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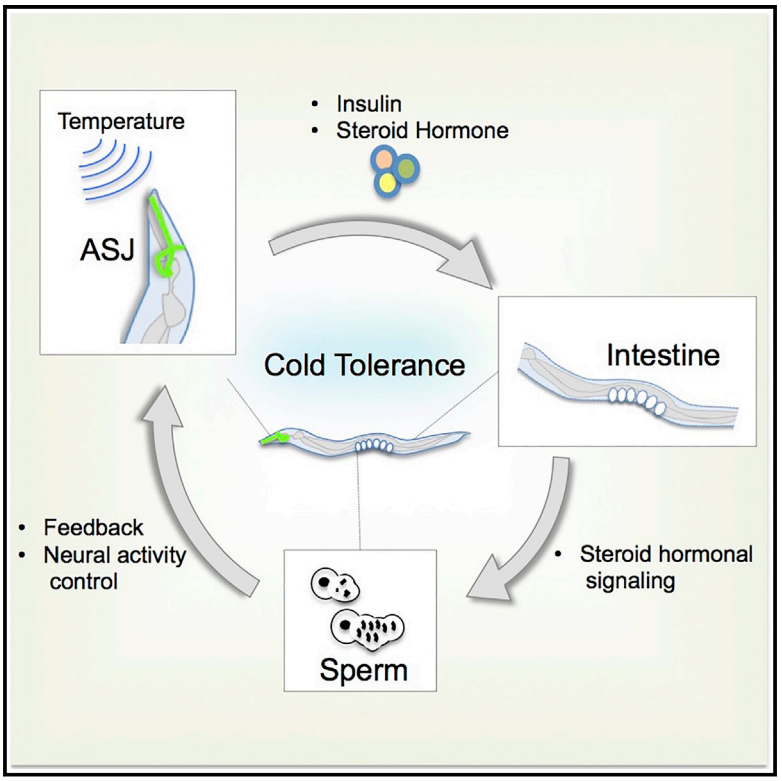
Sperm Affects Head Sensory Neuron in Temperature Tolerance of *Caenorhabditis elegans*

Graphical Abstract



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In Brief

Sonoda et al. find a feedback network between sperm and neurons in cold tolerance of *Caenorhabditis elegans*. ASJ temperature-sensing neurons regulate the intestine through insulin and steroid hormone, which then affects sperm, which, in turn, controls ASJ neuronal activity.

Highlights

- PP1 in sperm is required for cold tolerance
- Sperm is downstream of intestinal insulin signaling in cold tolerance
- Sperm affects the activity of ASJ temperature-sensing neurons
- Steroid-hormone receptors are required for tissue network of cold tolerance

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Sperm Affects Head Sensory Neuron in Temperature Tolerance of *Caenorhabditis elegans*

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SUMMARY

Tolerance to environmental temperature change is essential for the survival and proliferation of animals. The process is controlled by various body tissues, but the orchestration of activity within the tissue network has not been elucidated in detail. Here, we show that sperm affects the activity of temperature-sensing neurons (ASJ) that control cold tolerance in *Caenorhabditis elegans*. Genetic impairment of sperm caused abnormal cold tolerance, which was unexpectedly restored by impairment of temperature signaling in ASJ neurons. Calcium imaging revealed that ASJ neuronal activity in response to temperature was decreased in sperm mutant *gsp-4* with impaired protein phosphatase 1 and rescued by expressing *gsp-4* in sperm. Genetic analysis revealed a feedback network in which ASJ neuronal activity regulates the intestine through insulin and a steroid hormone, which then affects sperm and, in turn, controls ASJ neuronal activity. Thus, we propose that feedback between sperm and a sensory neuron mediating temperature tolerance.

INTRODUCTION

Temperature is an important factor in the survival and proliferation of animals. Growth rates and reproductive efficiencies of invertebrates and many other animals are affected by environmental temperature change. Thus, animals tend to tolerate temperature change using behavioral mechanisms, or they may acclimate by endogenous homeostatic mechanisms, e.g., by changing the fatty acid composition of total lipids and by changing phospholipid saturation (Murray et al., 2007).

Caenorhabditis elegans is a useful model for studying the mechanism of temperature tolerance because of its well-established molecular genetics (Barr, 2003; Brenner, 1974). *C. elegans* is able to change its morphology and behavior in response to environmental temperature change. It is able to alter its temperature-seeking behavior (thermotaxis), depending on cultivation temperature and feeding state (Aoki and Mori, 2015; Ohta and Kuhara, 2013). High temperature induces formation of the dauer larva, which trades arrested development for high-temperature tolerance (Hu, 2007). In contrast to dauer larva formation, *C. elegans* acquires cold tolerance without observable morphological change. A previous report described a pair of temperature-sensing neurons (ASJ) in the head. These neurons are also known to be light- and pheromone-sensing neurons and have been shown to release insulin. Insulin targets the intestine and other neurons. Furthermore, it has been shown that insulin-signaling regulates cold acclimation (Cornils et al., 2011; Liu et al., 2010; Ohta et al., 2014) (Figure 2A). The roles of other tissues in the regulation of cold tolerance in *C. elegans*, however, remain unknown.

One of the most important functions of organisms is reproduction; in many animals, the reproductive machinery is controlled by environmental input via the nervous system (McKnight et al., 2014). For instance, sensory input modulates sperm motility critical for fertilization in *C. elegans*. High population density and food reduction increase synthesis of ascaroside pheromone, which is sensed by ASI sensory neurons, leading to decrease transforming growth factor β (TGF-β) endocrine signaling from ASI neurons. TGF-β directly and indirectly affects prostaglandin synthesis in oogonia, and prostaglandin regulates sperm motility, guiding them to the spermatheca (McKnight et al., 2014). In contrast to dauer larva formation, this sperm-targeting mechanism is not sensitive to temperature (McKnight et al., 2014).

Feedback from somatic cells to neural cells is important for a number of phenomena. The neural circuit underlying aerotaxis in *C. elegans* is modulated by signaling from uterine-vulval cells of the somatic gonad (uv1) through hypoxia-induced factor HIF-1 (Chang and Bargmann, 2008). In thermotaxis of *C. elegans*, neuronal activity of temperature-sensing neuron AFD is modulated by steroid-hormone signaling of estrogen from muscle and intestine (Sugi et al., 2011), in which a nuclear hormone receptor (NHR) acts as a putative estrogen receptor in AFD neurons. However, a feedback system from sperm to sensory neurons has not been reported.

Sperm motility and development are regulated by protein phosphatase 1 (PP1) in mammals (Koch et al., 2015). In *C. elegans,* GSP-4 and GSP-3 encode PP1, which is an ortholog of human PP1-gamma. Both GSP-4 and GSP-3 are expressed specifically in sperm and are required for sperm motility and development (Wu et al., 2012). The motility of nematode sperm depends on the pseudopod rather than flagella. The pseudopod contains major sperm proteins (MSPs), which form the central cytoskeletal element required for actin-independent motility

(Italiano et al., 1996; Smith, 2006). GSP-3 and GSP-4 regulate the normal rate of pseudopodial treadmilling in sperm motility and pseudopod development and also regulate the localization of the MSPs in sperm development (Wu et al., 2012). Translation of GSP-4 and GSP-3 is regulated by IFE-1 encoding a component of a translation initiation complex, which is specifically required for sperm function (Amiri et al., 2001; Jankowska-Anyszka et al., 1998).

In this study, we investigated the tissue complex underlying cold tolerance in *C. elegans*. Genetic impairment revealed that sperm are involved in cold tolerance. Abnormal cold tolerance of a sperm mutant was restored by the exogenous replenishment of wild-type sperm. Genetic epistasis and quantitative gene expression measurements showed that temperature-sensing neurons affect sperm via steroid-hormonal signaling targeted to the intestine. In addition, optogenetic calcium-imaging analyses demonstrated that the activity of a temperature-sensing neuron is altered by sperm condition, suggesting that the sperm affects neuronal activity by a feedback system. Our study investigates the orchestration of a tissue network associated with cold tolerance in a living animal.

RESULTS AND DISCUSSION

Sperm Is Required for Normal Cold Tolerance

A previous study demonstrated that cold tolerance is regulated by neurons and the intestine. Temperature is sensed by a pair of sensory neurons in the head, the ASJ neurons (Ohta et al., 2014). A temperature stimulus induces insulin secretion from the synaptic region of the ASJs; the insulin is received by the intestine and neurons, whereby insulin signaling controls cold tolerance (Ohta et al., 2014). Based on these observations, the neurons and intestine are essential for cold tolerance. However, little is known about the roles of other tissues and downstream events in the insulin pathway for cold tolerance. To identify downstream molecules and tissues involved in the insulin-signaling pathway for cold tolerance, we performed DNA microarray analysis, comparing the wild-type with *daf-2* mutants lacking the insulin receptor following temperature change (Figure 1A, S1A, S1B, S1D, and S1F; Table S1). We found that the expression levels of about 1,500 genes were altered in *daf-2* mutants (Table S1). The expression patterns of about 400 of these genes have been analyzed already and are described in the gene database of *C. elegans*, WormBase (http://www.wormbase.org). We categorized these expression patterns and found that about 40% of the expressions were in neurons and/or intestine, which are known to be important tissues for cold tolerance (Ohta et al., 2014) (Figure 1A). Approximately 15% of the expressions were in reproductive tissues, mainly sperm-related genes rather than genes associated with oocytes and gonads (Figures 1A and S1A). Because the role of sperm in cold tolerance has not been reported, we attempted to analyze sperm genes in cold tolerance.

To investigate whether sperm is required for cold tolerance, we measured the cold-tolerance phenotype of many mutant animals defective in development or function in sperm (Figure 1B). After cultivation at 20°C, wild-type animals did not survive at 2°C. By contrast, 15°C-cultivated wild-type survived at 2°C. We found that mutants impairing sperm (*gsp-3*, *gsp-4*, *ife-1*) showed abnormal enhancement of cold tolerance after cultivation at 20°C (Chu et al., 2006; Kawasaki et al., 2011; Wu et al., 2012) (Figure 1B). Abnormalities observed in sperm mutants were similar to the phenotypes of mutants in which temperature-sensory signaling in the ASJ neuron, or insulin signaling, were impaired (Ohta et al., 2014) (*daf-2* in Figure 2B, *gpa-3* in Figure 2C, and *odr-1* in Figure 2D). Previous immunoblotting analysis identified that GSP-4 and GSP-3, which encode PP1s, were specifically expressed in sperm (Wu et al., 2012). IFE-1 is a translational factor for GSP-4/3 (Amiri et al., 2001; Jankowska-Anyszka et al., 1998).

To determine whether the abnormal cold tolerance of *gsp-4* mutant animals with impaired sperm was restored by exogenous supplementation with normal sperm, we introduced wild-type sperm into hermaphrodites of a sperm-defective mutant (Figure 1C). The abnormal enhancement of cold tolerance in the sperm mutant *gsp-4* was restored to normal in *gsp-4* hermaphrodites with an exogenous supply of wild-type sperm obtained by mating with wild-type males (Figure 1C). These results suggest that normal sperm is required for normal cold tolerance.

Sperm Function Is Required for Cold Tolerance

To investigate which aspects of sperm abnormality affect cold tolerance, we analyzed mutants defective in spermatogenesis and sperm function (L'Hernault, 2006). We found that *spe-15* and *spe-39* mutant animals showed abnormal enhancement of cold tolerance after cultivation at 20°C (Figure 1D). *spe-15* encodes myosin VI, important for transportation of cellular components—particularly, the movement of FB-MOs (fibrous-body-membranous organelles) and mitochondria to spermatids (Kelleher et al., 2000). *spe-39* encodes novel hydrophilic proteins essential for making FB-MOs, which are required for spermatogenesis and sperm functions. The fibrous bodies of the FB-MOs are constructed by assembled MSPs that are involved in the actin-independent motility of nematode sperm (Italiano et al., 1996; Kelleher et al., 2000; Smith, 2006). As previously reported, GSP-4 and GSP-3 (PP1s) are essential for sperm motility and development through MSPs (Wu et al., 2012). Because the expression levels of many *msp* genes were changed by temperature stimuli in the DNA microarray analysis in this study (Figures S1B, S1D, and S1F; Table S1), we measured the cold tolerance of MSP-impaired animals. We found that *msp*-impaired animals showed abnormal enhancement of cold tolerance (Figure 1E), which was a similar abnormality to that displayed by *gsp-3*, *gsp-4*, and *ife-1* mutant animals (Figure 1B). These results, at least, suggest that an MSP-dependent pathway in sperm is required for cold tolerance. Since other *spe* mutant animals showed weak abnormalities in cold tolerance (Figure 1D), it is also likely that other aspects of sperm function or development are required for cold tolerance.

Previous reports suggest that, under environmental stress, there is a tradeoff between reduced reproductive capacity and resource allocation toward somatic maintenance, e.g., relationships between reproduction and lifespan (Thondamal et al., 2014) and among growth, reproduction, and stress resistance (Jobson et al., 2015). To analyze whether a correlation existed between reproductive capacity and cold tolerance in sperm

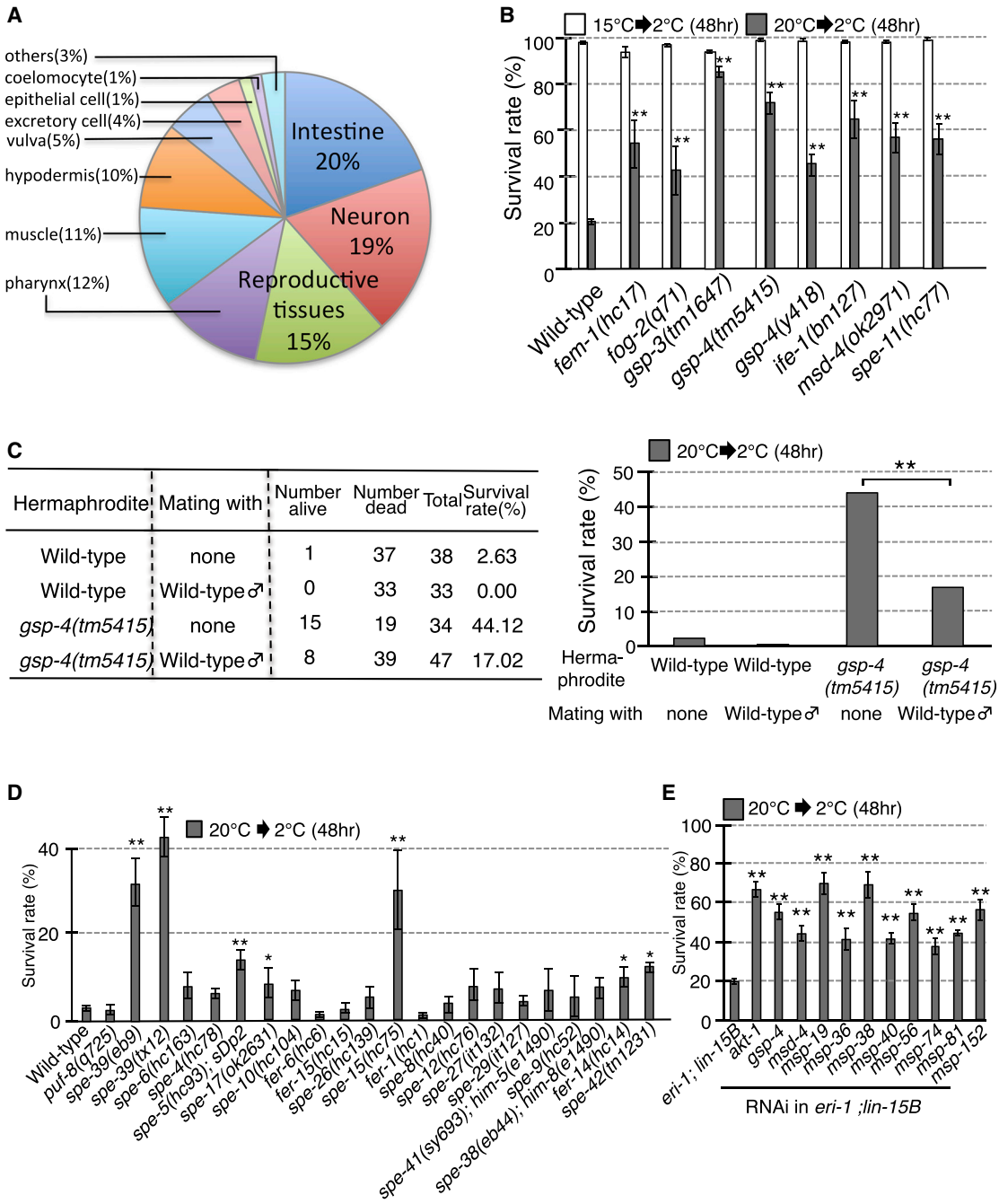


Figure 1. Sperm Is Involved in Cold Tolerance

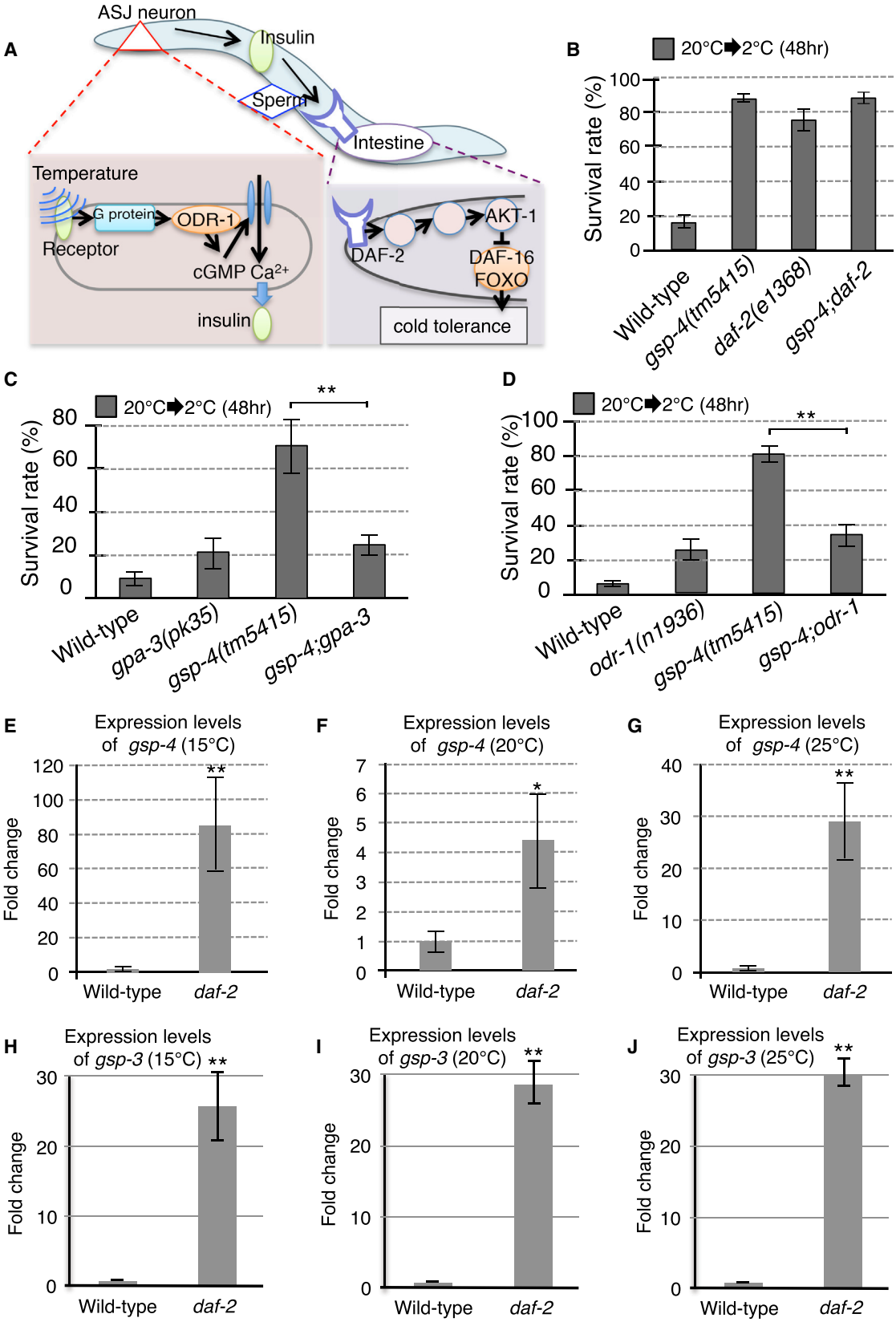
(A) Pie chart showing tissues and cells in which the genes extracted from DNA microarray analysis are mainly expressed. These genes were identified from the differences in expression from DNA microarray analysis between the insulin receptor (*daf-2*) mutant and wild-type. Both strains were cultivated at 15°C until the adult stage and then were transferred to 25°C and cultivated for 12 hr ($q < 0.05$). About half of the genes are expressed in neuron (20%), intestine (19%), or reproductive tissues (15%).

(B) Cold tolerance of wild-type and mutants. In each assay, $n \geq 9$.

(C) Abnormal enhancement of cold tolerance in *gsp-4(tm5415)* hermaphrodites with impaired sperm-specific PP1 is restored by mating with wild-type males. The table and the graph show the same data as in the cold tolerance test.

(D) Cold tolerance of wild-type and *spe* mutants. In each assay, $n \geq 4$.

(E) *msp* genes were knocked down by RNAi. The feeding RNAi technique was used with *eri-1;lin-15B* mutations that enhance the sensitivity to RNAi. *akt-1* (RNAi) impairing AKT kinase in insulin signaling was used as a positive control of cold tolerance, as previously reported (Ohta et al., 2014). In each assay, $n \geq 5$. Error bars indicate SEM. *$p < 0.05$; **$p < 0.01$. See also Figure S1.



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mutants, we constructed reproductive profiles by counting the total number of eggs laid (fertilized and unfertilized). Most of the sperm mutants showed a reduction in reproductive capacity (Figure S2), which is consistent with previous reports (L'Hernault, 2006). However, we did not observe a strong correlation between reproductive capacity and cold tolerance (Figure S2).

gsp-4 in Sperm Is Downstream of *daf-2* Insulin Signaling in Cold Tolerance

To investigate how sperm affects cold tolerance, and whether sperm interacts with the known signaling pathways of cold tolerance in the ASJ sensory neurons and the intestine, we carried out an epistasis analysis (Figure 2A). It is thought that, in the ASJ neuron, temperature is received by an unidentified receptor, the signal is transmitted by a trimeric G-protein-alpha (Gα) subunit encoded by *gpa-3*, and guanylyl cyclase (GCY) encoded by *odr-1* (L'Etoile and Bargmann, 2000; Lochrie et al., 1991; Ohta et al., 2014; Zwaal et al., 1997) (Figure 2A). The temperature signal from ASJ neurons is transmitted by insulin and received by the insulin receptor encoded by the *daf-2* gene (Kimura et al., 1997; Ohta et al., 2014) (Figure 2A). To analyze epistasis between mutation in the sperm and mutation in the neuron or intestine, we constructed their double mutants and measured cold tolerance. The *gsp-4* mutant lacking PP1 in sperm survived at 2°C after cultivation at 20°C (Figures 1B and 2B). Likewise, the *daf-2* mutant lacking insulin receptors showed this abnormal enhancement of cold tolerance (Figure 2B). The *gsp-4;daf-2* double mutant showed a similar abnormality of cold tolerance (Figure 2B), suggesting that *gsp-4* and *daf-2* are genetically involved in the same pathway. Furthermore, qPCR analysis revealed that the expression levels of the *gsp-4* and *gsp-3* genes encoding PP1 in sperm were significantly increased in *daf-2* mutant animals (*gsp-4* and Figures 2E–2G and *gsp-3* in Figures 2H–2J). The increased expression levels of *gsp-4* and *gsp-3* genes in *daf-2* mutants probably result from a compensating increase in expression of downstream signaling in the same pathway. Similar compensating increases of gene expression are sometimes observed in other signaling pathways (Sergina et al., 2007). These results are consistent with a model in which *gsp-4* in sperm is downstream of *daf-2* insulin signaling.

gsp-4 Mutation Is Suppressed by Mutation in ASJ Temperature Signaling

Next, we constructed double mutants between *gsp-4* in sperm and *gpa-3* or *odr-1* in the ASJ temperature-sensing neuron (Figures 2A, 2C, and 2D). Both *gpa-3* mutants lacking the trimeric G-protein-alpha (Gα) subunit and *odr-1* mutants lacking GCY showed a small increase in cold tolerance, as previously reported (Ohta et al., 2014) (Figures 2C and 2D). Unexpectedly, abnormally enhanced cold tolerance of the *gsp-4* mutant was significantly suppressed by *gpa-3* or *odr-1* mutations in the ASJ neuron (Figures 2C and 2D). From the results of these genetic analyses of epistasis, we hypothesized that *gpa-3* encoding Gα and *odr-1* encoding GCY are genetically downstream of *gsp-4* encoding PP1 in sperm. qPCR analysis indicated that the expression levels of *gpa-3* or *odr-1* genes were disturbed in *gsp-4* mutant animals (*gpa*-3 in Figures S3A–S3C and *odr-1* in Figures S3D–S3F). These analyses of genetic epistasis and quantitative gene expression suggest that GSP-4 in sperm affects gene expression in the sensory neuron.

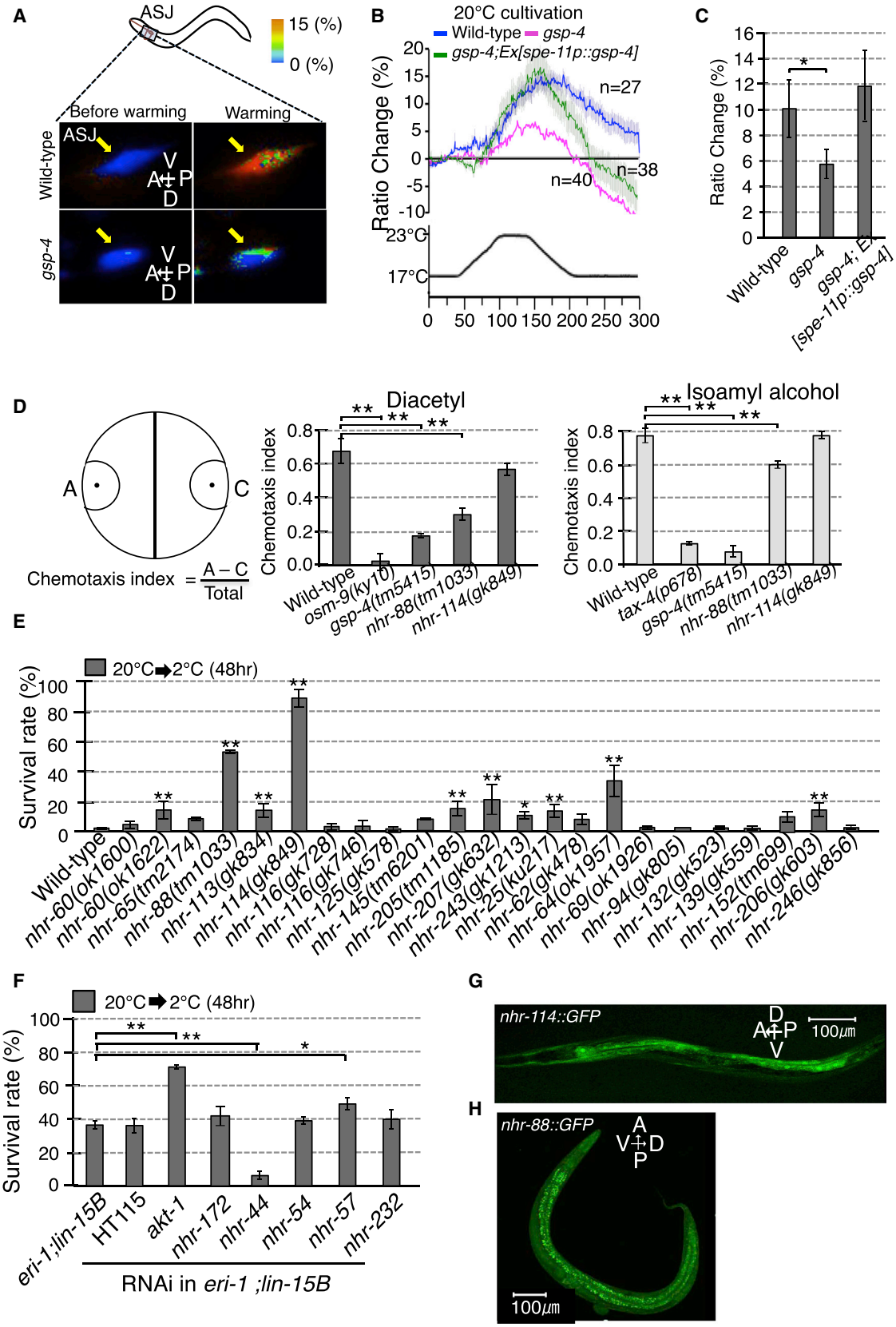
Sperm Affects Neuronal Activity of the Sensory Neurons

Genetic analysis implied that the ASJ neuron is downstream of sperm in the cold tolerance pathway. To test this hypothesis, we measured physiological activity optically in ASJ neurons during temperature stimulation, using a genetically encoded calcium indicator, yellow cameleon encoded by the *yc3.6* gene (Kuhara et al., 2011; Miyawaki et al., 1997; Ohta et al., 2014). We expressed the *yc3.6* gene specifically in ASJ neurons of *gsp-4* mutants with impaired PP1 in sperm. In a previous calcium-imaging analysis, we showed that the neuronal activity of ASJ neurons is changed by temperature stimuli (Ohta et al., 2014). Similarly, in this study, the calcium concentrations of ASJ neurons of wild-type animals changed reproducibly with temperature change (Figures 3A and 3B). By contrast, the response of ASJ neurons to temperature stimuli was strongly decreased in *gsp-4* mutants (Figures 3A–3C). We found that the abnormal temperature response of ASJ sensory neurons in *gsp-4* mutants was rescued by the specific expression of *gsp-4 cDNA* in the sperm driven by *spe-11* promoter (Frøkjaer-Jensen et al., 2008) (Figures 3B and 3C). These results suggest that PP1/GSP-4 in sperm affects neuronal activity in ASJ temperature-sensing neurons.

Genetic and physiological analyses in this study indicated that sperm affects neuronal activity associated with temperature tolerance. We investigated whether sperm abnormality induces other abnormal neuronal events. We tested *gsp-4* mutants showing abnormal cold tolerance for chemotaxis to the volatile attractants diacetyl, sensed by AWA, and isoamyl alcohol, sensed by AWC sensory neurons (Bargmann et al., 1993; Kuhara et al., 2002). Wild-type animals were attracted to diacetyl and isoamyl alcohol (Figure 3D). By contrast, the *gsp-4* mutant with impaired PP1 in sperm was defective in chemotaxis to volatile diacetyl and isoamyl alcohol (Figure 3D). Thus, *gsp-4* affects olfactory responses in AWA- and AWC-mediated chemotaxes.

Figure 2. Sperm Interacts with ASJ Sensory Neurons and Intestine in Cold Tolerance

(A) A molecular and cellular model for cold tolerance that we have previously reported (Ohta et al., 2014). Temperature is detected by the ASJ neuron, in which a trimeric G-protein-dependent temperature signal controls insulin secretion. *gpa-3* encodes a trimeric G-protein-alpha (Gα) subunit, and *odr-1* encodes a guanylyl cyclase (GCY). Insulin is received by the insulin receptor DAF-2 in intestine and neuron, where insulin signaling may regulate gene expression for cold tolerance.
(B–D) Epistasis analyses between mutation in sperm and mutations in neuron or intestine in the cold tolerance test. Cold tolerance tests were performed after cultivation at 20°C. In each, $n \geq 5$.
(E–J) Relative expression levels of *gsp-4* or *gsp-3* genes in *daf-2* mutants by qPCR analysis after cultivation at 15, 20, or 25°C. Expression levels of *gsp-4* and *gsp-3* genes were increased relative to the wild-type. Each bar represents the fold change relative to the wild-type. In each assay, $n \geq 3$.
Error bars indicate SEM. *$p < 0.05$; **$p < 0.01$.



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Steroid-Hormone Receptors Are Required for the Tissue Network Involved in Cold Tolerance

The analyses of genetic epistasis, quantitative gene expression, and calcium imaging all suggested that insulin signaling affects sperm, which, in turn, affects neuronal activity of the ASJ temperature-sensing neuron. To identify the molecules regulating this tissue complex in cold tolerance, we focused on secretory signaling. Because we previously reported that hydroxysteroid 17β dehydrogenase, an enzyme involved in steroid-hormone synthesis, encoded by the *dhs-4* gene, is involved in cold tolerance (Ohta et al., 2014), we measured the cold tolerance phenotypes of animals with impaired steroid-hormone receptors of the NHR superfamily. We screened *nhr* genes among the genes listed in the DNA microarray analysis in this study (Figures 3E and 3F), comparing gene expression before and after temperature change from 15°C to 25°C in wild-type and between wild-type and *daf-2* mutants (Figures S1B–S1G). DNA microarray analysis showed that the expression levels of many *nhr* genes were changed by temperature stimuli (Tables S1 and S2). We measured cold tolerance phenotypes of animals with impaired *nhr* genes. Mutants defective in *nhr-88* and *nhr-114* showed strong abnormalities in cold tolerance (Figure 3E). *nhr-88* mutants also showed decreased chemotaxis to AWA- and AWC-sensed odorants (Figure 3D). Previously, it was reported that *nhr-114* is expressed in the intestine and that NHR-114 is involved in dietary metabolic signaling between the intestine and germ cells (Gracida and Eckmann, 2013). However, the function and expression of *nhr-88* have not been identified. Expression pattern analysis of *nhr-88* and *nhr-114* identified that full-length or partial genomic genes fused with GFP were observed in intestine (Figures 3G and 3H). It is probable that NHR-114 and NHR-88 in intestine affect sperm. To investigate whether NHR-114 is upstream of sperm, we performed epistasis analysis (Figure 4A). The abnormal increase of cold tolerance of *nhr-114* mutant was suppressed by the *gsp-4* mutation in sperm (Figure 4A). Also, the abnormality of the *nhr-88* mutant was suppressed by the *gsp-4* mutation (Figure 4A). Additionally, expression levels of *gsp-4* were disturbed in *nhr-88* and *nhr-114* mutants (Figures 4B, S3G, and S3H). These results suggest that *gsp-4* in sperm is genetically downstream of both *nhr-88* and *nhr-114*.

To analyze epistasis between the mutation in *odr-1* that impairs temperature signaling of the ASJ neuron and *nhr* mutations in the intestine, we constructed double mutants and measured their cold tolerance (Figures 4C and 4D). The *odr-1* mutant lacking GCY showed a partial increase in cold tolerance, while wild-type animals were unable to survive at 2°C, after cultivation at 20°C (Ohta et al., 2014) (Figures 4C and 4D). The *nhr-88;odr-1* double mutant showed abnormal enhancement of cold tolerance, which was similar to the abnormality in the *nhr-88* single mutant (Figure 4C). Epistasis analysis indicated that *nhr-88* is epistatic to *odr-1*; a similar phenomenon was observed in the *nhr-114;odr-1* double mutant (Figure 4D). Genetic analysis indicated that NHR-114 and NHR-88 in the intestine are genetically downstream from ODR-1 in the sensory neuron. These results suggest that the steroid hormone receptors NHR-114 and NHR-88 may directly or indirectly receive signals from the ASJ temperature-sensing neuron. Previously, it was reported that the temperature signal releases insulin molecules from ASJ neurons, which is received by DAF-2 insulin receptors in the intestine (Ohta et al., 2014). We analyzed epistasis between *daf-2* and *nhr* mutations to identify whether hormonal signaling interacts with or functions in parallel with insulin signaling. Double mutants *nhr-88;daf-2* and *daf-2;nhr-114* showed stronger abnormalities in cold tolerance than in either single mutant (Figures 4E and 4F), implying that *nhr-88* and *nhr-114* genetically function in parallel with *daf-2*.

A Model for Regulation of Cold Tolerance through Multiple Tissues

Exploration of the mechanisms within the complex of tissues underlying acclimation to environmental change in an animal is an important challenge in biology. We have shown that cold tolerance in *C. elegans* is affected by the sperm, which controls temperature-sensing neurons in the head (Figure 4G). Sperm-specific PP1 mutant showed abnormal ASJ neuronal activity, which was rescued by expressing the PP1 gene specifically in sperm. Based on genetic and physiological data, we propose a genetic and physiological model for the tissue network and a feedback system involved in cold tolerance. Temperature activates the ASJ sensory neurons, which regulate the intestine through insulin and steroid-hormonal signaling; this affects the sperm, which, in turn, indirectly or directly affects temperature signaling in the ASJ neurons, perhaps through secretory signaling. This simple feedback model plausibly accounts for the observed physiological and molecular aspects of temperature tolerance in the tissue network, although it is also possible that

Figure 3. Tissue Network between Sperm and Neuron and between Sperm and Intestine

(A–C) Optical calcium imaging of ASJ neurons in wild-type, PP1 (*gsp-4*) mutants, and *gsp-4* mutants specifically expressing *gsp-4* cDNA in the sperm under-driven by *spe-11* promoter. Animals expressing yellow cameleon driven by ASJ-specific *trx-1* promoter were cultivated at 20°C and then tested under temperature change, indicated in the bottom chart in (B). (A) shows a schematic diagram of an ASJ neuron in the head, and corresponding pseudo-color images depicting the fluorescence ratio of cameleon before and during temperature changes. Arrows indicate ASJ cell body. (B) A relative increase or decrease in the intracellular calcium concentration was measured as an increase or decrease in the fluorescence ratio of yellow fluorescent protein to CFP chameleon during temperature change. (C) The bar chart shows the average ratio change during 20 s from 130 to 150 s of the experiment in (B).

(D) Chemotaxis to AWA-sensed diacetyl and AWC-sensed isoamyl alcohol (Bargmann et al., 1993). *gsp-4* and *nhr-88* mutants showed decreased chemotaxis. In each assay, $n \geq 4$. $^{**}p < 0.01$ compared to wild-type. In each assay, $n = 4$.

(E and F) Cold tolerance of animals with impaired nuclear hormone receptor (*nhr*). 20°C-cultivated *nhr* mutants (E) and *nhr*-knocked-down animals (F) were subjected to 2°C for 48 hr. *nhr-88* and *nhr-114* mutants showed abnormal cold tolerance after cultivation at 20°C. In each assay, $n \geq 9$ (using mutants) and $n \geq 5$ (RNAi).

(G and H) Wild-type animals expressing GFP 4.0 kb upstream of *nhr-114* and full-length *nhr-114* gene (G) or 4.0 kb upstream of *nhr-88* promoter and partial *nhr-88* gene (H). Fluorescence of NHR-114(*full*)::GFP and NHR-88(*partial*)::GFP is visible in the intestine. Scale bars represent 100 μm.

Error bars indicate SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

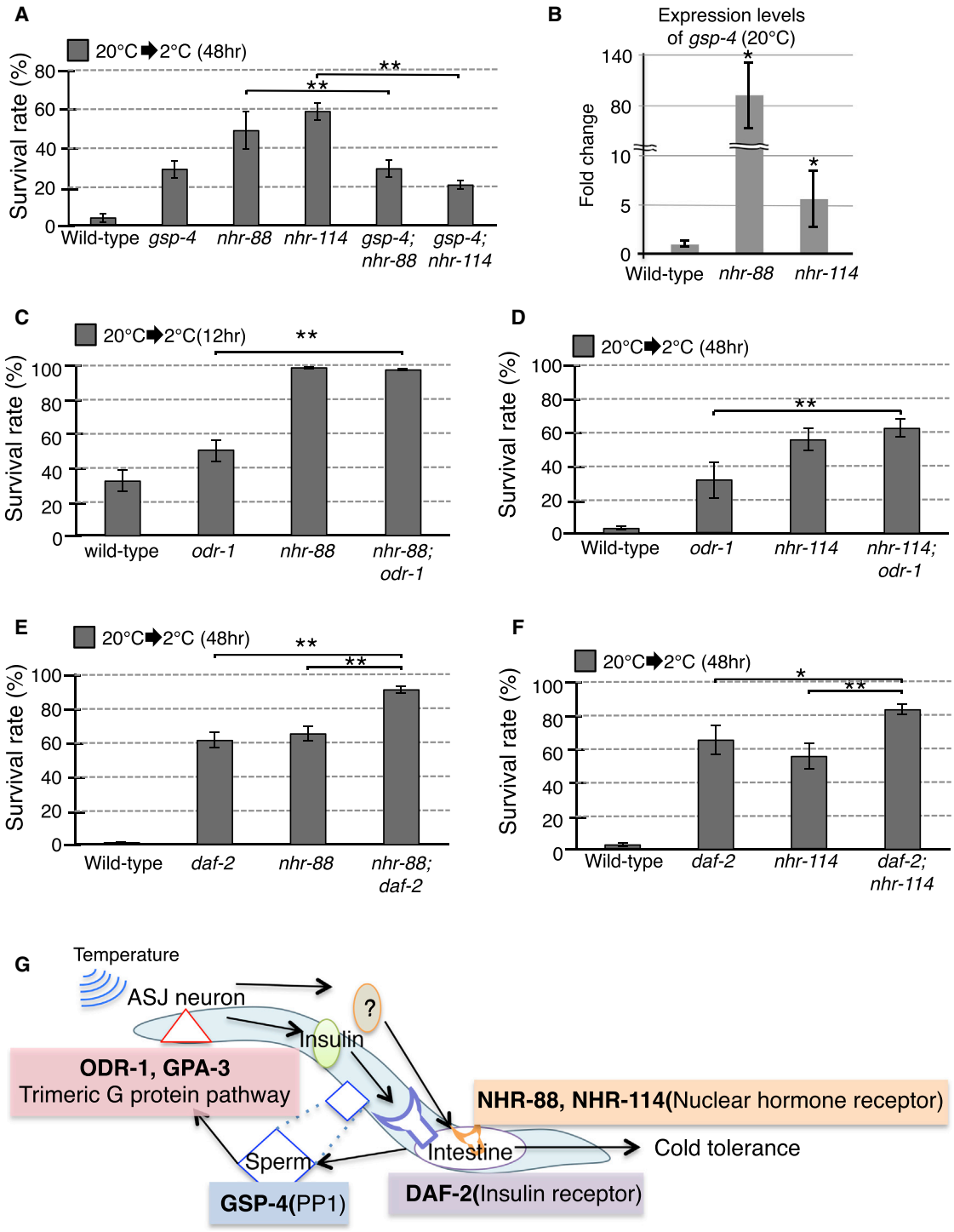

Figure 4. Tissues Network in Cold Tolerance

(A) Epistasis analyses between mutations in sperm and mutations in NHR in relation to cold tolerance ($n \geq 12$).

(B) Relative expression levels of the *gsp-4* gene in *nhr* mutants by qPCR analysis after cultivation at 20°C. Each bar represents the fold change relative to the wild-type ($n = 6$).

(C–F) Epistasis analyses between mutations in NHR and other tissues involved in cold tolerance. The mutants impairing NHR-88 or NHR-114 in the intestine; impairing guanylyl cyclase (*odr-1*) in neuron; and impairing the insulin receptor (*daf-2*) in intestine were used in these experiments. We used 2°C for 12 hr as a cold stimulus to more clearly display the phenotype of *odr-1* mutants (C) (see Experimental Procedures) ($n \geq 8$).

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cold tolerance is accomplished by a more complicated mechanism. (Figure 4G).

In many organisms, including nematodes, the proportions of different fatty acids in the body is an important aspect of cold tolerance (Murray et al., 2007; Ohta et al., 2014). Therefore, we measured the fatty-acid composition of total lipids in the sperm mutant *gsp-4*, defective in protein phosphatase. We found that the fatty-acid composition of total lipids was slightly different between wild-type and *gsp-4* mutants (Figure S4A).

This study helps to explain a complex mechanism of temperature tolerance in a tissue network. Fundamental physiological and molecular biological mechanisms are mainly conserved from *C. elegans* to humans, and the conceptual systems elucidated in this study may, therefore, provide a useful paradigm to study tissue network systems, including feedback mechanisms from sperm to neurons, in other animals.

EXPERIMENTAL PROCEDURES

Strains

We used the following *C. elegans* strains: wild-type N2 Bristol, BA17 *fem-1(hc17)*, CB4108 *fog-2(q71)*, FX1647 *gsp-3(tm1647)*, FX05415 *gsp-4(tm5415)*, *gsp-4(y418)*, SS712 *ife-1(bn127)*, RB2191 *msd-4(ok2971)*, BA819 *spe-11(hc77)*, DR1572 *daf-2(e1368)*, IK038 *daf-2(e1370)*, NL335 *gpa-3(pk35)*, CX2336 *odr-1(n1936)*, VC1290 *nhr-125(gk578)*, FX00886 *nhr-19(tm886)*, MH1955 *nhr-25(ku217)*, FX01033 *nhr-88(tm1033)*, VC1759 *nhr-95(gk836)*, FX016661 *nhr-121(tm6661)*, VC1554 *nhr-155(ok2026)*, FX01819 *nhr-156(tm1819)*, VC2567 *nhr-209(gk1135)*, RB1407 *nhr-60(ok1600)*, RB1425 *nhr-60(ok1622)*, FX02174 *nhr-65(tm2174)*, VC1760 *nhr-114(gk849)*, VC1565 *nhr-116(gk728)*, VC1591 *nhr-116(gk746)*, FX01185 *nhr-205(tm1185)*, VC2761 *nhr-243(gk1213)*, VC1095 *nhr-62(gk478)*, RB1592 *nhr-64(ok1957)*, RB1578 *nhr-69(ok1926)*, VC1140 *nhr-132(gk523)*, FX01698 *nhr-136(tm1698)*, FX00699 *nhr-152(tm699)*, VC1364 *nhr-206(gk603)*, VC1751 *nhr-246(gk856)*, JK3231 *puf-8(q725)*, SL754 *spe-39(eb9)/nT1[unc-?(n754) let-?]*, SL753 *spe-39(tx12)/nT1[unc-?(n754) let-?]*, *spe-6(hc163)*, BA714 *sDf5/spe-4(hc78)*, BA763 *spe-5(hc93); sDp2*, VC2253 *spe-17(ok2631)*, BA744 *spe-10(hc104)*, BA6 *fer-6(hc6)*, BA15 *fer-15(hc15)*, BA840 *spe-26(hc139)*, SL3 *spe-15(hc75)*, BA1 *fer-1(hc1)*, BA785 *spe-8(hc40)*, BA783 *spe-12(hc76)*, BA963 *spe-27(it132)*, BA962 *spe-29(it127)*, PS4330 *spe-41(sy693); him-5(e1490)*, BA708 *spe-9(hc52)*, AD271 *spe-38(eb44)*; *him-5(e1490)*; *asEx78[spe-38p::spe-38(cDNA)::spe-38 30UTR + myo-3p::GFP]*, BA14 *fer-14(hc14)*, SL1138 *spe-42(tn1231)/nT1[unc-?(n754) let-? qIs50]*.

Statistical Analysis

All error bars in the figures indicate the SEM. All statistical analyses in the figures were performed by one-way ANOVA, followed by Dunnett's post hoc tests for multiple comparisons, except for Figure 1C (Data S1). For Figures 1C and S4B, statistical analyses were performed by Fisher's exact test (Data S1). Single and double asterisks in the figures indicate $p < 0.05$ and $p < 0.01$, respectively (see also Data S1).

Cold Tolerance Assay

Flesh adult animal(s) were placed on a nematode growth medium (NGM), on which *Escherichia coli* OP50 was seeded; the animals were transferred to the outside of the plate after 8–12 hr. The plates containing their progenies at the fresh adult stage were transferred to 2°C in a refrigerated cabinet for the designated time (Ohta et al., 2014; Ujisawa et al., 2014). See also the Supplemental Experimental Procedures.

In Vivo Calcium Imaging

In vivo calcium imaging was performed essentially according to previous reports (Kuhara et al., 2011; Ohta et al., 2014). Samples from animals expressing yellow cameleon 3.60 in ASJ neurons were glued onto a 2% (w/v) agar pad on glass, immersed in M9 buffer, and covered with a coverslip. Fluorescence images of donor and acceptor fluorescent protein in yellow cameleon were simultaneously captured using an EM-CCD camera EVOLVE512 (Photometrics). Relative changes in intracellular calcium concentration were measured as the change in the acceptor/donor fluorescence ratio of yellow cameleon protein. See also the Supplemental Experimental Procedures.

Germline Transformation

Germline transformations were performed essentially as described previously (Mello et al., 1991), with co-injection mixes consisting of experimental DNA at various concentrations (5–50 ng/μl) and pRF4 *rol-6gf* as a transgenic marker.

DNA Microarray

DNA microarray analyses were performed essentially as described previously (Sugi et al., 2011). For DNA microarray, we isolated total RNAs from three groups: (1) wild-type N2 strain cultivated at 15°C; (2) wild-type N2 strain cultivated at 25°C for 12 hr after cultivation at 15°C for 5 days; and (3) *daf-2(e1370)* strain cultivated at 25°C for 12 hr after cultivation at 15°C for 5 days. See also the Supplemental Experimental Procedures.

Chemotaxis to Volatile Odorant Assay

Chemotaxis to volatile odorants was assayed according to a previous report (Bargmann et al., 1993).

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE81409.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.078.

AUTHOR CONTRIBUTIONS

S.S., A.O., T.U., A.M., and A.K. performed the experiments. S.S., A.O., and A.K. designed and interpreted the experiments and wrote the paper.

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(G) A molecular and cellar model for the tissue network involved in cold tolerance, including sperm. The insulin pathway in the intestine receives temperature signaling, detected by the ASJ neuron through insulin. Our data suggest that sperm affects temperature signaling in the ASJ neuron by a feedback system; steroid hormone receptors NHR-88 and NHR-114 affect sperm and function, in parallel with the insulin pathway in the intestine.
Error bars indicate SEM. *$p < 0.05$; **$p < 0.01$.

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