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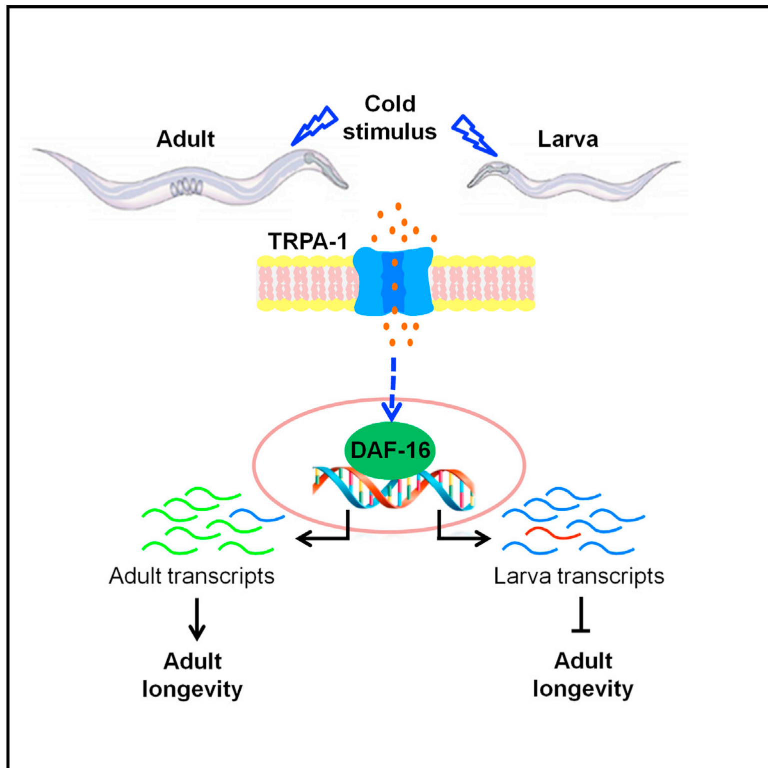
# Environmental Temperature Differentially Modulates *C. elegans* Longevity through a Thermosensitive TRP Channel

## Graphical Abstract




## Highlights

- Worms experiencing low temperatures during development are short-lived
- TRPA-1 acts through DAF-16 during development to shorten lifespan at low temperatures
- DAF-16 differentially regulates gene expression during development and adulthood

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## In Brief

Zhang et al. show that, although exposure of *C. elegans* to low temperatures during adulthood prolongs lifespan, low-temperature treatment during development reduces lifespan. This differential temperature effect is mediated by the thermosensitive channel TRPA-1.

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# Environmental Temperature Differentially Modulates *C. elegans* Longevity through a Thermosensitive TRP Channel

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## SUMMARY

**Temperature profoundly affects aging in both poikilotherms and homeotherms. A general belief is that lower temperatures extend lifespan, whereas higher temperatures shorten it. Although this "temperature law" is widely accepted, it has not been extensively tested. Here, we systematically evaluated the role of temperature in lifespan regulation in *C. elegans*. We found that, although exposure to low temperatures at the adult stage prolongs lifespan, low-temperature treatment at the larval stage surprisingly reduces lifespan. Interestingly, this differential effect of temperature on longevity in larvae and adults is mediated by the same thermosensitive TRP channel TRPA-1 that signals to the transcription factor DAF-16/FOXO. DAF-16/FOXO and TRPA-1 act in larva to shorten lifespan but extend lifespan in adulthood. DAF-16/FOXO differentially regulates gene expression in larva and adult in a temperature-dependent manner. Our results uncover complexity underlying temperature modulation of longevity, demonstrating that temperature differentially regulates lifespan at different stages of life.**

## INTRODUCTION

Both environmental factors and genes affect aging (Fontana et al., 2010; Kenyon, 2010). Temperature and food are the two primary environmental factors that modulate longevity (Fontana et al., 2010; Kenyon, 2010). Reduction in either food intake (dietary restriction) or temperature can extend lifespan (Conti, 2008). Although the effect of diet on longevity has been extensively characterized, very little is known about how temperature regulates lifespan.

It was documented a century ago that, with the exception of extreme temperatures, which may threaten animal survival, lower environmental temperatures generally extend the lifespan of poikilotherms such as worms, flies, and fish, whereas higher temperatures shorten their lifespan (Loeb and Northrop, 1916). Recent studies demonstrate that this also appears to be the case in homeothermic animals. For example, lowering the core body temperature of mice extends lifespan (Conti et al., 2006); exposing rats to lower environmental temperatures also promotes longevity (Holloszy and Smith, 1986), indicating that both body and environmental temperatures affect lifespan in rodents. Interestingly, lower body temperatures are also associated with longer human lifespan in the Baltimore Longitudinal Study of Aging (Roth et al., 2002). These observations highlight a general role of temperature in lifespan regulation in both poikilotherms and homeotherms.

Traditionally, the rate-of-living theory has been adopted to explain the effect of temperature on lifespan (Loeb and Northrop, 1916). Namely, low temperatures reduce the rate of chemical reactions, thereby slowing down the pace of aging, and vice versa. Recent work in *C. elegans*, however, shows that genes can actively regulate lifespan in response to temperature changes (Lee and Kenyon, 2009; Xiao et al., 2013). Specifically, the cold-sensitive TRPA-1 channel can detect temperature drop in the environment to initiate a pro-longevity signaling cascade to extend lifespan (Xiao et al., 2013). At high temperatures, the heat-sensitive neuron AFD antagonizes the detrimental effect of high temperature to increase lifespan via neuroendocrine signaling (Lee and Kenyon, 2009). Additional genes are also found to regulate lifespan in a temperature-dependent manner (Horikawa et al., 2015; Mizunuma et al., 2014). Despite these observations, the "temperature law" remains: low temperatures increase lifespan, whereas high temperatures decrease it.

In this report, we re-visited the temperature law in lifespan regulation in *C. elegans*. Our data show that, whereas low temperature exposure at the adult stage extends lifespan, similar


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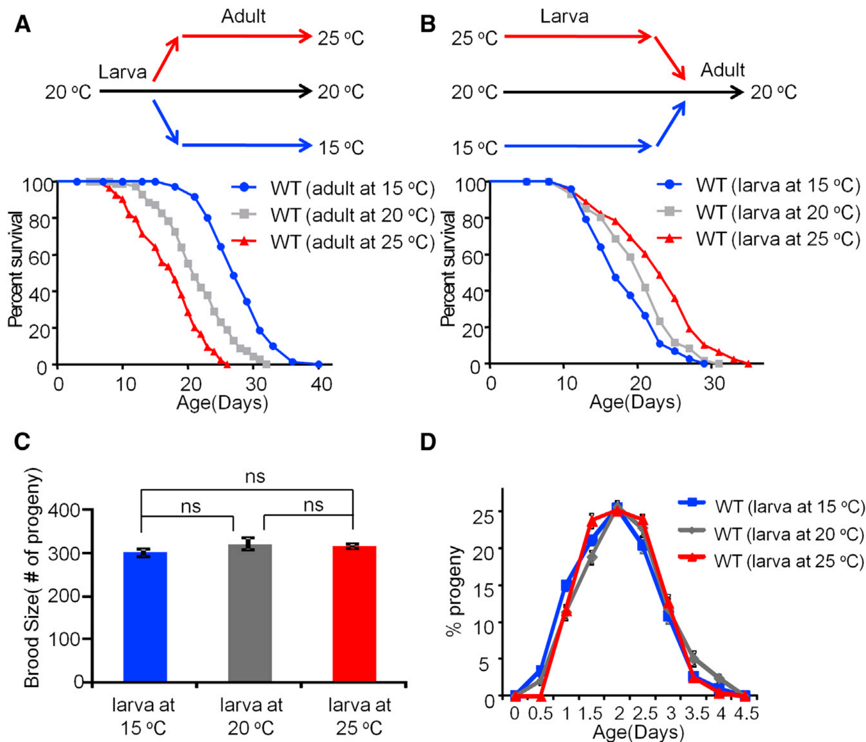



**Figure 1. Temperature Treatment at Different Stages of Worm Life Differentially Affects Lifespan, but Not Fertility**

(A) Exposure to low and high temperatures at the adult stage extends and shortens lifespan, respectively ($p < 0.001$; log rank test). The top panel describes the protocol of temperature treatment.

(B) Exposure to low and high temperatures at the larval stage shortens and extends lifespan, respectively ($p < 0.001$; log rank test). The top panel describes the protocol of temperature treatment.

(C and D) Exposure to different temperatures at the larval stage does not have a notable effect on fertility in adulthood. (C) Overall fertility is not notably affected by temperature treatment at the larval stage. (D) Egg-laying pattern is not notably affected by temperature treatment at the larval stage. The percentage of eggs laid every 12 hr (half a day) was plotted. Larvae were reared at 15°C, 20°C, and 25°C, and their adults were moved to 20°C to lay eggs. n = 30. Error bars: SEM.

See also Figure S1. Representative data are shown here, and replicates and detailed statistics are shown in Table S1.

treatment at the larval stage, remarkably, shortens lifespan. Interestingly, this differential effect of temperature on lifespan requires the cold-sensitive TRPA-1 channel and its downstream transcription factor DAF-16/FOXO, which is a key regulator in lifespan control (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). Both DAF-16/FOXO and TRPA-1 promote longevity at the adult stage but surprisingly play an inhibitory role on longevity at the larval stage. We further demonstrate that DAF-16/FOXO differentially regulates gene expression at the larval and adult stages in a temperature-dependent manner, which is consistent with its role in mediating the differential effect of temperature on longevity in larvae and adults. Our studies demonstrate that temperature modulation of longevity is more complex than previously thought, suggesting that caution needs to be exercised when applying the temperature law to aging studies. More importantly, we show that temperature exerts a differential effect on lifespan at different stages of life.

## RESULTS

### Temperature Treatment at Different Stages of Worm Life Differentially Affects Lifespan

Previous work mainly focused on the impact of temperature on lifespan at the adult stage (Klass, 1977; Lee and Kenyon, 2009; Wu et al., 2009; Xiao et al., 2013). As a result, how temperature treatment at the larval stage influences lifespan has not been explored. The cultivation temperature for *C. elegans* in the laboratory spans from 15°C to 25°C, with three temperatures (15°C, 20°C, and 25°C) being most commonly used. To isolate the effect of temperature on adults, we first let three groups of larval worms grow at the same temperature (20°C) until they reached the last larval stage L4 and then shifted them to three different temperatures (15°C, 20°C, and 25°C) throughout the adult stage (Figure 1A). By fixing the temperature at the larval stage, we were able to interrogate how exposure to different temperatures at the adult stage affects lifespan. We found that adult worms exposed to 15°C and 25°C lived the longest and shortest lifespan, respectively (Figure 1A). These data are consistent with the view that low temperatures extend lifespan, whereas high temperatures shorten it.

To characterize how temperature treatment at the larval stage affects longevity, we exposed eggs at three different temperatures until they developed as L4 larvae and then shifted them to the same temperature to score adult lifespan (Figure 1B). Surprisingly, larvae developed at 15°C exhibited the shortest lifespan, whereas those grown at 25°C lived the longest life (Figure 1B). Similar results were obtained with worms that had been maintained at different temperatures for several generations (Figure S1A) and with other temperature treatment protocols (Figures S1B–S1D). Apparently, low-temperature treatment at the larval stage shortened adult lifespan, whereas exposure to high temperature at this stage extended adult lifespan. We also checked fertility but found that similar temperature treatments at the larval stage did not have a notable effect on fertility in adulthood (Figures 1C and 1D). These results demonstrate that temperature differentially affects longevity at different stages of worm life. This also shows that temperature treatment at the larval stage has a long-lasting effect on lifespan in adulthood.

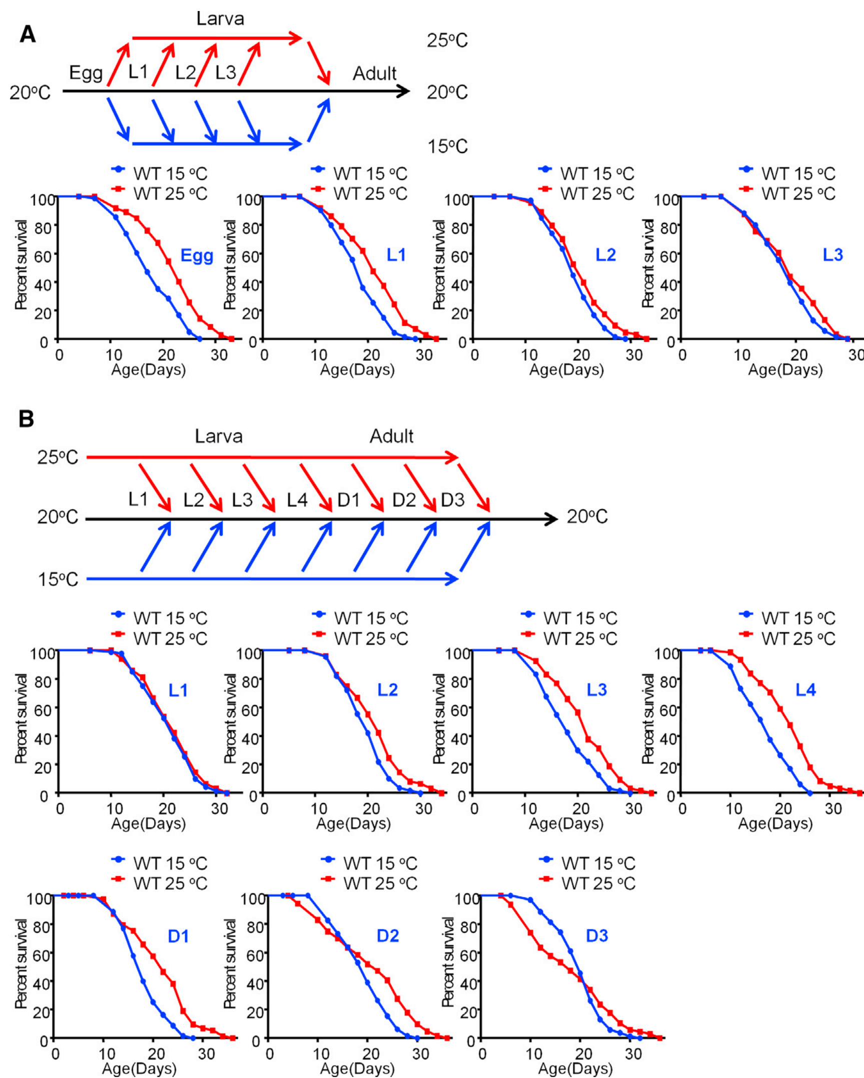



**Figure 2. The Temporal Window Critical for Temperature Modulation of Lifespan**

(A) The L1 to L2 stage is a critical window for temperature modulation of lifespan at the larval stage. The protocol was described in the upper panel. Hermaphrodite mothers were allowed to lay eggs at 20°C. The specific stage (egg, L1, L2, and L3) at which animals were up- or downshifted from 20°C to 25°C or 15°C was labeled in each panel. At the L4 stage, animals were all shifted back to 20°C to score adult lifespan. (egg: $p < 0.001$, log rank test; L1: $p < 0.001$, log rank test; L2: $p = 0.066$, log rank test; L3: $p = 0.198$, log rank test).

(B) A second protocol also shows that the L1 to L2 stage is a window critical for temperature modulation of lifespan at the larval stage. The protocol was described in the upper panel. Eggs were allowed to hatch at 15°C and 25°C and developed to different stages of larvae or adults, which were shifted to 20°C to score lifespan. The specific stage (L1, L2, L3, L4, D1 [day 1 adult], D2 [day 2 adult], and D3 [day 3 adult]) at which animals were shifted to 20°C was labeled in each panel (L1: $p = 0.399$, log rank test; L2: $p = 0.013$, log rank test; L3: $p = 0.001$, log rank test; L4: $p < 0.001$, log rank test; D1: $p < 0.001$, log rank test; D2: $p = 0.007$, log rank test; D3: $p = 0.788$, log rank test).

Representative data are shown here; replicates and detailed statistics are described in Table S1.

## The Temporal Window Critical for Temperature Modulation of Lifespan

We decided to focus on characterizing the effect of temperature treatment at the larval stage given that much is known about how such treatment at the adult stage affects lifespan. Because 15°C and 25°C treatment elicits the greatest difference, for simplicity, we mainly focused on these two temperatures. We first asked whether the effect of temperature is uniform across all the larval stages or there is a specific time window during which temperature treatment elicits the greatest effect.

To test this, we employed two strategies. In the first protocol, we shifted eggs and different stages of larvae (i.e., L1, L2, and L3) from 20°C to 15°C and 25°C until they reached L4 followed by assaying their adult lifespan at the same temperature 20°C (Figure 2A). We found that, if animals were shifted after the L1 stage, temperature can no longer elicit a notable effect, indicating that the L1 to L2 stage is a critical time window (Figure 2A). In the second protocol, we let eggs hatch at 15°C and 25°C and then shifted different stages of larvae and adults to 20°C (Figure 2B). Similarly, if animals were shifted to 20°C before the L2 stage, we no longer observed a notable effect of temperature (Figure 2B). This protocol also revealed the L1 to L2 stage as a critical window. Interestingly, if larvae were allowed to develop into adulthood at 15°C and 25°C and then shifted to 20°C, the temperature effect at the larval stage began to fade and finally disappeared at day 3 of the adult stage (Figure 2B). This is consistent with the notion that temperature treatment in the larval and adult stages elicits opposite effects and antagonizes each other. These experiments revealed a specific temporal window during which temperature elicits the greatest effect on longevity, demonstrating that the impact of temperature is not uniform across all the larval stages.

## TRPA-1 Is Required for Temperature Modulation of Longevity at the Larval Stage

The observation that low temperature exposure shortens rather than extends lifespan is inconsistent with the temperature law. This also suggests that such a phenomenon is probably regulated by genes. Therefore, we set out to identify genes that regulate this process.

At the adult stage, temperature modulation of longevity is regulated by the cold-sensitive channel TRPA-1, which presumably detects temperature drop in the environment to

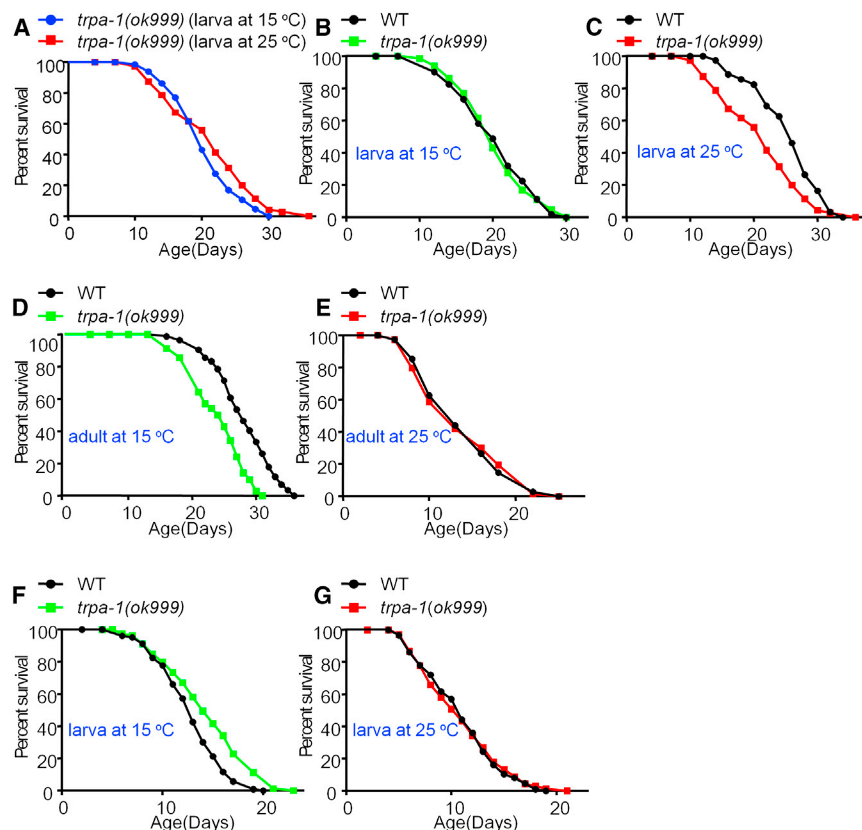



**Figure 3. TRPA-1 Is Required for Temperature Modulation of Longevity at the Larval Stage**

(A) Loss of *trpa-1* nearly eliminated the effect of larval temperature treatment on lifespan ($p = 0.104$; log rank test).

(B and C) *trpa-1* mutant worms show a normal lifespan following treatment at 15°C at the larval stage (B; $p = 0.945$; log rank test) but are short lived if their larvae were treated at 25°C (C; $p < 0.001$; log rank test). Temperature treatment was performed as shown in Figure 1B, and the adult lifespan was scored at 20°C.

(D and E) *trpa-1* mutant worms are short lived when adult animals are reared at 15°C (D; $p < 0.001$; log rank test) but show a normal lifespan when reared at 25°C (E; $p = 0.934$; log rank test). Temperature treatment was performed as shown in Figure 1A, and the adult lifespan was scored at 15°C and 25°C in (D) and (E), respectively.

(F and G) *trpa-1* mutant worms are long lived following treatment at 15°C at the larval stage (F; $p < 0.001$; log rank test) but show a normal lifespan if their larvae were treated at 25°C (G; $p = 0.878$; log rank test). Temperature treatment was performed as shown in Figure 1B, but the adult lifespan was scored at 25°C instead of 20°C.

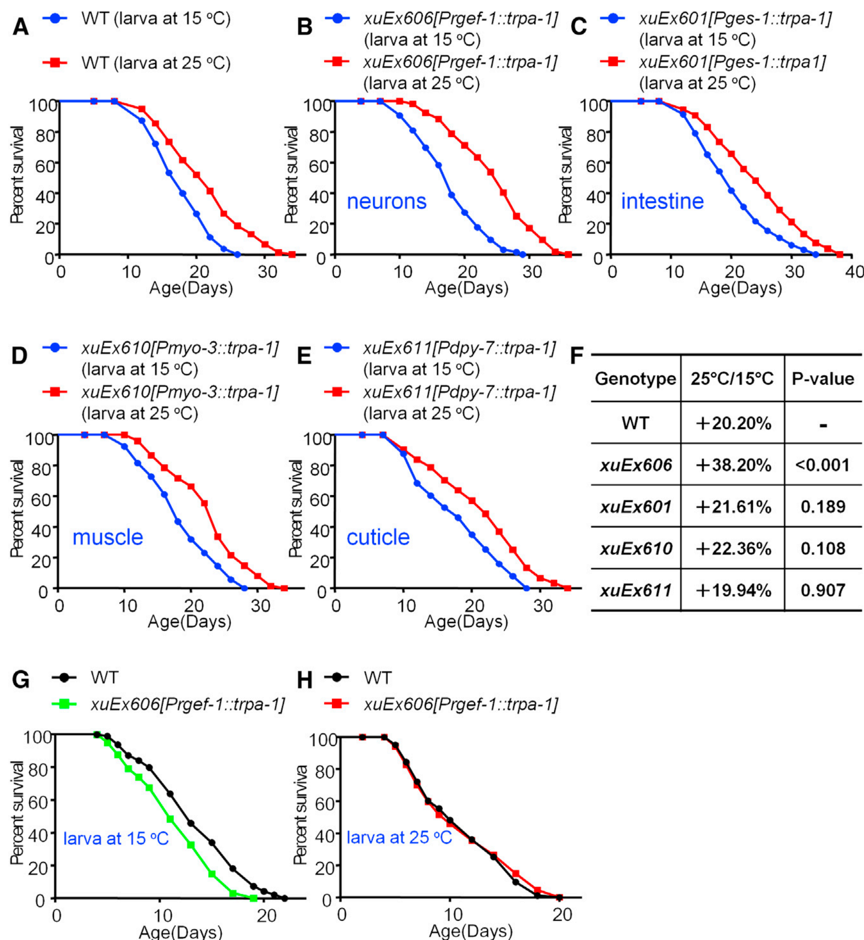
See also Figure S2. Representative data are shown here; replicates and detailed statistics are described in Table S1.

initiate a pro-longevity genetic program (Xiao et al., 2013). We thus tested whether TRPA-1 plays a role in mediating temperature modulation of longevity at the larval stage. We found that loss of *trpa-1* gene nearly eliminated the effect of temperature (Figure 3A), indicating that TRPA-1 is also important for mediating temperature modulation of longevity at the larval stage.

## TRPA-1 Shortens Lifespan at the Larval Stage while Promoting Lifespan at the Adult Stage in a Temperature-Dependent Manner

How might TRPA-1 regulate lifespan at the larval stage? TRPA-1 is a cold-sensitive channel that is active at 15°C (opens at ≤20°C) but remains closed at 25°C (Chatzigeorgiou et al., 2010; Xiao et al., 2013). At the adult stage, TRPA-1 extends lifespan at 15°C but has no effect on lifespan at 25°C (Xiao et al., 2013). Because low-temperature treatment at the larval stage shortens lifespan, one would expect that cold activation of TRPA-1 at the larval stage shall shorten lifespan but that, at high temperatures, this channel should have no effect on lifespan because it is inactive. If so, *trpa-1* mutant worms should be long lived or exhibit a normal lifespan when their larvae were reared at low or high temperatures, respectively. However, this does not appear to be the case (Figures 3B and 3C). In fact, the opposite was observed (Figures 3B and 3C). We realized that TRPA-1 also functions in adulthood to prolong lifespan at low temperatures (Xiao et al., 2013). This adult effect of TRPA-1 may obscure our results when we examined its role at the larval stage. To overcome this difficulty, we treated larvae under 15°C and 25°C but scored their adult lifespan at 25°C instead of 20°C. As TRPA-1 shows no effect on lifespan at 25°C at the adult stage (Figures 3D and 3E), this protocol should selectively restrict the effect of TRPA-1 to the larval stage. Using this protocol, we found that *trpa-1* mutants were long lived when their larvae were reared at low temperatures (Figure 3F) but showed a normal lifespan when their larvae were grown at high temperatures (Figure 3G). Though HSF-1 is best known to be activated at ≥30°C in most organisms, it could be turned on in *C. elegans* when animals are up shifted to 25°C (Sugi et al., 2011). Thus, we knocked down *hsf-1* by RNAi at the adult stage to exclude its potential contribution to lifespan and obtained a similar result (Figures S2A and S2B). These data suggest that TRPA-1 shortens lifespan at low temperatures at the larval stage, a function opposite to that observed at the adult stage.

To provide further evidence, we expressed TRPA-1 as a transgene in wild-type worms to ascertain whether its overexpression would enhance the effect of temperature treatment on lifespan at the larval stage. As TRPA-1 is expressed in multiple tissues, including neurons, intestine, muscle, and cuticle (Chatzigeorgiou et al., 2010; Xiao et al., 2013), we selectively expressed TRPA-1 in different tissues using tissue-specific promoters. Although treatment at low and high temperatures at the larval stage elicited ~20% difference in lifespan in




| Genotype | 25°C/15°C | P-value |
|---|---|---|
| WT | +20.20% | - |
| *xuEx606* | +38.20% | <0.001 |
| *xuEx601* | +21.61% | 0.189 |
| *xuEx610* | +22.36% | 0.108 |
| *xuEx611* | +19.94% | 0.907 |

**Figure 4. Overexpression of TRPA-1 Enhances the Effect of Larval Temperature Treatment on Lifespan**

(A) The effect of larval temperature treatment on lifespan in wild-type worms ($p < 0.001$; log rank test). Temperature treatment was performed as described in Figure 1B.

(B–F) Overexpression of TRPA-1 as a transgene in neurons (B; $p < 0.001$; log rank test), but not in the intestine (C; $p < 0.001$; log rank test), muscle (D; $p < 0.001$; log rank test), or cuticle (E; $p = 0.001$; log rank test), augments the effect of larval temperature treatment on lifespan. Data in (A)–(E) were summarized in (F). p values are listed (paired t test). The *ges-1*, *rgef-1*, *myo-3*, and *dpy-7* promoter drives expression in the intestine, neurons, muscle, and cuticle, respectively (Aamodt et al., 1991; Altun-Gultekin et al., 2001; Fire and Waterston, 1989; Gilleard et al., 1997).

(G and H) Overexpression of TRPA-1 as a transgene shortens lifespan following temperature treatment at 15°C at the larval stage (G; $p < 0.001$; log rank test), but the same transgene has no effect on lifespan if larvae are treated at 25°C (H; $p = 0.637$; log rank test). Temperature treatment was performed as shown in Figure 1B, but the adult lifespan was scored at 25°C instead of 20°C.

See also Figure S3. Representative data are shown here; replicates and detailed statistics are described in Table S1.

wild-type (Figures 4A and 4F), a neuronal *trpa-1* transgene augmented the difference to ~38% (Figures 4B and 4F). By contrast, transgenic expression of TRPA-1 in other tissues had no obvious effect (Figures 4C–4F), indicating that TRPA-1 may act in neurons to mediate its temperature-dependent longevity effect at the larval stage. The observation that overexpression of TRPA-1 enhanced the temperature effect further supports a role of TRPA-1 in regulating lifespan at the larval stage.

We wondered how TRPA-1 overexpression might augment the temperature effect on lifespan at the larval stage. If TRPA-1 regulates lifespan at the larval stage in a temperature-dependent manner, then one would predict that its overexpression should enhance the temperature effect by shortening lifespan at low, but not high, temperatures. To test this, we restricted the contribution of *trpa-1* transgene to the larval stage by treating larvae at 15°C and 25°C and then scored adult lifespan at 25°C, a temperature under which *trpa-1* transgene is no longer active (Xiao et al., 2013). Indeed, we found that *trpa-1* transgene shortened lifespan at low, but not high, temperatures at the larval stage in both wild-type and *hsf-1*(*RNAi*) worms (Figures 4G, 4H, S3A, and S3B). It is worth noting that the same transgene extended lifespan at low, but not high, temperatures at the adult stage, an effect opposite to that observed at the larval stage (Figures S3C and S3D). Thus, both mutant and overexpression data support that TRPA-1 shortens lifespan at the larval stage in a temperature-dependent manner.

## DAF-16 and HSF-1 Mediate the Temperature Effect at the Larval Stage

As a temperature sensor, TRPA-1 cannot regulate lifespan on its own. Typically, longevity signals converge on a small group of transcription factors (Fontana et al., 2010; Kenyon, 2010). We therefore searched for the transcription factors that may act downstream of TRPA-1 to mediate its effect on temperature modulation of longevity. To this end, we examined those well-characterized transcription factors known to regulate lifespan. We found that loss of DAF-16/FOXO, a master regulator of lifespan, nearly abolished the effect of temperature treatment at the larval stage on lifespan (Figure 5A; $p = 0.021$; Cox proportional hazard regression analysis). Namely, *daf-16* mutant worms exhibited similar lifespans following larval temperature treatment at 15°C and 25°C (Figure 5A), revealing a critical role of *daf-16* in mediating the temperature effect at the larval stage. Consistent with this model, RNAi of *daf-16* at the adult stage alone had no notable effect ($p = 0.804$; Cox proportional hazard regression analysis), as the difference in adult lifespan between individuals developed as larva at 15°C (mean lifespan 14.16 ± 0.28 days) and 25°C (mean lifespan 16.89 ± 0.33 days) remained in these

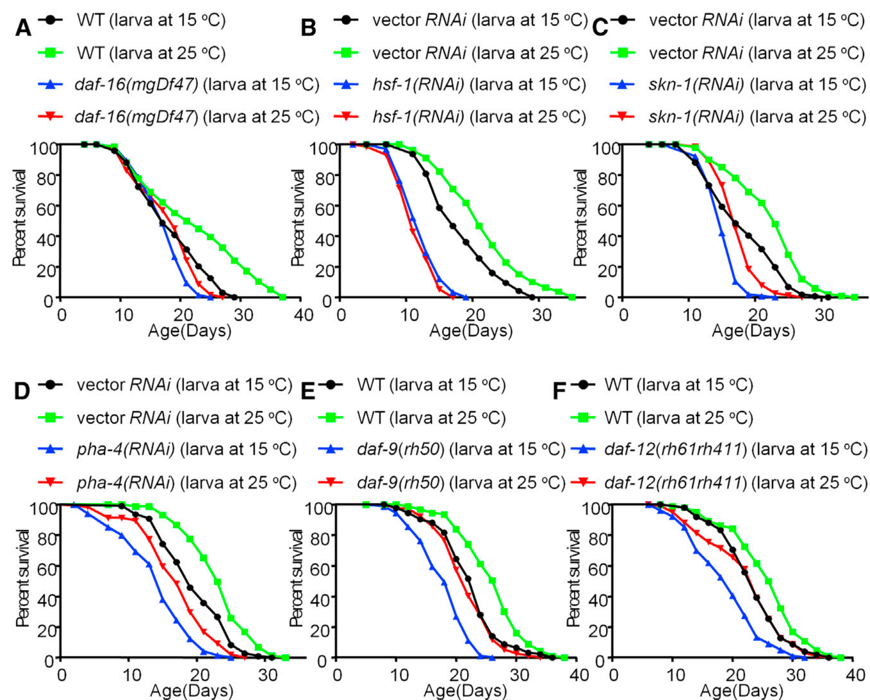



**Figure 5. DAF-16 and HSF-1 Mediate the Temperature Effect at the Larval Stage**

(A) Loss of *daf-16* nearly eliminated the effect of larval temperature treatment on lifespan ($p = 0.021$; Cox proportional hazard regression analysis). The temperature treatment protocol was performed as described in Figure 1B.

(B) RNAi of *hsf-1* blocked the effect of larval temperature treatment on lifespan ($p = 0.002$; Cox proportional hazard regression analysis). The temperature treatment protocol was performed as described in Figure 1B.

(C–F) SKN-1 ($p = 0.788$; Cox proportional hazard regression analysis), PHA-4 ($p = 0.390$; Cox proportional hazard regression), DAF-9 ($p = 0.179$; Cox proportional hazard regression analysis), and DAF-12 ($p = 0.190$; Cox proportional hazard regression analysis) are not required for larval temperature treatment to affect lifespan.

Representative data are shown here; replicates and detailed statistics are described in Table S1.

*daf-16* RNAi animals ($p < 0.001$; log rank). On the other hand, these animals were short lived, indicating an effect of *daf-16* RNAi (see also Figures 6C and 6D). These data suggest that *daf-16* may act at the larval stage.

Deficiency in HSF-1, another prominent longevity regulator (Hsu et al., 2003; Satyal et al., 1998), also eliminated the temperature effect (Figure 5B; $p = 0.002$; Cox proportional hazard regression analysis). By contrast, other transcription factors, such as SKN-1 ($p = 0.788$; Cox proportional hazard regression analysis), PHA-4 ($p = 0.390$; Cox proportional hazard regression analysis), DAF-12 ($p = 0.190$; Cox proportional hazard regression analysis), and its regulator DAF-9 ($p = 0.179$; Cox proportional hazard regression analysis; Antebi et al., 2000; Bishop and Guarente, 2007; Jia et al., 2002; Panowski et al., 2007; Tullet et al., 2008), were not required (Figures 5C–5F). These experiments identify DAF-16 and HSF-1 as key regulators mediating the temperature effect at the larval stage.

## DAF-16 Is Required for TRPA-1 to Regulate Lifespan at the Larval Stage

We then asked whether DAF-16 and/or HSF-1 mediate the function of TRPA-1 in temperature modulation of longevity at the larval stage. Although TRPA-1 overexpression greatly enhanced the effect of larval temperature treatment (15°C and 25°C) on lifespan, loss of *daf-16* abrogated such an effect (Figure 6A; $p < 0.001$; Cox proportional hazard regression analysis). Namely, transgenic worms harboring a *daf-16* mutation exhibited similar lifespans in response to larval temperature treatment at 15°C versus 25°C (Figure 6A). By contrast, such a temperature effect remained unchanged in *hsf-1*-deficient worms (Figure 6B; $p = 0.824$; Cox proportional hazard regression analysis). These data suggest that DAF-16, but not HSF-1, is required for TRPA-1 to regulate lifespan at the larval stage in a temperature dependent manner.

To provide further evidence, we selectively examined the role of DAF-16 in mediating TRPA-1-dependent temperature effect at the larval stage. Again, we restricted the effect of *trpa-1* transgene to the larval stage by treating larvae at 15°C and 25°C and then shifted them to 25°C to score adult lifespan. We found that loss of *daf-16* blocked the ability of *trpa-1* transgene to shorten lifespan at low temperatures in wild-type and *hsf-1*(*RNAi*) worms (Figure S4), providing further evidence that DAF-16 is required for TRPA-1 to regulate lifespan at the larval stage. We also obtained a similar result with worms lacking *sgk-1* (Figures S4E–S4G), a gene which has been previously shown to act upstream of DAF-16 but downstream of TRPA-1 to regulate lifespan in a temperature-dependent manner in adulthood (Mizunuma et al., 2014; Xiao et al., 2013). These experiments suggest that TRPA-1 acts upstream of DAF-16 to regulate lifespan at the larval stage.

## DAF-16 Shortens Lifespan at the Larval Stage while Promoting Lifespan at the Adult Stage

How might DAF-16 mediate the effect of TRPA-1 on longevity at the larval stage? The finding that TRPA-1 shortens lifespan in response to low-temperature treatment at the larval stage raises the possibility that DAF-16 may also shorten lifespan in larvae. This model, however, is confounded by the general view that DAF-16 promotes lifespan (Kenyon, 2010). Interestingly, a previous report showed that DAF-16 promotes lifespan by acting in adulthood (Dillin et al., 2002). This prompted an intriguing question: does DAF-16 play a different role in lifespan regulation at the larval stage?

To test this, we first selectively knocked down *daf-16* by RNAi in adults and found that RNAi of *daf-16* in adulthood shortened adult lifespan upon larval temperature treatment (Figures 6C and 6D), consistent with a role of DAF-16 in promoting lifespan

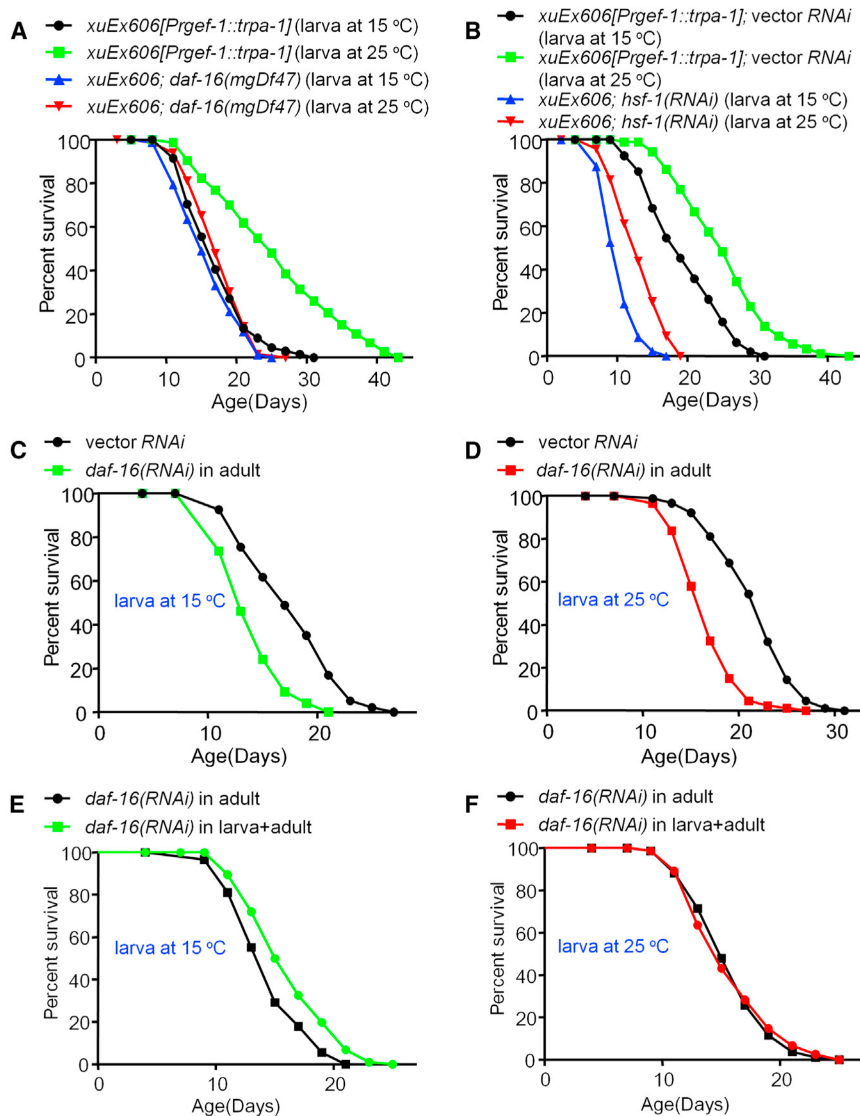



**Figure 6. DAF-16 Mediates the Effect of TRPA-1 on Lifespan at the Larval Stage**

(A) Loss of *daf-16* blocks the effect of *trpa-1* transgene on temperature modulation of lifespan at the larval stage ($p < 0.001$; Cox proportional hazard regression analysis). Temperature treatment protocol was performed as described in Figure 1B.

(B) *hsf-1* RNAi cannot block the effect of *trpa-1* transgene on temperature modulation of lifespan at the larval stage ($p = 0.824$; Cox proportional hazard regression analysis).

(C and D) RNAi of *daf-16* in adulthood shortens lifespan, independently of the cultivation temperature at the larval stage. Larvae were cultured at 15°C (C; $p < 0.001$; log rank test) and 25°C (D; $p < 0.001$; log rank test) until L4 and then shifted to 20°C to score lifespan. *daf-16* RNAi was delivered at the adult stage only. At the larval stage, the same worms were fed vector RNAi. Control, worms were fed vector RNAi throughout the larval and adult stages.

(E and F) RNAi of *daf-16* in larvae extends lifespan in a temperature-dependent manner. Larvae were cultured at 15°C (E; $p < 0.001$; log rank test) and 25°C (F; $p = 0.877$; log rank test) until L4 and then shifted to 20°C to score lifespan. *daf-16* RNAi was delivered throughout the larval and adult stages (larva+adult) or adult stage only (adult). For those worms treated with *daf-16* RNAi at the adult stage only (adult), their larvae were fed vector RNAi.

See also Figure S4. Representative data are shown here; replicates and detailed statistics are described in Table S1.

in adults. RNAi is fairly potent in worms, and once triggered, its effect is long lasting and can be amplified through RdRP (Pak et al., 2012). As such, it is difficult to selectively knock down *daf-16* by RNAi only in larva without affecting the later adult stage. To overcome this technical difficulty, we knocked down *daf-16* by RNAi throughout the larval and adult stages (larva+adult RNAi). By comparing the outcome of this larva+adult RNAi and adult-only RNAi, it would provide an alternative means of examining the role of *daf-16* in larvae (Figures 6E and 6F). Strikingly, worms deficient in *daf-16* at both the larval and adult stages (larva+adult RNAi) lived longer than those deficient in *daf-16* in adults only (adult-only RNAi), suggesting that loss of *daf-16* at the larval stage extends lifespan (Figure 6E). This phenomenon is temperature dependent, as it was only observed in worms subjected to low (15°C), but not high (25°C), temperature treatment at the larval stage (Figures 6E and 6F). These results suggest that DAF-16 shortens lifespan upon low-temperature treatment at the larval stage.

## DAF-16 Differentially Regulates Gene Expression in Larvae and Adults at Different Temperatures

How might the same transcription factor DAF-16 mediate the opposite effects of temperature on lifespan in larvae and adults? One possibility is that DAF-16 differentially regulates gene expression in larvae and adults in response to temperature changes. To test this model, we first conducted a genome-wide microarray analysis to determine whether temperature treatment differentially affects gene expression in larvae and adults. We compared two temperatures: 15°C and 25°C. Genes differentially regulated by temperature are rather diverse, including those involved in the synthesis, modification, and/or processing of lipids, carbohydrates, carboxylic acids, and peptides (Figure S5). Genes regulating temperature responses (e.g., HSPs), electron transport chain, ion transport, and body morphogenesis were also picked up (Figure S5). Overall, 303 genes were found to be upregulated by low temperature in larvae (15°C versus 25°C; Figure 7A; Table S2). Among them, only ~15% (44 genes) were upregulated by low temperature in adults; the rest ~85% genes, however, became downregulated or remained unchanged in adults (Figure 7A; Table S2). Similarly, 391 genes were downregulated by low temperature in larvae (15°C versus 25°C), of which merely ~43% (167 genes) were downregulated in adults and the

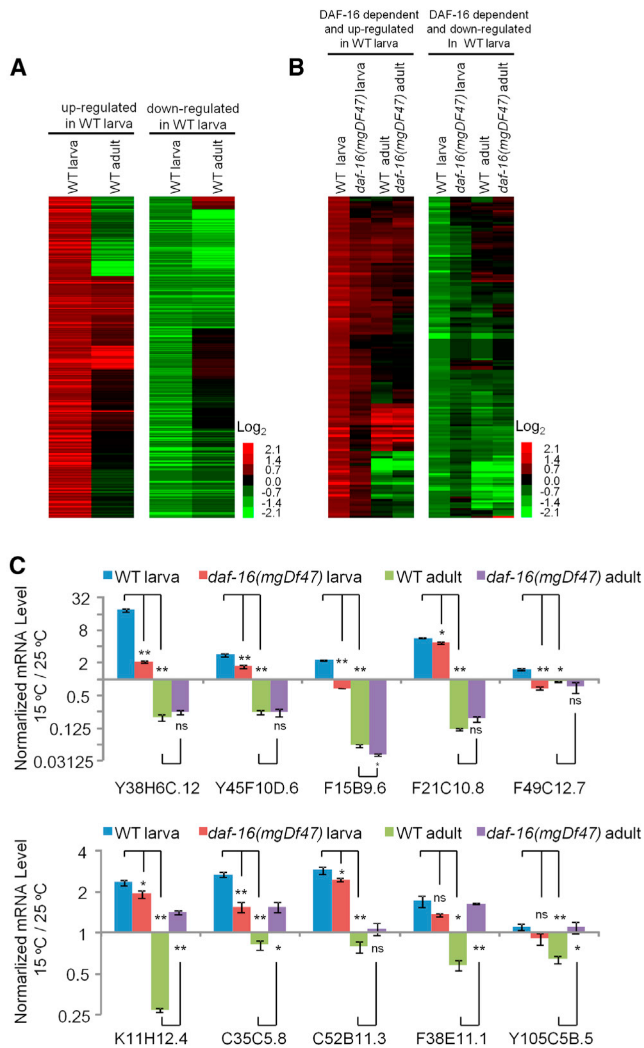



**Figure 7. DAF-16-Dependent Genes Are Differentially Regulated by Temperature in Larvae and Adults**

(A) Genes are differentially regulated by temperature in larvae and adults of wild-type (WT). Genes that were upregulated (15°C versus 25°C) in wild-type larvae are shown in the left panel, whereas those genes that were downregulated in wild-type larvae are shown in the right panel. As a comparison, the relative expression levels of the same set of genes in wild-type adults are also shown. Only differentially regulated genes are included in the panels. FDR < 0.05; $\log_2$ FC > 0.7.

(B) DAF-16-dependent genes are differentially regulated by temperature in larvae and adults (15°C versus 25°C). The left and right panels list up- and downregulated genes in larvae, respectively. Specifically, the first lane in the left panel lists DAF-16-dependent genes that were upregulated by low temperature (15°C versus 25°C) in wild-type larvae. The relative expression levels of the same set of genes in *daf-16*(*mgDf47*) mutant background are listed in the second lane. Each gene showed significant difference in expression level ($p < 0.05$) between wild-type and *daf-16* mutant, indicative of DAF-16 dependence. The third and fourth lanes list the relative expression levels of the same set of genes in wild-type adults and *daf-16* adults, respectively. Some of these genes become DAF-16 independent in adults. The lanes in the right panel show downregulated genes and are organized in a similar manner. FDR < 0.05; $\log_2$ FC > 0.7.

(C) qPCR quantification of DAF-16-dependent larval genes upregulated by low temperature (15°C versus 25°C) in wild-type and *daf-16* mutant worms. The expression level of each gene (8/10) was significantly different in *daf-16*(*mgDf47*) mutant larvae compared to wild-type, indicative of DAF-16 dependence. Their expression (10/10) is also significantly different in wild-type adults. Some of these genes (5/10), however, are no longer DAF-16 dependent in adults, indicating a possible switch in regulatory mechanisms. These genes were picked from Table S2A based on their high FC values. Experiments were repeated three times. Error bars: SEM. *$p < 0.05$; **$p < 0.005$ (ANOVA).
See also Figure S5 and Table S2.

remaining (~57%) genes were instead upregulated or unchanged in adult animals (Figure 7A; Table S2). Apparently, temperature differentially regulates gene expression in larvae and adults.

We also performed a similar analysis on *daf-16* mutant worms. By comparing data from *daf-16* mutant and wild-type animals, we found that 135 DAF-16-dependent genes were upregulated by low temperature (15°C versus 25°C) in larvae (Figure 7B; Table S2). Among these DAF-16-dependent larval genes, only 17% (23 genes) were upregulated in adults by low temperature, and the rest were, however, either downregulated or unaffected (Figure 7B; Table S2). Even among those 23 genes that were also upregulated in adults by low temperature, 13 became DAF-16 independent, indicating a possible switch in regulatory mechanisms (Figure 7B; Table S2). A similar result was obtained with DAF-16-dependent genes that were downregulated by low temperature (Figure 7B; Table S2). Apparently, a large portion of DAF-16-dependent genes were differentially regulated by temperature in larvae and adults. Lastly, we sampled a few such genes and verified the microarray data by qPCR analysis (Figure 7C). These results suggest that DAF-16 differentially regulates gene expression in larvae and adults at different temperatures.

## DISCUSSION

Temperature has long been thought to regulate lifespan by globally altering the rate of chemical reactions and hence the pace of aging (Loeb and Northrop, 1916). It is generally believed that lower temperatures extend lifespan, whereas higher temperatures shorten it. With the exception of extreme temperatures that may jeopardize an animal's survival, this temperature law, thus far, has been consistent with empirical observations, particularly in poikilotherms. Although recent work demonstrated that genes play an active role in temperature modulation of longevity (Lee and Kenyon, 2009; Xiao et al., 2013), the temperature law remains valid. In the current study, we systemically interrogated how temperature affects lifespan in *C. elegans*. By examining the commonly used cultivation temperature range (15°C–25°C), we found with surprise that exposure to low temperatures at the larval stage in fact shortens adult lifespan, whereas high temperature treatment prolongs it. This observation is inconsistent with the temperature law. Apparently, temperature differentially regulates lifespan at different stages of worm life, unveiling an unexpected layer of complexity underlying temperature modulation of longevity.

The fact that temperature treatment at the larval stage affects adult lifespan also uncovers an interesting phenomenon: temperature experience in the early life of an animal can induce a

long-lasting effect on its late life. We found that the cold-sensitive channel TRPA-1 plays a key role in regulating lifespan at the larval stage. Interestingly, recent work shows that thermosensitive TRP channels also have a role in longevity in mammals (Riera et al., 2014). As a well-characterized temperature-sensitive channel, TRPA-1 may act as thermosensor to detect temperature drop in the environment to regulate lifespan at low temperatures through DAF-16, a master regulator of longevity. TRPA-1 is probably not the only thermosensor that mediates temperature modulation of lifespan, at least at the larval stage. Indeed, we found that HSF-1, another master regulator of lifespan, also mediates the effect of temperature on lifespan in larvae. HSF-1 is not required for TRPA-1 to regulate lifespan, suggesting that HSF-1 acts independently of and probably in parallel to TRPA-1 and DAF-16. Given that it has been well established that HSF-1 promotes longevity and can be activated by high temperatures through heat shock signaling (Baird et al., 2014; Hsu et al., 2003; Satyal et al., 1998), it may potentially act as a heat-sensor, albeit indirectly, to detect temperature rise in the environment to promote lifespan at the larval stage. This points to an interesting model that TRPA-1 acts as a cold sensor to shorten lifespan, whereas HSF-1 responds to heat to extend lifespan at the larval stage.

Interestingly, TRPA-1 also regulates lifespan at the adult stage, although the outcome is the opposite of that observed at the larval stage (Xiao et al., 2013). Specifically, TRPA-1 acts through DAF-16 to prolong lifespan in adults while reducing lifespan in larvae at low temperatures. Notably, SGK-1, which is known to act upstream of DAF-16 to mediate the temperature effect of TRPA-1 in adulthood (Mizunuma et al., 2014; Xiao et al., 2013), is also required for TRPA-1 to regulate lifespan at the larval stage. Apparently, the TRPA-1 genetic pathway is employed to regulate lifespan in both larvae and adults through DAF-16.

An intriguing question is how DAF-16 might produce two opposite lifespan outcomes in larvae and adults. As a master regulator of lifespan in *C. elegans*, DAF-16 is known to activate/repress the expression of hundreds of genes directly and indirectly (Lee et al., 2003; Murphy et al., 2003). One possibility is that DAF-16 differentially regulates gene expression at the larval and adult stages in response to temperature. This indeed appears to be the case, as shown by gene profiling and qPCR analysis. Such DAF-16-dependent differential regulation of gene expression in larvae and adults may contribute to the distinct effects of temperature on lifespan at the larval and adult stages.

DAF-16 has been long thought to be a pro-longevity transcription factor. Our results suggest that DAF-16 can also inhibit lifespan, depending on the stage of worm life (larva versus adult) and context (low versus high temperatures). Interestingly, a separate study showed that overexpression of DAF-16 in hypodermis results in tumorigenic activity and shortens lifespan (Qi et al., 2012). Overexpression of mammalian FOXO1 in myocytes during development causes lethality (Evans-Anderson et al., 2008). These observations uncover a multifaceted role of DAF-16/FOXO in lifespan regulation. In summary, our studies demonstrate for the first time that temperature differentially regulates lifespan, establishing a framework for investigating this interesting phenomenon in a powerful genetic model organism.

## EXPERIMENTAL PROCEDURES

### Strains

Strains are as follows: wild-type, N2. TQ1516: *trpa-1*(*ok999*) X6 outcrossed; TQ1643: *xuEx601*[*Pges-1*::*trpa-1*::*SL2*::*yfp*+*Punc122*::*DsRed*]; TQ1648: *xuEx606* [*Prgef-1*::*trpa-1*::*SL2*::*yfp*+*Punc-122*::*DsRed*]; TQ1657: *xuEx610*[*Pmyo-3*::*trpa-1*::*SL2*::*yfp*+*Punc-122*::*DsRed*]; TQ1658: *xuEx611*[*Pdpy-7*::*trpa-1*::*SL2*::*yfp*+*Punc-122*::*DsRed*];TQ1654: *daf-16*(*mgDF47*); TQ2012: *xuEx606*[*Prgef-1*::*trpa-1*::*SL2*::*yfp*+*Punc-122*::*DsRed*]; *daf-16*(*mgDF47*); TQ6068: *daf-9*(*rh50*); and TQ6069: *daf-12*(*rh61rh411*).

### Lifespan

Lifespan was performed as described previously (Hsu et al., 2009; Liu et al., 2013). Wild-type (N2) worms were maintained at 20°C unless indicated otherwise. To test the effect of larval temperature treatment, parents (L4 stage) reared at 20°C were moved to 15°C, 20°C, and 25°C to mature and lay eggs at these temperatures. Eggs were allowed to hatch and develop at these three temperatures until reaching L4 and then shifted back to 20°C to score adult lifespan. To test the effect of adult temperature treatment, eggs were allowed to hatch and develop into L4 at 20°C and then shifted to 15°C, 20°C, and 25°C to score adult lifespan. The first day of adulthood was recorded as day 1 in all experiments. Worms that crawled off the plate, exploded, or bagged were censored at the time of the event. All lifespans were performed on OP50 bacteria with the exception of those involving RNAi, in which case were conducted on HT115 bacteria. It should be noted that different diets may affect lifespan differently (Mizunuma et al., 2014).

For the lifespan experiments involving RNAi, fresh single colonies of HT115 bacteria containing empty vector L4440 or RNAi plasmid were cultured overnight at 37°C in LB with carbenicillin (100 μg/ml). Two days prior to the experiments, freshly grown RNAi bacteria were seeded on NGM plates containing carbenicillin (25 μg/ml) and IPTG (1 mM). Most RNAi clones were obtained from the Ahringer library (Kamath and Ahringer, 2003). *hsf-1* RNAi clone was generated as described previously (Walker et al., 2003).

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software) and IBM SPSS Statistics 19 (IBM). p values were calculated with the log rank (Kaplan-Meier) method and Cox proportional hazard regression as indicated.

### Microarray Procedure and Data Analysis

For microarray sample preparation, synchronous populations of worms were generated by making 25–30 adult worms to lay eggs on newly seeded NGM plates for 2 or 3 hr. To obtain the larval sample, 300 worms were harvested when they reached late L4 stage. To obtain the adult sample, 120 worms were harvested when they became day 3 adults. Biological samples were prepared on separate days, and total RNA was extracted with TRI Reagent (Life Technologies). The concentrations and quality of total RNA samples were checked with Thermo NanoDrop 2000c (Thermo Scientific) and Agilent 2100 Bio-Analyzer (Agilent Technologies). Microarrays were done on Affymetrix *C. elegans* Gene 1.1 ST Array Strips. Preparation of cDNA, hybridization, quality controls, and scanning of arrays were performed according to the manufacturer's protocol (Affymetrix) at the Microarray Core Facility of University of Michigan.

All microarray analysis was performed with bioconductor implemented in R statistical environment (Gentleman et al., 2004). Expression values of each gene were calculated with a robust multi-array average (RMA) method (Irizarry et al., 2003). Differentially expressed genes were identified using weighted linear models designed specifically for microarray analysis (Ritchie et al., 2006; Smyth, 2004). p values were adjusted for multiple comparisons using false discovery rate (FDR) (Benjamini and Hochberg, 1995). Hierarchical clustering was performed with an uncentered correlation similarity metric by Cluster using Centroid linkage clustering method (de Hoon et al., 2004). Gene ontology analysis was analyzed using DAVID, and functional clusters were identified using Functional Annotation Chart tool (Huang et al., 2009).

### qRT-PCR

Total RNA was extracted from 100 to 300 worms with TRI Reagent (Life Technologies). qPCR was carried out with CYBR Green (Life Technologies)

according to the protocol provided by the manufacturer. We used *act-1* (actin) as an internal reference for normalization, and ΔΔCt method was used to analyze qPCR data.

## ACCESSION NUMBERS

The microarray data have been deposited in the NCBI GEO database and are available under accession number GEO:GSE62297.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.066.

## AUTHOR CONTRIBUTIONS

B.Z., R.X., and E.A.R. performed the experiments and analyzed the data. Y.H. assisted B.Z. on data analysis. B.Z., A.-L.H., J.L., and X.Z.S.X. wrote the paper.

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