



Caenorhabditis elegans HSF-1 is an essential nuclear protein that forms stress granule-like structures following heat shock

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Summary

The heat shock transcription factor (HSF) is a conserved regulator of heat shock-inducible gene expression. Organismal roles for HSF in physiological processes such as development, aging, and immunity have been defined largely through studies of the single *Caenorhabditis elegans* HSF homolog, *hsf-1*. However, the molecular and cell biological properties of *hsf-1* in *C. elegans* are incompletely understood. We generated animals expressing physiological levels of an HSF-1::GFP fusion protein and examined its function, localization, and regulation *in vivo*. HSF-1::GFP was functional, as measured by its ability to rescue phenotypes associated with two *hsf-1* mutant alleles. Rescue of *hsf-1* development phenotypes was abolished in a DNA-binding-deficient mutant, demonstrating that the transcriptional targets of *hsf-1* are critical to its function even in the absence of stress. Under nonstress conditions, HSF-1::GFP was found primarily in the nucleus. Following heat shock, HSF-1::GFP rapidly and reversibly redistributed into dynamic, subnuclear structures that share many properties with human nuclear stress granules, including colocalization with markers of active transcription. Rapid formation of HSF-1 stress granules required HSF-1 DNA-binding activity, and the threshold for stress granule formation was altered by growth temperature. HSF-1 stress granule formation was not induced by inhibition of IGF signaling, a pathway previously suggested to function upstream of *hsf-1*. Our findings suggest that development, stress, and aging pathways may regulate HSF-1 function in distinct ways, and that HSF-1 nuclear stress granule formation is an evolutionarily conserved aspect of HSF-1 regulation *in vivo*.

Key words: aging; heat shock factor; longevity.

Introduction

The heat shock transcription factor (HSF) plays essential and evolutionarily conserved roles in the activation of heat shock-inducible gene expression. While HSFs are best recognized as regulators of stress-induced gene expression, they also contribute to more complex organismal physiological processes such as development, growth, aging, immunity, and reproduction. HSFs are also central to many pathophys-

iological processes and contribute to the tumorigenic phenotype as well as to the pathology underlying diseases of protein misfolding, such as Huntington's and Alzheimer's diseases (Hsu *et al.*, 2003; Morley & Morimoto, 2004; Cohen *et al.*, 2006; Dai *et al.*, 2007). Given the importance of HSFs to these physiological and pathophysiological states, understanding the mechanisms that regulate HSF function could provide new insights and therapeutic strategies for a variety of diseases (Westerheide & Morimoto, 2005).

In mammals, HSF1 encodes the master regulator of the heat shock response (Akerfelt *et al.*, 2010). Regulation of HSF1 is thought to occur at several levels. In the absence of heat shock, mammalian HSF1 exists primarily as a monomeric protein that is bound and repressed by heat shock protein 90 (HSP90) (Voellmy & Boellmann, 2007). Following an acute heat shock, HSP90 binds to misfolded client proteins, releasing HSF1 so that it can trimerize, bind sequence-specific heat shock elements (HSEs), and transactivate gene expression. In human (and other primate but not rodent) cells, HSF1 also responds to stress by localizing to subnuclear structures termed nuclear stress granules (or nuclear stress bodies; Biamonti & Vourc'h, 2010). HSF1 stress granules represent the binding of HSF1 to heterochromatic, pericentromeric repeat regions of DNA, leading to transcription of noncoding RNAs (ncRNAs) (Jolly *et al.*, 1997, 1999, 2004; Denegri *et al.*, 2002; Eymery *et al.*, 2010). Stress granule formation seems to be determined by specific DNA sequences, as rodent HSF1 can be induced to form stress granules when it is in the presence of human chromosomes (Denegri *et al.*, 2002). Despite intensive study, the functional role of HSF1 stress granules and their associated ncRNAs remains one of the most mysterious aspects of HSF1 regulation. As HSF1 stress granule formation has only been observed in primate cells, their study has been hampered by the lack of a suitable system in which to manipulate stress granule formation and ncRNA synthesis. Moreover, while the biochemical and cell biological properties of HSF1 are well described in isolated cells, studies investigating whether similar regulation occurs in a multicellular organism are more limited.

Recent studies in *C. elegans* have played a major role in understanding how HSF contributes to organismal physiology and pathophysiology. While vertebrates express four major HSFs, worms express a single HSF homolog, *hsf-1*. By sequence homology, *C. elegans* HSF-1 contains N-terminal DNA binding and trimerization domains, with a putative transactivation domain at the C-terminus (Hajdu-Cronin *et al.*, 2004). RNAi-mediated knockdown of *hsf-1* gives rise to a progeric phenotype (Garigan *et al.*, 2002), while *hsf-1* overexpression promotes longevity and delays age-related protein misfolding and proteotoxicity (Hsu *et al.*, 2003; Morley & Morimoto, 2004; Cohen *et al.*, 2006). *hsf-1* also plays important functional roles in *C. elegans* innate immunity (Singh & Aballay, 2006), where it functions in the intestine to inhibit pathogen-induced protein aggregation (Mohri-Shiomi & Garsin, 2008). Despite these many roles, the localization pattern and dynamic regulation of the *C. elegans* HSF-1 protein expressed at physiological levels have not been reported.

Here, we generated *C. elegans* expressing a functional single-copy HSF-1::GFP transgene driven by the native *hsf-1* promoter. We find that *in vivo*, HSF-1::GFP is a ubiquitously expressed, predominantly nuclear protein. Following heat shock, HSF-1::GFP does not exhibit further nuclear enrichment but does undergo rapid and reversible reorganization

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into subnuclear structures that share many characteristics with human HSF1 stress granules. The rapid formation of these structures in *C. elegans* required DNA-binding activity but was not induced by inactivation of insulin/IGF signaling. These studies are among the first to demonstrate the dynamic nuclear behavior of HSF in native tissues in a live animal setting, suggesting that nuclear stress granule formation may be an evolutionarily ancient mechanism for regulating HSF *in vivo*.

Results

HSF-1 constitutively localizes to the nucleus and forms nuclear granules upon heat shock

Many HSF-1-dependent processes have been studied in *C. elegans* and the properties of overexpressed *C. elegans* HSF-1 have recently been investigated (Chiang *et al.*, 2012). However, overexpressed *C. elegans* HSF-1 produces gain-of-function phenotypes (Hsu *et al.*, 2003; Morley & Morimoto, 2004; Singh & Aballay, 2006; Chiang *et al.*, 2012) and may not accurately reflect the physiological properties of endogenous HSF-1. To characterize these properties, we generated a fluorescent reporter that fused GFP to the C-terminus of the HSF-1 protein and integrated this transgene at single-copy level into the *C. elegans* genome (Fig. 1A) (Frøkjær-Jensen *et al.*, 2008). Quantitative real-time PCR confirmed that this transgenic line expressed *hsf-1* at near physiological levels (Fig. 1B). In contrast with previous studies of overexpressed HSF-1::GFP, single-copy HSF-1::GFP localized predominantly to the nucleus under basal conditions (20 °C) (Fig. 1C). Following heat shock (35 °C for 1 or 20 min), HSF-1::GFP did not exhibit further accumulation of fluorescence intensity within the nucleus (Fig. S1, Supporting Information), suggesting that during the earliest phases of heat shock, nuclear translocation of HSF-1::GFP is a minor component of its regulation in *C. elegans*. However, heat shock did result in the redistribution of HSF-1::GFP into distinct subnuclear structures (Fig. 1C,D), a property reminiscent of human HSF1, which forms structures termed HSF1 nuclear stress granules upon heat shock (Cotto *et al.*, 1997; Jolly *et al.*, 1997). HSF-1::GFP granule formation also occurred in the *hsf-1(ok600)* deletion mutant, indicating that stress granule formation does not require the endogenous HSF-1 protein (data not shown). A PCR fusion-derived HSF-1::YFP protein lacking gateway adaptor sequences also showed nuclear enrichment and stress granule formation (Fig. S2, Supporting Information). Quantification of the size of *C. elegans* HSF-1 stress granules revealed an average diameter of $0.6 \pm 0.2 \mu\text{m}$, slightly smaller than human HSF1 stress granules, which distribute among two populations of structures with diameters of 0.5–1.6 and 1.6–3.0 μm (Fig. 1E) (Cotto *et al.*, 1997). The *C. elegans* HSF-1 structures were also similar in number to human HSF1 granules (7.1 ± 2.7 granules per nucleus in *C. elegans* (Fig. 1F) vs. 6.8 ± 2.4 granules per nucleus in HeLa cells (Cotto *et al.*, 1997)). Stress-induced HSF-1::GFP granules were observed in all examined tissue types (Fig. S3, Supporting Information). Polyploid intestinal nuclei appeared to contain more granules per nucleus than other cell types, but intestinal autofluorescence hindered precise quantification (data not shown). Granules formed within 1 min during heat shock at 35 °C and dispersed following an hour of recovery at 20 °C (Fig. S3). After recovery, HSF-1::GFP could reform nuclear granules with subsequent heat shocks, although reformation required a longer heat shock suggesting that stress granule formation exhibits adaptation (Fig. S4, Supporting Information). These granules formed in locations similar to the initial granules, suggesting that another existing nuclear structure, such as DNA or RNA, acts as a scaffold for granule formation. When animals were grown at 16 °C, HSF-1::GFP formed granules at

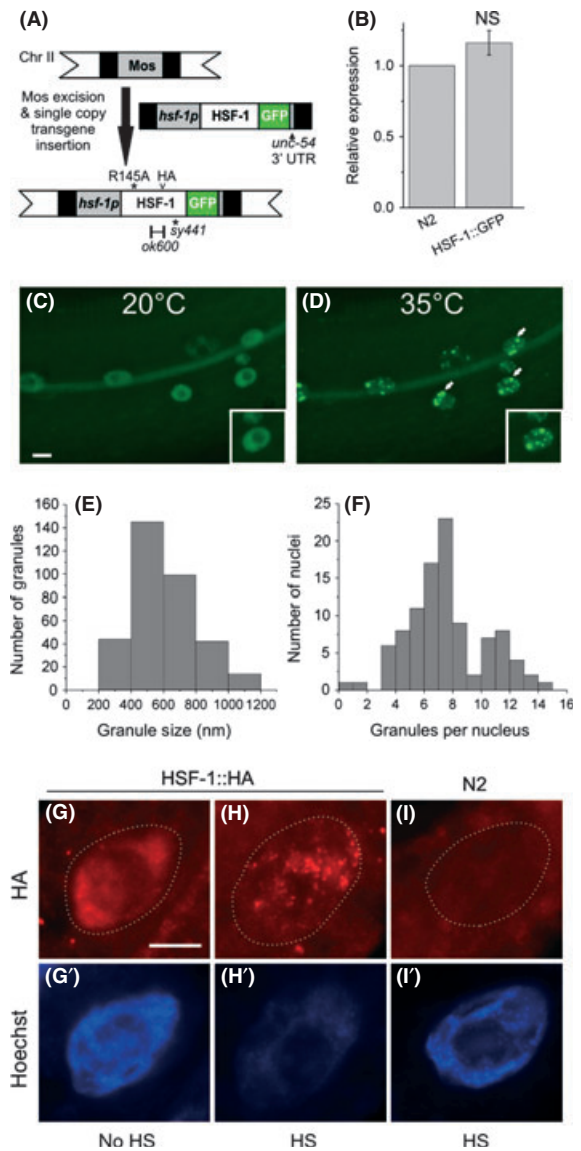


Fig. 1 HSF-1::GFP is broadly expressed and condenses into nuclear granules following heat shock. (A) Diagram of the *hsf-1p::hsf-1(cDNA)::GFP::unc-54 3'UTR* expression construct (*drSi13*) used in this study. *hsf-1p* is 4 kb of sequence upstream of the *hsf-1* start ATG. Notations above the construct diagram indicate changes made to the transgene (R to A at residue 145 and HA insertion after residue 370); notations below indicate relative positions of mutations in the endogenous gene (diagram not to scale). (B) qRT-PCR comparing wild-type (N2) and HSF-1::GFP *hsf-1* mRNA levels relative to actin mRNA, normalized to N2. Shown is the mean relative expression \pm SEM in *drSi13* for three independent experiments. *hsf-1* mRNA in a wild-type background is less than double control wild-type, suggesting compensatory mechanisms acting on *hsf-1* expression. (C) HSF-1::GFP localizes primarily to the nucleus at 20 °C. (D) After 1 min of 35 °C heat shock, HSF-1::GFP collects into nuclear puncta (arrows). Shown are four merged (Z-dimension) deconvolved slices depicting hypodermal nuclei. Scale bar = 5 μm . (E) HSF-1::GFP granule size in hypodermal cells after 1 min 35 °C heat shock ($N = 349$ granules). (F) Number of HSF-1::GFP granules per nucleus in hypodermal cells after 1 min 35 °C heat shock ($N = 100$ nuclei). (G–I) *drSi41*, a single-copy line expressing *hsf-1p::hsf-1::HA::unc-54 3'UTR*, in which the HA tag is inserted into the region between the putative trimerization and transactivation domains of the *hsf-1* cDNA. *drSi41* worms were either heat shocked (G, 30 min at 35 °C) or not (F, 30 min at 25 °C), dissected, fixed, probed for HA (red, G–I), and stained with Hoechst dye (blue, G'–I'). Heat-shocked N2 worms are shown as a control (I). Shown are nine merged (Z-dimension) deconvolved slices. Dotted line indicates outline of nuclei as determined by Hoechst. Scale bar = 5 μm .

heat shock temperatures ≥ 28 °C (Fig. S5, Supporting Information). Animals grown at 20 or 25 °C formed few or no granules at this temperature, but did form stress granules when exposed to ≥ 33 °C (Fig. S5). This suggests that the induction of HSF-1::GFP granules by heat shock is adaptable and regulated by growth temperature. Together, these data suggest that stress-induced HSF-1::GFP nuclear granules in *C. elegans* resemble human HSF1 stress granules in their number, kinetics, reformation, and reversibility.

We considered the possibility that the behavior of HSF-1::GFP may represent an artifact of the GFP tag. We therefore attempted to immunolocalize endogenous worm HSF-1 using a human antibody previously reported to recognize worm HSF-1 by immunoblot (Alavez *et al.*, 2011; Volovik *et al.*, 2012). Although we could detect human HSF1::GFP expressed in worms, we were unable to immunolocalize *C. elegans* HSF-1::GFP with this antibody following heat shock (Fig. S6, Supporting Information). Furthermore, we were also unable to detect a bacterially expressed *C. elegans* 6XHis-HSF-1 fusion protein by Western blot with either this antibody or another reported cross-reactive anti-human HSF-1 antibody (Chiang *et al.*, 2012), although both antibodies robustly detected human 6XHis-HSF1 (Fig. S7, Supporting Information). As an alternative approach, we generated single-copy transgenic animals expressing an HA-tagged form of HSF-1. We inserted the HA tag into the HSF-1 protein between the predicted DNA binding and transactivation domains (Hajdu-Cronin *et al.*, 2004). Like HSF-1::GFP, HSF-1::HA localized to the nucleus and redistributed into granule-like structures following heat shock (Fig. 1G–H). HSF-1::HA retained function, as measured by its ability to rescue an *hsf-1* mutant (Fig. 2A). These data show that HSF-1 is a predominantly nuclear protein that undergoes rapid and reversible changes in subnuclear distribution in response to acute heat shock in *C. elegans*.

HSF-1::GFP is functional

We considered the possibility that the GFP fusion might disrupt HSF-1 protein function. We tested this by assessing the ability of HSF-1::GFP to rescue *hsf-1* mutant phenotypes. We also investigated the ability of a human HSF1::GFP protein to functionally substitute for *C. elegans* HSF-1. The *hsf-1(sy441)* allele introduces a stop codon at position 585 preceding the predicted C-terminal transactivation domain, and mutant animals exhibit temperature-sensitive growth arrest, reduced expression of HSP-16.2 after heat shock, shortened lifespan, and sensitivity to pathogens (Hajdu-Cronin *et al.*, 2004; Singh & Aballay, 2006). A second *hsf-1* allele, *ok600*, encodes a frameshifting deletion that eliminates potential regulatory and transactivation domains at the C-terminus of the HSF-1 protein (Fig. 1A) and causes a 100% penetrant larval arrest phenotype at all growth temperatures, suggesting that *ok600* is a more severe allele than *sy441*. Whether the lethality of *ok600* is due to loss of *hsf-1* or another linked mutation has not been described. Single-copy worm *hsf-1p::hsf-1::GFP* rescued the temperature-dependent growth defects, temperature-dependent induction of HSP-16.2, lifespan reduction, and enhanced pathogen sensitivity of *hsf-1(sy441)* mutants (Fig. 2A–D). Unlike *hsf-1* overexpression transgenes, single-copy HSF-1::GFP did not cause increased longevity or enhanced pathogen resistance, suggesting that this transgene does not produce gain-of-function phenotypes like that observed for *hsf-1* overexpression (Fig. 2C,D). HSF-1::GFP was also able to rescue the larval lethality associated with the *hsf-1(ok600)* allele, indicating that the *ok600* lethal phenotype is indeed due to loss of *hsf-1* function (Fig. 2E,F). Human HSF1::GFP under the control of the *C. elegans* *hsf-1* promoter was expressed, properly localized to the nucleus, and capable of forming granules following heat

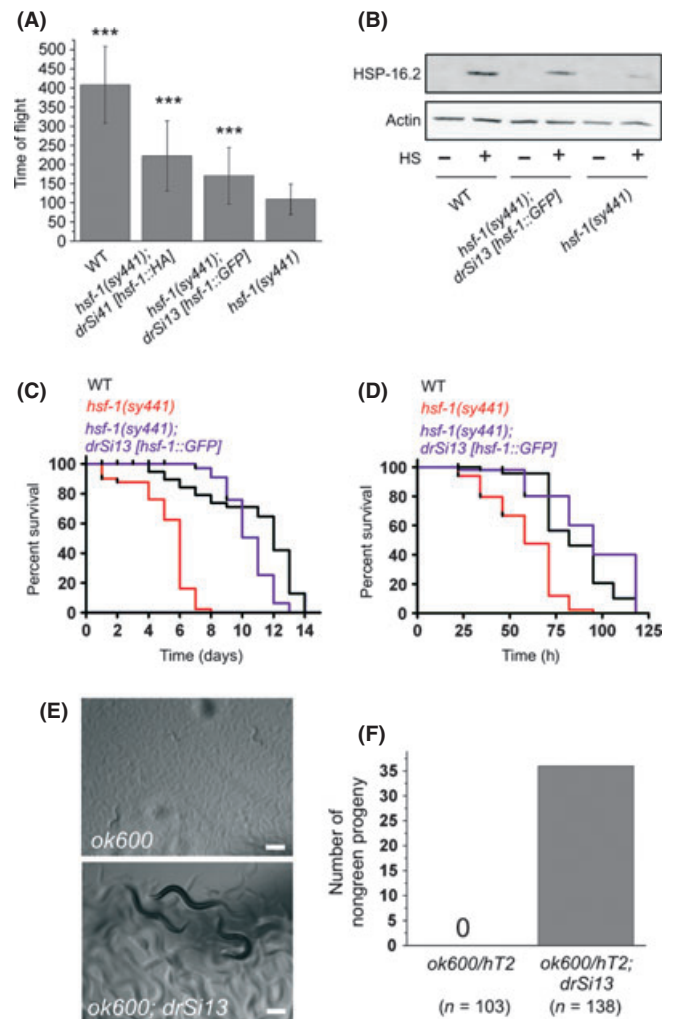


Fig. 2 HSF-1::GFP is functional. (A) Wild-type (WT), *hsf-1(sy441); drSi41 [hsf-1p::hsf-1::HA::unc-54 3'UTR]*, *hsf-1(sy441); drSi13 [hsf-1p::hsf-1::GFP::unc-54 3'UTR]*, and *hsf-1(sy441)* eggs were placed at 25 °C and allowed to grow until wild-type was L4/young adult. Worms were analyzed for size (time of flight) in a COPAS Biosort ($N \geq 89$ animals. Mean \pm SD, *** $P < 0.001$ as compared with *hsf-1(sy441)*). (B) Representative Western against HSP-16.2 (top panel) and β -actin (bottom panel) on WT, *hsf-1(sy441); drSi13*, and *hsf-1(sy441)* worms \pm a 35 °C 3 h heat shock followed by 3 h recovery at 16 °C. Relative HSP-16.2:actin ratio for WT: *hsf-1(sy441); drSi13*: *hsf-1(sy441)* is 1.0:0.67:0.22. (C) Lifespan of WT, *hsf-1(sy441)*, and *hsf-1(sy441); drSi13* animals at 25 °C ($N = 50$ for all) ($P < 0.0001$ between *hsf-1(sy441)* and *hsf-1(sy441); drSi13*; $P = 0.0036$ between WT and *hsf-1(sy441); drSi13*). (D) Survival of WT, *hsf-1(sy441)*, and *hsf-1(sy441); drSi13* animals on *Pseudomonas aeruginosa* PA14 at 25 °C ($N = 50$ for all) ($P < 0.0001$ between *hsf-1(sy441)* and *hsf-1(sy441); drSi13*; $P = 0.498$ between WT and *hsf-1(sy441); drSi13*). (E) Images of *ok600* homozygous animals with (lower) or without (upper) the *drSi13* HSF-1::GFP transgene. Scale bar = 100 μ m. (F) Quantification of the number of *ok600* homozygous animals that reach L4 stage or later within 3 days at 20 °C with or without the *drSi13* HSF-1::GFP transgene. n = total number of progeny.

shock (Figs. S6 and S8A, Supporting Information), albeit with substantially slower kinetics than those observed with worm HSF-1::GFP (1 h of heat shock required for human HSF1::GFP vs. 1 min for *C. elegans* HSF-1::GFP). Despite this, human HSF1::GFP was unable to rescue *hsf-1(sy441)* mutant phenotypes (Fig. S8B–F), suggesting that worm and human HSF1 are not functionally interchangeable. These data show that, in addition to its roles in aging, *C. elegans* *hsf-1* is an essential gene required for larval development and that worm HSF-1::GFP, but not

human HSF1::GFP, is functional and capable of rescuing *hsf-1*-associated mutant phenotypes in *C. elegans*.

HSF-1::GFP granules are dynamic and induced by specific environmental stressors

We next considered the possibility that HSF-1::GFP granules may simply be the result of protein aggregation caused by heat shock. We used fluorescence recovery after photobleaching (FRAP) to examine the mobility of the HSF-1::GFP protein within stress-induced nuclear granules. In contrast to protein aggregates induced by either stress or aging (Moronetti Mazzeo *et al.*, 2012), puncta of HSF-1::GFP exhibited rapid recovery after photobleaching (Video S1, Supporting Information). Similar results have been shown for human HSF1 stress granules (Jolly *et al.*, 1999). These data demonstrate that the HSF-1::GFP granules are not aggregates but rather are composed of dynamic HSF-1::GFP molecules. In addition to heat shock, other environmental stressors, including cadmium and azetidine, induce the formation of HSF1 granules in human cells (Jolly *et al.*, 1999). We asked whether *C. elegans* HSF-1::GFP also forms nuclear granules in response to various environmental stressors. Exposure of HSF-1::GFP worms to osmotic stress (219 mM NaCl), heavy metals (100- μ m cadmium), or ethanol (100 mM) did not induce granule formation (Fig. 3D–F). However, exposure to sodium azide, a well-known inhibitor of cytochrome oxidase and cellular ATP production and a commonly used anesthetic in the *C. elegans* field (Sulston & Hodgkin, 1988), induced robust HSF-1::GFP granule formation similar to that observed with heat shock (Fig. 3B, C). Conditions that induced granule formation also activated *hsf-1*-dependent gene expression, as measured using a reporter transgene (*hsp-16.2p::GFP*) (Fig. 3H–M). HSF-1::GFP granule formation can therefore be induced by elevated temperature or through azide-dependent inhibition of ATP production, demonstrating that heat shock *per se* is not required for granule formation.

Post-translational modification of HSF-1 temporally lags granule formation

Previous data from several species demonstrate that upon heat shock, HSF-1 is subject to a number of post-translational modifications (Akerfelt *et al.*, 2010). We asked whether *C. elegans* HSF-1::GFP was similarly modified after heat shock. Consistent with other recent observations of HSF-1 in *C. elegans* (Chiang *et al.*, 2012), HSF-1::GFP shifted toward a higher molecular weight in response to heat shock (Fig. S9A, Supporting Information), suggesting it is the target of stress-inducible post-translational modification (PTM). While previous studies showed that this PTM is due to phosphorylation (Chiang *et al.*, 2012), we were unable to confirm this by phosphatase assay due to the extremely low abundance of the single-copy HSF-1::GFP protein (data not shown). The molecular weight shift was not apparent after 1 min of heat shock but was detectable after 10 min of heat shock, thus temporally lagging the observed kinetics of HSF-1 stress granule formation (Fig. S9B).

DNA binding promotes HSF-1 stress granule formation

Studies in human cells have established that human HSF1 nuclear stress granules do not represent the binding of HSF1 to HSEs in canonical HSF1 targets like HSP70 (Jolly *et al.*, 1997), but rather HSF1 binding to and transcription of noncoding satellite II and III repeats, elements specific to primate genomes (Jolly *et al.*, 2002; Eymery *et al.*, 2010). Supporting this model, the DNA-binding domain of HSF1 is required for stress

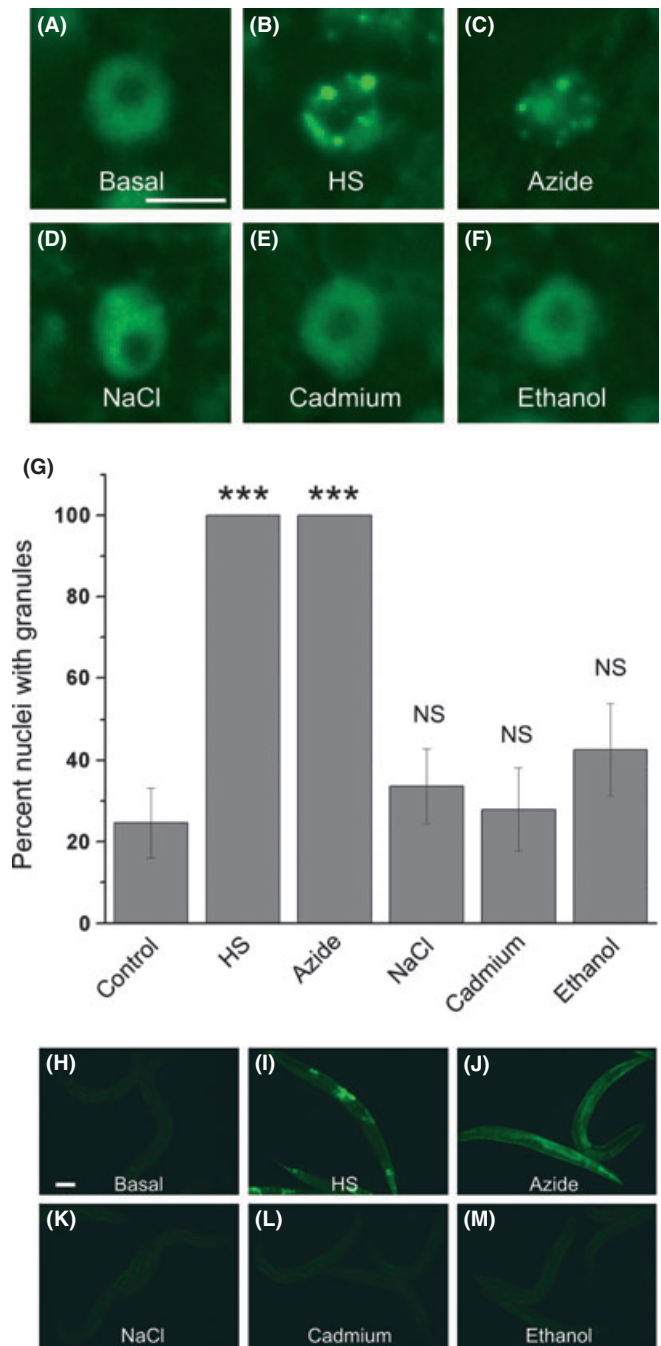


Fig. 3 Nuclear granules form in response to heat shock and sodium azide. Worms expressing *drSi13* HSF-1::GFP were anesthetized 30 min in 1 mM levamisole (except for 'C' and 'J', which were anesthetized by azide) at either room temperature (A), 35 °C (HS) (B), 5 mM sodium azide (C), 219 mM NaCl (D), 100 μ M CdCl₂ (E), or 100 mM ethanol (F). Scale bar = 5 μ m. (G) Percent of hypodermal nuclei with ≥ 1 visible granule were quantified for each condition ($N \geq 10$ worms per condition, representing ≥ 85 nuclei. Mean \pm SEM, *** $P < 0.001$ vs. control; n.s., not significant). (H–M) TJ375 (*hsp-16.2p::GFP*) worms were subjected to 30 min of the same conditions as in (A)–(F), followed by recovery at 20 °C for 4 h before imaging. Scale bar = 100 μ m.

granule formation in human cells (Jolly *et al.*, 2002). To ask whether DNA binding was also required for *C. elegans* HSF-1::GFP stress granule formation, we generated a point mutation (R145A, equivalent to human R71A, Fig. 4A) in a completely conserved amino acid within the DNA-

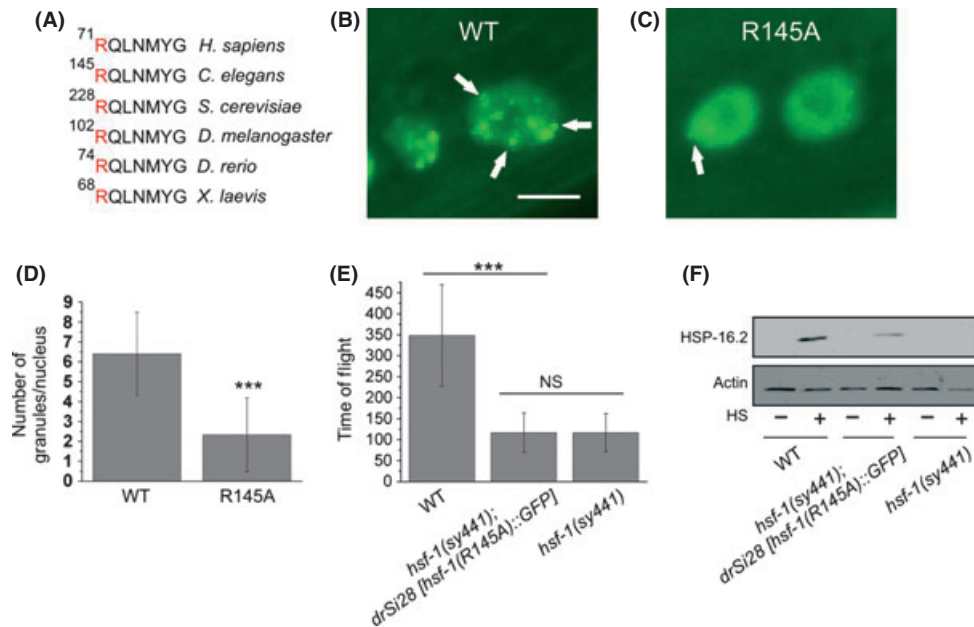


Fig. 4 HSF-1 DNA binding promotes stress granule formation and developmental rescue of *hsf-1(sy441)*. (A) Alignment of the region of the HSF1 DNA-binding domain containing R145 (red) from the indicated species. (B, C) Images of *drSi13* HSF-1::GFP (WT) and *drSi28* HSF-1(R145A)::GFP (R145A) taken after a 1 min 35 °C heat shock, showing granule formation (arrow). Scale bar = 5 μ m. Shown are four merged (Z-dimension) deconvolved slices. (D) Number of granules per nucleus was quantified for WT and R145A. ($N \geq 18$ worms, representing ≥ 140 nuclei for each line. Mean \pm SD, *** $P < 0.001$). (E) N2 wild-type (WT), *hsf-1(sy441)*, and *hsf-1(sy441);drSi28* eggs were placed at 25 °C and allowed to grow until wild-type was L4/young adult. Worms were analyzed for size (time of flight) in a COPAS Biosort ($N \geq 30$ animals. Mean \pm SD, *** $P < 0.001$, n.s., not significant). (F) Representative Western against HSP-16.2 (top panel) and β -actin (bottom panel) on young adult wild-type, *hsf-1(sy441)*; *drSi28*, and *hsf-1(sy441)* worms \pm a 3 h 35 °C heat shock followed by 3 h recovery at 16 °C.

binding domain that has been previously shown to be required for HSF-1-HSE DNA binding (Inouye *et al.*, 2003; Morley & Morimoto, 2004). We expressed this HSF-1(R145A) as a fusion with GFP under the native *hsf-1* promoter at single-copy level. Like wild-type HSF-1::GFP, HSF-1(R145A)::GFP was expressed and localized to the nucleus; however, the ability of HSF-1(R145A)::GFP to form nuclear granules in response to acute heat shock was significantly reduced (Fig. 4B–D). This suggests that DNA binding promotes HSF-1 granule formation in *C. elegans*.

As HSF-1 mutant phenotypes are probably due to an inability of HSF-1 to transactivate target gene expression, we examined the ability of HSF-1(R145A)::GFP to rescue the *hsf-1* mutant alleles *ok600* and *sy441*. Because the *sy441* point mutation is viable and the *ok600* deletion allele lethal, *sy441* likely results in the expression of a partially functional protein, which is predicted to contain intact DNA binding and oligomerization domains but lack the putative C-terminal transactivation domain. Given that the active form of HSF-1 is thought to be a trimer (Baler *et al.*, 1993), we hypothesized that the DNA-binding-deficient HSF-1(R145A), which contains a functional transactivation domain, might be able to interact with the truncated *hsf-1(sy441)* protein and provide the missing transactivation function. Consistent with this model, *hsf-1(sy441)* animals expressing the HSF-1(R145A)::GFP transgene produced a marked increase in stress-inducible gene expression as compared with the *hsf-1(sy441)* background alone (Fig. 4F), although quantification of this was difficult due to the very low levels of HSP-16.2 expression in *hsf-1(sy441)*. However, HSF-1(R145A)::GFP was unable to rescue the developmental functions of HSF-1 in either the *sy441* or *ok600* backgrounds (Fig. 4E and data not shown). The ability of HSF-1(R145A)::GFP to partially rescue the stress-inducible phenotype of *hsf-1(sy441)* but not the developmental phenotype suggests that the functional requirements for *hsf-1* in stress responses and development may be genetically separable.

HSF-1 granules in *C. elegans* colocalize with markers of active transcription

In human cells, HSF1 granules represent sites of HSF1 binding at satellite II and III repeats, colocalization with markers of active transcription like phosphorylated RNA polymerase II and acetylated histones, and transcription of ncRNAs (Denegri *et al.*, 2002; Jolly *et al.*, 2002, 2004; Metz *et al.*, 2004). Because *C. elegans* contains little if any satellite repeat DNA sequences (Sulston & Brenner, 1974), the putative site(s) of HSF-1 stress granule binding are unknown. Therefore, we asked whether worm HSF-1 stress granules also colocalized with general markers of active transcription. We stained control and heat-shocked animals for either the phosphorylated Serine 2 form of RNA polymerase II (RNA polII Ser2p) or the acetylated Lysine 5 form of histone H2A (H2Aac), both of which have been used as markers of active transcription in human HSF1 stress granules (Jolly *et al.*, 2004). Both of these markers showed colocalization with some (but not all) stress-induced HSF-1::GFP granules (Fig. 5A–C, I–K, arrows). The overall number of H2Aac foci was significantly increased by heat shock, consistent with transcriptionally active HSF-1 granules being induced by stress (Fig. S10, Supporting Information). Although these data do not directly demonstrate transcriptional activity of HSF-1 within nuclear stress granules, they strongly suggest that active transcription is occurring at some sites of HSF-1 granule formation.

IGF and neuronal genetic pathways influence HSF-1 granule formation

Previous studies have shown that several genetic and physiological pathways require HSF-1 function. For example, extension of lifespan via activation of insulin signaling requires HSF-1 activity (Hsu *et al.*, 2003;

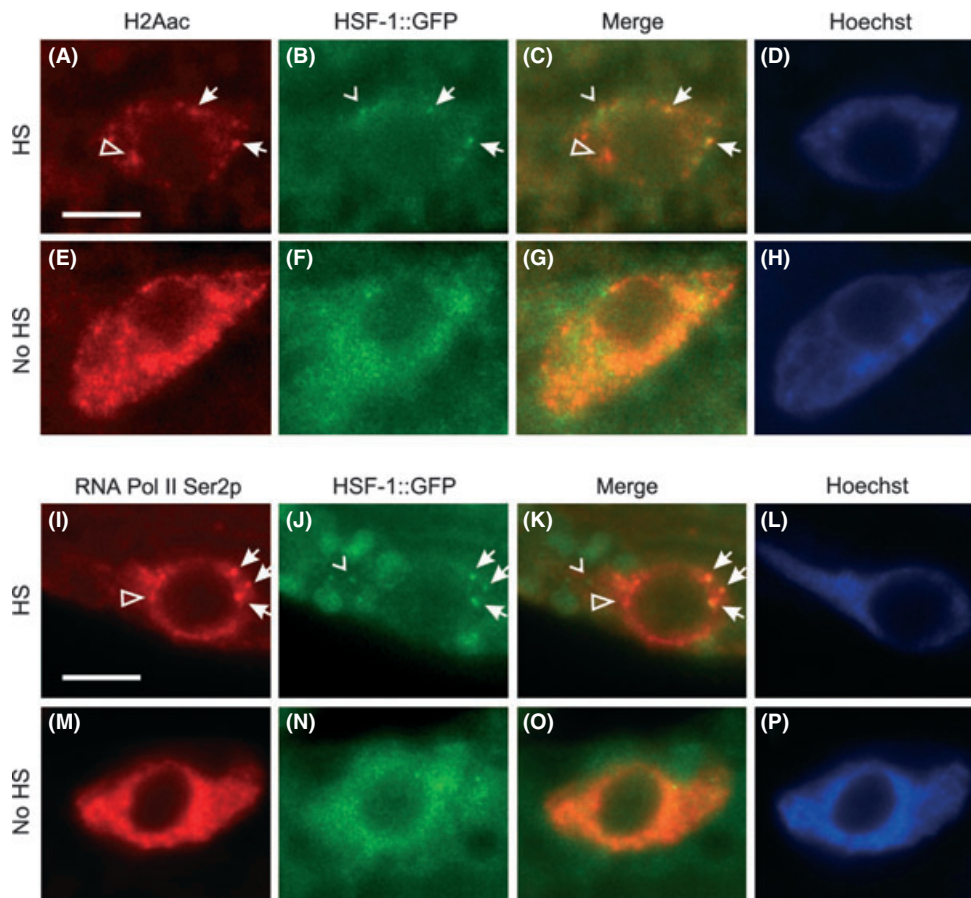


Fig. 5 HSF-1::GFP granules colocalize with markers of active transcription. *drSi13* HSF-1::GFP worms grown at 25 °C were heat shocked for 1.5 h at 35 °C (HS) or put at 25 °C 1.5 h (no HS) and intestinal nuclei of fixed worms were probed for GFP (green, B, F, J, N) histone H2A acetylated on Lysine 5 (red, A, E), or RNA polymerase II phosphorylated on Serine 2 (red, I, M). (C, G) - Merged image from H2Ac and HSF-1::GFP staining. (K, O) - Merged image from RNA Pol II Ser2p and HSF-1::GFP staining. Exposure times in I and M were different because we observed substantially reduced RNA polII Ser2p staining post-heat shock. Nuclei (intestinal) were detected by Hoechst staining (D, H, L, P). Nuclear staining showed puncta of fluorescence, some of which show colocalization of GFP and an active transcription marker (arrows). Other puncta exhibit GFP-only (carrot) or active transcription marker-only (open arrowhead) staining. Scale bar = 5 μ m.

Morley & Morimoto, 2004). Likewise, a recently described neuronal pathway regulates HSF-1-dependent gene expression (Prahlad *et al.*, 2008; Prahlad & Morimoto, 2011). To determine whether these pathways regulate HSF-1 in a way similar to that of heat shock, we examined the expression, localization, and stress-inducible behavior of HSF-1::GFP in the IGF mutant *daf-2(e1370)* and in the AIY neuron mutant *ttx-3(ks5)*. In *daf-2(e1370)*, in the absence of stress, HSF-1::GFP localized to and was evenly distributed within the nucleus (of hypodermis, intestine, and other cell types), as was previously observed in nonstressed wild-type animals (Fig. S11E,F, Supporting Information and data not shown). Granule formation could still be induced, but quantification of granule formation revealed a statistically significant decrease in the number of granules induced by heat shock in a *daf-2(e1370)* background (Fig. S11H) in hypodermal nuclei. The effect of *daf-2(e1370)* on granule formation in other tissues was more difficult to quantify due to the small size of the nuclei and autofluorescence, although stress-induced granule formation was observed (data not shown). Likewise, in the AIY interneuron mutant *ttx-3(ks5)*, (previously shown to be required for non-neuronal heat shock-inducible gene expression) HSF-1::GFP remained localized to the nucleus in the absence of stress. Following heat shock of *ttx-3*, HSF-1::GFP continued to form stress granules in hypodermal nuclei (Fig. S11D) and other cell types

(data not shown). However, as with *daf-2*, the number of granules induced by heat shock in *ttx-3* animals was reduced (Fig. S11G). These findings show that IGF and AFD signaling promote proper stress-inducible HSF-1::GFP granule formation but do not alter HSF-1::GFP nuclear localization in nonstressful environments, suggesting that the mechanism(s) by which IGF signaling regulates HSF-1 is distinct from the mechanisms that regulate HSF-1 in response to heat shock.

Discussion

While most of the work demonstrating a functional role for HSF-1 in the regulation of aging and diseases of protein aggregation has been carried out in *C. elegans*, there is still much to learn regarding the molecular properties of HSF-1 in this system or any organismal context. Our findings fill important gaps in our knowledge of HSF biology, provide new resources with which to study this conserved transcription factor, and describe insights into HSF-1 regulation in *C. elegans* that both concur with and contradict other recent studies (Chiang *et al.*, 2012).

The major model for HSF1 regulation predicts it to be predominantly cytoplasmic under control conditions, constrained by interactions with cytoplasmic heat shock proteins (HSPs) (Voellmy & Boellmann, 2007). Following heat stress, HSPs are competed away by interactions with

misfolded client proteins, allowing HSF1 to trimerize and translocate to the nucleus. While all studies agree that HSF1 is localized to the nucleus following stress, the localization of HSF1 prior to stress has been controversial. For example, *Drosophila* HSF1 is considered a constitutively nuclear protein (Westwood *et al.*, 1991; Yao *et al.*, 2006), but some studies have suggested that it is predominantly cytoplasmic and undergoes nuclear translocation with heat shock (Zandi *et al.*, 1997). Likewise, human HSF1 has been reported both as a nuclear protein under all conditions (Martinez-Balbas *et al.*, 1995; Mercier *et al.*, 1999) and as a predominantly cytoplasmic protein that undergoes stress-induced nuclear translocation (Baler *et al.*, 1993; Sarge *et al.*, 1993). The reasons for these inconsistencies are unclear but may be due to artifacts of overexpression or biochemical preparations that artificially place inactive HSF1 in the cytoplasm (Mercier *et al.*, 1999). Recently, Chiang *et al.* (2012) reported that an HSF-1::GFP protein in *C. elegans* exhibited diffuse nucleocytoplasmic fluorescence under control conditions, converting to weak nuclear localization after heat shock. This finding contrasts with our observations that HSF-1::GFP is a nuclear protein before and after stress. How can this discrepancy be explained? One possibility is that the HSF-1::GFP fusion protein differs between the two studies and these differing sequences alter localization. Both fusion proteins are derived from an HSF-1 cDNA fused with C-terminal GFP via different linker sequences. The presence of the GFP tag does not affect localization because an HSF-1::HA protein exhibits similar nuclear localization to our HSF-1::GFP protein. Likewise, our linker sequences also do not affect localization because an HSF-1::YFP protein lacking linkers also localized to the nucleus and formed granules with stress. It is possible that overexpression of HSF-1::GFP in the Chiang *et al.* study alters localization. High-level expression may overwhelm HSF-1 regulatory mechanisms, driving dysregulated HSF-1 into the cytoplasm. Such dysregulation would be unlikely to affect our HSF-1 reporter due to its physiological level of expression. This discrepancy could be best resolved by examining the localization of the endogenous HSF-1 protein, but such studies will require the generation of new antibodies that are compatible with immunofluorescence techniques in *C. elegans*.

One important new finding of our work is that heat shock induces *C. elegans* HSF-1 to form discrete subnuclear foci that are similar to structures previously thought to occur only in primate cells. While *C. elegans* and human HSF-1 foci share many characteristics (Table S1, Supporting Information), we have not shown that all properties are shared between the two structures. For example, human HSF1 granules do not colocalize with standard HSF1 targets (Jolly *et al.*, 1997) but rather with heterochromatic pericentromeric satII and satIII repeats (Jolly *et al.*, 2004; Metz *et al.*, 2004; Eymery *et al.*, 2010), where they promote the transcription of ncRNAs that remain associated with the stress granule. While we have shown the *C. elegans* HSF-1 granules involve DNA binding and are (in a subset of granules) associated with transcription, we have not shown that such binding is distinct from the binding of HSF-1 to target promoters or that these binding sites are associated with the transcription of ncRNAs. Additionally, worms do not have centromeric sequences or satellite repeat DNA, so it remains possible that the specific properties of stress granule binding sites in *C. elegans* may be distinct from those in human cells. Despite these potential differences, it is also possible that the *C. elegans* stress granules are sites of centromere-independent ncRNA transcription. If so, *C. elegans* could provide important insights regarding the role of such ncRNAs in *hsf-1*-dependent processes. This is currently an important but unanswered question in the HSF1 field to which studies in *C. elegans* could make an important contribution.

Our data also suggest that the mechanisms regulating HSF-1 may not be the same in all contexts. In *C. elegans*, *hsf-1* has been primarily studied at the phenotypic level where it has roles in aging, immunity, and development. Prior to our work, it was not clear if these processes acted through mechanisms similar to those by which temperature regulates HSF-1 activity. We found that activation of HSF-1 via heat shock induces granule formation, but inactivation of insulin signaling via mutation of *daf-2* does not. It bears noting that we were able to induce granule formation with sodium azide, demonstrating that heat shock itself is not required for granule formation. Like inhibition of insulin signaling, inhibition of AFD signaling via *ttx-3* mutation also does not constitutively induce or prevent granule formation, although it does reduce number of granules visible per nucleus. This observation could be due to a requirement of neuronal signaling for stress-induced granule formation, or possibly due to a decrease in total HSF-1, as HSF-1 levels were not quantified in this line. Altogether, our findings provide an alternative model to that proposed by Chiang *et al.* (2012) and suggest that insulin signaling and temperature might employ distinct mechanisms to control HSF-1 activity.

Consistent with the idea that multiple mechanisms may regulate HSF-1, we also found evidence that the activity of HSF-1 in development and heat shock are not two outcomes of a single activation pathway, but rather two mechanistically different activation pathways (Fig. 6). Similar observations have been noted for *Drosophila hsf-1* (Jedlicka *et al.*, 1997) and *C. elegans hsf-1* (Walker *et al.*, 2003). We have built on these observations by showing that the DNA-binding-deficient HSF-1(R145A) molecule could partially complement the transactivation-deficient *hsf-1* (*sy441*) mutant for stress-inducible gene expression but not development. This could be explained through stress-specific oligomerization of HSF-1(R145A) and HSF-1(*sy441*) molecules, both of which possess intact putative trimerization domains, leading to an oligomer containing functional domains for both transactivation and DNA binding. An alternative possibility is that the R145A mutant may retain low levels of DNA-binding activity that are sufficient to rescue *hsf-1* stress functions

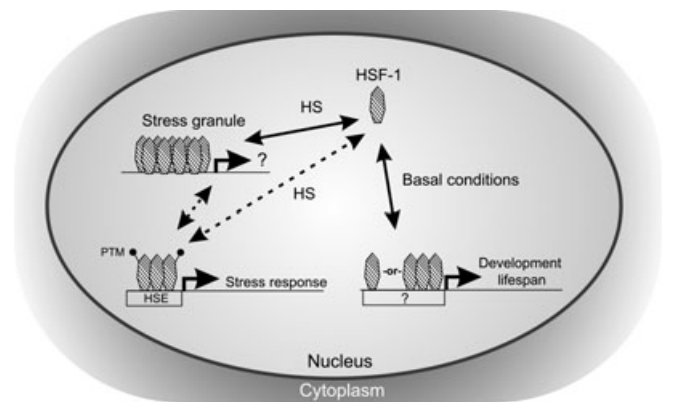


Fig. 6 Model for HSF-1 regulation in *Caenorhabditis elegans*. HSF-1 is a predominately nuclear protein in *C. elegans*, and its modes of activity under basal conditions and stress conditions (HS) differ. Stress-inducibility is distinguished by stress granule formation, oligomerization, and post-translational modification of HSF-1. Due to its oligomeric nature, we hypothesize that physiological levels of the DNA-binding-deficient HSF-1(R145A) can still associate with the active HSF-1 complex and contribute transactivation function to stress-inducible targets *in trans*. The observation that HSF-1(R145A) cannot rescue developmental defects in the *sy441* transactivation-deficient background suggests that HSF-1 activity in the context of development may not operate *in trans*. Basal targets of HSF-1, including genes involved in development and possibly lifespan, require DNA-binding activity, but may not involve stress granule formation or oligomerization.

but not developmental functions. Regardless, the fact that HSF-1(R145A) is incapable of rescuing development strongly suggests that HSF-1 has transcriptional targets even in the absence of stress. Such targets may even be transcribed by monomeric HSF-1, likely the predominant form in the absence of stress (Fig. 6). Further testing of this model, using more precise deletion alleles that eliminate specific HSF-1 functional domains, is called for.

In conclusion, we have provided new *in vivo* insights into the regulation of HSF-1 in *C. elegans* and its mechanism of regulation by heat shock, including a potentially evolutionarily conserved subnuclear behavior that was previously thought to be present only in primates. Expression of epitope-tagged, physiological levels of HSF-1 *in vivo* offers new experimental opportunities to understand how this protein integrates development, stress, aging, and metabolic pathways in a live organism setting to determine condition-specific gene expression.

Experimental procedures

Caenorhabditis elegans strains and culture

Strains were cultured on standard NGM media with OP50 bacteria. For a list of all strains and alleles used in this study, see 'Supplemental Methods'.

Molecular biology and transgenics

Detailed description of the cloning methods and expression constructs used in this study can be found in the 'Supplemental Methods'. Single-copy-compatible constructs were integrated into the chromosome II locus (ttTi5605) using the direct insertion MosSCI technique (Frøkjær-Jensen *et al.*, 2008). The single-copy nature of the insertion was verified by PCR using primers flanking the recombination interval (OG967 and OG970). The HSF-1(R145A)::GFP construct (pOG124) was created by site-directed mutagenesis (Agilent Technologies, Santa Clara, CA, USA) of pOG34. The sequences of all primers used in this study can be found in Table S1.

Statistical analysis

Survival studies were analyzed using the Kaplan–Meier log-rank function (GraphPad Software). Comparisons of means were analyzed with either a two-tailed Student's *t*-test (two groups) or ANOVA (three or more groups) using Bonferroni post-test analysis. A paired *t*-test was used in Fig. S1, others were unpaired. $P < 0.05$ was considered significant.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 The levels of nuclear HSF-1::GFP are not significantly increased in response to heat shock.

Fig. S2 HSF-1::YFP localization is not affected by absence of Gateway *att* linker sequences.

Fig. S3 HSF-1::GFP is expressed and forms granules in multiple cell types.

Fig. S4 HSF-1::GFP granules reform in similar locations with subsequent heat shocks.

Fig. S5 The threshold for HSF-1::GFP stress granule formation is influenced by growth temperature.

Fig. S6 Human HSF1 antibody does not detect heat-shocked worm HSF-1 by immunofluorescence.

Fig. S7 Commercially available antibodies against human HSF-1 do not recognize a bacterially expressed *Caenorhabditis elegans* 6X-His-HSF-1 fusion protein.

Fig. S8 Human HSF1 is expressed and localizes to the nucleus but does not rescue *Caenorhabditis elegans hsf-1(sy441)* mutant phenotypes.

Fig. S9 HSF-1::GFP is post-translationally modified following heat shock.

Fig. S10 H2Aac is distributed in a greater number of discrete puncta after heat shock.

Fig. S11 *ttx-3* and *daf-2* mutations reduce the number of HSF-1 granules.

Video S1 HSF-1::GFP granules are not aggregates.

Table S1 Comparison between *Caenorhabditis elegans* and human HSF-1 stress granules

Table S2 Primers

Table S3 Lifespan statistics