translated during osmotic stress when protein synthesis is inhibited, and if so, how?

Can mechanisms that protect proteins from hypertonic stress be therapeutically exploited to combat protein folding diseases?

What is the nature of the putative extracellular osmotic sensor, and is it also present in vertebrates?

ACKNOWLEDGMENTS

My young scientific career has benefited greatly from excellent mentors: James B. Claiborne and David H. Evans, who introduced me to salt and water homeostasis in fishes and Kevin Strange, who introduced me to C. elegans as a model for understanding molecular mechanisms of physiology. I thank Dr. Kelly Hyndman for the initial invitation to present this subject at the 2012 Experimental Biology meeting and the Comparative and Evolutionary Physiology Section of the American Physiological Society for continued support throughout my training and career. I thank Dr. David Hall for providing some of the micrographs of the excretory system. Lastly, I thank Dr. David Evans for providing comments on a draft version of this review. Writing of this paper was supported by National Science Foundation grant IOS-1120130 to K. P. Choe.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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