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RESEARCH ARTICLE

Inhibition of the oxidative stress response by heat stress in *Caenorhabditis elegans*

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ABSTRACT

It has long been recognized that simultaneous exposure to heat stress and oxidative stress shows a synergistic interaction that reduces organismal fitness, but relatively little is known about the mechanisms underlying this interaction. We investigated the role of molecular stress responses in driving this synergistic interaction using the nematode *Caenorhabditis elegans*. To induce oxidative stress, we used the pro-oxidant compounds acrylamide, paraquat and juglone. As expected, we found that heat stress and oxidative stress interact synergistically to reduce survival. Compared with exposure to each stressor alone, during simultaneous sublethal exposure to heat stress and oxidative stress the normal induction of key oxidative-stress response (OxSR) genes was generally inhibited, whereas the induction of key heat-shock response (HSR) genes was not. Genetically activating the SKN-1-dependent OxSR increased a marker for protein aggregation and decreased whole-worm survival during heat stress alone, with the latter being independent of HSF-1. In contrast, compared with wild-type worms, inactivating the HSR by HSF-1 knockdown, which would be expected to decrease basal heat shock protein expression, increased survival during oxidative stress alone. Taken together, these data suggest that, in *C. elegans*, the HSR and OxSR cannot be simultaneously activated to the same extent that each can be activated during a single stressor exposure. We conclude that the observed synergistic reduction in survival during combined exposure to heat stress and oxidative stress is due, at least in part, to inhibition of the OxSR during activation of the HSR.

KEY WORDS: Multiple stressors, Synergistic interaction, SKN-1, HSF-1, Survival

INTRODUCTION

Environmental stressors can interact to produce additive, synergistic or even antagonistic effects on the growth, performance and survival of organisms (Crain et al., 2008; Darling and Côté, 2008; Folt et al., 1999). Temperature and oxidative stress can interact synergistically, but the underlying mechanisms remain poorly understood (Holmstrup et al., 2010). Potential mechanisms include insufficient metabolic capacity to simultaneously respond to both stressors (Cherkasov et al., 2006; Sokolova, 2013), and direct effects of heat on the chemistry or bioavailability of oxidants, which could increase oxidant uptake (Jones and Candido, 1999; Scheil and Köhler, 2009). Less studied are potential interactions between the molecular stress responses. Combinations of stressors can radically modulate the expression of various stress-responsive genes relative to single-stress exposures (Rasmussen et al., 2013), raising the possibility that the altered expression of stress-responsive genes during combined stress is maladaptive, contributing to synergistic effects that reduce organism growth, performance and survival.

Animals exposed to potentially deleterious elevations in body temperature activate the heat-shock response (HSR) (Lindquist, 1986), a program that protects cells from perturbations in proteostasis via the induction of molecular chaperones known as heat shock proteins (HSPs) (van Oosten-Hawle and Morimoto, 2014). Similarly, animals exposed to oxidative stress activate the oxidative-stress response (OxSR), a program that protects cells from oxidative damage by the induction of antioxidant and detoxification enzymes and their reducing equivalents (Zhang et al., 2015). The HSR in eukaryotes is transcriptionally regulated by heat shock factor 1 (HSF-1) (Anckar and Sistonen, 2011; Verghese et al., 2012; Xiao et al., 1999), which is itself regulated by interactions with chaperone complexes (Schöffl et al., 1998; Voellmy and Boellmann, 2007), other HSF-1 monomers and various post-translational modifications, including phosphorylation and acetylation (Mager and De Kruijff, 1995; Raynes et al., 2013). The OxSR in eukaryotes is largely controlled by Cap'n'collar (CNC) transcription factors, a family of conserved master regulators of OxSRs (Pitoniak and Bohmann, 2015; Sykiotis and Bohmann, 2010). CNC transcription factors are themselves regulated by complex signals, including ubiquitin ligases, insulin/IGF-1-like signaling (IIS), glycogen synthase kinase-3 and TOR signaling, and seem to regulate diverse physiological processes (Blackwell et al., 2015; Robida-Stubbs et al., 2012; Tullet et al., 2008; Wang et al., 2010). The presence of multiple signaling inputs to the HSR and OxSR allows for the integration of diverse signals that direct the cellular response to stress, but whether this also results in complex interactions during simultaneous exposure to multiple stressors is poorly understood.

In this study, we tested the hypothesis that interactions between the HSR and OxSR signaling pathways alter the expression of stress-responsive genes and contribute to synergistic interactions between heat stress and oxidative stress that impact organism survival. We selected the nematode *Caenorhabditis elegans* as a convenient model organism. *Caenorhabditis elegans* is a widely distributed opportunistic colonizer of microbe-rich habitats (Frézal and Félix, 2015), where it is likely exposed to daily fluctuations in temperature and to both naturally occurring and anthropogenic toxins that increase oxidative stress (Isaksson, 2010; Watanabe et al., 2004). Its small size (~1 mm), short lifespan (~3–4 weeks) and large brood size simplify its culture in the lab (Brenner, 1974). Furthermore, its genome is fully sequenced and well-annotated, relatively simple techniques are available for knockout or knockdown of specific genes (Kamath et al., 2001), and transgenic green fluorescent protein (GFP)-tagged reporter strains are available for quantitative assessment of gene expression

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Received 28 November 2015; Accepted 9 May 2016

Journal of Experimental Biology (2016) 219, 2201-2211 doi:10.1242/jeb.135327

patterns. Finally, the molecular responses to single stressors in *C. elegans* have been well-characterized (Fig. 1).

The HSR in *C. elegans* is largely mediated by a single heat shock factor, HSF-1, that induces expression of multiple classes of HSPs. HSF-1 is itself negatively regulated by Hsp70 and Hsp90 chaperone complexes that bind HSF-1 during non-stress conditions and reduce its transcriptional activity (Morton and Lamitina, 2013; Schöffl et al., 1998; Voellmy and Boellmann, 2007). The OxSR in *C. elegans* is largely mediated by a single CNC transcription factor, SKN-1, that translocates into nuclei during oxidative stress to activate the transcription of over 2000 genes, including more than 100 antioxidant and detoxification genes (An and Blackwell, 2003; Choe et al., 2012; Oliveira et al., 2009; Park et al., 2009). Important OxSR genes upregulated by SKN-1 activation include those encoding glutathione S-transferases (e.g. *gst-4*, *gst-12* and *gst-30*), which detoxify xenobiotic toxins by conjugating them with glutathione, and γ-glutamine cysteine synthetase (*gcs-1*), which encodes the rate-limiting enzyme in glutathione synthesis. SKN-1 is negatively regulated by the WDR-23 protein, which is part of a CUL-4–DDB-1 ubiquitin ligase complex that reduces the nuclear accumulation of SKN-1 and thus its transcriptional activity (Choe et al., 2009). The OxSR can be induced in *C. elegans* with pro-oxidant compounds, including juglone, paraquat and acrylamide. Juglone is a naturally occurring electrophile and redox-cycling compound produced by black walnut trees and other members of the Juglandacea family. It is exuded into the soil by roots or decomposing leaves and fruit, where it can be encountered by *C. elegans* at concentrations as high as 6.4 µmol l^{-1} (von Kiparski et al., 2007). Paraquat is a synthetic, contact herbicide used throughout the world in agricultural applications (Powles and Yu, 2010). Acrylamide is not likely to affect *C. elegans* in nature, but it is a strong inducer of SKN-1 transcriptional activity (Hasegawa et al., 2008) and produces oxidative stress in other organisms (Prasad and Muralidhara, 2012).

MATERIALS AND METHODS

Caenorhabditis elegans strains and culture

The following *C. elegans* strains were used (note that the P prefix in a gene construct indicates that only the promoter region for the gene is present): (1) wild-type N2 Bristol, (2) VP596 *vsIs33 [Pdop-3::RFP]; dvIs19 [Pgst-4::GFP]*, (3) QV9 *wdr-23(tm1817)*, (4) AM140 rmIs132 *[Punc-54::Q35::YFP]*, (5) PS3551 *hsf-1(sy441)*, (6) QV212 *skn-1(k1023)*, and (7) QV216 *wdr-23(k1002)*. Except in specific protocols noted below, worms were cultured at 20°C using standard methods with OP50 or NA-22 *Escherichia coli* as a food source (Brenner, 1974). Stressor exposures were conducted with worms on agar plates with bacteria or in multiwell plates containing nematode growth medium (NGM) without bacteria (and therefore unfed), as described below.

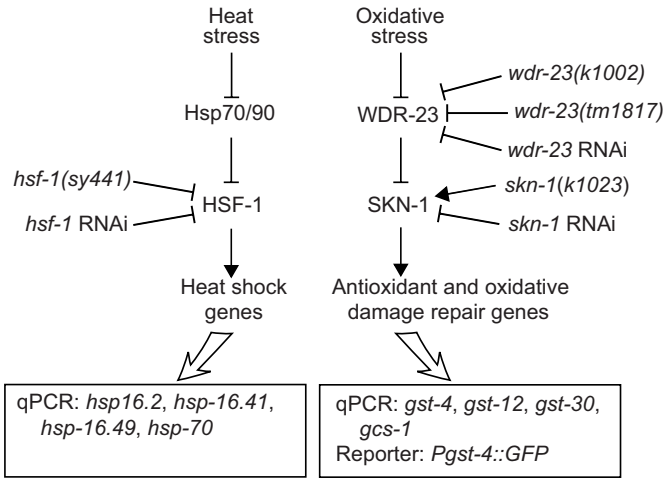

Fig. 1. *Caenorhabditis elegans* heat-shock response (HSR) and oxidative-stress response (OxSR) pathways. Also shown are the molecular tools used in this study to increase or decrease the signaling and quantify changes in the products of the response pathways.

Survival of N2 worms following heat stress and pro-oxidant exposure

Age-synchronized worms at the L4 to young-adult stage were washed from their growth plates using NGM buffer, the *E. coli* were removed by serial dilution, and 0.05% w/v tryptone was added to prevent worms from sticking to plasticware. The worm density was adjusted to 0.4 worms $µl^{-1}$ with NGM buffer and 25 µl of the worm suspension was transferred to each well of a clear 384-well microplate (Corning Inc., Corning, NY, USA) using a 96-channel manual pipette (Liquidator, Mettler Toledo, Columbus, OH, USA) to achieve an average of 10 worms per well. A stock solution of 25 mmol l^{-1} juglone (Sigma-Aldrich, St. Louis, MO, USA) in dimethyl sulfoxide (DMSO) was prepared fresh and used to create 2× working stocks of juglone in NGM+0.05% tryptone. The DMSO concentration of working stocks was adjusted to 0.8% v/v to control for any effect of the DMSO vehicle on the worms. Paraquat and acrylamide (Sigma-Aldrich, St Louis, MO, USA) 2× working stocks were prepared in NGM+0.05% tryptone without DMSO. Worms were exposed to the pro-oxidants by adding 25 µl of 2× working stock 1:1 by volume to wells of the 384-well plate. The plates were then covered with gas-permeable sealing tape and transferred into air-tight aluminum incubation chambers described previously (Ortega et al., 2008). The chambers were placed on a shaker table in a large incubator at 20, 25, 30 or 35°C, with thermosensor data loggers (ibutton, Maxim Integrated, San Jose, CA, USA) used to monitor temperatures. After 4 h, the pro-oxidants were removed by serial dilution with NGM buffer using a 16-channel microplate washer (ELx50, BioTek Instruments, Winooksi, VT, USA) and the 384-well plates were placed in recovery condition (20°C, unfed). After 16 h, the worms were then imaged at 30× magnification using an automated epifluorescence microscope and CCD camera system described previously (Ortega et al., 2008). The images were later analyzed manually, with worms being scored as alive if they appeared sinusoidal and scored as dead if they appeared straight and rigid (Moy et al., 2009).

To test the effect of feeding on survival, N2 (wild type) worms were exposed to 20°C or 32°C with or without acrylamide (0, 50 or 100 mmol l^{-1}) for 4 h followed by 16 h in recovery conditions, as described above, except that each treatment group was further subdivided into unfed and fed groups. The unfed worms were kept in NGM buffer (without bacteria) throughout the 20 h experiment, as above, whereas the fed treatments were provided killed HB101 *E. coli*.

Quantification of *Pgst-4::GFP* reporter intensity

The transgenic reporter line VP596 (expressing *Pgst-4::GFP*) was used to estimate SKN-1 transactivation activity *in vivo*. Age-synchronized L4 VP596 worms were placed in 384-well microplates with ∼10 worms per well in 50 µl of NGM buffer, as described above. The worms were imaged after exposure to heat stress and pro-oxidants using one of three protocols: (1) exposure to heat (20–33°C) simultaneously with or without 20 µmol l^{-1} juglone for 4 h, followed by assessment of reporter intensity; (2) exposure to 20°C or 33°C simultaneously with or without 20 µmol l^{-1} juglone for 4 h

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followed by 0, 4, 8 or 16 h recovery; or (3) sequential exposure to temperature pre-treatment (20 or 33°C) for 4 h, followed by recovery at 20°C for 0, 1, 2, 4 or 8 h, which was then followed by exposure to 0 or 20 µmol l^{-1} juglone at 20°C for 4 h. Immediately prior to imaging, worms were paralyzed with 10 mg ml^{-1} levamisole. Bright-field and GFP fluorescence images (470±20 nm excitation, 525±25 nm emission) were captured at 30× magnification for each well. The mean fluorescence intensity of individual worms was measured with ImageJ software (National Institutes of Health). Regions of interest (ROIs) were obtained by manually adjusting the threshold of bright-field images then overlaying those outlines on the fluorescence images.

Quantitative PCR

Reverse transcription quantitative real-time PCR (RT-qPCR) was used to measure mRNA levels in age-synchronized L4 worm populations, as described previously (Choe et al., 2009). Owing to small differences in the developmental rate of worms and experimental timing, some trials included a small fraction of young adult worms. All expression data were normalized to the internal reference gene *rpl-2*.

RNA interference

RNA interference (RNAi) was performed by feeding the worms strains of *E. coli* [HT115(DE3)] engineered to transcribe double-stranded RNA (dsRNA) homologous to a target gene (Kamath et al., 2001). Bacteria with plasmid pPD129.36 (LH4440) were used as a control for non-specific RNAi effects. This control plasmid expresses 202 bases of dsRNA that are not homologous to any predicted *C. elegans* gene. Bacteria producing dsRNA were grown in lysogeny broth containing selective antibiotic and then transferred to agar plates containing 0.2% β-lactose.

Longitudinal survival following heat stress and pro-oxidant exposure

Wild-type N2 or mutant strain worms were grown on agar plates with *E. coli* [HT115(DE3)] (for RNAi experiments) or OP50 *E. coli* (for non-RNAi experiments). Age-synchronized worms at the L4 to young-adult stage were transferred to fresh agar plates with OP50 *E. coli*, then exposed to heat shock by placing the plates in a 33°C incubator for 12 h, and then at 20°C for recovery. For oxidative stress assays, L4 to young-adult worms were washed from growth plates with NGM buffer and transferred to 96-well multiwell plates (Corning Inc., Corning, NY, USA). The wells were filled with 100 mmol l^{-1} paraquat or 175 µmol l^{-1} juglone in NGM buffer (without bacteria) and death was recorded every 2–3 h for 8–16 h. For all assays, worms were considered dead if they did not display any movement in response to repeated prodding with a thin wire or tapping of the plate.

Polyglutamine aggregation

Transgenic worms with *Punc-54::Q35::YFP*, which express polyglutamine::YFP fusion proteins (polyQ::YFP) in the body wall muscles, were exposed on agar plates to 34°C for 12 h and then allowed to recover for 12 h at 20°C. PolyQ::YFP aggregates in individual worms were counted using a fluorescence stereo microscope (Zeiss Discovery 12, Thornwood, NY, USA) before heat shock, immediately after 12 h of heat shock and after 12 h recovery.

Statistical analysis

A generalized linear model with a binomial error distribution and a logit link function was used to test for effects of heat, pro-oxidants and feeding on worm survival. The full model was tested to identify two-way interactions between heat and pro-oxidants. A trial was treated as a random effect when testing for three-way interactions between heat, pro-oxidant and feeding. The effect of stress exposure on GFP reporter expression and qPCR data were analyzed by two-way analysis of variance (ANOVA), with Tukey HSD or Dunnett's *post hoc* multiple comparison tests, as indicated in the figure legends. Analyses were performed using R (R Core Team, 2014) and JMP Pro, v. 12 (SAS Institute Inc., Cary, NC, USA). Longitudinal survival was analyzed with Kaplan–Meier survival analysis followed by the log-rank test and the Holm–Šídák multiple comparison test using SigmaPlot (Systat Software, San Jose, CA, USA).

RESULTS

Heat stress and oxidative stress synergistically decrease survival

To test for interactions between heat stress and oxidative stress, we exposed age-synchronized pre-reproductive wild-type N2-strain *C. elegans* in multiwell plates to fully factorial combinations of heat (20, 25, 30, 35°C) and one of three pro-oxidants: juglone (0, 0.02, 0.04, 0.06, 0.08, 0.1 mmol l^{-1}), paraquat (0, 1, 3, 10, 33, 100 mmol l^{-1}) or acrylamide (0, 1, 3, 10, 33, 100 mmol l^{-1}) for 4 h. Worms were then allowed to recover for 16 h under control conditions (20°C without pro-oxidant, unfed), after which we assessed survival by worm morphology. We found that heat stress alone (up to 35°C) did not reduce nematode survival, and exposure to juglone alone (i.e. at 20°C) produced a dose-dependent decrease in survival, although even at the maximum juglone concentration of 0.1 mmol l^{-1} survival was reduced by only 36±21% (means±s.d.) (Fig. 2A). However, the combined exposure to heat and juglone (35°C and 0.1 mmol l^{-1} juglone) reduced survival by 90±4%, with a significant two-way interaction (P<0.0001). This is consistent with a synergistic interaction between heat stress and juglone because the effect size of the combined treatment (35°C and 0.1 mmol l^{-1} juglone) is larger than the sum of the effect sizes for either stressor. Similar statistically significant interactions occurred between heat stress and paraquat, and heat stress and acrylamide (P<0.0001 for each; Fig. 2B,C). The largest interaction effect was for acrylamide, which did not affect survival alone at any of the doses tested but, in combination with heat stress (35°C and 100 mmol l^{-1} acrylamide), reduced survival by 99±1%.

Feeding does not reduce the interaction between heat stress and oxidative stress

To determine whether the strength of the interaction between heat stress and oxidative stress was due at least in part to the absence of feeding, we measured the survival of worms in multiwell plates after a 4 h exposure to heat (32°C) and oxidative stress (50 or 100 mmol l^{-1} acrylamide), followed by a 16 h recovery (20°C without acrylamide), as described above, except that we also tested the effect of adding killed HB101 *E. coli* as a food source. We found that, compared with unfed worms, feeding decreased the survival following exposure to heat stress at 50 mmol l^{-1} acrylamide (P=0.029) but not at 100 mmol l^{-1} acrylamide (Fig. 2D).

The OxSR is inhibited during combined heat stress and oxidative stress

Induction of the *gst-4* transcriptional reporter by juglone is inhibited by heat stress

To investigate potential interactions between the OxSR and HSR pathways, we tested whether the activity of the OxSR is modulated by a sublethal exposure to heat stress. We assessed OxSR induction activity by measuring the mean fluorescence intensity of transgenic worms carrying a *Pgst-4::GFP* reporter construct (Fig. 1), in which

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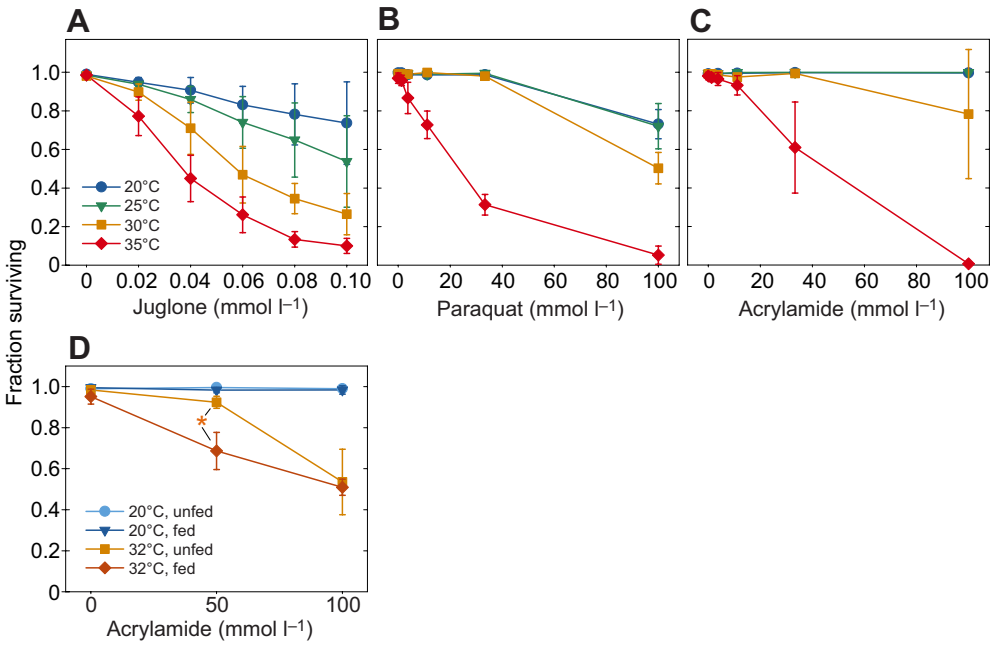

Fig. 2. Survival after 4 h simultaneous exposure to heat stress and pro-oxidant compounds followed by 16 h recovery. (A) Survival of worms after exposure to juglone and elevated temperature (*N*=5 trials with 4046–12,938 worms per trial). (B) Survival of worms after exposure to paraquat and elevated temperature (*N*=5 trials with 982–3058 worms per trial). (C) Survival of worms after exposure to acrylamide and elevated temperature (*N*=5 trials with 1068–3042 worms per trial). (D) Survival of worms after exposure to acrylamide and elevated temperature in the unfed or fed state. Fed worms were provided killed HB101 *E. coli*, with the asterisk indicating a significant difference (*P*=0.029) between the unfed and fed treatments (*N*=3 trials with 5763 worms and trials as a random effect). Data are means±s.d., with each replicate trial being a *C. elegans* subculture. Note that the maximum juglone concentration was 1/1000 that of paraquat or acrylamide.

increased GFP fluorescence indicates increased activation of the OxSR (Choe et al., 2009). We found that 4 h exposure in multiwell plates to heat stress alone had no effect on *Pgst-4::GFP* reporter expression relative to the control condition (20°C, no juglone), whereas exposure to 20 µmol l^{-1} juglone significantly increased reporter expression at 20–31°C ($P<0.0001$ for all), but did not significantly increase expression at 33°C (Fig. 3A). Therefore, juglone-induced activation of the OxSR – as measured by the *Pgst-4::GFP* reporter construct – can be inhibited by simultaneous exposure to heat stress.

Inhibition of *gst-4* reporter induction by heat stress persists during heat-stress recovery

To examine whether the inhibitory effect of heat stress on the OxSR persists after a return to non-heat-stress conditions, we measured the fluorescence intensity of the *Pgst-4::GFP* reporter during recovery from combined stress exposure. We exposed *Pgst-4::GFP*-containing *C. elegans* in multiwell plates to 20 or 33°C with or without juglone (20 µmol l^{-1}) for 4 h and then allowed the worms to recover for up to 16 h in control conditions (20°C without juglone). We found that worms exposed to juglone at 20°C had increased expression of the *Pgst-4::GFP* reporter throughout the recovery period relative to worms exposed to the control treatment (Fig. 3B). In contrast, worms that had been exposed to juglone at 33°C for 4 h did not have significantly increased *Pgst-4::GFP* expression relative to the control treatment until 8 h into the recovery period, and this increase was much smaller than in worms that had been exposed to juglone at 20°C (Fig. 3B). Overall, the effect of juglone on *Pgst-4::GFP* reporter expression was dependent on heat exposure (two-way ANOVA: $F=637$, $P<0.0001$), with *Pgst-4::GFP* reporter expression being inhibited by high temperatures. Thus, the inhibitory effect of heat on OxSR activity persists for at least 16 h after worms are transferred back to control conditions.

Inhibition of *gst-4* reporter induction by heat stress does not require simultaneous stressor exposure

To determine whether the inhibitory effect of heat stress on the OxSR can occur even when the heat stress precedes oxidative stress, we pre-exposed worms carrying the *Pgst-4::GFP* reporter to 33°C heat stress for 4 h and then exposed the worms to juglone at 20°C up to 8 h later. As expected, worms pre-exposed for 4 h to either the control temperature (20°C) or heat stress (33°C) alone (i.e. without a subsequent juglone exposure) showed consistently low *Pgst-4::GFP* expression across all time points (Fig. 3C). Worms that had been pre-exposed to 20°C and then subsequently exposed to juglone (20 µmol l^{-1}) showed a similar, large increase in *Pgst-4::GFP* expression regardless of the delay before juglone exposure. However, in worms pre-exposed to 33°C and then exposed to juglone up to 8 h later, the *Pgst-4::GFP* expression varied: expression remained low when juglone exposure occurred immediately after the heat stress, but then increased with recovery time, eventually exceeding the expression level observed in worms that had not been exposed to heat stress. Therefore, inhibition of OxSR by heat occurs even without simultaneous exposure, and the degree of inhibition decreases over time and even reverses after an 8 h delay between the stresses. These results also rule out a direct physicochemical effect of high temperature on juglone (e.g. heat degrading the juglone) as the mechanism of the heat-induced inhibition of the OxSR, because the juglone exposures in these experiments all occurred at 20°C.

Heat stress alone or in combination with oxidative stress inhibits key OxSR genes regulated by SKN-1

We used qPCR to test the expression patterns of OxSR and HSR target genes immediately after 4 h of combined stress. We assessed OxSR activity by measuring four SKN-1 targets: three isoforms of GST (*gst-4*, *gst-12*, *gst-30*), and γ-glutamine cysteine synthetase (*gcs-1*). Measuring mRNA of *gst-4* also provided the opportunity to validate the *Pgst-4::GFP* reporter assays. We assessed HSR activity by measuring four HSF-1 targets: *hsp-16.2*, *hsp-16.41*, *hsp-16.49* and *hsp-70*. Exposure to oxidative stress (20 µmol l^{-1} juglone) at the control temperature (20°C) significantly increased expression of *gst-4*, *gst-12* and *gst-30* by 18-, 34- and 243-fold, respectively ($P<0.05$ for all), compared with the control treatment (Fig. 4A). However, when juglone exposure was performed at 33°C, the increases in expression of *gst-4*, *gst-12* and *gst-30* were only 5.9-, 2.5- and 107-fold, which were each significantly lower than the increased expression seen with exposure to juglone at 20°C ($P<0.05$

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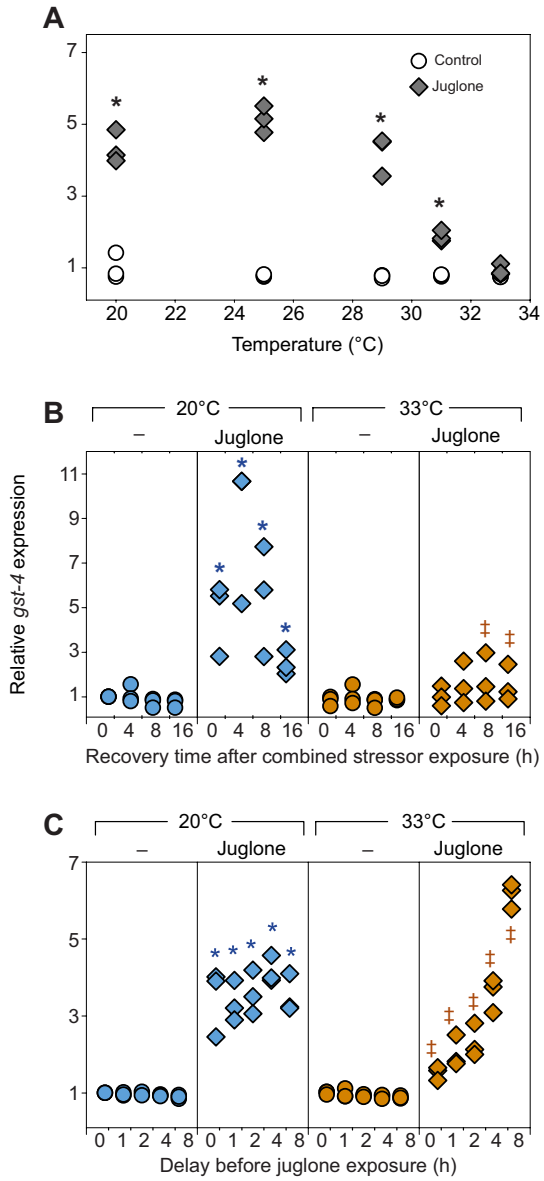

Fig. 3. *Pgst-4::GFP* reporter expression after simultaneous or sequential exposure to heat stress and pro-oxidant compounds. Relative *gst-4* expression was measured with the fluorescent reporter construct *Pgst4::GFP*. Each data point represents the mean fluorescence intensity of the *gst-4* reporter *Pgst4::GFP* per worm per trial (*C. elegans* subculture) normalized to the mean intensity of the control treatment (20°C, without juglone). The fraction of worms surviving was >96% for all trials. (A) Reporter expression immediately after simultaneous exposure to heat (20–33°C) with or without 20 μmol l^{-1} juglone for 4 h. Three trials were conducted, with fluorescence individually assessed for 2752 worms. There was no main effect of trial (P=0.107, F=2.24), so trials were pooled (*P<0.0001 compared with control). (B) Reporter expression after simultaneous exposure to 20°C or 33°C with or without 20 μmol l^{-1} juglone for 4 h followed by 0, 4, 8 or 16 h recovery. Three trials were conducted, with fluorescence individually assessed for 4821 worms. There was no main effect of trial (P=0.175, F=1.75), so trials were pooled (*P<0.0001 compared with the 20°C treatment; ‡P<0.0001 compared with the 20°C and 20°C+juglone treatment). Only two data points are shown for the 20°C+juglone treatment at the 4 h recovery time point owing to camera failure for that trial. (C) Reporter expression after a sequential exposure to temperature pre-treatment (20 or 33°C), followed by recovery at 20°C for 0, 1, 2, 4 or 8 h, followed by exposure to 0 or 20 μmol l^{-1} juglone at 20°C for 4 h. Three trials were conducted, with fluorescence individually assessed for a total of 1745 worms. There was no main effect of trial (P=0.366, F=1.01), so trials were pooled (*P<0.0001 compared with the 20°C treatment at that time point; ‡P<0.0001 compared with the 20°C and 20°C+juglone treatment at that time point).

for all). Furthermore, exposure to heat stress alone (i.e. 33°C without juglone) significantly decreased expression of *gst-12* and *gst-30* relative to control conditions (P<0.05 for each). By comparison, the expression of *gcs-1* was increased by juglone but, in contrast to the glutathione S-transferase isoforms, expression of *gcs-1* was not significantly reduced by heat stress in combination with juglone exposure or by heat stress alone. Therefore, heat stress alone or in combination with oxidative stress inhibits a subset of the OxSR genes regulated by SKN-1, consistent with those results obtained in the *Pgst-4::GFP* reporter experiments above (Fig. 3).

Exposure to heat stress (33°C) in the absence of juglone significantly increased the expression of *hsp-16.2*, *hsp-16.41*, *hsp-16.49* and *hsp-70* by 179-, 345-, 314- and 26-fold, respectively (P<0.05 for all), compared with the control treatment of 20°C without juglone (Fig. 4B). Oxidative stress in the absence of heat stress (i.e. 20°C and 20 μmol l^{-1} juglone) also increased the expression of *hsp-16.2*, *hsp-16.41* and *hsp-16.49* (P<0.05 for each), but not *hsp-70* (P=0.907), compared with the control treatment. Combined exposure to heat and oxidative stress (i.e. 33°C and 20 μmol l^{-1} juglone) further increased the expression of *hsp-16.2*, *hsp-16.41* and *hsp-70* (P<0.05 for each), but not *hsp-16.49* (P=0.094), relative to heat stress alone. Based on these results, exposure to oxidative stress does not seem to inhibit the HSR either alone or when combined with heat stress, and in most cases increases expression of HSPs.

Genetic and environmental activation of SKN-1 decreases tolerance to heat stress

That exposure to heat stress inhibits the expression of OxSR genes in response to juglone, whereas co-exposure to both stressors does not inhibit HSR genes, suggests that high temperature interacts either directly or indirectly with the OxSR pathway to reduce its activity. To further examine the potential for interactions between the OxSR and the HSR, we tested whether genetic manipulation of SKN-1 activity could influence heat tolerance. In *C. elegans*, the WD40-repeat protein WDR-23 acts as a principal suppressor of SKN-1 activity (Choe et al., 2009). As shown previously (Tang and Choe, 2015), SKN-1 activity is lowest in wild-type N2 worms, higher in *wdr-23(k1002)* mutants with a *wdr-23* loss-of-function allele, higher still in *skn-1(k1023)* mutants with a *skn-1* gain-of-function allele, and highest in *wdr-23(tm1817)* mutants with a *wdr-23* deletion allele (Fig. 1). We exposed these four strains to 34°C heat stress for 12 h and then to recovery conditions (20°C) for up to 60 h, during which we measured survival every 12–24 h for a total of 72 h (Fig. 5A). We found that survival following heat stress was inversely proportional to constitutive SKN-1 activity: N2>*wdr-23(k1002)*>*skn-1(k1023)*>*wdr-23(tm1817)*. These results suggest that increased SKN-1 activity increases susceptibility to heat stress.

To determine whether the inhibition of SKN-1 activity decreases susceptibility to heat stress, we used *skn-1* RNAi to genetically suppress SKN-1 activity and then exposed the worms to 34°C heat stress for 12 h followed by recovery conditions for 60 h, as above. We found that control worms treated with empty vector (EV) RNAi had a survival probability of 0.61±0.02 (means±s.e.m.) at 72 h, whereas worms that had been pre-treated with *skn-1* RNAi showed a significantly increased survival probability of 0.90±0.02 (means±s.e.m.) at 72 h (Fig. 5B). This supports the conclusion that even basal SKN-1 activity increases the susceptibility to heat stress. We next tested whether SKN-1 activity is at least partially responsible for the observed synergistic interaction between oxidative stress and heat stress on survival. We pre-exposed worms for 48 h to 1.4 mmol l^{-1} acrylamide, which is sufficient to

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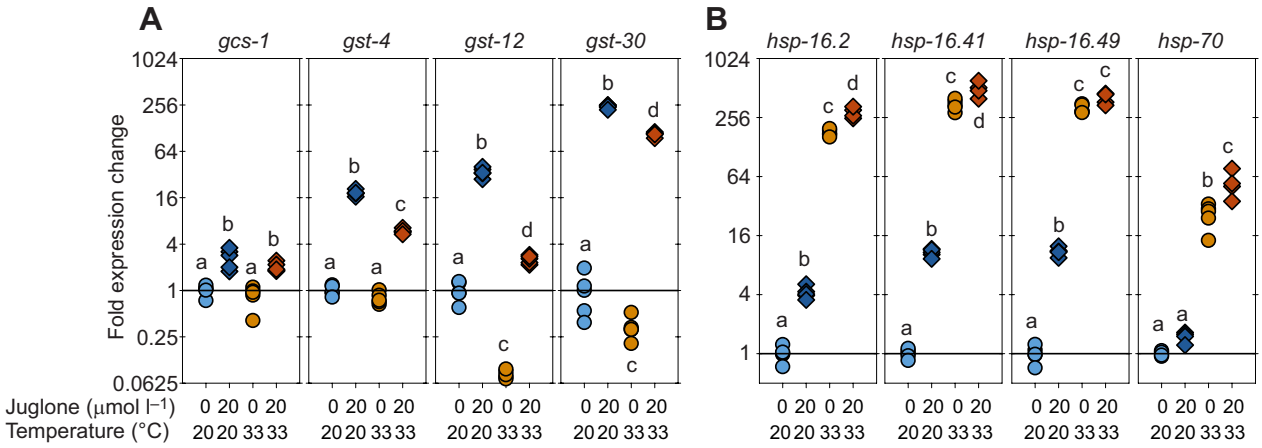

Fig. 4. Relative expression of OxSR and HSR genes following simultaneous exposure to heat stress and a pro-oxidant. Worms were exposed for 4 h to 20°C or 33°C, with or without 20 μmol l^{-1} juglone. (A) OxSR genes. (B) HSR genes. Each data point represents the fold expression change relative to the control treatment (20°C, without juglone) per trial (*C. elegans* subculture), with *N*=4–5 trials per treatment and ca. 400 worms per trial at each treatment. All gene expression data are normalized to the internal reference gene *rpl-2*. Different letters represent significant differences (*P*<0.05) between treatments within genes (Tukey HSD *post hoc*).

upregulate SKN-1 activity without having a significant effect on survival. These worms were then exposed to heat (34°C for 12 h) followed by recovery conditions for 60 h. As expected, the acrylamide pre-exposure significantly decreased the survival probability (*P*<0.0001) to 0.42±0.03 (means±s.e.m.) at 72 h after the initiation of the 12 h heat stress and recovery (Fig. 5B). Importantly, inhibiting SKN-1 activity by treatment with *skn-1* RNAi during the acrylamide pre-exposure completely eliminated the negative effects of acrylamide pre-exposure and significantly increased the survival probability after heat stress relative to even the control treatment (*P*<0.0001), with a survival probability of 0.85 ±0.02 (means±s.e.m.). Taken together, these data are consistent with SKN-1 activity decreasing tolerance to heat stress regardless of whether SKN-1 is at basal levels or increased by genetic manipulation or exposure to pro-oxidants.

SKN-1 decreases heat tolerance independently of HSF-1

HSF-1 regulates HSP expression, but also regulates many other genes that contribute to the HSR (Baird et al., 2014). To test whether the effect of SKN-1 activation on decreased heat tolerance is dependent on HSF-1, we tested the effects of heat stress on *hsf-1 (sy441)* mutants. These worms have an *hsf-1* loss-of-function allele (*sy441*) containing an early stop codon in the transactivation domain, preventing the induction of HSPs (Hajdu-Cronin et al., 2004). As expected, we found that *hsf-1(sy441)* mutants had significantly decreased survival following exposure to heat stress (34°C for 12 h) compared with wild-type worms (Fig. 5C). Treating *hsf-1(sy441)* mutants with *wdr-23* RNAi to activate SKN-1 further decreased survival, and this effect was larger than in wild-type worms treated with *wdr-23* RNAi. Because *hsf-1(sy441)* mutants have strongly reduced HSF-1 activity, this finding is consistent with activation of SKN-1 increasing the susceptibility of *C. elegans* to heat stress through a mechanism that is independent of HSF-1.

Activation of SKN-1 increases a protein aggregate marker during heat stress

If SKN-1 activation decreases the tolerance to heat stress, then genetic activation of SKN-1 should increase the accumulation of heat-associated pathology. Heat and oxidative stress can disrupt proteostasis and cause accumulation of protein aggregates (Moronetti Mazzeo et al., 2012; Vabulas et al., 2010). Under stress conditions, proteins containing polyQ repeats are structurally unstable and often self-assemble into aggregates (van Dellen et al., 2005). In *C. elegans*, the expression of polyQ::YFP proteins have been used as a biomarker for *in vivo* protein homeostasis (Brignull et al., 2006; Ignatova and Gierasch, 2006). To assess protein homeostasis following heat stress, we used *C. elegans* transgenic for polyQ::YFP driven by a muscle-specific promoter. We exposed these worms to 34°C heat stress for 12 h and assessed the number of polyQ::YFP aggregates at three time points: immediately preceding heat stress (0 h), immediately after heat stress (12 h) and after 12 h of recovery at 20°C (24 h total time). To determine whether the accumulation of polyQ is affected by SKN-1 activation, prior to the heat stress we treated the worms with RNAi for *wdr-23* or *skn-1* to increase or decrease SKN-1 activity, respectively (Fig. 1). We found that the mean number of polyQ::YFP aggregates per worm did not differ between RNAi treatments immediately preceding heat stress (Fig. 5D). However, worms pre-treated with *wdr-23* RNAi had significantly more polyQ::YFP aggregates relative to the EV RNAi control group, both immediately after the heat stress (12 h, *P*=0.044) and after the recovery (24 h, *P*<0.0001). In contrast, the number of polyQ::YFP aggregates in worms pre-treated with *skn-1* RNAi prior to heat stress did not differ significantly from the EV RNAi control at any time point (*P*>0.751 for all).

Inhibition of the HSR (*hsf-1* RNAi) increases oxidative stress tolerance

To determine whether basal HSR activity affects the tolerance to oxidative stress, we genetically knocked down *hsf-1* with RNAi in N2 *C. elegans* and then exposed the worms to juglone or paraquat. Compared with worms pre-treated with control RNAi, worms pre-treated with *hsf-1* RNAi showed significantly increased tolerance to 100 mmol l^{-1} paraquat (*P*<0.001, Fig. 5E) or 175 μmol l^{-1} juglone (*P*=0.017, Fig. 5F), suggesting that basal HSR activity reduces the tolerance to oxidative stress.

Activation of SKN-1 does not inhibit HSP gene expression in response to acute heat stress

To test whether the effect of OxSR activation on heat-stress susceptibility is due to inhibition of the HSR, we used qPCR to

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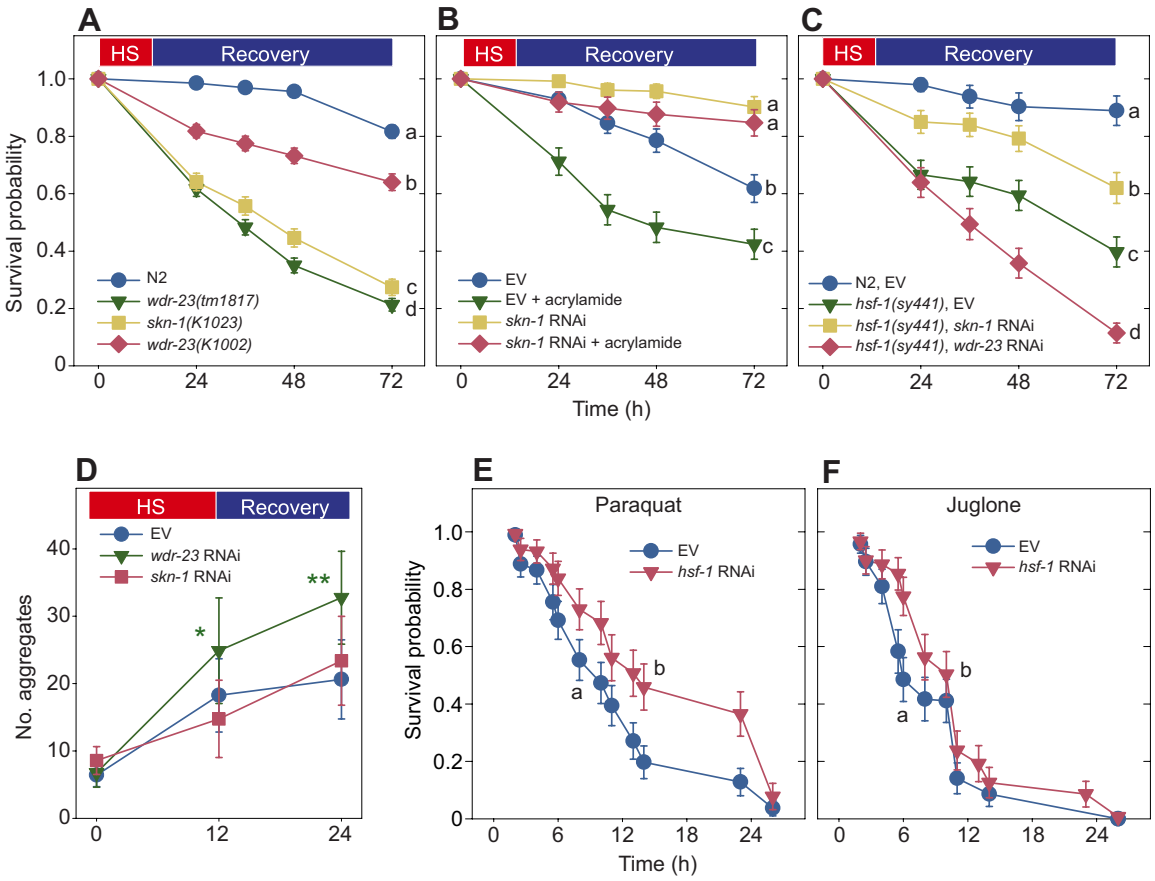

Fig. 5. Genetic manipulation of stress-response pathways. (A–C,E,F) Longitudinal survival as Kaplan–Meier survival curves, with error bars representing 95% confidence intervals. (A) Effect of increased SKN-1 activity on survival following heat stress (HS). Wild-type N2 worms and mutant strains with different levels of SKN-1 activity (*tm1817*>*k1023*>*k1002*>N2) were exposed to heat (34°C) for 12 h starting at time 0, then survival was recorded for up to 60 h recovery at 20°C (i.e. 72 h total). Different letters represent significant differences between survival curves (P<0.001); N=1107 for N2; N=1049 for *k1002*; N=945 for *k1023*; and N=1330 for *tm1817*. (B) Effect of reduced SKN-1 activity on survival following heat stress and pro-oxidant exposure. Wild-type N2 worms that had been pre-treated with empty vector (EV) control RNAi or *skn-1* RNAi were exposed to 1.4 mmol l^{-1} acrylamide for 48 h. The worms were then exposed to heat (34°C) for 12 h starting at time 0, after which survival was recorded for up to 60 h recovery at 20°C (i.e. 72 h total). Different letters represent significant differences between survival curves (P<0.001); N=395 for EV; N=344 for EV+acrylamide; N=255 for *skn-1* RNAi; and N=236 for *skn-1* RNAi+acrylamide. Statistical comparisons between curves made with log-rank test and Bonferonni adjustments. (C) Effect of reduced HSF-1 activity with and without SKN-1 activation on survival following heat stress. Wild-type N2 worms and worms containing the *sy441* allele (which have reduced *hsf-1* transactivation) that had been pre-treated with EV control RNAi or *skn-1* RNAi were exposed to heat (34°C) for 12 h starting at time 0, then survival was recorded for up to 60 h recovery at 20°C (i.e. 72 h total). Different letters represent significant differences between survival curves (P<0.001); N=144 for N2, EV; N=335 for *hsf-1(sy441)*, EV; N=313 for *hsf-1 (sy441)*, *skn-1* RNAi; and N=330 for *hsf-1(sy441)*, *wdr-23* RNAi. (D) Effect of SKN-1 activity on protein aggregation induced by heat stress. Wild-type N2 worms that had been pre-treated with EV control RNAi, *wdr-23* RNAi or *skn-1* RNAi were exposed to heat stress (34°C) for 12 h followed by 12 h of recovery at 20°C. Data represent the number of polyQ::YFP protein aggregates per worm (N=12, 12 and 16 worms at 0, 12 and 24 h, respectively). Data points represent means±s.d. *P<0.05, **P<0.0001 relative to N2 EV at the same time point. (E,F) Effect of reduced HSF-1 activity on survival during pro-oxidant exposure. Wild-type N2 worms that had been pre-treated with EV control RNAi or *hsf-1* RNAi were continuously exposed to 100 mmol l^{-1} paraquat (E) or 175 μmol l^{-1} juglone (F) pro-oxidant for up to 26 h. Different letters represent significant differences between survival curves (P<0.05); N=188 for EV with paraquat; N=148 for *hsf-1* RNAi with paraquat; N=163 for EV with juglone; and N=151 for *hsf-1* RNAi with juglone.

compare the expression of HSF-1 target genes following an acute (30 min) heat stress (34°C) in worms with different levels of SKN-1 activity. For reference, we also measured the expression of *gst-4* as an indicator of OxSR activation. We chose a short heat exposure because *wdr-23(tm1817)* worms with high levels of SKN-1 activity suffered significant mortality in longer heat exposures. As expected, and consistent with the results presented above (Fig. 4), mRNA levels of five HSPs (*hsp-70*, *hsp-16.2*, *hsp-16.1*, *hsp-16.49* and *hsp-16.41*) increased by 70- to 180-fold in control worms following an acute heat shock of 34°C for 30 min, whereas the expression of *gst-4* was unchanged (Fig. 6). HSP gene expression following heat stress was slightly – but significantly – larger in *wdr-23(tm1817)* mutants (which have increased SKN-1 activity) compared with the control worms for all HSPs measured except *hsp-16.1* (Fig. 6). Expression of *gst-4* increased ca. 30-fold in *wdr-23(tm1817)* mutants regardless of heat stress, but pre-treatment with *skn-1* RNAi before the heat stress suppressed the HSP mRNA induction back toward wild-type levels and reduced the expression of *gst-4* to about fivefold above the wild-type level. Surprisingly, under non-heat-stress conditions (i.e. in worms exposed to 20°C), expression of all *hsp-16* isoforms was significantly suppressed in *wdr-23(tm1817)* mutants relative to the control (P<0.05 for all). Taken together, these data indicate that genetic activation of SKN-1 does not inhibit HSP gene expression in response to acute heat stress, and even increases it slightly, but that SKN-1 activation decreases basal expression levels of some HSP genes.

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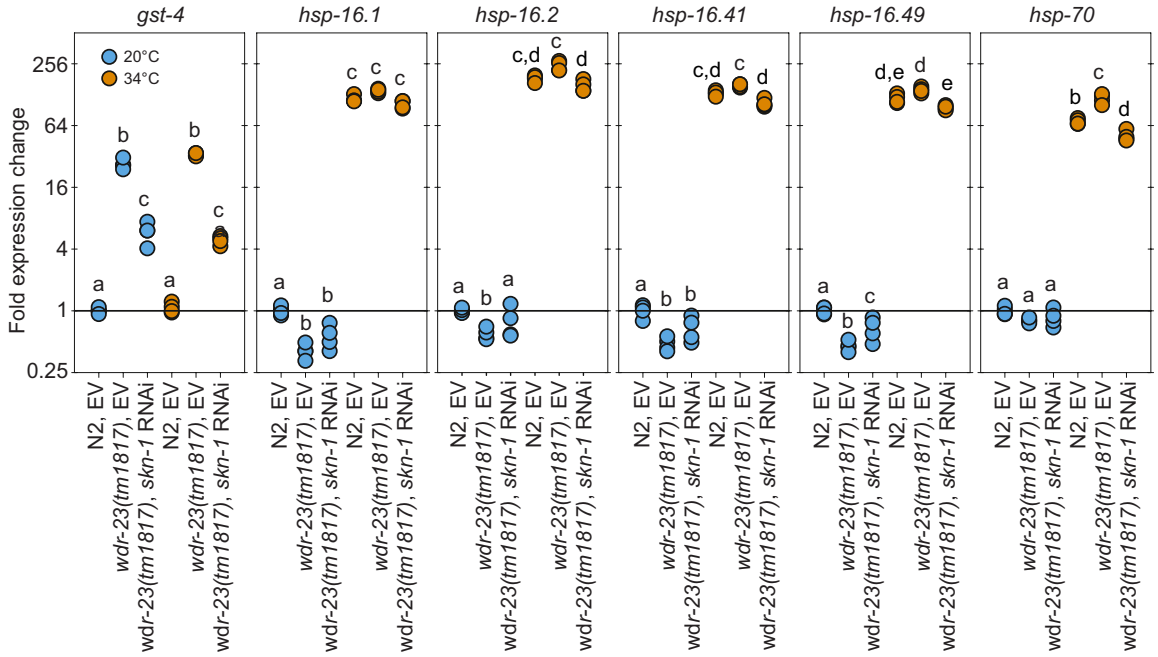

Fig. 6. Relative expression of HSR genes following acute heat stress. Worms were exposed to heat (34°C) for 30 min. Each data point represents the fold expression change relative to empty vector RNAi control (EV) at 20°C per trial (*C. elegans* subcultures), with *N*=4 trials and ca. 400 worms per trial at each treatment. All gene expression data are normalized to the internal reference gene *rpl-2*. Different letters represent significant differences ($P<0.05$) between treatments within the same gene (Tukey HSD *post hoc*). The expression of *gst-4* is included to show the effect of the deletion mutant *wdr-23(tm1817)* and *skn-1* RNAi on OxSR target gene expression.

DISCUSSION

Most animals mount robust and specialized molecular responses to heat stress and oxidative stress (i.e. the HSR and OxSR), and therefore it might seem reasonable that robust induction of both the HSR and the OxSR would occur during exposure to combined stressors. However, using *C. elegans* as a model, we found that heat stress and oxidative stress interact synergistically to decrease survival, and that, during combined exposure to both stressors, the expression of OxSR genes is generally inhibited, whereas the expression of HSR genes is not. We also found that activation of the OxSR during heat stress alone increases protein aggregation and decreases survival, and that the effect on survival is independent of HSF-1. By contrast, inhibition of basal HSR activity increases survival during exposure to pro-oxidants. We did not determine whether the synergistic interaction increased death via heat stress, oxidative stress, or both.

Synergy between heat stress and oxidative stress

Our finding that heat stress interacted synergistically with pro-oxidants to reduce survival is consistent with reports of synergies between heat stress and other oxidative-stress-inducing toxins. Heavy metals are relatively common anthropogenic stressors that can cause oxidative stress by generating reactive oxygen species (ROS) through Fenton-like chemistry (Valko et al., 2005). In a review, Holmstrup et al. (2010) found that 19 of 24 studies on the combined effects of heat and heavy-metal exposure identified synergistic interactions. Proposed mechanisms for the interactions include elevated energy costs and impaired aerobic energy production (Cherkasov et al., 2006), increased metal absorption at higher temperatures (Scheil and Köhler, 2009), and lipid peroxidation and increased membrane destabilization at high temperatures (Slotsbo et al., 2009). Any of these associated mechanisms could have contributed to the synergistic interactions we observed in our study, but none exclude the possibility that alterations in stress-responsive gene expression further contribute to these interactions.

In mammalian cell culture, pre-exposure to the pro-oxidant hydrogen peroxide (H_2O_2) increases the susceptibility of cells to a subsequent heat stress and inhibits the expression of *hsp-70*, a key HSR target gene, whereas no changes occur in the OxSR-related mRNAs or proteins for catalase, glutathione peroxidase or heme oxygenase-1 (Adachi et al., 2009). In both mammalian cells and *C. elegans*, pre-exposure to H_2O_2 can inhibit acquired thermotolerance and is linked to reduced HSP-70 levels (Spiró et al., 2012). In contrast, we found increased *hsp-70*, *hsp-16.2* and *hsp-16.41* expression following combined exposure to heat and oxidative stress. However, Adachi et al. and Spiró et al. performed their experiments with a pre-exposure to oxidative stress followed by heat stress, whereas, in our experimental conditions for assessing expression changes, we exposed worms to heat stress and oxidative stress simultaneously. Nonetheless, the findings are all consistent in showing that combinations of stressors can reduce the expression of stress-response genes that would normally be induced by exposure to a single stressor.

In the freshwater mussel *Unio tumidus*, Falfushynska et al. (2015) recently showed that heat stress in combination with exposure to pro-oxidant zinc-oxide nanoparticles (n-ZnO) decreased metallothionein protein levels and activity of superoxide dismutase compared with n-ZnO without heat stress, and synergistically increased oxidative damage to lipids, proteins and DNA. These data are consistent with our observation that heat stress inhibits expression of at least some components of the OxSR. However, we did not measure oxidative damage in *C. elegans*, and therefore it is unknown whether the addition of heat stress increases the damage caused by exposure to pro-oxidants, and we did not measure antioxidant protein content or activity, so we cannot determine whether these were correlated with the changes we observed in mRNA expression.

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To avoid potential interactions between the heat and pro-oxidants on the *E. coli* food source, worms were kept unfed during many of our stressor exposure protocols. In *C. elegans*, starvation reduces IIS and activates the FOXO transcription factor DAF-16 (Ogg et al., 1997), which in turn upregulates the expression of genes conferring resistance to heat and oxidative stress (Hsu et al., 2003). The time course of the proteomic response to starvation was recently measured by Larance et al. (2015), who found that, after 16 h of starvation, some stress-response proteins regulated by DAF-16, as well as proteins associated with the gene ontology (GO) term 'response to heat', were upregulated. Therefore, a starvation effect, possibly driven by IIS, may influence the synergy we found between heat and oxidative stress. Consistent with this, we observed that starvation had a protective effect on worms exposed to heat (32°C) in combination with an intermediate concentration of pro-oxidant (50 mmol l^{-1} acrylamide). However, starvation had no effect on worms exposed to heat at a higher pro-oxidant concentration (100 mmol l^{-1} acrylamide).

Stress-response prioritization during multiple-stress exposure

If the complete HSR cannot be activated simultaneously with the complete OxSR then it may be advantageous to prioritize the most general stress response. The HSR prevents protein aggregation during mild stress, but also enables organized aggregation during moderate to severe stress via small HSPs (Tyedmers et al., 2010), which facilitates the removal of aggregates when conditions improve (Ratajczak et al., 2009). Formation of protein aggregates can be caused by either heat stress or oxidative stress (Squier, 2001), and aggregates can themselves cause oxidative stress by reacting with redox-active metals to generate H_2O_2 (Tabner et al., 2005). Therefore, by increasing general proteome stabilization and promoting organized aggregation, the HSR provides some protection against oxidative stress (Préville et al., 1999). We observed upregulation of small HSPs with oxidative stress (Fig. 4B), as has been shown before (Shin et al., 2011), but we also found that inactivation of HSF-1 increased survival of oxidative stress alone (Fig. 2E,F), suggesting that any potential benefit from activation of the HSF-1-dependent HSR is not outweighed by its costs during oxidative stress alone.

Alternatively, proteome stability may be the highest priority during combined heat stress and oxidative stress. Sublethal heat stress can denature a variety of proteins (Feder and Hofmann, 1999), potentially including newly synthesized OxSR proteins. Therefore, delaying the induction of the OxSR until the proteome is stabilized by the HSR may ensure that the OxSR proteins can be functional. We found that the OxSR is repressed for at least 2 h following termination of acute heat stress (Fig. 3C). Although these data are based on a GFP-reporter construct using the *gst-4* promoter, which may have different kinetics than *gst-4* mRNA, our RT-qPCR data also showed repression of *gst-4* mRNA immediately following 4 h of co-exposure to heat and oxidative stress (Fig. 4A). Nonetheless, during combined exposure to heat stress and oxidative stress, any inhibition of the OxSR – however small or transient – would increase susceptibility to oxidative stress, potentially contributing to the observed synergistic decrease in worm survival.

Potential mechanisms underlying inhibition of the OxSR during heat stress

An and Blackwell (2003) observed substantial accumulation of SKN-1::GFP in cell nuclei upon heat stress, indicating that heat stress does not compromise OxSR transcription factor accumulation. Therefore, the reduction of core SKN-1 target gene transcription by heat stress observed in our study suggests a decoupling of transcription factor accumulation and target gene transcription during heat stress. The mechanism for this decoupling could include misfolding of functional domains in the SKN-1 protein, chromatin modifications that alter promoter accessibility, and changes to RNA processing or splicing (Goh et al., 2014; Ip et al., 2011; Sailer et al., 1997). In addition, IIS inhibits the activity of both HSF-1 (Chiang et al., 2012) and SKN-1 (Tullet et al., 2008) via independent mechanisms, and thus upregulation of IIS may contribute to inhibition of the OxSR during heat stress. It would be valuable to characterize the role of IIS in the modulation of both SKN-1 and HSF-1 target genes during combined stress exposures.

SKN-1 activation is detrimental to survival under heat stress

We found that *C. elegans* with genetically activated SKN-1 showed decreased survival during heat stress (Fig. 5A) and increased formation of polyQ aggregates (Fig. 5D), consistent with increased disruption of proteostasis. The effect on survival was independent of HSF-1, but the mechanism by which SKN-1 activation affects survival during heat stress is otherwise unknown. Activation of SKN-1 moderately reduced the expression of HSP genes in non-heat-stressed conditions (Fig. 6), suggesting that SKN-1 plays a role in modulating the basal expression of HSF-1 targets. Interestingly, mammalian HSF-1 is sensitive to redox status and can be post-translationally activated by disulfide-bond formation between cysteine residues near the HSF-1 DNA-binding domain (Ahn and Thiele, 2003). Therefore, a shift to a more reducing environment due to activation of SKN-1 may decrease the activity of the HSR. Additional mechanisms could include disruption of the redox signaling that is required for some protein chaperones (Jang et al., 2004), and competition between the OxSR and the HSR for ATP and other cellular resources necessary for gene expression and protein translation. The loss of *wdr-23* increases longevity and resistance to oxidative stress, but decreases growth and reproduction in a SKN-1-dependent manner (Tang and Choe, 2015). The fact that stress resistance and longevity necessitate trade-offs with development and reproduction is well known (Flatt, 2011; Jenkins et al., 2004). Our findings suggest, however, that the increased oxidative-stress resistance and longevity associated with increased SKN-1 activation under laboratory conditions may not be relevant in natural habitats if oxidative stress occurs in combination with heat stress.

Conclusion

SKN-1 is the *C. elegans* ortholog to NF-E2-related factor (Nrf) in mammals. To our knowledge, this is the first whole-animal study to characterize the role of the conserved SKN-1/Nrf factors in heat-stress survival. We found that exposure of *C. elegans* to heat stress in combination with oxidative stress caused a synergistic decrease in survival, that the HSR was prioritized over the OxSR, and that genetic activation of the OxSR decreased the tolerance of heat stress. We conclude that, in *C. elegans*, the HSR and OxSR cannot be simultaneously activated to the same extent that each can be activated during exposure to a single stressor. We propose that this is at least partially responsible for the synergistic effect of heat stress and oxidative stress on worm survival. If prioritization of stress responses is also characteristic of natural populations of *C. elegans* taxa and other animals, this would represent an important mechanism by which increased environmental temperatures can decrease the survival of organisms when they also experience oxidative stress, and potentially other stressors.

Acknowledgements
The authors thank Stanley David Stupski, Alexandra Alberdi and Joon Hyung Kim for their contribution to the laboratory procedures, José Miguel Ponciano for providing help with the statistical methods, and Stephanie Wohlgemuth and the anonymous reviewers for their insightful comments and suggestions.

Competing interests
The authors declare no competing or financial interests.

Author contributions
All authors conceptualized and designed the study; the experiments and analyses were performed by T.A.C. and L.T. under the supervision of K.P.C. and D.J.; the text was written primarily by T.A.C. and D.J., and all authors approved the manuscript.

Funding
This research was supported in part by National Science Foundation (NSF) grants IOS-1120130 and IOS-1452948 to K.P.C.

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