

DnaJ chaperones contribute to canalization

Short running title: Role of ER-associated DnJs in canalization

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Key words: *C. elegans*, DnaJs, developmental robustness, seam cell, lifespan

Abstract

Canalization, an intrinsic robustness of development to external (environmental) or internal (genetic) perturbations, was first proposed over half a century ago. However, whether the robustness to environmental stress (environmental canalization) and to genetic variation (genetic canalization) are underpinned by the same molecular basis remains elusive. The recent discovery of the involvement of two Endoplasmic Reticulum-associated DnaJ genes in developmental buffering, orthologues of which are conserved across Metazoa, indicates that the role of Endoplasmic Reticulum-associated DnaJ genes might be conserved across the animal kingdom. To test this, we surveyed the Endoplasmic Reticulum-associated DnaJ chaperones in the nematode *Caenorhabditis elegans*. We then quantified the phenotype, in the form of variance and mean of seam cell counts, from RNAi knock-down of DnaJs under three different temperatures.

We find that seven out of eight ER-associated DnaJs are involved in either environmental canalization or microenvironmental canalization. Moreover, we also found two DnaJ genes not specifically associated with Endoplasmic Reticulum (DNAJC2/*dnj-11* and DNAJA2/*dnj-19*) were involved in canalization. Protein expression pattern showed that these DnaJs are upregulated by heat stress, yet not all of them are expressed in the seam cells. Moreover, we found that most of the buffering DnaJs also control lifespan. We therefore conclude that a number of DnaJ chaperones, not limited to those associated with the ER,, are involved in canalization as a part of the complex system that underlies development.

Research highlights

- A number of DNAJs, not limited to ER-associated chaperones, are involved in canalization in *C. elegans*.
- DnaJ chaperones buffers seam cell development as a part of a complex genetic network.

1. Introduction

Organismal embryonic development is robust to the impact of environmental stress as well as genetic variation or mutation. Conrad Hal Waddington famously coined the term ‘canalization’ to describe an evolved state of robustness (Waddington, 1957). Understanding the mechanisms of canalization is a central theme in developmental biology as well as evolutionary biology. Waddington described development as a ball falling down a ‘canalized’ slope on metaphoric hills called the ‘epigenetic landscape’ (Waddington, 1957). The epigenetic landscape is underpinned by ‘a complex system’ which ultimately determines the slope that the ball will fall down (Waddington, 1957). From a theoretical perspective, canalization for cryptic genomic variation (Genetic Canalization, GC) and environmental fluctuations (Environmental Canalization, EC) are modeled with different inputs (Kaneko, 2007), yet GC is a byproduct of EC (Wagner and Gibson, 2000). However, the molecular basis underpinning GC and EC remains elusive.

Several proteins and cellular systems have been proposed for the molecular and cellular basis of GC. The implication of Hsp90 in masking cryptic genetic variation in *Drosophila* provided the first empirical insights (Rutherford and Lindquist, 1998). Hsp90 is a heat shock protein that buffers impact of changes on proteins to ensure proteostasis (Schopf et al., 2017). However, when the function of Hsp90 is compromised, phenotypic variability appears (Rutherford and Lindquist,

1998; Quentisch et al., 2002; Rohner et al., 2013). Rutherford and Lindquist coined the term 'capacitor' to describe this effect, proposing that Hsp90 suppresses cryptic genetic variation (Rutherford and Lindquist, 1998). Indeed, impairment of Hsp90 function increases destabilizing variants with increased aggregation propensity and hydrophobicity in viruses (Geller et al., 2018). Moreover, recent evidence showed that Hsp90 is a capacitor of transcriptional regulation (Hummel et al., 2017). On the other hand, examining the impact of impairment of Hsp90 function in yeast demonstrated that Hsp90 acts as potentiator for a small number of phenotypes, but not in all the mutation accumulation lines (Geiler-Samerotte et al., 2016). This result agreed with previous studies in *Drosophila* that failed to detect phenotypes buffered by Hsp90 (reviewed by Takahashi, 2018). Such studies led to another hypothesis; that Hsp90 might not be a capacitor, but a 'genetic modifier' that has many interacting clients and can modify the genetic interaction network when the function is impaired (Geiler-Samerotte et al., 2018). A recent study comparing effects of different genetic backgrounds has revealed that there are potentially numerous 'genetic modifiers' in a genome (Paaby et al., 2015). Such a notion is consistent with Conrad Hal Waddington's original views on this, that it was not correct to assign a single functional role for each gene (Loison, 2018).

Consistent with this hypothesis, an increasing amount of evidence has shown that other molecules are involved in canalization (for more details see reviews by Lachowiec et al., 2017; Takahashi et al. 2018). Most of these studies used model organisms that have been kept in a laboratory for many generations. However, a recent study using wild populations of the tunicate *Ciona intestinalis* revealed that two ER-associated DnaJ chaperone genes, DNAJC3 and DNAJC10, are associated with developmental buffering under increased temperature stress, and have orthologues across Metazoa (Sato et al., 2015). No DnaJs or ER-associated chaperones had been studied in relation to developmental robustness before this. Subsequently, this prompted us to undertake a more general analysis of the role of ER-associated DnaJ genes in EC.

The DnaJ proteins are the largest chaperone families in humans. They are involved in diverse biological processes and are an integral part of the Hsp70 machinery. The human genome contains 49 genes encoding DnaJ proteins, united by the possession of a DnaJ domain (Kampinga and Craig, 2010). They are classified into Class I (DNAJA), Class II (DNAJB) and Class III (DNAJC) DnaJs (Ohtsuka, 2000), yet their molecular size, domain composition and function cannot be determined from these classifications (Kampinga and Craig, 2010). DnaJs

show variation in expression patterns, with some having tissue-specific expression, while others are confined to specific cellular compartments, such as the mitochondria or endoplasmic reticulum (Hageman and Kampinga 2009). In the human genome, there are eight ER-associated DnaJ chaperones. Amongst them, while DNAJB11, DNAJC10, DNAJC3 and SEC63 are involved in both ER-associated folding (ERAF) and ER-associated degradation (ERAD) as a response to the unfolded protein response (UPR) (Oyamadori et al., 2006; Dong et al., 2008; Oka et al., 2013; Tan et al., 2014; Servas et al. 2013; Oka et al., 2013; Yamamoto et al., 2010), DNAJC10, DNAJB9 and DNAJB12 are known only in ERAD (Ushioda et al., 2008; Lai et al., 2012; Yamamoto et al., 2010; Grove et al., 2010).

To investigate the roles of ER-associated DnaJs in canalization, we turned to the model nematode *Caenorhabditis elegans*, where isogenic lines are available and therefore variation in genetic background can be controlled. Although DnaJ homologs have been identified for the majority of DnaJ proteins in *C. elegans*, most have yet to be fully investigated. Overall, *C. elegans* has 34 DnaJ genes of which 9 are predicted to be associated with the ER, and we focus our analysis on these. To test whether buffering molecules are limited to ER-associated chaperones, or are a more general feature of DnaJ chaperones, we used seam cell number as a phenotypic readout and examined phenotypic variation following DnaJ gene RNAi knockdown under thermal stress. *C. elegans* seam cells are lateral neuroectodermal cells that are formed on both sides along the length of the animal. *C. elegans* hatch from an egg with 10 seam cells per side, which pass through a series of reiterative asymmetric divisions at each larval stage, in addition to a single symmetrical division resulting in an adult worm with 16 seam cells per side (Sulston and Horvitz, 1977). The development of the seam cells is canalized to the point of eutely, so that during normal development the balance of asymmetric and symmetric divisions maintains a constant seam cell number (Sulston and Horvitz, 1977). Variation in the number of seam cells can be used as a metric for phenotypic variation in animals subjected to genetic perturbation in addition to environmental stress (Boukhibar and Barkoulas, 2016; Katanos et al., 2017). In *C. elegans*, we find that a number of DnaJs, not limited to ER-associated chaperones, are involved in canalization. Consequently, we conclude that genetic and environmental canalization are underpinned by multiple chaperones with different protein structures and cellular functions, as part of a complex gene network.

2. Materials and Methods

2.1 Strains and maintenance of worms

Strains were derived from the wild type *N2* Bristol strain and maintained at the 'control' temperature of 19°C, as described previously (Brenner, 1974). The following strains were used in this study: *JR667 unc-119(e248)III;wls51[pMF1(scm::gfp)+pDP#MM016β(unc-119*)V; BC14473 dpy-5(e907)I; sEx14473[dnj-20::gfp+pCeh361]; BC14321 dpy-5(e907)I;sls11063[dnj-11::gfp+pCeh361]; BC10178 dpy-5(e907)I;sEx10178[dnj-7::gfp+pCeh361]; RB1784 dnj-27(ok2302)I; VC1642 dnj-11(gk125)IV.*

2.2 RNAi

RNAi clones for DNAJB12/*dnj-1*, DNAJC3/*dnj-7*, DNAJC2/*dnj-11*, DNAJB9/*dnj-18*, DNAJA2/*dnj-19*, SEC63/*dnj-29* and DNAJC1/*F54F2.9* were obtained from the Ahringer RNAi library (Kamath and Alinger, 2003), while DNAJC25/*dnj-2*, and DNAJC9/*dnj-23* were obtained from the Vidal ORFeome-based RNAi library (Rual et al., 2014).

Three clones were not available in either library and were constructed separately by PCR amplification from wild type *C. elegans* gDNA using Phusion Taq (NEB). DNAJC10/*dnj-27* was amplified with primer pair 5'- CATGCTCCACACAGTAGCCA-3' and 5'- TCTCCTCGGCTGCTTAGTCT-3') generating a 466 bp amplicon. Using primers 5'- GATGAAGCTGCTTCAGGTCG-3' and 5'- GGAGTCAATCCGCATCGG-3', a 480 bp amplicon of DNAJC16/*dnj-8* was amplified, and a 487 bp amplicon of DNAJB11/*dnj-20* was generated using primer pair 5'- CACCCCGATAGGAACCAG-3' and 5'- CAACCTCCACTTCAACCTCC-3').

Each amplicon was cloned into pCR2.1®-XL-TOPO® (Invitrogen) and then digested with *Pst*I and *Sac*I. The resulting fragment was sub-cloned into Fire Lab RNAi vector *L4440* (Timmos and Fire, 1998), generating pAW803 (DNAJC10/*dnj-27*), pAW841 (DNAJC16/*dnj-8*) and pAW842 (DNAJB11/*dnj-20*) respectively. All plasmids were confirmed by sequencing before being transformed into *HT115* bacteria.

All nematode experiments were conducted using the strain *JR667*, which carries the integrated *scm::gfp* transgene allowing seam cells to be visualized (Koh et al., 2001). dsRNA was delivered by feeding to L4 stage *JR667* animals, which are incubated at 15°C, 19°C or 24.5°C. In all cases,

seam cell number was counted in L4 offspring. Control RNAi was performed using *HT115* bacteria transformed with an empty *L4440* vector.

Comparison of the means (*t*-test), and variations (*var.* test) were conducted using a statistical package, R (Crawley, 2005). Due to multiple testing, *P*-values of 0.00416 (0.05 divided by 12 for Bonferroni correction) or under was considered as significant.

2.3 Lifespan assay

To assess lifespan, we surveyed *N2*, *RB1784* (*DNAJC10/dnj-27*) and *VC1642* (*DNAJC2/dnj-11*) animals or *N2* animals with their DnaJ genes silenced by RNAi compared to the empty RNAi vector, *L4440*, as a control. Animals were reared on *OP50* seeded NGM at either 15°C, 19°C or 24.5°C following standard protocols. When gravid, animals were bleached and the resulting age-synchronized L1 animals were placed onto seeded RNAi NGM and grown at the specified temperatures. When animals reached L4, this was counted as Day 0 of the survival assay. During the reproductive period, the worms were transferred to a new plate and observed every day for death. Animals were scored as dead if they failed to respond to a touch by a platinum wire. Survival curves were generated and data analyzed using the OASIS software (Yang et al., 2011).

2.4 Microscopy

To take images, worms were mounted onto 2% agarose pads in 0.1% sodium azide. Fluorescent imaging was carried out using a Zeiss Imager.M2 microscope and photomicrographs were taken using a x40 objective (Zeiss) and Zeiss Zen 2012 Blue Software. In addition, the images for tissue localization images were taken using a x63 oil immersion objective on a Zeiss AxioSKOP2 microscope with a Leica AxioCamMR digital camera and analyzed with Axiovision software (Release 4.5). All images of animals were compiled using Adobe Photoshop 7.0 and backgrounds merged.

3. Results

3.1 Experimental system to distinguish EC and MEC using *C. elegans*.

The literature contains various definitions of canalization (Gibson and Wagner, 2000), making understanding canalization in empirical experiments challenging and in need of explicit definition. In this manuscript, we use the definition where an increase in variance of a trait refers to canalization, while a change in its mean value reflects developmental construction (Dworkin, 2005; Félix and Barkoulas, 2015). We first considered what seam cell number would be expected if robustness was compromised by stress and/or genetic manipulation. Compromising a specific aspect of the development of the seam cell lineage should result in animals consistently developing either more or less seam cells, thus changing the mean number of cells per animal (Fig. 1a), and indicating that the gene is involved in phenotypic construction (PC) (Félix and Barkoulas, 2015). However, if robustness were compromised, we would expect more developmental errors but for these to be random in whether they increased or decreased the number of cells. Hence the mean number of cells would stay the same, but the variation in seam cell number would increase (Fig. 1b).

Exposing animals to gene knockdown, and gene knockdown in combination with a stress that by itself is insufficient to compromise development, should determine whether a gene is involved in microenvironmental canalization (MEC), EC or PC. Specifically, we predict that if a gene is involved in buffering microenvironmental variation (Siegal and Liu, 2014) then its knockdown at the control temperature will lead to an increased variation in seam cell number, but not a change in the mean seam cell number; this is MEC (Fig. 1c). In contrast, when genes are silenced in the presence of a macroenvironmental stress i.e. temperature variation, resulting in an increase in variation, but not a change in the mean number of seam cells, this is indicative of a role for the gene in EC (Fig. 1c).

3.2 Canalization by DnaJ chaperones in *C. elegans*

To test for a canalizing role of DnaJs, animals were grown at control (19°C), elevated (24.5°C) and lowered (15°C) temperatures and fed dsRNA for the different DnaJs, or a control dsRNA. The lowered and elevated temperatures were selected as at these temperatures seam cell number does not change in either mean or variance with control RNAi and are the limits of temperature at which *C. elegans* can survive normally. At this point, it is important to note that for clarity and consistency with published literature DnaJs will be referred to with hybrid names, with the human gene name first and the nematode gene name second (e.g. DNAJB11/*dnj-20*). We have chosen three non-ER associated DnaJ genes (DNAJC2/*dnj-11*, DNAJC9/*dnj-23* and DNAJA2/*dnj-19*), in addition to the nine known ER-associated DnaJ genes, so as to test whether a role in canalization

is limited to ER-associated DnaJ chaperones. Seam cell number data were compared for mean (using *t*-test) and variance (*var* test). Three of the tested DnaJ genes, DNAJC1/*F54F2.9*, DNAJC25/*dnj-2* and DNAJC9/*dnj-23*, showed no change in mean or variance under RNAi, whether at normal or experimental temperatures (Table 1, Supplementary Table S1, Supplementary Figure S1). Three genes, DNAJC16/*dnj-8*, DNAJC2/*dnj-11* and DNAJC10/*dnj-27*, showed evidence for MEC, in that variation (but not mean) of seam cell number changed significantly with RNAi knockdown in the absence of temperature stress (Fig. 2, Table 1, Supplementary Table S1). This might seem contradictory to the fact that we used an isogenic strain for these experiments, however, we suggest that this reflects penetrance in that effect of mutation varies between individuals despite their isogenic nature, as has been previously considered (Casanueva et al., 2012). Of note, DNAJB9/*dnj-18* decreased variance with RNAi at all temperatures, suggesting that DNAJB9/*dnj-18* is involved in decanalization, acting as a 'potentiator' (Jarosz and Lindquist, 2010) rather than increasing the level of canalization (Table 1, Supplementary Table S1, Supplementary Figure S1).

Four DnaJ genes showed evidence for EC, where variance of seam cell number changed under temperature stress (Fig. 3, Table 1, Supplementary Table S1). Two of these (DNAJC3/*dnj-7* and DNAJB11/*dnj-20*) changed only at elevated temperature, while DNAJA2/*dnj-19* showed EC at both elevated and lowered temperatures. In addition, DNAJB12/*dnj-1* and SEC63/*dnj-29* showed significant increase in variance at both 19°C and 24°C or 15°C respectively, suggesting roles in both MEC and EC. Consequently, DNAJB12/*dnj-1* and SEC63/*dnj-20* are difficult to place, and we have chosen to show the effect of silencing these genes on seam cell number with the other DnaJs involved in EC (Fig. 3). Furthermore, DNAJB12/*dnj-1*, DNAJB11/*dnj-20* and SEC63/*dnj-29* showed evidence for a role in PC, in that as well as variance, the mean number of seam cells also changed.

Together, we found that five of the *C. elegans* ER-associated chaperones were involved in canalization, but MEC and EC were underpinned by different DnaJ chaperones. In addition, our data also suggests that DnaJs involved in canalization are not limited to ER-associated chaperones, as shown by DNAJC2/*dnj-11* which is located in the ribosome, and DNAJA2/*dnj-19*.

3.3 DNAJs control developmental robustness as a part of gene interaction networks

We next aimed to gain some mechanistic insights into the function of DnaJs in canalization. To this end, we tested two hypotheses: One is that DnaJs are directly involved in developmental pathways, and the other is that DnaJs are a part of a large, complex gene interacting network, and they act as 'genetic modifiers'. If those DnaJs involved in canalization control seam cell development directly, they should be specifically expressed in the seam cells. However, if DnaJs act as a modifier of a complex network, they may not be expressed in the seam cells. To test this, we examined 3 GFP reporter strains, DNAJC3/*dnj-7*, DNAJB11/*dnj-20* and DNAJC2/*dnj-11* (Strains, *BC10178*, *BC14473*, *BC14321* respectively), to observe the tissue specificity of DnaJ expression. Importantly, we found that they are not always expressed in the seam cells (Figure 4). In animals reared at the control temperature (19°C), tissue specific expression of DNAJC3/*dnj-7* was found in the germline (distal tip cell, vulval muscle and spermatheca), head (pharynx) and body wall muscle (Fig. 4a-d), but not in seam cells. Previous report also showed expression of DNAJC10/*dnj-27* was unrelated to seam cell (Muñoz-Lobato et al. 2014). A similar expression pattern to DNAJC3/*dnj-7* was observed for DNAJC2/*dnj-11*, with additional expression in the seam cells and anal depressor muscles (Fig. 4e-h). Reporter expression in DNAJB11/*dnj-20* animals was mainly restricted to the intestine, but expression could also be observed in the hypodermis and seam cells (Fig. 4i-l). In all strains, expression was minimal during early embryonic development, and once the tissue types were specified, GFP expression could be observed (data not shown).

The expression pattern of these three DnaJ proteins remained constant throughout development, from hatching through to adulthood (data not shown). Also, we observed that these GFP expression patterns were visible but very low at 15°C (Fig. 5), whereas slightly increased at 24.5°C compared to the control temperature (Fig. 5), even after 2 weeks of rearing at the same temperature. We note that expression pattern at tissue level was not altered by rearing temperature.

Since not all of the DnaJs involved in canalization were expressed in the seam cells, it is more likely that these DnaJs buffer developmental robustness as 'genetic modifiers' in a complex gene network. If this is true, we would be able to see the impact of functional impairment of these DnaJs in other phenotypes that involve the whole organism, and not just limited to the seam cells. For example, a previous study showed that overexpression of HSF-1, a master regulatory gene (a 'hub') of the heat shock response, controls penetrance of mutational effect in an isogenic line of *C. elegans* (Casanueva et al., 2013). Overexpression of HSF-1 increased susceptibility to stress

and extended lifespan (Casanueva et al., 2013) while functional impairment of HSF-1 shortened lifespan (Morley and Morimoto, 2004). Therefore, it is likely that functional impairment of DnaJs involved in canalization might also affect lifespan. To test this, the lifespan of animals in the absence of different DnaJs was observed. Of those DnaJs involved in MEC (Fig. 6), knockdown of DNAJC2/*dnj-11* showed an increase in lifespan at all temperatures compared to controls although in only about 10% of all animals examined (strain, N2); hence the 50% mortality of DNAJC2/*dnj-11* is similar to control animals, but maximum lifespan significantly increased (Supplementary Table S2). The lifespan of animals without DNAJC10/*dnj-27* was not dissimilar to that of the wild type animals, however, at 24.5°C, lifespan was significantly reduced (Fig. 6, Supplementary Table S2). The maximum lifespan of animals exposed to DNAJC16/*dnj-8*(RNAi) was extended at all temperatures compared to those animals on control RNAi (N2 L4440).

In contrast, in those DnaJs involved in EC, such as DNAJC3/*dnj-7* and DNAJB11/*dnj-20*, knockdown of these genes showed pronounced extension of maximum lifespan at all temperatures compared to the lifespan of control animals (Fig. 7, Supplementary Table S2). Indeed, the 50% mortality of knockdown of DNAJC3/*dnj-7* and DNAJB11/*dnj-20* is increased compared to the controls at all temperatures, and this is reflected in the shape of the lifespan curve which is much extended. However, in RNAi knockdown of the ER-related chaperone DNAJB12/*dnj-1* there was only a significant reduction to maximum lifespan at 24.5°C while for SEC63/*dnj-29*, also an ER-associated chaperone, lifespan was significantly extended at 15 and 19°C, but reduced at 24.5°C (Fig. 7, Supplementary Table S2).

Together, knockdown of DnaJs involved in canalization generally affect lifespan, although some of the effects are temperature-dependent. We conclude that these DnaJs control developmental robustness not specifically in seam cells, but as a part of a complex gene network.

4. Discussion

Canalization is the intrinsic robustness of development to environmental or genetic perturbations. Two Endoplasmic Reticulum-associated DnaJ genes in developmental buffering were recently discovered in an ascidian species (Sato et al., 2015), but whether this is limited to ascidians or conserved more broadly across the animal kingdom is not known. To test this, we have surveyed

the ER-associated DnaJ chaperones in the nematode *Caenorhabditis elegans*. Using the number of the stem cell-like seam cells as a phenotypic readout of robustness, our results demonstrate that a repertoire of ER-associated DnaJ genes are involved in EC, namely DNAJB12/*dnj-1*, DNAJC3/*dnj-7*, DNAJB11/*dnj-20* and SEC63/*dnj-29* while four of the ER-associated DnaJ genes (DNAJC16/*dnj-8*, DNAJC2/*dnj-11*, DNAJC10/*dnj-27* and SEC63/*dnj-29*) play a role in MEC in *C. elegans*. Unlike these, DNAJB9/*dnj-18* is likely to be involved in decanalization at all temperatures. We also found that DnaJ genes not associated with the ER can also be involved in canalization, for example, DNAJA2/*dnj-19* in EC and DNAJC2/*dnj-11* in MEC. These results are summarized in Fig. 8. We conclude that chaperones involved in canalization are not limited to being ER-associated, and there are potentially many DnaJs involved in canalization.

Amongst the DnaJs involved in EC in this study, DNAJA2 is involved in refolding of disordered proteins (Baaklini et al. 2012), while others are mostly involved in ERAF. The ER-associated chaperones (DNAJB12/*dnj-1*, DNAJC3/*dnj-7*, DNAJB11/*dnj-20* and SEC63/*dnj-29*) have been shown to have a function in ERAD (Grove et al., 2010; Oka et al., 2013; Otero et al., 2014; Ushioda et al., 2008) (Fig. 8). Developmentally relevant proteins, such as signaling molecules and transcription factors, are known to be highly disordered (Ward et al., 2004), and such intrinsically disordered proteins are tightly regulated by degradation (Gsponer et al., 2008). Thus, degradation might also be an important aspect of robustness in addition to a previous hypothesis suggesting that folding is the key in thermal susceptibility (Tyedmers et al, 2010). Similarly, amongst DnaJs involved in MEC, DNAJC10/*dnj-27* is also involved in ERAD while DNAJC2/*dnj-11* is involved in ribosome-associated nascent chain folding (Willmund et al., 2013). Hence, we propose that canalization is underpinned by various chaperones with different protein structures and cellular functions, but that the chaperones share roles for the different types of canalization. Defects in these genes can result in serious diseases, including, diabetes (Ladiges et al., 2005), hepatic steatosis (Rutkowski et al., 2008) and neurodegenerative disease (Muñoz-Lobato et al., 2014). Consequently, the outcome of our DnaJ study will contribute to opening up new avenues of clinical research.

Intriguingly, our data on DnaJ expression patterns show that the three *C. elegans dnj* genes are not all expressed in the seam cells, and that expression is only moderately increased under elevated temperatures, while tissue specificity does not alter. We also showed that those DnaJs involved in canalization also alter lifespan, a phenotype at the whole organismal level, and not just seam cells specifically. This evidence suggests that these DnaJ genes are involved in

canalization as part of a genetic network having multiple interacting clients, as Waddington had originally described canalization (Loison et al. 2018), rather than being involved in the seam cell development pathway. Our finding that the decrease in lifespan by some of the DnaJs, such as DNAJC3/*dnj-7*, DNAJB11/*dnj-20* and DNAJC2/*dnj-11*, is particularly intriguing, since an increase in expression of heat shock proteins such as HSF-1 (Morley and Morimoto, 2004), *daf-21*/Hsp90 (Somogyvári et al., 2018), *hsp70* (Tatar et al., 1997) and *hsp-16.2* (Rea et al., 2005), has been shown to increase lifespan. In these DnaJs, expression decrease might decanalize the phenotype whilst the population might increase lifespan and propagate. This could explain the evolutionary drive of phenotypic diversity by level of chaperone expression.

Consequently, a future important challenge will be to identify the interactome for each DnaJ so as to reconstruct how the environment may have affected the evolution of organism specific DnaJs and decipher the molecular mechanisms of developmental buffering. Previous studies have shown that signaling molecules play a pivotal role in developmental robustness (Milloz et al., 2008; Braendle and Félix, 2008). Indeed, analysis of human DNAJC10 shows that it interacts with signaling molecules that are important in developmental processes, including EGF, TGF- β , and Notch (Oka et al., 2013), which have an integral role in morphogenesis. It has been suggested that DNAJB11, DNAJC3 and DNAJB9 can also bind a variety of unfolded and/or misfolded proteins (Craig and Marszalek, 2017), but the extent of binding remains elusive. Knockdown of SEC63/*dnj-29* showed a marked increase in variance, which might be due to the large number of interactions it might have as a translocon. Importantly, some DnaJ proteins, for example DNAJC3, have been shown to interact with its client protein depending on the status of its protein structure *i.e.* if it is nascent, mature etc. (Oyamadori et al., 2006). Therefore, it is essential to investigate how fluctuations in temperature affect such proteins and how ER-associated DnaJ chaperones rescue such disordered proteins under thermal stress. Recent advances in mass spectrometry analysis has enabled monitoring of protein structural change under various temperatures (Leuenberger et al., 2017). Applying such technology to reveal the interactome of these DnaJ chaperones will further test the molecular mechanisms underpinning canalization.

Acknowledgements

We are extremely grateful to Danny Godfrey, Michalis Bakoulas and Marie-Anne Felix for their insightful suggestions for our experimental design and analysis. We also thank Gina Miku Oba for helping making figure 8 and Shohei Mitani for technical advice. Strains were obtained from the *Caenorhabditis* Genetics Centre, funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). This work was supported by KAKENHI grant number 15K18584 and 17K15167 to AS.

Author Contributions

Conceptualization; SH, SS, and AS. Investigation; SH, IV, JR, SS. Methodology; SS, SH, AS, AW. Formal Analysis; SH, AS. Funding Acquisition; AS. Project Administration; SH, AS. Resources; CF, AW. Writing; SH, SS, AS.

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Table Legends

Table 1. *P*-values shown in statistical testing of seam cell counts in RNAi screening. *P*-values which showed significance ($P < 0.00416$) after Bonferroni correction are bold. Nomenclature is based on that by Ohtsuka and Hatta (2000).

Figure Legends

Figure 1. Explanation of environmental canalization (EC) and microenvironmental canalization (MEC). (a) Experimental design using seam cell number in *C. elegans*. (i) At the L4 stage, the nuclei of seam cells are easily visible with a GFP marker (Strain, JR667) and all animals have 16 per side with little variation (blue graph). Scale bar, 100 μ m. However, if a gene functions as an environmental buffer, gene silencing and thermal stress will change the mean and/or variation in number of seam cells in an animal. (ii) RNAi knockdown of genes involved in phenotypic construction results in animals consistently developing either more or less seam cells, thus changing the mean number of seam cells. (iii) If a gene involved in canalization is compromised, the mean number of seam cells remains the same, but the variation in seam cell number would increase. (b) Changes in phenotypic mean and variation in seam cell counts. Wild type animals have 16 seam cells at the L4 stage (blue). However, gene silencing may affect only variation (green), only the mean (pink), or both variation and mean (orange). (c) Definition of microenvironmental canalization (MEC), environmental canalization (EC) and phenotypic construction (PC) in relation to mean and variation in phenotype. ND, not determined. Decanalization which would result in decreasing phenotypic variance, is not shown in this figure.

Figure 2. Seam cell number following silencing of DnaJ genes involved in microenvironmental canalization. Genetic canalization is observed when there is an increase in the variation of seam cell number, but not a change in the mean at the control temperature (19°C) in the absence of a DnaJ gene. The seam cell counts are presented as histograms. In control animals (top panel), the majority of animals have 16 seam cells per side. RNAi knockdown of three genes; DNAJC16/*dnj-8*, DNAJC2/*dnj-11* and DNAJC10/*dnj-27*, showed an increase in the variation but not the mean, at the control temperature (19°C). This suggests that these DnaJs

function in microenvironmental canalization (MEC). For each gene at each temperature, over 50 individual worms were counted ($n>50$), with more than 100 animals observed for the *L4440* control ($n>100$). The *P*-values can be found in Table 1 with average seam cell count data is shown in Supplementary Table S1.

Figure 3. Seam cell number following silencing of DnaJ genes involved in environmental canalization. Environmental canalization is observed when the variance of seam cell number changed under temperature stress. This figure presents the seam cell counts as histograms. Control animals, exposed to *L4440* RNAi (top panel) showed no change in seam cell number at any temperature. Of the five DnaJ genes that were silenced by RNAi, three changed only at the elevated temperature of 24.5°C; DNAJB12/*dnj-1*, DNAJC3/*dnj-7*, DNAJB11/*dnj-20*, while SEC63/*dnj-29* displayed a variation in seam cell number at 15°C. In contrast, DNAJA2/*dnj-19* showed variation in seam cell number at all temperatures. At each temperature, over 50 individual worms were counted ($n>50$) for DNAJC2/*dnj-11* and SEC63/*dnj-29*, with more than 100 animals observed for the *L4440* control, DNAJB12/*dnj-1*, DNAJC3/*dnj-7* and DNAJB11/*dnj-20* ($n>100$). The *P*-values can be found in Table 1 and average seam cell count data is shown in Supplementary Table S1.

Figure 4. GFP expression of DnaJ genes for canalization. Representative images of tissue specific expression of DnaJs. Top panel shows the expression pattern of DNAJC3/*dnj-7* (strain, BC10178). **(a)** There is broad expression of *dnj-7::gfp* in the head, tail, reproductive system and body wall muscle. **(b)** Significant expression of *dnj-7::gfp* is observed in the head, **(c)** the vulva and **(d)** spermatheca. Expression pattern of DNAJC2/*dnj-11* (strain, BC14321) is shown in the middle panel. **(e)** *dnj-11::gfp* is expressed in the head, tail and reproductive system to a low level. **(f)** Strong expression of *dnj-11::gfp* is present in the tail. **(g)** In the germline, the distal tip cell and spermatheca have strong *dnj-11::gfp* expression. **(h)** The cells of the vulva also show strong *dnj-11::gfp*. Here, expression is observed in the seam cells. The bottom panel are representative images of the expression pattern of DNAJB11/*dnj-20* (strain, BC14473). **(i)** *dnj-20::gfp* is mainly observed in the intestine, but also the seam cells and germline **(j)** Expression in the intestine is localized to the nuclei, but some is observed in the cytosol of intestinal cells. **(k)** There is faint expression in the germline. **(l)** *dnj-20::gfp* has expression in the nuclei and cytosol of the seam cells. Scale bar in all images, 100µm.

Figure 5. GFP expression of DnaJ genes at environmental temperature extremes. Representative images to show the change in DnaJ expression at different temperatures. DNAJC3/*dnj-7::gfp* (Strain, BC10178) has minimal expression at 15°C and expression is elevated in the same tissues to a similar extent at 19°C and 24.5°C. The expression of DNAJC2/*dnj-11::gfp* (Strain, BC14321) at 15°C is very low but elevated in all tissues at 24.5°C, and at 19°C there is an intermediate level of expression. Expression of DNAJB11/*dnj-20::gfp* (Strain, BC14473) in the intestine is very low at all temperatures tested. Scale bar, 100µm. All images are taken at the same magnification, with the same excitation power of the UV lamp as well as the same gain and exposure settings on the camera.

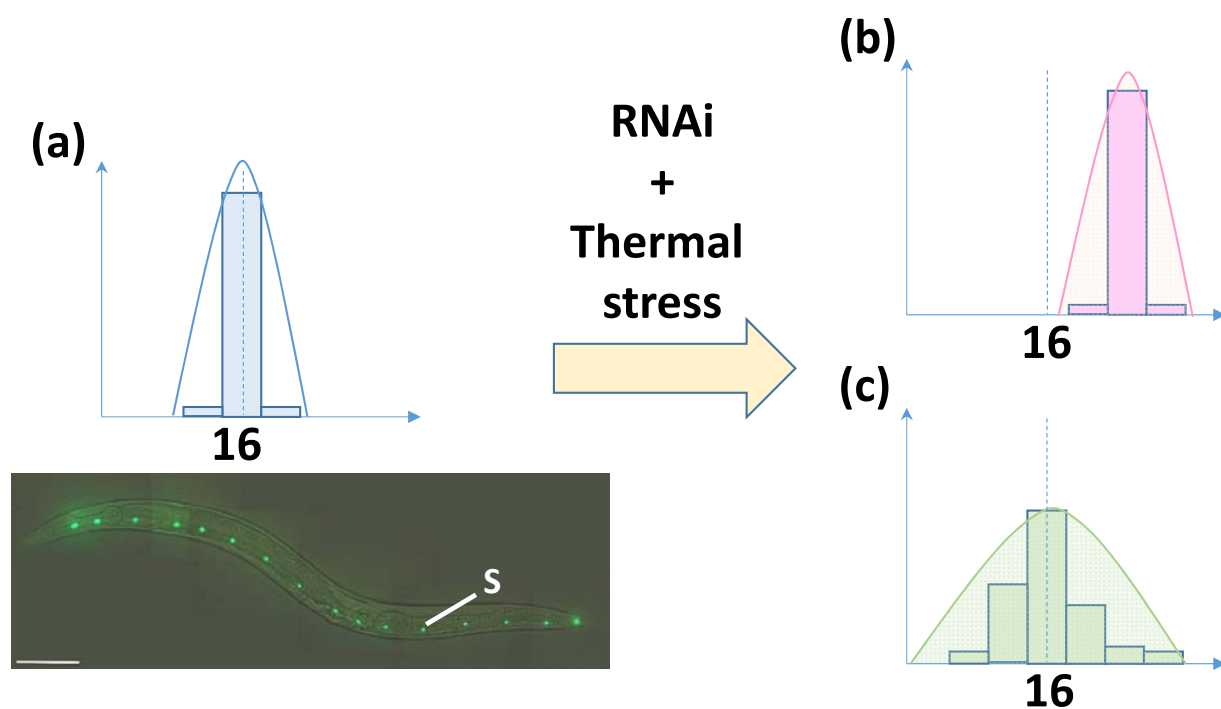
Figure 6. DnaJs involved in microenvironmental canalization affect lifespan of *C. elegans* at different temperatures. Age-synchronized animals were observed every day throughout life for death, with day 0 being the day animals were L4. Survival was assessed as a percentage, with more 100 animals assessed at each temperature. **(a)** Survival at 15°C, **(b)** Survival at 19°C and **(c)** Survival at 24.5°C. Survival of DNAJC2/*dnj-11* mutant animals (strain, VC1642) is shown as a solid grey line and survival of DNAJC10/*dnj-27* (strain, RB1784) animals are shown as a dashed grey line with the corresponding control, *N2*, is shown as a solid black line. The lifespan of *dnj-8*(RNAi) is shown as a grey dotted line, with the corresponding control of *N2* (L4440) as a dotted black line. OASIS analysis (Yang et al. 2011) of lifespan related to these DnaJs are shown in Supplementary Table S2.

Figure 7. Lifespan of DnaJs involved in environmental canalization is altered at different temperatures. Age synchronized animals were observed every day throughout life for death, with day 0 being the day animals were L4. Survival was assessed as a percentage, with more 100 animals assessed at each temperature. **(a)** Survival at 15°C, **(b)** Survival at 19°C and **(c)** Survival at 24.5°C. The control, L4440, is shown as a solid black line, survival of DNAJB12/*dnj-1*(RNAi) animals are displayed as a solid grey line. Lifespan of DNAJC3/*dnj-7*(RNAi) and SEC63/*dnj-29*(RNAi) animals are black and grey dashed lines respectively, while the grey dotted line shows the survival of DNAJB11/*dnj-20*(RNAi) animals. OASIS analysis of lifespan (Yang et al. 2011) related to these DnaJs are shown in Supplementary Table S2.

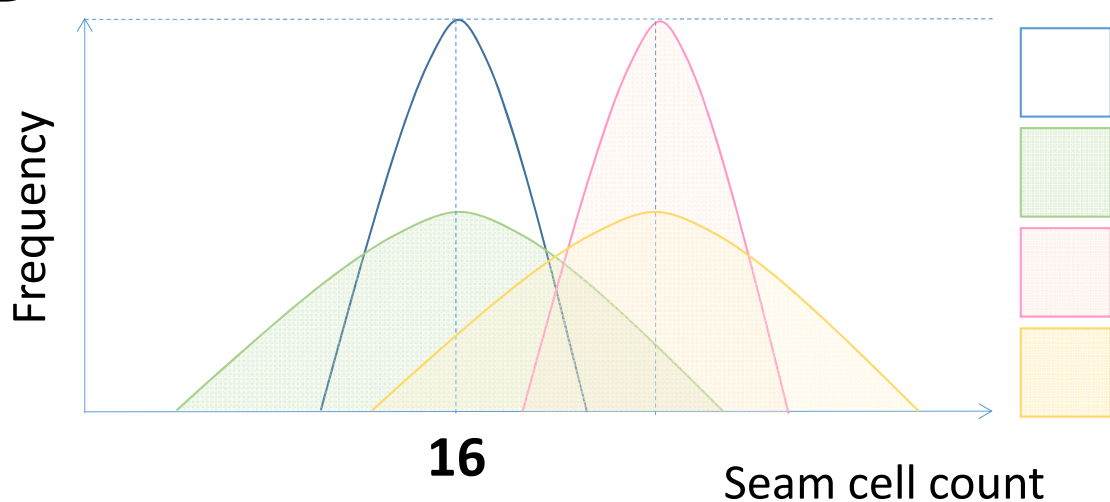
Figure 8. Summary of DnaJs in canalization investigated in this study. Protein structures are based on analysis of the sequence of *C. elegans* orthologues. Protein domains, including a PFAM

675 domain, signal peptide and internal repeats, were searched using SMART program (Schultz et
676 al. 1998). Purple box, DnaJ domain; Blue rectangle, transmembrane region; Red pentagon, SANT
677 domain; Green rectangle, coiled coil region; Black rectangle, DNAJ_C domain (Pfam) or
678 RAC_head domain (Pfam); Yellow box, TPR domain; Brown box, Sec63; Pink blocks, low
679 complexity region. F, folding; D, degradation; ND, not determined; -, no up or down regulation.
680 The change in maximal lifespan compared to controls is shown.

A



B

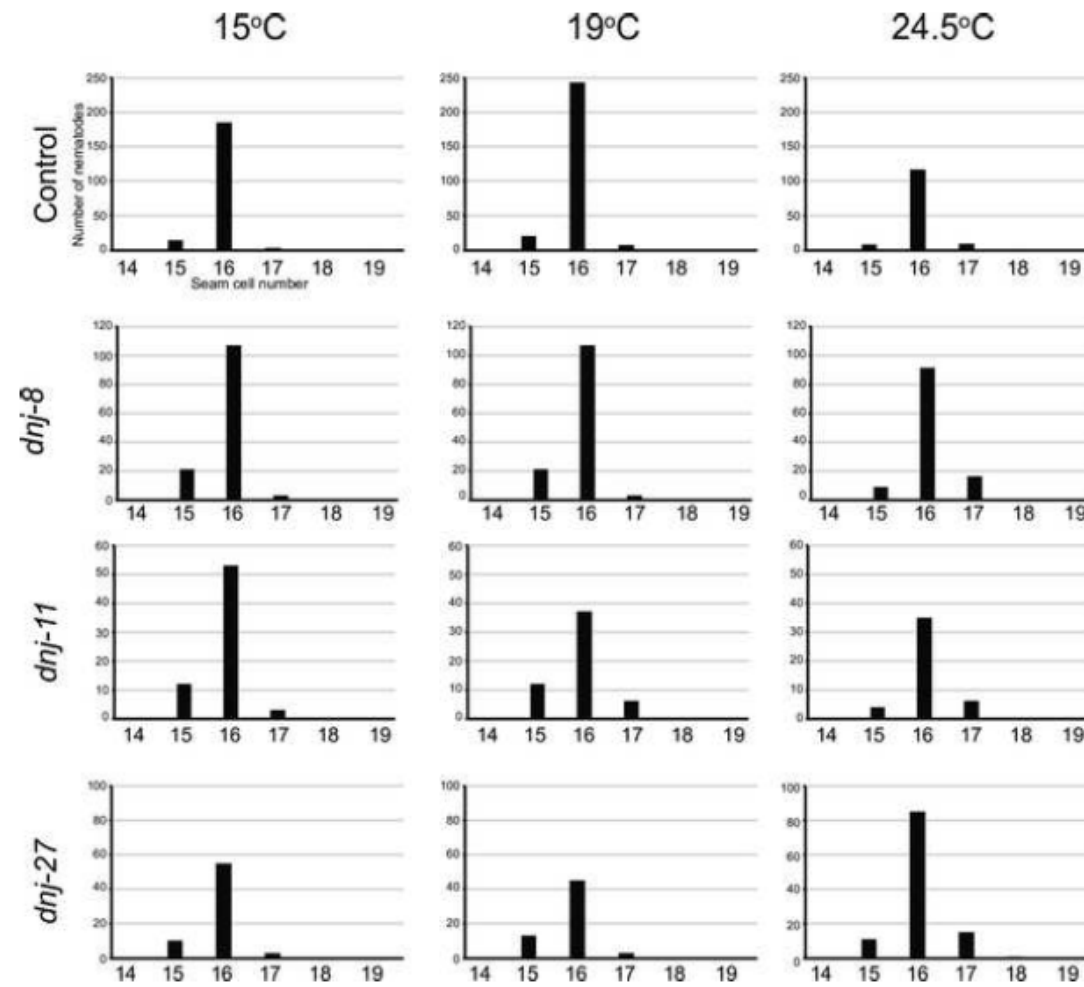


C

RNAi (19°C) vs RNAi (24/15°C)

Control vs RNAi (Both at 19°C)				
	WT	EC	PC	PC + EC
	MEC	MEC + EC	ND	ND
	PC	PC + EC	PC	ND
	PC + MEC	ND	ND	ND

Figure 2



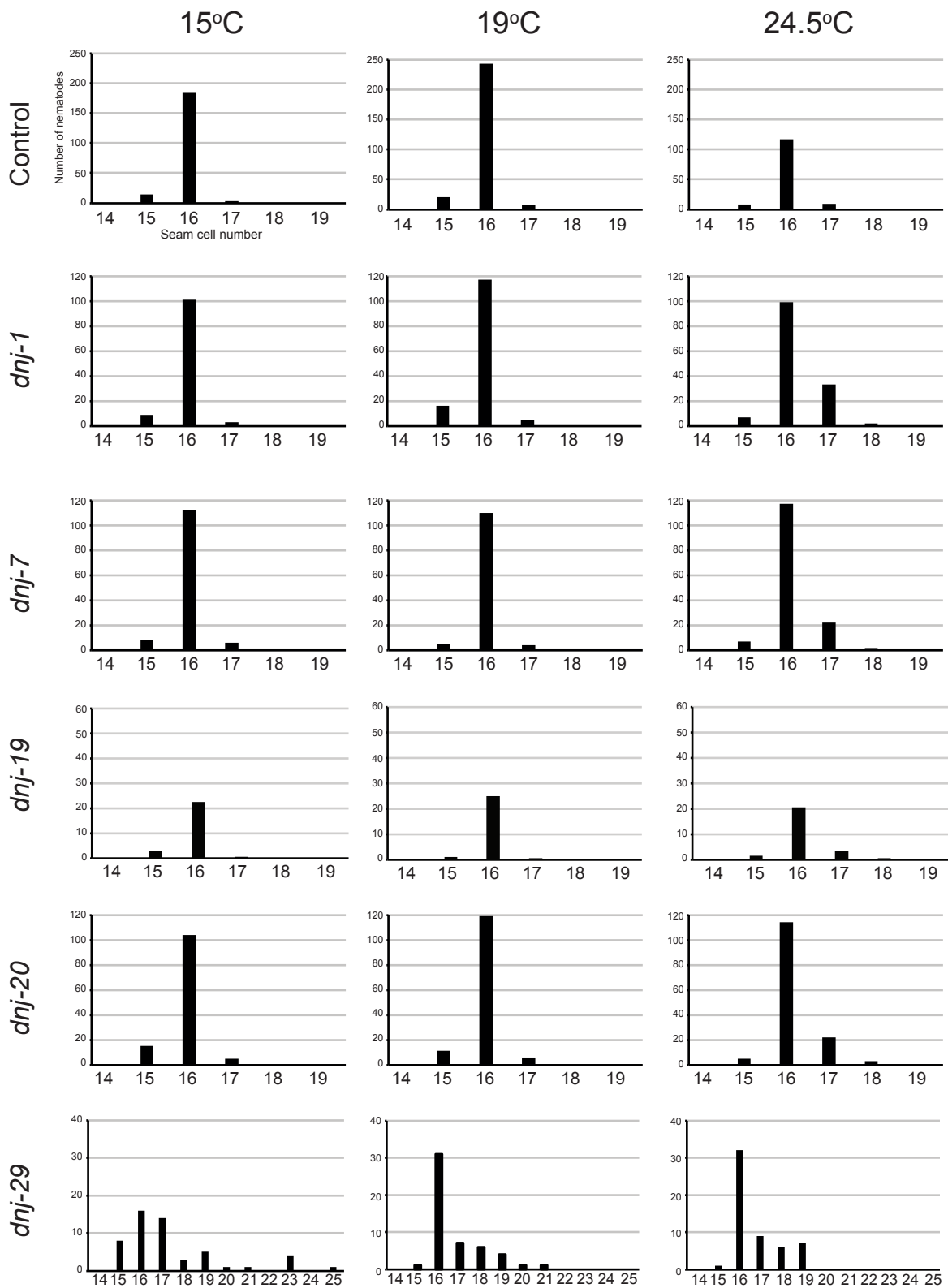


Figure 4

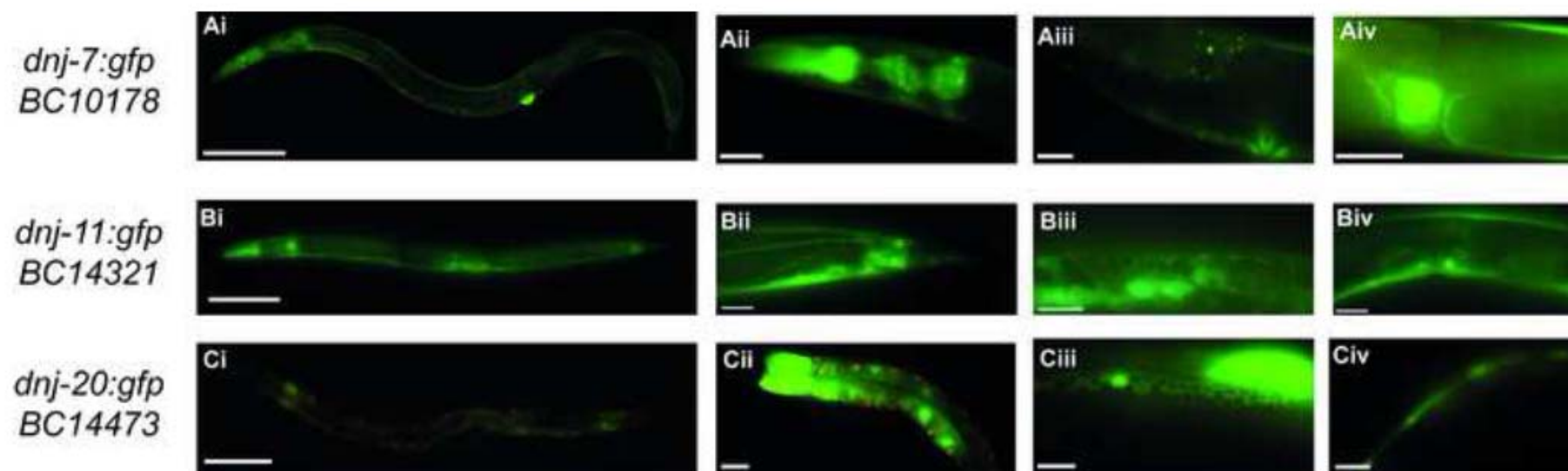
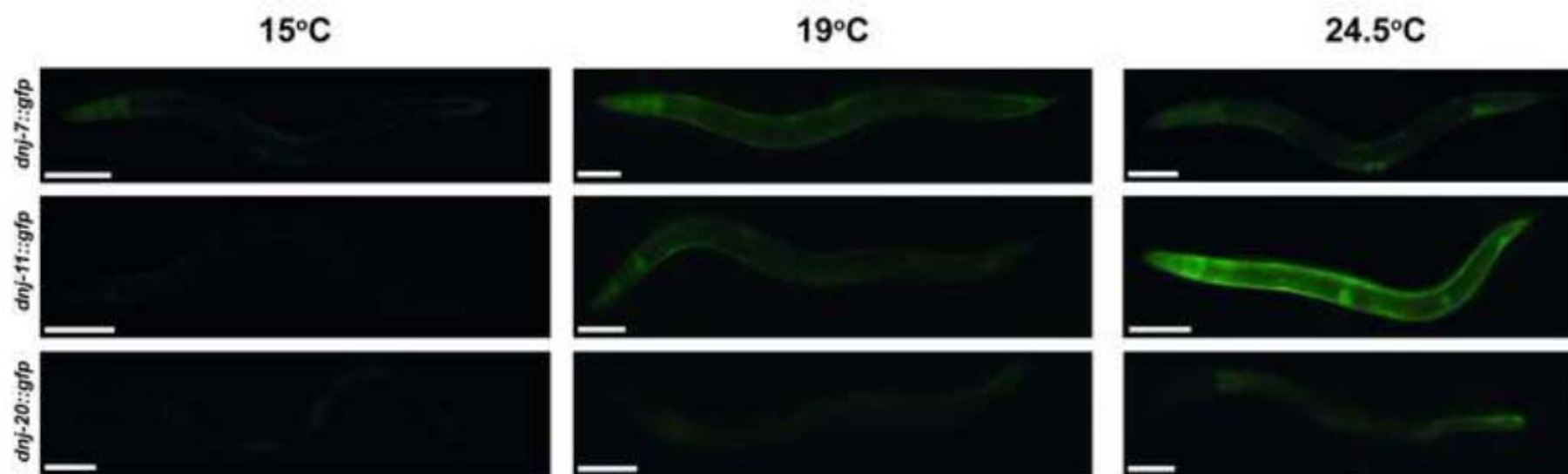
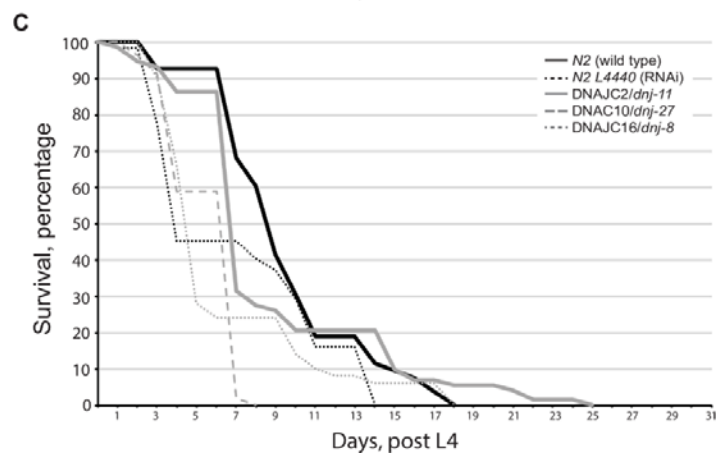
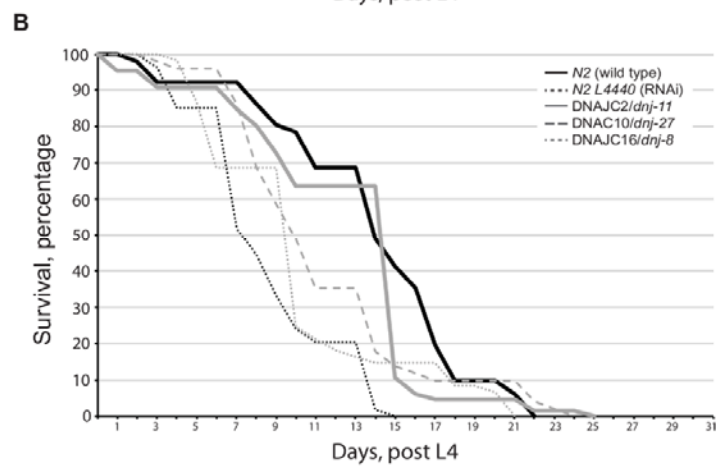
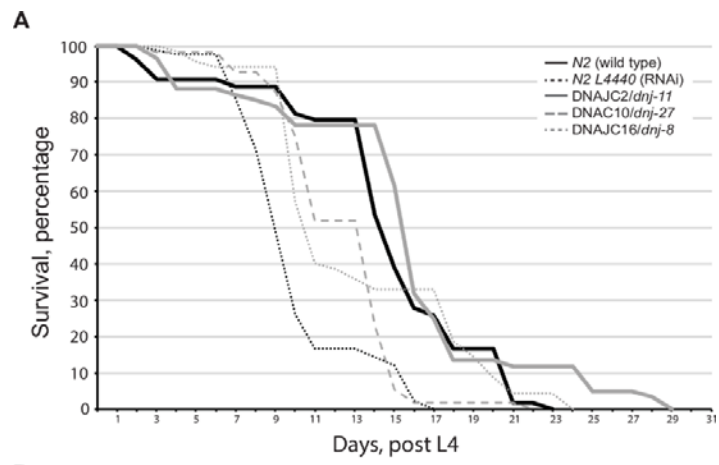
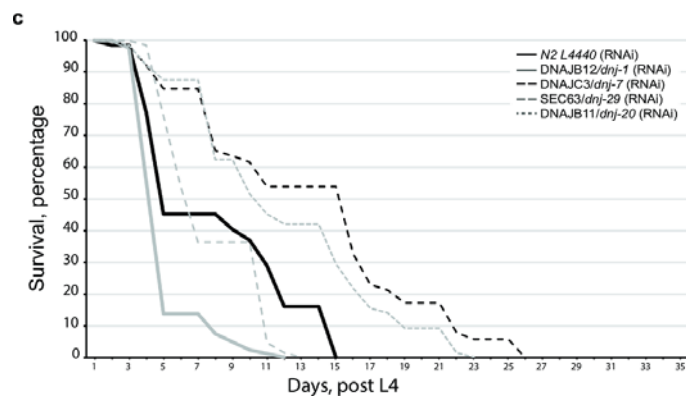
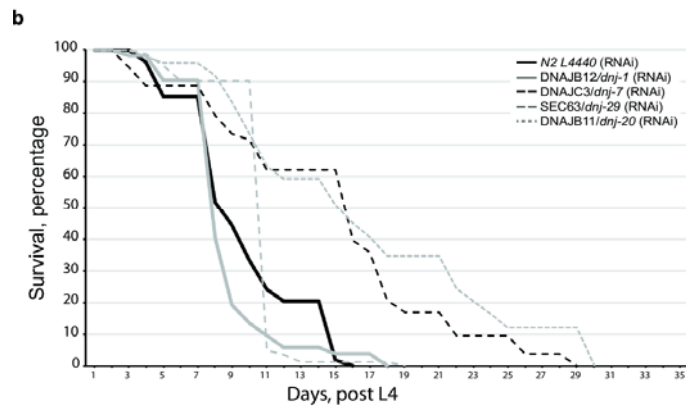
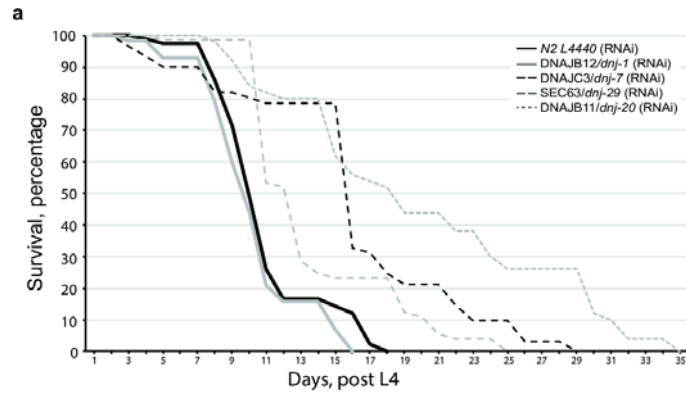
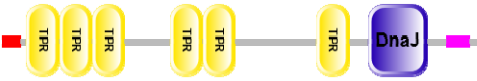










Figure 5







Canalization involved	Name in human	Name in worm	Protein structure	Cellular function [See text for Refs]	Protein expression pattern	Lifespan			Cellular localization
						15°C	19°C	24.5°C	
EC at 24.5°C	DNAJC3 (ERdj6)	<i>dnj-7</i>		F + D	Germline, head, body wall, muscle,	↑	↑	↑	ER
EC at both 24.5°C/15°C	DNAJA2	<i>dnj-19</i>		F	ND	ND			Cytosol
PC + EC at 24.5°C	DNAJB11 (ERdj3)	<i>dnj-20</i>		F + D	Intestine, germline, seam cell	↑	↑	↑	ER
PC + MEC + EC at 24.5°C	DNAJB12	<i>dnj-1</i>		D	ND	-	-	↓	ER, but J domain in cytosol
PC + MEC + EC at 15°C	SEC63 (ERdj2)	<i>dnj-29</i>		F + D	ND	↑	↑	↓	ER
MEC	DNAJC10 (DRdj5)	<i>dnj-27</i>		D	ND	-	-	↓	ER
	DNAJC2	<i>dnj-11</i>		F	Germline, head, tail, vulva, seam cell, anal depressor muscles	↑	↑	↑	Ribosome
	DNAJC16 (ERdj7)	<i>dnj-8</i>		ND	ND	↑	↑	↑	ND
Decanalization at all temperatures	DNAJB9 (ERdj4)	<i>dnj-18</i>		D	ND	ND			ER

Human Gene	Nomenclature*	Worm Gene	Control (19°C) vs RNAi (19°C)		RNAi (19°C) vs RNAi (24.5°C)		RNAi (19°C) vs RNAi (15°C)		Remarks
			Mean	Variance	Mean	Variance	Mean	Variance	
		<i>HT115</i>	-	-	0.12670	0.07107	0.82	0.07805	
ERdj1	DNAJC1	<i>F54F2.9</i>	0.02648	0.90980	0.74520	0.51570	0.16060	0.10410	
ERdj2	SEC63	<i>dnj-29</i>	0.00003	< 2.2e-16	0.93380	0.36340	0.10380	0.00001	PC + MEC + EC at 15°C
ERdj3	DNAJB11	<i>dnj-20</i>	0.75050	0.10120	0.00016	0.00007	0.34760	0.20080	PC + EC at 24.5°
ERdj4	DNAJB9	<i>dnj-18</i>	0.01209	< 2.2e-16	0.32180	< 2.2e-16	0.56850	< 2.2e-16	Decanalization at all temperatures
ERdj5	DNAJC10	<i>dnj-27</i>	0.08125	0.00000	0.00705	0.64420	0.45490	0.28760	MEC at 19°C
ERdj6/ P58 ^{IPK}	DNAJC3	<i>dnj-7</i>	0.21080	0.11780	0.00708	0.00000	0.84860	0.03635	EC at 24.5°C
ERdj7	DNAJC25	<i>dnj-2</i>	0.32220	0.30870	0.07359	0.03716	0.08514	0.70150	
ERdj8	DNAJC16	<i>dnj-8</i>	0.93260	0.00503	0.06445	0.06733	0.07086	0.62200	MEC at 19°C
	DNAJB12	<i>dnj-1</i>	0.40440	0.00531	0.00000	0.00004	0.55110	0.06073	MEC + PC + EC at 24.5°C
	DNAJC2	<i>dnj-11</i>	0.44210	0.00000	0.14360	0.22660	0.80540	0.08461	MEC at 19°C
	DNAJC9	<i>dnj-23</i>	0.34360	0.02143	0.89960	0.10320	0.07744	0.42280	
	DNAJA2	<i>dnj-19</i>	0.44310	0.02131	0.08991	0.00000	0.19730	0.00464	EC for both 15°C and 24.5°C

The DnaJ ‘capacitor’ chaperones contribute to environmental and genetic canalization

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Supplemental Table 1: Raw seam cell data. This table shows the raw values for mean and variance of seam cell number. For each gene at each temperature, over 50 individual worms were counted ($n>50$), with more than 100 animals observed for the *L4440* control ($n>100$). The p-values can be found in Table 1. The nomenclature is based on that by Ohtsuka and Hatta [23].

Human Gene	Nomenclature*	Worm Gene	RNAi (15°C)		RNAi (19°C)		RNAi (24.5°C)	
			Mean	Variance	Mean	Variance	Mean	Variance
		<i>HT115</i>	15.95	0.08	15.98	0.10	16.01	0.13
ERdj1	DNAJC1	<i>F54F2.9</i>	15.98	0.06	16.06	0.09	16.08	0.11
ERdj2	SEC63	<i>dnj-29</i>	17.38	5.70	16.76	1.58	16.75	1.23
ERdj3	DNAJB11	<i>dnj-20</i>	15.92	0.16	15.96	0.12	16.16	0.25
ERdj4	DNAJB9	<i>dnj-18</i>	15.90	0.06	16.00	0.00	15.98	0.02
ERdj5	DNAJC10	<i>dnj-27</i>	15.90	0.18	15.84	0.24	16.05	0.27
ERdj6/ P58 ^{IPK}	DNAJC3	<i>dnj-7</i>	15.98	0.11	15.99	0.08	16.12	0.21
ERdj7	DNAJC16	<i>dnj-8</i>	15.86	0.17	15.96	0.21	16.06	0.21
	DNAJB12	<i>dnj-1</i>	15.95	0.10	15.92	0.15	16.21	0.30
	DNAJC25	<i>dnj-2</i>	15.91	0.11	15.99	0.12	16.08	0.17
	DNAJC2	<i>dnj-11</i>	15.87	0.21	15.89	0.32	16.04	0.23
	DNAJC9	<i>dnj-23</i>	15.90	0.18	16.00	0.15	16.01	0.21
	DNAJA2	<i>dnj-19</i>	15.90	0.13	15.98	0.06	16.12	0.26

Supplemental Table 2: OASIS lifespan. Lifespan data was inputted into the OASIS software [47]. For each gene knockdown and at each temperature, the age in days at 50% and 100% mortality is given. The statistical analysis is shown using the Fishers exact Test. For each condition, at least 100 individual animals were observed for survival. Note that those DnaJs involved in GC, were genetic mutants (DNAJC2/*dnj-11*, strain VC1642 and DNAJC10/*dnj-27*, strain, RB1784) and thus were compared to *N2* wild type animals, while those involved in EC, and *dnj-8*, were investigated using RNAi and consequently compared to *N2* animals exposed to the empty RNAi feeding vector, *L4440*.

Name	temperature °C	Age in days at % mortality		Fishers exact Test <i>p</i> value
		50	100	
<i>N2</i>	15	15	23	-
<i>N2</i>	19	15	22	-
<i>N2</i>	24.5	9	18	-
<i>dnj-11</i>	15	16	28	1.000
<i>dnj-11</i>	19	15	25	0.023
<i>dnj-11</i>	24.5	8	25	0.015
<i>dnj-27</i>	15	14	22	0.203
<i>dnj-27</i>	19	11	24	1.000
<i>dnj-27</i>	24.5	7	8	0.089
<i>L4440</i>	15	10	17	-
<i>L4440</i>	19	9	16	-
<i>L4440</i>	24.5	10	14	-
<i>dnj-1</i>	15	10	16	0.012
<i>dnj-1</i>	19	8	16	0.777
<i>dnj-1</i>	24.5	4	11	0.000
<i>dnj-7</i>	15	16	28	0.000
<i>dnj-7</i>	19	15	28	0.209
<i>dnj-7</i>	24.5	15	25	0.003
<i>dnj-8</i>	15	11	24	0.000
<i>dnj-8</i>	19	10	21	0.000
<i>dnj-8</i>	24.5	5	18	0.000
<i>dnj-20</i>	15	29	32	0.000
<i>dnj-20</i>	19	15	28	0.047
<i>dnj-20</i>	24.5	10	22	0.251
<i>dnj-29</i>	15	11	24	0.002
<i>dnj-29</i>	19	11	18	0.000
<i>dnj-29</i>	24.5	6	12	0.201

Supplemental Figure S1: *dnj-2*, *dnj-23* and *F54F2.9* do not display significant changes in seam cell number due to environmental changes. Three of the tested DnaJ genes, DNAJC1/*F54F2.9*, DNAJC25/*dnj-2* and DNAJC9/*dnj-23*, showed no change in mean or variance under RNAi, whether at normal (19°C) or experimental temperatures (of 15°C or 24.5°C). This suggests that these genes do not function in genetic or environmental canalization. DNAJB9/*dnj-18* decreased variance with RNAi at all temperatures, suggesting that DNAJB9/*dnj-18* is involved in decanalization. For all genes and for all temperatures, seam cells from 100 or more different animals were observed ($n > 100$), while for *F54F2.9*, 50 animals were observed ($n \geq 50$).

