

# Temporal requirements of heat shock factor-1 for longevity assurance

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## Summary

**Reducing the activity of the insulin/IGF-1 signaling pathway (IIS) modifies development, elevates stress resistance, protects from toxic protein aggregation (proteotoxicity), and extends lifespan (LS) of worms, flies, and mice. In the nematode *Caenorhabditis elegans*, LS extension by IIS reduction is entirely dependent upon the activity of the transcription factors DAF-16 and the heat shock factor-1 (HSF-1). While DAF-16 determines LS exclusively during early adulthood, it is required for proteotoxicity protection also during late adulthood. In contrast, HSF-1 protects from proteotoxicity during larval development. Despite the critical requirement for HSF-1 for LS extension, the temporal requirements for this transcription factor as a LS determinant are unknown. To establish the temporal requirements of HSF-1 for longevity assurance, we conditionally knocked down *hsf-1* during larval development and adulthood of *C. elegans* and found that unlike *daf-16*, *hsf-1* is foremost required for LS determination during early larval development, required for a lesser extent during early adulthood and has small effect on longevity also during late adulthood. Our findings indicate that early developmental events affect LS and suggest that HSF-1 sets during development of the conditions that enable DAF-16 to promote longevity during reproductive adulthood. This study proposes a novel link between HSF-1 and the longevity functions of the IIS.**

**Key words:** aging; DAF-16; development; heat shock factor 1; insulin/IGF-1 signaling.

## Introduction

The insulin/IGF-1 signaling pathway (IIS) is a key regulator of development, stress resistance, metabolism, and longevity of worms (Kenyon *et al.*, 1993), flies (Giannakou *et al.*, 2007), mice (Bluhner *et al.*, 2003;

Holzenberger *et al.*, 2003; Taguchi *et al.*, 2007; Selman *et al.*, 2008), and presumably humans (Suh *et al.*, 2008; Willcox *et al.*, 2008; Flachsbarth *et al.*, 2009). In the nematode *Caenorhabditis elegans* DAF-2, the sole insulin/IGF-1 receptor, initiates a cascade of events that promotes the phosphorylation of its downstream forkhead-like transcription factor, DAF-16 (Paradis & Ruvkun, 1998; Henderson & Johnson, 2001). Phosphorylated DAF-16 is prevented from entering the nucleus and from regulating the expression of its target gene networks (Lin *et al.*, 2001). Thus, *daf-2* knockdown by either mutation or RNA interference (RNAi) hyperactivates *daf-16*, creating long-lived, youthful, and stress-resistant worms. These longevity and stress resistance effects of *daf-2* knockdown are entirely dependent upon *daf-16* (Ogg *et al.*, 1997; Lee *et al.*, 2001).

In *C. elegans*, the highly conserved (Liu *et al.*, 1997), leucine zipper containing (Rabindran *et al.*, 1993) transcription factor, heat shock factor 1 (HSF-1) is an additional critical and specific player in the IIS longevity pathway. Accordingly, the knockdown of *hsf-1* prevents IIS reduction from extending lifespan (LS) and *hsf-1* over-expression extends LS in a *daf-16* dependent manner (Hsu *et al.*, 2003; Morley & Morimoto, 2004). Similarly to DAF-16, HSF-1 exhibits various biological functions in addition to LS regulation. It is critically required for heat stress response (Sarge *et al.*, 1993), innate immunity (Singh & Aballay, 2006), and for proper development (Walker *et al.*, 2003; Akerfelt *et al.*, 2010).

In *C. elegans*, IIS reduction by *daf-2* RNAi regulates LS solely during reproductive adulthood (days 1–6 of adulthood). Consistently, conditional *daf-16* reduction during reproductive adulthood, but not larval development, has the reciprocal effect of shortening LS (Dillin *et al.*, 2002a). Similarly, in the fruit fly *Drosophila melanogaster*, conditional gene expression of dFOXO (DAF-16 ortholog) during the reproductive phase of adulthood results in an increased longevity (Giannakou *et al.*, 2007). Despite its critical roles in the IIS regulated longevity mechanism, the temporal requirements for HSF-1 as a LS determinant are unknown.

Insulin/IGF-1 signaling reduction provides protection from an additional aging-associated phenomenon, toxic protein aggregation (proteotoxicity; David *et al.*, 2010). This protection is entirely dependent upon both DAF-16 and HSF-1 (Hsu *et al.*, 2003; Morley & Morimoto, 2004; Cohen *et al.*, 2006; Zhang *et al.*, 2011). In the case of the aggregation of the Alzheimer's disease associated peptide A $\beta$ , DAF-16, and HSF-1 were shown to promote opposing activities, DAF-16 mediates protective active aggregation while HSF-1 facilitates disaggregation (Cohen *et al.*, 2006). The central role of HSF-1 in countering proteotoxicity was further established by the finding that it is critically needed for dietary restriction (DR) to promote protection from A $\beta$  aggregation (Steinkraus *et al.*, 2008). Recently, we tested the timing requirements for *daf-16* and *hsf-1* in reduced IIS mediated protection from A $\beta$  proteotoxicity and found that while *daf-16* is required during both early and late adulthood but not during development, *hsf-1* is predominantly required for protection from proteotoxicity during development (Cohen *et al.*, 2010). These surprising findings raised the question of whether HSF-1 protects from proteotoxicity and mediates longevity during the same time window during the nematode lifecycle or whether it functions as a LS determinant concurrently with DAF-16. To address this issue, we studied the temporal requirements for *hsf-1* for longevity assurance using the nematode *C. elegans* and conditional RNAi knockdown technique.

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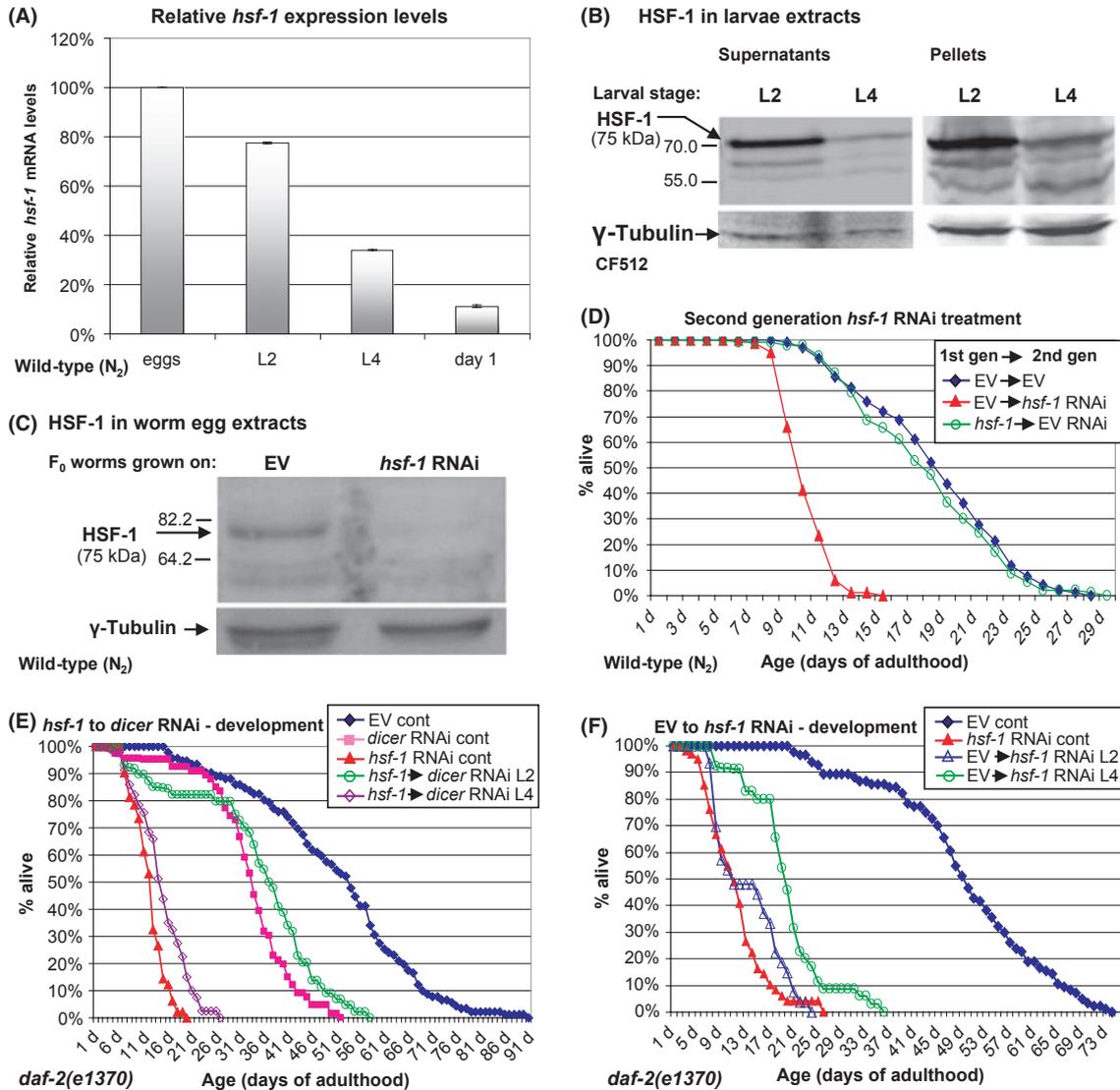
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**Results**

We determined the natural expression levels of *hsf-1* during the worm's larval development, using two separate sets of specific *hsf-1* primers and quantitative real-time PCR (qPCR). Our results indicate that *hsf-1* expression steadily declines during development as *hsf-1* mRNA transcripts were most abundant during embryonic development (in eggs), less in L2, even less in L4 larvae, while the least amount of *hsf-1* mRNA was detected in young adults (Fig. 1A and S1). To establish these results, we asked whether the relative HSF-1 protein levels in L2 larvae and L4 stages correlate with the mRNA levels. To directly compare the HSF-1 protein

amounts, we harvested and homogenized worms (strain *CF512*) that were grown on control bacteria carrying the empty RNAi plasmid (EV) up to either L2 or L4 larval stages. The homogenates were spun to separate the soluble (supernatant) and insoluble (pellet) fractions and equal amounts of total protein were subjected to Western Blot analysis (WB) using HSF-1 antibody. Our results (Fig. 1B) showed that HSF-1 is more abundant in L2 larvae than in their L4 counterparts in both supernatant and pellets, indicating that not only *hsf-1* expression level is elevated in L2 larval stage but also the amounts of HSF-1 protein. Therefore, if the expression pattern of *hsf-1* was correlative with the requirement for *hsf-1* to mediate longevity we would expect *hsf-1* to be required primarily



**Fig. 1** *hsf-1* is required foremost during development but also during adulthood to promote longevity. (A) Quantitative PCR revealed that *hsf-1* expression is maximal during embryonic as well as early larval development and declines thereafter. (B) The levels of HSF-1 protein in both soluble and insoluble fractions of L2 larvae are notably higher than in their L4 counterparts as detected by WB that was probed with an HSF-1 antibody. (C) WB analysis revealed that eggs of wild-type worms grown on *hsf-1* RNAi bacteria contained remarkably less HSF-1 than eggs of their EV-grown counterparts. (D) Reduced HSF-1 levels only during embryogenesis had no effect on lifespan (LS). Eggs obtained from *hsf-1* RNAi-grown worms placed on EV bacteria to enable hatching larvae to restore *hsf-1* expression. The hatched worms (green line) and control worms (blue line) exhibited indistinguishable LSs. (E) *daf-2(e1370)* mutant worms were hatched on *hsf-1* RNAi and were transferred onto *dcr-1* RNAi bacteria at either L2 (green open circles) or L4 (purple open diamonds) stages. Unlike L2 transferred animals, those transferred at L4 had exceptionally short LSs. For statistical data see Table S1 (Supporting Information). (F) Reciprocally to E, *daf-2(e1370)* mutant worms were hatched on EV bacteria and transferred onto *hsf-1* RNAi bacteria at either larval stage L2 (blue open triangles) or L4 (green open circles). As in E, worms which had reduced *hsf-1* levels during L2, but not L4, exhibited exceptionally short LSs. For statistical data see Table S2 (Supporting Information).

during embryogenesis, secondarily during larval development, and finally, weakly required during adulthood to mediate longevity.

We tested whether the temporal expression pattern of *hsf-1* was predictive of the temporal requirements of *hsf-1* for longevity by determining whether embryonic expression of *hsf-1* was needed for LS determination. Parental wild-type worms (strain N2) were cultured on *hsf-1* RNAi bacteria to deplete HSF-1 in the F1 generation of embryos. Western blot analysis confirmed that HSF-1 quantities were greatly reduced in the eggs of *hsf-1* RNAi-treated worms (Fig. 1C). HSF-1 reduced eggs were either placed on *hsf-1* RNAi bacteria to maintain compromised *hsf-1* mRNA levels or on bacteria harboring an empty RNAi vector (EV) to enable the hatching larva to restore *hsf-1* expression. The restoration of *hsf-1* in worms that were hatched from *hsf-1* reduced eggs and placed on EV bacteria was tested by functional analysis employing animals that express the Green Fluorescent Protein (GFP) under the regulation of the promoter of *hsp-16.2*, a well-established HSF-1 target gene (strain *CL2070*; Link et al., 1999). Eggs of *CL2070* worms were placed on *hsf-1* RNAi bacteria, hatched, grown to reproductive adulthood, and were bleached to obtain HSF-1 reduced eggs. These eggs were placed on EV bacteria and worms of the second generation were exposed to heat stress (33 °C, 3 h) at day 1 of adulthood to examine whether they restored the ability to induce the expression of *hsp-16.2* in response to heat. Our results (Fig. S2) indicate that unlike worms that were fed *hsf-1* RNAi bacteria for one or two generations (lanes 2 and 4), animals that were hatched from HSF-1 reduced eggs and grown on EV bacteria have restored the ability to induce the expression of *hsp-16.2* (lane 3). This observation indicates that these worms have restored HSF-1 to functional levels.

While worms that were grown for one generation on *hsf-1* RNAi lived an extremely short life (Fig. 1D, red line), restoration of *hsf-1* expression after hatching resulted in indistinguishable LSs compared to those of animals grown continuously on the EV bacteria (Fig. 1D green and blue lines, respectively, and Table S1). These results indicate that *hsf-1* is not required for longevity assurance during embryogenesis.

Next we sought to test whether *hsf-1* was required for longevity during larval development, a time in which the IIS pathway is dispensable for longevity regulation (Dillin et al., 2002a). To conditionally inactivate *hsf-1*, we used RNAi toward *hsf-1* (to knockdown its expression) and RNAi toward *dcr-1* to restore the expression of *hsf-1*. *dcr-1* (*dicer*) encodes an RNase III enzyme that plays critical roles in RNA interference (Knight & Bass, 2001). Accordingly, the knockdown of *dcr-1* reduces the endogenous RNAi response, enabling RNAi-inactivated genes to return to near normal expression levels (Bernstein et al., 2001; Dillin et al., 2002b). First, we tested whether the knockdown of *dcr-1* during development affects LS. To define the temporal requirements for *dicer* as a LS determinant, we transferred temperature-sensitive sterile worms (strain *CF512*) that were hatched and developed on control bacteria (EV) at 25 °C [in this temperature HSF-1 does not induce the heat shock response (Fig S3, lane 2)], onto *dcr-1* RNAi bacteria in three-hour intervals during early development (up to 18 h after hatching), or 36 h after hatching (late development) and recording their LSs. Our results (Fig. S4A) indicated that the knockdown of *dcr-1* during larval development shortened LS but this effect was progressively less prominent in worms that had natural *dicer* levels for longer time from hatching. Similarly, we tested whether the knockdown of *dcr-1* during adulthood affects LSs and found that *dcr-1* RNAi treatment has no effect on LS when applied during adulthood (Fig S4B).

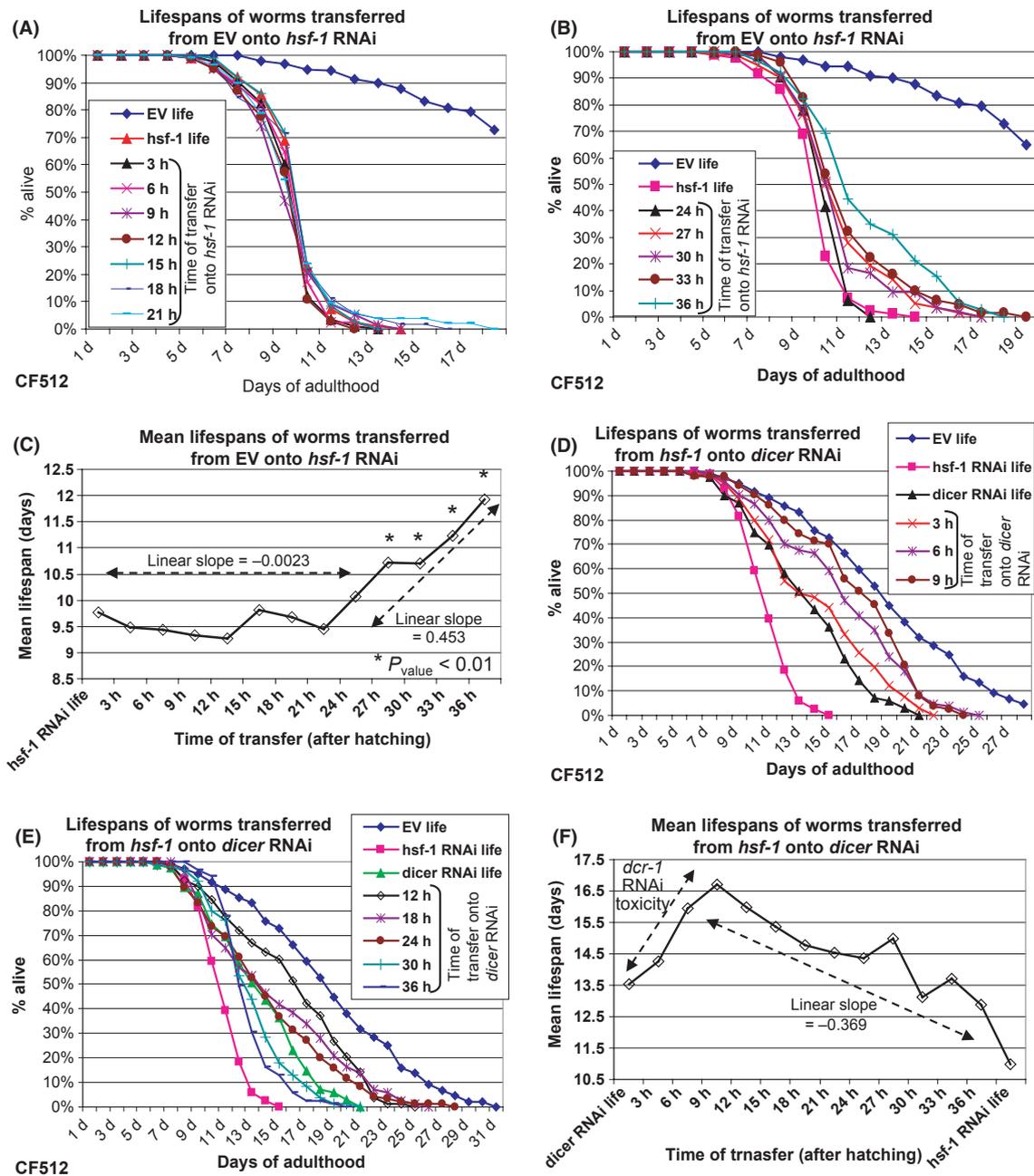
To assess the temporal requirement for *hsf-1* in the longevity phenotype associated with reduced *daf-2* activity, we utilized the long-lived *daf-2(e1370)* mutant strain (Kenyon et al., 1993). To conditionally inactivated *hsf-1* during specific times of larval development, the worms were grown on *hsf-1* RNAi bacteria and transferred onto plates spotted with

*dcr-1* RNAi bacteria at either larval stage L2 or L4 (20 or 36 h after hatching, respectively). Quantitative real-time PCR indicated that in L2 larvae, *hsf-1* expression was remarkably reduced within 4 h after transferring the worms from control bacteria (EV) onto *hsf-1* RNAi bacteria (Fig. S5A). A similar lag time was required for the worms to restore the expression of *hsf-1* after transferring them from *hsf-1* onto *dcr-1* RNAi bacteria (Fig. S5C). L4 larvae exhibited reduced *hsf-1* mRNA levels within 6 h after transferring from EV bacteria onto *hsf-1* RNAi (Fig. S5B). The restoration of *hsf-1* expression occurred in L4 larvae within one and a half hours (Fig. S5D; possibly due to higher rate of food consumption of L4 larvae compared to their L2 counterparts). Therefore, in this set of experiments the animals had reduced *hsf-1* expression from hatching up to the L2 or L4 larval stages, respectively, but restored expression quickly after transfer onto *dcr-1* RNAi. Reduction of *hsf-1* expression from hatching through the L4 larval stage resulted in a decreased longevity (Fig. 1E, mean LS  $14.7 \pm 5.49$  days), only slightly longer than that of control animals grown on *hsf-1* RNAi throughout life ( $12.29 \pm 3.34$  days). In a striking contrast, animals in which *hsf-1* was inactivated from hatching until the L2 larval stage lived remarkably longer (mean LS  $35.3 \pm 13.41$  days). This mean LS was very similar to that of animals grown on *dcr-1* RNAi throughout life ( $33.63 \pm 8.8$  days, Fig. 1E and Table S2). These results indicate that unlike *daf-16*, *hsf-1* executes an essential longevity function during larval development, predominantly between the L2 and L4 larval stages.

To determine if the expression of *hsf-1* during early larval development can set the rate of the animal's aging for the rest of its life or whether *hsf-1* expression also affects LS during later developmental stages, we performed a reciprocal experiment to the one described above. Long-lived *daf-2(e1370)* mutant worms were grown on EV bacteria and transferred onto *hsf-1* RNAi bacteria at either the L2 or L4 larval stages (Fig. 1F and Table S3). Therefore, these animals had natural *hsf-1* expression until the time of transfer to *hsf-1* RNAi bacteria, either the L2 or L4 larval stages. Consistent with our prior results, worms transferred onto *hsf-1* RNAi at the beginning of L2 larval stage had short LSs (mean LS  $12.97 \pm 5.74$  days), while those transferred at L4 lived considerably longer (mean LS  $19.85 \pm 6.37$  days) but much shorter than their counterparts that had an active *hsf-1* during adulthood (Fig. 1E). Together these results indicate that *hsf-1* is foremost necessary between the L2 and L4 larval stages for the extended LS of *daf-2* mutant animals. However, the observation that *daf-2* mutant worms transferred onto *hsf-1* RNAi at the L4 stage did not live as long as *daf-2* mutant animals grown on control bacteria indicates that *hsf-1* is also needed during adulthood to promote the full longevity effect of reduced IIS.

To accurately determine the critical timing requirements of *hsf-1* for longevity assurance during larval development, we tested the LSs of *CF512* worm populations which were hatched and grown on control bacteria (EV) at 25 °C and transferred onto *hsf-1* RNAi at 3-h intervals, beginning from 3 h until 36 h after hatching (12 time points in total). Worms that were transferred onto *hsf-1* RNAi 24 h after hatching or earlier had short mean LSs almost identical to these of animals treated with *hsf-1* RNAi bacteria throughout their lives (Fig. 2A,C and Table S4). Distinctly, the LSs of animals transferred onto *hsf-1* RNAi bacteria at 27 h after hatching or later lived progressively and significantly longer than their counterparts transferred earlier (Fig. 2B,C and Table S4) but still much less than animals that had active *hsf-1* up until adulthood. These results point to the late L2/early L3 developmental stages as the end of the time window in which *hsf-1* is necessary during development for longevity.

The last set of experiments defined the end of the time window in which *hsf-1* exhibits its most prominent effect on LS during larval development. To define the beginning of this time window, we performed the



**Fig. 2** Functions of *hsf-1* during L2 developmental stage are critical to enable development and promote longevity. (A–C) CF512 worms that were hatched on EV bacteria transferred onto *hsf-1* RNAi bacteria at the indicated times. Transfer at 24 h after hatching or earlier did not reverse the short lifespan (LS) phenotype associated with *hsf-1* RNAi, whereas worms transferred 27 h after hatching or later had longer LSs. (D–F) CF512 worms were hatched on *hsf-1* RNAi bacteria and transferred onto *dcr-1* RNAi at the indicated times. Transferring at either 3 or 6 h after hatching resulted in short LSs owing to interference with the developmental functions of *dicer*. Transferring 9 h after hatching had relatively long, comparable to that of EV-grown worms. Worms that were transferred onto *dcr-1* RNAi 12 h after hatching or later showed progressively shorter LSs owing to *hsf-1* RNAi toxicity.

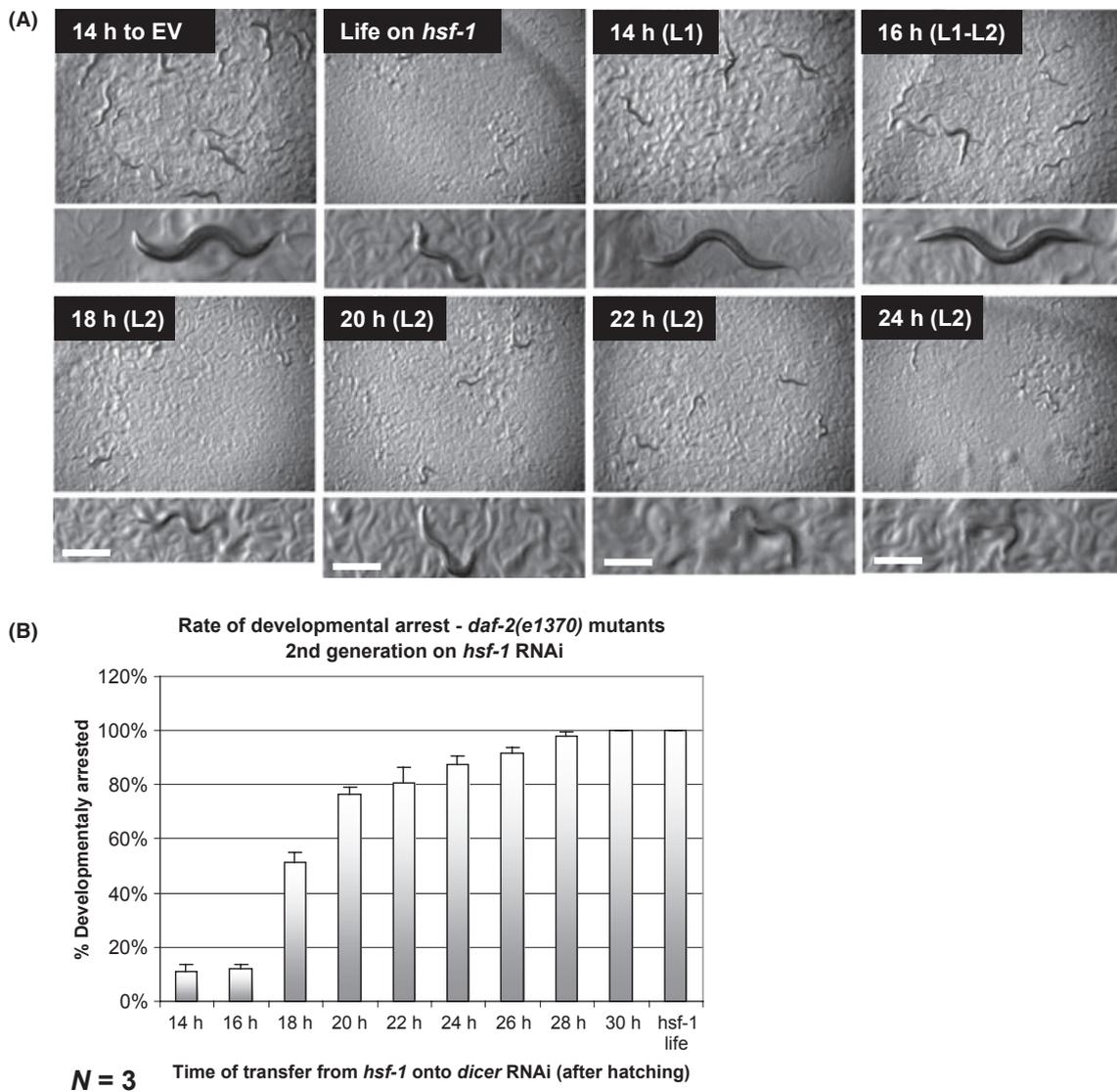
reciprocal inactivation experiment. Animals were hatched on *hsf-1* RNAi bacteria and transferred onto *dcr-1* RNAi bacteria at the same 3-h intervals 3–36 h after hatching to enable the restoration of *hsf-1* and rescue the worms from their extremely short LS phenotype. Functional *dicer* is required for appropriate worm development (Knight & Bass, 2001), and thus, RNAi toward *dicer* is predicted to shorten LSs of worms when applied during early development. Our results (Fig. S4A) confirm that the knockdown of *dcr-1* shortens LS only if it applied up to 12 h after hatch-

ing. Indeed, worms transferred onto *dcr-1* RNAi during the L1 developmental stage exhibited slightly reduced LSs and this effect was weakened as the worms were transferred later in development (Figs 2D,F and S6 and Table S5). We found that the LS shortening effect of *hsf-1* RNAi appeared in worms that were transferred 12 h after hatching, a time point corresponding to the early L2 developmental stage. The ability of the *dcr-1* RNAi to partially rescue the worms from the short LS phenotype associated with *hsf-1* RNAi declined with time (Figs 2E,F and S6 and

Table S5). Taken together, the role of *hsf-1* in longevity determination during larval development appears to begin 12 h after hatching (early L2 larval stage) and to extend for additional 12 h into the late L2/early L3 larval stages. Interestingly, even worms that were transferred at either 30, 33, or 36 h after hatching had longer LSs compared to their counterparts that were grown on *hsf-1* RNAi throughout life, supporting the idea that *hsf-1* is also required, albeit for a lesser extent, during adulthood.

As an independent measure of the temporal requirement for *hsf-1* during larval development, we utilized a unique phenotype of *daf-2(e1370)* mutant worms. When grown for two generations on *hsf-1* RNAi, *daf-2(e1370)* mutant worms were developmentally arrested at the L2 larval stage [wild-type and weak *daf-2(e1368)* mutant worm strains completed development even if grown on *hsf-1* RNAi for three generations (Fig. S7A,B)]. A similar observation of developmental arrest was observed in *age-1* mutant worms (that also exhibit reduced IIS) following *hsf-1* knockdown (Morley & Morimoto, 2004). Using this phenotype, we pin-

pointed the time window in which the development of *daf-2(e1370)* mutant worms cultured for two generations on *hsf-1* RNAi bacteria can be rescued from the L2 developmental arrest. Groups of *daf-2(e1370)* mutant worms of the second generation on *hsf-1* RNAi were transferred onto *dcr-1* RNAi bacteria every 2 h, starting 14 h after hatching, to reinstate *hsf-1* expression and rescue development (Fig. S7C). The animals that were transferred from *hsf-1* RNAi onto *dcr-1* RNAi at the beginning of the L2 larval stage or later exhibited a dramatic increase in the percentage of larval arrest from about 10% (16 h after hatching) to > 50% arrest a mere 2 h later (18 h after hatching) and nearly 78% after 20 h (Fig. 3A,B). Worms transferred at 30 h or later after hatching were 100% arrested. As *hsf-1* expression is restored quickly after transferring the worms onto *dcr-1* RNAi bacteria (Fig. S5C,D), our results indicate that the time window in which *hsf-1* is critical for the proper development and maturation of *daf-2(e1370)* mutant worms is also during the L2 larval stage, the time when *hsf-1* is critically needed for longevity.



**Fig 3** *hsf-1* is required during L2 larval stage for proper development of *daf-2* mutant worms. (A) Highly synchronized second generation on *hsf-1* RNAi, *daf-2(e1370)* mutant worms were transferred onto *dcr-1* RNAi bacteria at the indicated times after hatching to rescue the developmental arrest phenotype. Worms transferred 18 h after hatching or later showed increasing developmental arrest. (B) Bars represent the fraction of developmentally arrested worms at each time point in three independent experiments.

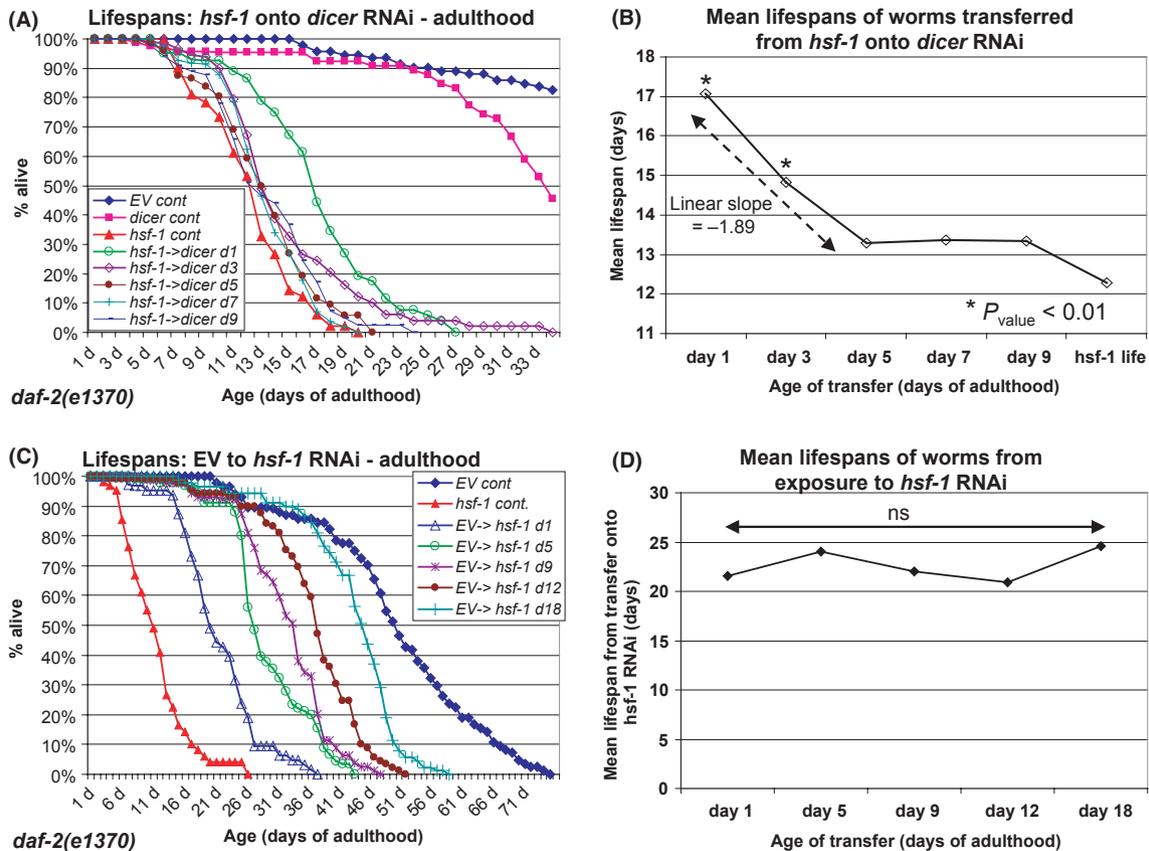
Two observations indicate that despite its relatively weak expression, *hsf-1* is also needed during adulthood for longevity. First, initiating *hsf-1* inactivation at the L4 larval stage still shortens the LS of *daf-2(e1370)* mutant animals (Fig. 1E). Secondly, worms transferred from *hsf-1* RNAi onto *dcr-1* RNAi later than 30 h after hatching lived longer than their counterparts which were grown on *hsf-1* RNAi throughout life (Fig. 2E,F). Thus, we sought to accurately define the secondary time window in which *hsf-1* determines LS during adulthood. *daf-2(e1370)* mutant worms were cultured on *hsf-1* RNAi throughout development and transferred onto *dcr-1* RNAi at either day 1, 3, 5, 7, or 9 of adulthood [the efficiency of *hsf-1* RNAi during adulthood was monitored and confirmed in *CF512* worms. The worms were grown to either day 1, 4, or 8 of adulthood, fed *hsf-1* RNAi bacteria for 24 h, exposed to heat stress (33 °C, 1 h), and their abilities to induce the expression of *hsp-70* were compared to the induction of this gene in their EV-fed counterparts of corresponding ages (Fig. S8)]. Inactivation of *hsf-1* during larval development and early adulthood (up to day 5 or later) reduced longevity of *daf-2(e1370)* mutant worms to similar extent as inactivation throughout life (Fig. 4A,B). However, when the worms were grown on *hsf-1* RNAi throughout development and transferred onto *dcr-1* RNAi earlier than day 5 of adulthood (day 1 or 3 of adulthood), their LSs were significantly longer than animals grown on *hsf-1* RNAi throughout life (Fig. 4A,B and Table S1). Taken together, *hsf-1* is prominently required during larval development and for a lesser extent up to day 5 of adulthood for LS determination. The role of *hsf-1* as LS determinant during reproductive adulthood is of particular

interest as *daf-16* executes its LS functions at the same time window (Dillin et al., 2002a).

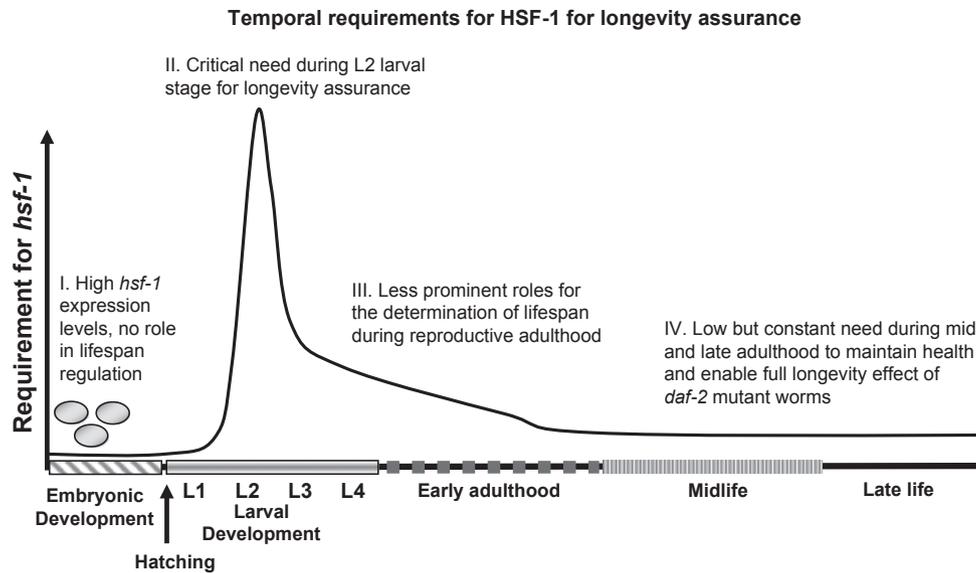
To test whether *hsf-1* plays any role as a LS determinant during mid and late stages of life, we performed the reciprocal experiment; *daf-2(e1370)* mutant worms were developed and grown on EV bacteria and then transferred onto *hsf-1* RNAi bacteria at either day 1, 5, 9, 12, or 18 of adulthood. This experiment (Fig. 4C,D) revealed that feeding worms with bacteria expressing *hsf-1* RNAi shortens life at any stage of life. This observation suggests that HSF-1 is required for the maintenance of health and to enable longevity even at late stages of life. Yet, the LS shortening effects of *hsf-1* RNAi at late stages of life were relatively mild and worms that were transferred at different ages exhibited similar average LSs from exposure to *hsf-1* RNAi of 20–24 days (Fig. 4D). For instance, worms that were transferred to *hsf-1* RNAi at day 9 of adulthood had a mean LS of  $31.06 \pm 7.16$  days (22.06 days from exposure to *hsf-1* RNAi) while their counterparts that were transferred to *hsf-1* RNAi at day 18 of adulthood exhibited mean LS of  $42.55 \pm 8.22$  days (24.55 days from exposure; Table S3). These observations indicate that *hsf-1* is required for the maintenance of health and promotion of full LS throughout life but its prominence declines over time.

## Discussion

Because *hsf-1* is foremost required for LS determination in two distinct times during the animal's lifecycle, the L2 larval stage, and days 1–5 of



**Fig 4** *hsf-1* determines lifespan (LS) also during early adulthood and required for full LS throughout life. (A and B) *daf-2(e1370)* mutant worms that developed on *hsf-1* RNAi were transferred onto *dcr-1* RNAi bacteria at either day 1, 3, 5, 7, or 9 of adulthood. Worms transferred at either day 1 or 3, but not those transferred at day 5 or later, lived longer than their counterparts grown on *hsf-1* RNAi throughout life. (C and D) *daf-2(e1370)* mutant worms were let hatch and develop on EV bacteria and transferred onto *hsf-1* RNAi at either day 1, 5, 9, 12, or 18 of adulthood. All worm groups exhibited similar average survival rates from the age of exposure to *hsf-1* RNAi.



**Fig 5** Schematic illustration of the temporal requirement for *hsf-1* as a lifespan (LS) determinant during the worm lifecycle. I. There is no need for *hsf-1* during embryogenesis for longevity assurance as *hsf-1* reduction exclusively during embryogenesis has no effect on LS. II. *hsf-1* is critically required during L2 developmental stage to enable longevity. *hsf-1* reduction at this time has severe irreversible short life phenotype. III. *hsf-1* functions as longevity determinant also during early adulthood yet, for a lesser extent compared to early development. IV. Although less prominently, *hsf-1* is constantly required during midlife and late adulthood for *daf-2* mutants to achieve their full longevity phenotype.

adulthood, we propose a model in which *hsf-1* functions in a two-step mechanism for longevity assurance (Fig. 5). During early larval development, it is critically required to set a competent state that enables IIS reduction to promote longevity later in life. Thus, *hsf-1* knockdown during this stage prevents the creation of pivotal components of the longevity mechanism that enables IIS reduction to extend LS. Later, during early adulthood and for a lesser extent during late adulthood, it is required to maintain and perhaps strengthen these cellular mechanisms. What might be the nature of the developmental *hsf-1* regulated mechanism? The well-established role of HSF-1 in various stress responses raises the prospect that during development it activates the expression of genes that encode molecular chaperones and other proteins which are required for the maintenance of proteostasis. Such developmental target genes might encode constitutive heat shock proteins such as HSP-1. The observation that treating worms with *hsp-1* RNAi for two generations results in a developmental arrest (our unpublished data) supports this notion. The identification of developmental HSF-1 transcriptional targets that function as DAF-16 co-factors during adulthood will be critically required for the examination of this putative linkage between HSF-1 and the IIS.

It is likely that the maintenance of proteostasis is a specific aspect of longevity. First, we have recently shown that HSF-1 is foremost required during development to protect from proteotoxicity (Cohen *et al.*, 2010). Moreover, as even a single protein aggregation event can destabilize the proteome (Gidalevitz *et al.*, 2006) the formation of an efficient proteostasis assurance mechanism appears to be required for IIS reduction to slow aging and promote longevity (Balch *et al.*, 2008). The key role of HSF-1 in mediating disaggregation and subsequently aggregate detoxification (Cohen *et al.*, 2006) strongly suggests that its activity during development is needed for the formation of tight protein integrity assurance machinery that supports longevity.

This study also provides an interesting insight into the temporal requirement for the worm's microRNA mechanism for longevity. The experiments which determine the temporal need for *dcr-1* as a LS determinant

(Fig. S4) show that the microRNA mechanism is important during early development for longevity. As this mechanism was previously shown to be required for proper development of *C. elegans* (Knight & Bass, 2001) our finding might point at an additional link, perhaps ancillary, between developmental events and longevity. This hypothesized link might be supported by the report that components of the microRNA processing mechanism are required for *daf-2* mutant worms to exhibit their full longevity phenotype (Boehm & Slack, 2005).

The requirement for *hsf-1* during day 1–5 of adulthood correlates with the time window in which *daf-16* is required to mediate the increased longevity of *daf-2* mutant worms (Dillin *et al.*, 2002a). This finding raises the prospect that HSF-1 is required for proper DAF-16 function. It is tempting to speculate that DAF-16 and HSF-1 acts in concert during reproductive adulthood to mediate the expression of specific genes. Accordingly, promoter regions of several small heat shock proteins were found to contain the canonical recognition sites of both DAF-16 and HSF-1 (Hsu *et al.*, 2003) and *daf-2* RNAi treatment was shown to increase the expression levels of these genes (Murphy *et al.*, 2003).

This study provides the temporal insights that enable the definition of the roles played by HSF-1 in the mediation of longevity, the identification of its downstream gene networks and biological functions that regulate aging and the characterization of the links between this transcription factor and the IIS.

## Experimental procedures

### Worm and RNAi strains

$N_2$ , *daf-2(e1370)* mutant *daf-2(e1368)* mutant worm strains as well as *hsp-16.2p::GFP* worms (strain CL2070) were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). The worms were grown at 20 °C. CF512 (*fer-15(b26)II*; *fem-1(hc17)IV*) worms are heat-sensitive sterile that were routinely grown at 15 °C. To avoid progeny,

CF512 worm eggs were incubated at 20 °C for 16 h to enable efficient hatching, larvae transferred to 25 °C for 48 h and back to 20 °C until harvested. To reduce gene expression, we used previously described (Dillin *et al.*, 2002a) bacterial strains expressing dsRNA: empty vector (pAD12), *daf-2* (pAD48), *daf-16* (pAD43), and *dicer*. *hsf-1* dsRNA expressing bacterial strain was from genomic RNAi library (J. Ahringer). Each RNAi bacteria colony was grown at 37 °C in LB with 100 µg mL<sup>-1</sup> carbenicillin, and then seeded onto NG-carbenicillin plates supplemented with 100 mM IPTG.

### Lifespan analysis

Synchronized worm eggs were placed on master NG-carbenicillin plates seeded with the indicated RNAi bacterial strain and supplemented with 100 mM IPTG. The eggs were incubated at 20 °C until transferred onto small NG-carbenicillin plates (10 animals per plate) at the indicated ages. Adult worms were transferred onto freshly seeded plates every 4 days. Worms that failed to move their noses when tapped twice with a platinum wire were scored as dead. Dead worms were scored daily. Lifespan analyses were conducted at 20 °C.

### Worm synchronization for developmental arrest experiment

Day 1 adult reproductive worms were transferred onto plates seeded with bacteria. The worms were removed after 30 min leaving highly synchronized eggs on the plates.

### Antibodies

Heat shock factor-1 antibody (SPA-901) was from Stressgen. GFP antibody (mAb #2956) was purchased from Cell Signalling and anti  $\gamma$ -tubulin antibody clone GTU-88 (T-6557) was from Sigma (St. Louis, MO, USA). Secondary antibodies conjugated to HRP were purchased from Jackson Immuno-Research (West Grove, PA, USA).

### Protein blotting

Worm eggs were purified by bleaching, boiled in loading buffer (10% glycerol, 125 mM Tris base, 1% SDS), loaded on 12% Tris-Glycine polyacrylamide, and proteins were separated and transferred onto PVDF membranes. Worms (either larvae or adults) were washed from plates with M9, homogenized using a dounce homogenizer, and centrifuged for 3 min at 850 g. Pellets were boiled in loading buffer (10% glycerol, 125 mM Tris base, 1% SDS). Protein concentration of supernatants was determined using Bio-Rad (Hercules, CA, USA) Protein assay (#500-0006). Hundred micrograms of the supernatant was boiled in loading buffer. Chemiluminescence was detected using a Luminescent Image Analyzer (Las-3000, Fujifilm, Tokyo, Japan). For reprobing, PVDF membranes were stripped by incubation in 300 mM NaOH (5 min, RT with agitation), followed by neutralization by several rinses in TBST (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.3% Tween-20).

### RNA isolation and quantitative RT-PCR

Total RNA was isolated from synchronized worm populations at the indicated ages. Total RNA was extracted using Qiazol reagent (Qiagen, Hilden, Germany; #79306) and purified using RNeasy kit (Qiagen #74104). cDNA was created using QuantiTect Probe RT-PCR Kit (Qiagen #204443). Quantification was completed using SDS2.1 software (Applied Biosys-

tems, Foster city, CA, USA), normalizing to control levels of *act-1* cDNA. SybrGreen real-time qPCR experiments were performed as described in the manual using ABI Prism7900HT (Applied Biosystems). *hsf-1* primer set 1: forward: TTGACGACGACAAGCTTCCAGT reverse: AAAGCTTGCAC CAGAATCATCCC *hsf-1* primer set 2: forward: GTCTCTGTCATGCAGC CAGG reverse: TTGGGTCCGGCAGTTCC. *act-1* primers: forward: GAG CACGGTATCGTCACCAA reverse: TGTGATGCCAGATCTTCCAT. *hsp-70* primers: forward: AGTGGATCCTCCGACAAGG reverse: CACCAAAG GCTACTGCTTCG.

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### Author contribution

EC and AD designed and initiated this study. YV, MM, TD, MBS, DJ, EK, and EC performed the experimental work. EC and AD wrote the manuscript.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1** (A and B) *act-1* quantitative PCR calibration for *hsf-1* mRNA quantification (corresponding Fig. 1A). (C–F) Raw data of Fig. 1A.

**Fig. S2** Worms expressing GFP under the *hsp-16.2* promoter were either grown on control bacteria (EV) or *hsf-1* RNAi for one generation and bleached.

**Fig. S3** To test whether exposure to 25 °C induces the heat shock response, *CL2070* worms that were grown to adulthood at 20 °C were exposed for 3 h to either 25 °C (lane 2), 33 °C (lane 3), or left at 20 °C (lane 1).

**Fig. S4** (A) To examine how *dcr-1* RNAi treatment affects lifespan, *CF512* animals were let hatched on EV bacteria and transferred onto *dcr-1* RNAi in 3-h intervals from 3 to 18 or 36 h after hatching and their lifespans were recorded. Our results show that *dcr-1* RNAi shortens lifespan when applied during larval development but this effect becomes less prominent when the worms had natural dicer levels for longer time from hatching. (B) Similarly we tested whether the knockdown of *dcr-1* during adulthood modifies lifespans of *CF512* worms. No effects on lifespan were observed when *dcr-1* RNAi was applied at either days 1, 5, or 9 of adulthood.

**Fig. S5** (A and B) Quantitative PCR indicates that the *hsf-1* expression levels are efficiently decreased (70–80% compared to control) in wild-type larvae within 4–6 h after exposure to *hsf-1* RNAi in L2 (A) and L4 (B) stages. (C and D) The expression levels of *hsf-1* are restored to nearly natural levels in L2 larvae ~6 h after transfer onto *dcr-1* RNAi (C) while in L4 larvae *hsf-1* restoration is apparent 1.5 h after exposure to *dcr-1* RNAi (D).

**Fig. S6** Worms transferred from *hsf-1* onto *dcr-1* RNAi later than 12 h after hatching exhibited progressively shorter lifespans (corresponding Fig. 2E, corresponding Table S5).

**Fig. S7** (A) *daf-2(e1370)* mutant, but neither wild-type nor *daf-2(e1368)* mutant worm are developmentally arrested when grown for two generations on *hsf-1* RNAi (white arrows point to progeny). (B) *daf-2(e1368)* grown for three generations on *hsf-1* RNAi completed development. (C) Experimental design of Fig. 3A,B.

**Fig. S8** *CF512* worms were grown on EV bacteria up to days 1, 4, or 8 of adulthood and either exposed for 24 h to *hsf-1* RNAi or left untreated prior to exposure to heat shock (33 °C, 1 h).

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