ORIGINAL RESEARCH COMMUNICATION

RNA interference links oxidative stress to the inhibition of heat stress adaptation

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Abstract

Increased oxidative stress is associated with various diseases and aging, while adaptation to heat stress is an important determinant of survival and contributes to longevity. However, the impact of oxidative stress on heat resistance remains largely unclear. **Aim:** In this study we investigated how oxidative stress impinges on heat stress responses. **Results:** We report that H_2O_2 pretreatment inhibits both acquired thermotolerance and heat-induced Hsp70 expression in mammalian cells, as well as acquired thermotolerance in the nematode *Caenorhabditis elegans*, *via* RNA interference. Moreover, we demonstrate that elimination of RNA interference by silencing key enzymes in microRNA biogenesis, *dcr-1* or *pash-1*, restores the diminished intrinsic thermotolerance of aged and H_2O_2-elimination compromised (*catalase-2* and *peroxiredoxin-2* deficient) worms. **Innovation and Conclusion:** These results uncover a novel post-transcriptional element in the regulation of heat stress adaptation under oxidative conditions which may have implications in disease susceptibility and aging.

Introduction

Basic physiological processes such as metabolism, cellular signaling and immunity are associated with the production of reactive oxygen species (ROS) (16). An accumulation of ROS, called oxidative stress plays a critical role in various diseases and in aging (13,16,34). Though an excess of ROS generates diverse molecular and cellular damages and evokes a plethora of signaling events, how it is involved in the induction or aggravation of these pathological states is not entirely understood.

Increased resistance to heat stress protects against degenerative diseases in mammals (9,32) and associates with longevity in *Caenorhabditis elegans* (10,26). Intrinsic thermotolerance is maintained by multiple mechanisms. A preconditioning (i.e. heat) stress induces acquired thermotolerance, mediated by the heat shock response *via* heat shock factor HSF1-dependent induction of heat shock proteins (Hsp-s) (30,47). Previous studies reported contrasting results of oxidative stress on HSF1 activation (2,28) and Hsp70 levels (14,22,43). However, the effect of oxidative stress on thermotolerance remains largely unexplored.

RNA interference is a powerful post-transcriptional regulator of gene expression that operates via ~22nt microRNAs (miRNAs) (27). Genomic miRNA precursors are processed by highly specific RNases: the nuclear Drosha/PASH-1 produces hairpin pre-miRNAs, which are transported to the cytoplasm and cleaved to mature miRNAs by Dicer/DCR-1 (capital names indicate the respective nematode orthologs). Hence, Dicer/Drosha knockout is a reliable tool to investigate the general role of miRNAs (5,41,44). miRNAs bind to the mRNA 3'untranslated region (3'UTR), repress translation or promote mRNA degradation (27). miRNAs modulate diverse biological processes. Their connection with stress is exemplified by imparting robustness to gene expression networks in response to environmental change (24) and by the profound alterations of miRNA expression upon heat and oxidative stresses (25,42,49) (reviewed in (23)). Heat and ischemic preconditioning-induced miRNAs induce Hsp70 and are cardioprotective during ischemia-reperfusion in mice (48,49). Moreover, miRNAs modulate the life span and stress resistance of *C. elegans* involving DAF-16 and HSF1 (6,11), underscoring a vital role of RNA interference in stress responses.

In this study we focused on the impact of oxidative stress on heat stress adaptation and found that H_2O_2 pretreatment inhibited acquired thermotolerance in both COS-7 mammalian cells and in *C. elegans*. As an underlying mechanism, H_2O_2 inhibited the heat-induction of Hsp70 in cells, consistent with a recent study (1). Moreover, H_2O_2 prevented the heat-induction of an Hsp70 3'UTR reporter. H_2O_2-induced effects required Dicer, a key enzyme in

miRNA biogenesis, in both cells and worms. We further found that RNAi against Dicer and Drosha orthologs restored the compromised thermotolerance of two worm strains deficient in H_2O_2 disposal. Finally, Dicer silencing delayed the decline of thermotolerance in aging worms and phenocopied the effect of the antioxidant N-acetylcysteine. Our results reveal RNA interference as a mediator of oxidative stress-induced inhibition of heat stress responses.

Results

H_2O_2 inhibits acquired thermotolerance and Hsp70 induction at the post-transcriptional level in COS-7 cells

The effect of a transient H_2O_2 exposure on thermotolerance of COS-7 cells was determined by subjecting cells to a lethal heat stress 24 hours after H_2O_2 and/or preconditioning heat treatments. Heat preconditioning elicited a large increase in survival (acquired thermotolerance, Fig 1A). A prior H_2O_2 treatment slightly increased intrinsic thermotolerance. Importantly, it potently inhibited acquired thermotolerance in a concentration-dependent manner (Fig 1A).

To examine whether the decrease in acquired thermotolerance is due to the inhibition of the heat shock response, we pretreated COS-7 cells with a series of H_2O_2 concentrations and monitored the heat-induction of Hsp70 by flow cytometry (Fig. 1B). Cells, exposed to heat shock, exhibited an approximately 10-fold induction of Hsp70, concordant with the induction of thermotolerance (cf. Fig. 1A). H_2O_2 treatment did not affect basal Hsp70 level, but inhibited Hsp70 heat-induction in a concentration-dependent manner (Fig. 1B). Western blots using a polyclonal anti-Hsp70 antibody showed a similar inhibition of Hsp70, but not of the specific chaperone Hsp90 (Fig. 1C and D). These results exclude an H_2O_2-induced modification or degradation of Hsp70 as well as a general, stress-induced transcriptional or translational block. The efficacy of H_2O_2 was verified by cells expressing the H_2O_2-sensor Hyper-C (Fig. 1E) (12). Thus, H_2O_2 pretreatment compromises both acquired thermotolerance and Hsp70 heat-induction in COS-7 cells.

Next, we investigated the site of action of H_2O_2 along the heat shock regulon. Upon heat, misfolded proteins activate HSF1, which binds to heat shock promoter elements (HSE) and induces *hsp* gene transcription (32). To assess the level of HSF1-dependent transactivation, we transfected COS-7 cells with a *hsp70pr*/luciferase vector and performed reporter gene assays after cells had either been oxidatively stressed and/or heat-shocked. Heat shock markedly induced reporter activity, while H_2O_2 treatment neither significantly affected basal nor heat-induced transactivation (Fig. 2A). Likewise, H_2O_2 treatment did not decrease *hsp70* mRNA level (Fig. 2B). Thus, a transcriptional inhibition does not seem to underlie the H_2O_2-induced decrease in Hsp70 protein expression.

To assess, if H_2O_2 could down-regulate Hsp70 post-translationally, we changed the order of stresses (i.e. employed H_2O_2 after heat shock) and followed the Hsp70 protein level

by flow cytometry (Fig. 2C). H_2O_2 did not change the heat shock-induced sustained elevation of Hsp70, which excluded the possibility of an accelerated Hsp70 turnover.

The 3' untranslated region (3'UTR) is intimately connected with the post-transcriptional regulation of mRNAs. To investigate the molecular events at the Hsp70 3'UTR, we took use of a reporter harboring the mouse *hsp70.1* 3'UTR fused to Firefly luciferase (18). Monitoring luciferase activity provided an estimate of the impact of the *hsp70* 3'UTR on the translation of luciferase mRNA following H_2O_2 and/or heat shock treatments. 3'UTR reporter activity displayed a time-dependent increase after heat-shock peaking at 6 hours (Fig. 2C). This finding is consistent with early reports in *Drosophila* and mammalian cells on the role of the 3'UTR in the regulation of Hsp70 protein synthesis during heat shock (33,38). Neither H_2O_2 nor the combination of H_2O_2 and heat shock increased luciferase activity above the baseline demonstrating that H_2O_2 entirely prevented the heat-induced activation by the *hsp70* 3'UTR (Fig. 2C).

RNA interference mediates H_2O_2-induced inhibition of Hsp70 induction and acquired thermotolerance in COS-7 cells

RNA interference is a powerful modulator of stress responses (23). To address whether RNA interference may mediate the events involving the Hsp70 3'UTR, we blocked miRNA maturation by anti-Dicer siRNA transfection. Only the siRNA, but not H_2O_2 led to a knock-down of Dicer (Fig. 3A). Intriguingly, anti-Dicer siRNA led to a large decrease in heat-induced 3'UTR reporter activity, suggesting that Dicer was necessary for the 3'UTR-mediated translational activation of the luciferase mRNA upon heat shock. This inhibition was comparable to that induced by H_2O_2, and a combination of anti-Dicer siRNA and H_2O_2 was not additive (Fig 3B). Thus, H_2O_2 prevents the heat-induced Hsp70 3'UTR activation primarily *via* RNA interference.

To investigate how the inhibition of the 3'UTR by RNA interference is reflected in Hsp70 translation, we determined Hsp70 protein levels in anti-Dicer siRNA-transfected cells undergoing H_2O_2 and heat shock treatments. Dicer silencing inhibited Hsp70 heat-induction to approximately two thirds of the control siRNA transfected value, comparable to the effect of Dicer silencing (Fig. 3C). Remarkably, H_2O_2 could not further reduce Hsp70 expression in Dicer-silenced cells suggesting that the effect of H_2O_2 required an intact RNA interference.

These results suggested that RNA interference might play a role in the H_2O_2-induced inhibition of acquired thermotolerance. Indeed, we found that Dicer siRNA reduced acquired thermotolerance in heat-preconditioned cells, which was similar to the effect of H_2O_2 (Fig.

3D). Moreover, H_2O_2 did not further diminish thermotolerance in Dicer-silenced cells, in agreement with our observations on Hsp70 induction (cf. Fig. 3C). Hence, we conclude that RNA interference mediates the H_2O_2-induced inhibition of heat stress adaptation in COS-7 cells.

H_2O_2 inhibits aquired thermotolerance through DCR-1 in *C. elegans*

To address if the effect of H_2O_2 on heat stress adaptation was conserved during evolution, we used *Caenorhabditis elegans*, a powerful model system exhibiting an organismal complexity. In search of an H_2O_2-exposure that did not cause significant damage in nematodes, we found that a treatment by 100 μM for 1 hour was below the threshold to induce oxidative tolerance to a lethal H_2O_2 challenge (Fig. 4A). This concentration induced a rapid signal elevation in the pharynx and intestine of worms ubiquitously expressing HyPer (Fig. 4B) (3). We used the 100-μM pretreatment to investigate its effect on nematodal thermotolerance. A preconditioning heat shock at 30ºC for 2 hours resulted in a 20 to 40% increase in thermotolerance (Fig. 4C). A prior H_2O_2 treatment did not affect intrinsic thermotolerance of worms, however, it entirely abolished acquisition of thermotolerance by the preconditioning heat shock.

To address whether H_2O_2 would affect an HSF1-dependent process, we employed the *hsf-1(sy441)* point mutant strain harboring a truncated transactivation domain that prevented the heat-induction of HSF1-target genes (15). In line with recently published data of McColl and colleagues (29), HSF1 was required for aquired, but not for intrinsic thermotolerance (Fig. 4D). H_2O_2 treatment was not additive to the *hsf-1(sy441)* background, it neither affected basal, nor heat-preconditioned survival. Moreover, in wildtype worms, H_2O_2, if applied after the preconditioning heat shock, was unable to inhibit acquired thermotolerance (Fig. 4E), suggesting that H_2O_2 needs to precede heat preconditioning. Thus, H_2O_2 specifically inhibits the acquisition of HSF1-dependent thermotolerance in *C. elegans*.

If, similarly to mammalian cells, H_2O_2 inhibited the heat shock response *via* RNA interference in *C. elegans*, then worms deficient in miRNA synthesis would escape from the H_2O_2-dependent inhibition of thermotolerance. Investigating this hypothesis we found that silencing the Dicer ortholog by *dcr-1(RNAi)* restored the acquired thermotolerance of H_2O_2-treated worms to levels comparable to heat shock alone (Figs. 5A and B). *dcr-1(RNAi) per se* did not affect thermotolerance (Fig. 5B). We obtained similar results using loss-of-function *dcr-1* mutant nematodes (Fig. 5C). The efficiency of *dcr*-1 silencing and the lack of a general disruption of RNA interference by H_2O_2, respectively, were demonstrated by an RNA

interference reporter strain (20) (Fig. 5D). We made attempts to investigate an analogous involvement of Hsp70 regulation. Unfortunately, a number of antibodies were unable to detect nematode Hsp70. Quantitative PCR measurements revealed a tendency of H_2O_2 preconditioning to augment heat-induced *hsp-70* mRNA expression. However, *dcr-1(RNAi)* neither significantly altered heat-induced mRNA level, nor the H_2O_2-induced elevation (Supplemental Fig. S1). Despite the unclear involvement of Hsp70, RNA interference is required for H_2O_2 to inhibit acquired thermotolerance in worms.

Inhibition of RNA interference restores thermotolerance in endogenous models of oxidative stress

Next we asked how thermotolerance might be affected by chronic genetic disturbances in antioxidant defense. Antioxidant enzymes provide protection against oxidative stress by removing ROS. *Catalase-2* is a peroxisomal enzyme involved in H_2O_2 elimination accounting for ~80% of total catalase activity in the worm (39). *ctl-2* loss-of-function elevates endogenous H_2O_2 levels (3), decreases oxidative tolerance and shortens lifespan (39). We observed that *ctl-2(ok1137)* animals exhibited impaired intrinsic thermotolerance compared to wildtype, which was completely restored by *dcr-1(RNAi)* (Fig. 6A). Silencing the Drosha ortholog PASH-1, the other key enzyme in miRNA biogenesis phenocopied the effect of *dcr-1(RNAi)* in the *ctl-2(ok1137)* strain (Fig. 6B) without affecting wildtype thermotolerance (Fig. 6C). Neither the survival decrease in *ctl-2(ok1137)*, nor the amelioration by *pash-1(RNAi)* was prevented by *hsf-1(RNAi)* (Fig. 6D). Hence, *ctl-2* loss-of-function modulates intrinsic thermotolerance, not involving the HSF1-Hsp axis.

To test whether the observed phenomena might be attributed to the general impairment of H_2O_2 elimination, we examined the lack of *peroxiredoxin-2*, involved in H_2O_2 reduction in the cytosol. *prdx-2(gk169)* worms, similarly to the *ctl-2(ok1137)* strain, are susceptible to H_2O_2 injury, and display a shortened lifespan (37). We found that *prdx-2* knockout also markedly decreased *C. elegans* thermotolerance (Fig. 6E). Importantly, *dcr-1(RNAi)* prevented thermotolerance inhibition in *prdx-2(gk169)* worms. Together these data suggest that genetic defects in H_2O_2 elimination compromise heat stress adaptation *via* RNA interference.

Inhibition of RNA interference delays age-dependent decline of thermotolerance

Aging is characterized by a collapse of proteostasis and an impairment of the heat shock response in *C. elegans* (4). Consistent with this, we observed a decline in *C. elegans*

thermotolerance during aging (Fig. 7A-C). Oxidative stress and H_2O_2 increases during aging and ROS are considered a major cause of aging (3,34). To address if oxidative stress affected thermotolerance during aging, we treated worms with the small molecular antioxidant, N-acetylcysteine (NAC). Intriguingly, NAC was able to reduce the decline of thermotolerance during aging resulting in a milder slope and a significant difference at the old worms at day 10 of age (Fig. 7A-C).

Then we asked, whether RNA interference was involved in the age-associated decline of thermotolerance of worms. *dcr-1(RNAi)* did not significantly influence the thermotolerance of young animals at day 1 (Fig. 5), but efficiently suppressed the age-induced decline of thermotolerance similarly to NAC treatment (Fig. 7A-C). Moreover, the combination of *dcr-1(RNAi)* with NAC was not significantly different from the effect of *dcr-1(RNAi)* at any time points tested. These findings indicate that RNA interference is involved in the oxidative stress-induced age-dependent decline of heat stress adaptation in *C. elegans*.

Discussion

In this study, we have presented evidence that oxidative stress inhibits the adaptive responses to heat stress in both mammalian cells and *C. elegans*. Silencing Dicer and Drosha orthologs, key enzymes specific to miRNA maturation reveals a conserved role for RNA interference. In mammalian cells H_2O_2 abolishes a positive action of RNA interference on acquired thermotolerance. Inhibition of RNA interference does not alter thermotolerance in young nematodes, suggesting H_2O_2 may induce miRNA(s) that inhibit the acquisition of thermotolerance. Intrinsic thermotolerance decrease of *prdx-2* and *ctl-2* knockouts and aged worms might require accumulation of miRNA(s) inhibiting HSF1-independent processes. Despite species-specific and context-dependent mechanisms our results provide support to the modulation of stress responses by RNA interference (Fig. 8) (23).

Our findings on the post-transcriptional inhibition of Hsp70 expression offer a potential molecular mechanism underlying the H_2O_2-induced compromise of acquired thermotolerance. Early reports demonstrating a heat-induced stabilization of *hsp70* mRNA by its 3'UTR (33,38) and the decrease in heat-induced *hsp70* mRNA by H_2O_2 in glioma cells (1) suggested that H_2O_2 may prevent mRNA stabilization. However, our results showing no impact of H_2O_2 on *hsp70* mRNA and inhibition of 3'UTR reporter, respectively, are consistent with a compromised translation by H_2O_2. Interestingly, inflammatory cytokines inhibit colonic Hsp70 translation by recruiting its mRNA to stress granules (17,18). Though it may be one plausible mechanism, our results using Dicer knockdown suggest the involvement of miRNA(s). Possible scenarios include an H_2O_2-induced decrease of activatory miRNA(s), or displacement/domination of heat-induced activatory miRNA(s) by H_2O_2-induced inhibitory/neutral miRNA(s) from the *hsp70* mRNA. Both mechanisms are generally employed by RNA interference (23,27). Moreover, recent papers provide evidence on miRNAs either inhibiting (miR-378*, miR-711, miR-146a, miR-146b-5b) (35,46), or ischemic preconditioning-induced miRNAs (miR-1, miR-21, miR-24 or others) (48,49) activating Hsp70 expression. Identification of the exact mechanism(s) and miRNA(s), as well as an analogous Hsp70 regulation in nematodes requires further studies. Nevertheless, our study raises the idea that pathophysiological oxidative conditions (inflammation, wound healing, aging) might employ RNA interference to post-transcriptionally regulate Hsp70 in various tissues (7,18,36).

Our use of mutants deficient in H_2O_2 elimination demonstrates that a chronic disturbance in ROS metabolism impairs intrinsic thermotolerance, independently of HSF1

(Fig. 6). This defect can entirely be reversed by blocking miRNA maturation, suggesting a profound post-transcriptional remodeling of heat stress adaptation by RNA interference in response to oxidative stress. McColl *et al.* elegantly showed that increased intrinsic thermotolerance in *daf-2* mutant worms is mediated by a *daf-16*-dependent translational response (29). A common motif in the two studies is that RNA interference or translation do not limit survival in young worms, however, they differentially condition heat resistance in both long-lived insulin-like signaling mutants and short-lived oxidative defense deficient mutants and aged worms, respectively ((29) and our study). It is tempting to speculate that the *daf-16*-regulated response of insulin signaling mutants might involve miRNAs. The clarification of a possible interaction of the translational response and RNA interference in the regulation of stress resistance remains the task of future studies.

Aging in the worm is characterized by an increased accumulation of ROS as well as a collapse of protein homeostasis (4). Our results on the age-induced decline of intrinsic thermotolerance support these observations, and use of the antioxidant NAC demonstrates a progressive causal role for ROS in decline of stress resistance during aging (Fig. 7). Importantly, both the comparable pattern of NAC and *dcr-1(RNAi)* protection and the lack of significant synergism imply a substantially overlapping mode of action. Moreover, increased protection by *dcr-1(RNAi)* suggests that RNA interference adversely affects heat resistance with aging. Single miRNAs do not seem to play an essential role in *C. elegans* development and growth, but both RNA interference and single miRNAs are indispensable to ensure proper development during environmental stress (20,24,31). Likewise, there is an extensive change in miRNA expression during *C. elegans* aging (11,19) and several individual miRNAs similarly modulate longevity and stress resistance in *C. elegans* (11). Inhibition of the entire RNA interference in adulthood provides strong evidence to the general dysregulation of miRNAs in aging and in oxidative stress with a negative impact on stress resistance (Figs. 6 and 7). It remains to be seen whether RNA interference would pose a trade-off between fine-tuning developmental programs and growth during stress in exchange for a self-maintenance later in life. Our results imply that beyond well-characterized stress-responsive HSF1 and DAF-16 pathways, RNA interference may offer a novel target to alleviate decline of stress responses during aging.

(Innovation)

Oxidative stress is a serious cause of cell and tissue damage associated with many human diseases. Our observations beyond demonstrating a novel crosstalk between various types of stresses via RNA interference, extend our understanding on how oxidative stress may debilitate physiological function. As RNA interference exhibits a significant functional conservation from nematodes to humans, we anticipate that the mechanism identified herein may be involved in human diseases and aging.

Materials and Methods

Materials. Reagents for cell culture were from Invitrogen. Solutions for flow cytometry were from BD Biosciences. Electrophoresis and blotting reagents were from Bio-Rad. N-acetyl-L-cysteine and H_2O_2 were from Sigma. All other reagents were from either from Sigma or Fluka.

Cell culture and survival. COS-7 cells were obtained from the ATCC. Cells were cultured as described (40). Cell survival was analyzed by Trypan Blue exclusion 24 hours after challenge.

Determination of protein levels. Flow cytometry using a FITC-conjugated monoclonal anti-Hsp70 antibody (StressGen), cell lysis and Western blotting using a polyclonal anti-Hsp70 antibody (21), or antibodies against Hsp90 (Stressgen), Dicer (CST), actin (Sigma) was carried out as previously described (40).

Transfection and reporter gene assays. Cells were transfected at a density of 40% using Lipofectamine (Invitrogen). Control/anti-Dicer siRNA (Quiagen) was introduced at 100 nM. Further treatments were applied at 48 hours post-transfection. For the hsp70-promoter reporter gene assay cells were transfected with 0.35 μg *hsp70.1pr*/Firefly luciferase plasmid (Rick Morimoto, Northwestern University) and CMV/β-galactosidase plasmids, while for the 3'UTR reporter assay 0.35 μg pGL3 basic or pGL3/*hsp70.1 3'-UTR* plasmid (Eugene Chang, (University of Chicago) and TK/Renilla luciferase plasmids were employed, respectively. Treatments were performed 24 hours post-transfection. 18 hours post-treatment reporter activities were measured using commercial assay kits (Promega) and expressed.

***C. elegans* strains and RNA interference.** Strains were obtained from the CGC, if not otherwise specified. The following strains were used in this study: wildtype (N2), jrIs[Prpl-17::HyPer], PS3551 *hsf-1(sy441)*, BB1 *dcr-1(ok247);unc-32(e189)* III, VC289 *prdx-2(gk169)* II, VC574 *ctl-2(ok1137)* II, and the GFP RNAi-reporter GR1401 (Gary Ruvkun, Harvard University). Strains were backcrossed to the wildtype at east three times to clear potential background mutations, and were maintained as described (8). RNAi was performed as described by feeding worms with HT115(DE3) bacteria transformed with empty vector, *dcr-1(RNAi)* Gary Ruvkun (Harvard University) or *pash-1(RNAi)* (Source BioScience) vectors, repectively (45). Experiments were carried out in the second generation. Experiments were performed in the second generation with synchronized young 1-day old adults, except for age-related thermotolerance.

Thermotolerance assay. Thermotolerance was performed on NGM plates at 35°C till complete extinction of the population using 25 animals per condition in at least two

independent trials. Viability was determined hourly by assaying for movement in response to gentle prodding.

mRNA expression analysis mRNA was prepared using the GeneJET RNA Purification Kit (Fermentas). mRNA was reverse transcribed using the RevertAid^{TM} cDNA Synthesis Kit (Fermentas). Quantitative PCR was performed in an ABI 7300 System by Taqman Gene Expression Assays: HSPA1A: Hs_00359147_s1; β-actin: Hs_99999903_m1 (Applied Biosystems). Relative amounts of *hsp70* mRNA were determined using the Comparative CT Method for quantitation and normalized to actin mRNA levels.

Analysis of H_2O_2 levels and fluorescence microscopy Fluorescence measurements in COS-7 cells transfected by HyPer-C (Miklós Geiszt, Semmelweis University) were performed as described (12). HyPer titration was achieved by sequential addition of increasing concentrations of H_2O_2. Mean fluorescence intensities over individual cells were calculated from 3-min recordings. H_2O_2 in worms was monitored using the jrIs[Prpl-17::HyPer] strain, ubiquitously expressing the H_2O_2-biosensor HyPer, was used. Worms were immobilized and imaged as described (3).

Statistical analysis. Data were analyzed using the SPSS software 15.0 (SPSS Inc., Chicago, IL, USA). Survival curves were compared by the log-rank test. If not stated otherwise, all experiments were repeated at least three times. Variables were expressed as mean ± standard deviation (SD). Statistical significance was indicated as follows: *p<0.05, **p<0.01, ***p<0.0001.

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Author Disclosure Statement

No competing financial interests exist.

Abbreviations Used

3'UTR = 3'untranslated region

CMV = Cytomegalovirus

DMEM = Dulbecco's Minimum Essential Medium

FITC= Fluorescein-Isothiocyanate

H_2O_2 = Hydrogen-peroxide

HSF1 = Heat Shock transcription Factor 1

Hsp = Heat shock protein

miRNA = microRNA

NAC= N-Acetyl-L-Cysteine

NGM = Nematode Growth Medium

ROS = Reactive Oxygen Species

MFI = Mean Fluorescence Intensity

TK = Thymidine Kinase

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Figure legends

Fig. 1. H_2O_2 impairs heat-preconditioned thermotolerance and Hsp70 heat-induction in COS-7 cells. (A) Effect of H_2O_2 on thermotolerance. Cells were treated by the indicated concentrations of H_2O_2 for 2 hours, then kept at 37ºC (control) or at 43ºC for 30 min (heat shock). 24 hours later cells were subjected to a lethal heat stress (45ºC, 60 min). Cell survival was analyzed 24 hours later by Trypan blue exclusion. Values are means ± SD-s of 3 experiments. **(B and C)** Effect of H_2O_2 on Hsp70 and Hsp90 protein levels. Cells were treated by H_2O_2 and heat shock as in panel A. 5 hours later Hsp70 levels were analyzed by flow cytometry using a monoclonal antibody **(B)** or by Western blot using a polyclonal anti-Hsp70 and monoclonal anti-Hsp90 and anti-actin antibodies, respectively **(C).** Values are means ± SD-s of 5 experiments compared to their respective controls, and image is a representative of 3 experiments. **(D)** Densitometric analysis of relative Hsp70 and Hsp90 levels from (C). **(E)** H_2O_2 titration curve of cytosolic HyPer-C in COS-7 cells. The 490/420-nm fluorescence excitation ratio of HyPer was calculated after background fluorescence subtraction from two experiments.

Fig. 2. H_2O_2 inhibition of Hsp70 involves a potential post-transcriptional regulation. (A) H_2O_2 does not affect *hsp70* promoter activation. Cells transfected with the *hsp70.1pr*/luc and control plasmids were treated as in Fig. 1. Enzyme activities were measured 18 hours later, and their ratios were expressed. **(B)** H_2O_2 does not diminish *hsp70* (HSPA1A) mRNA expression. Cells were treated as in Fig. 1. mRNA levels were determined 1-hour after treatments by quantitative RT-PCR and expressed relative to β-actin. **(C)** H_2O_2 does not affect Hsp70 protein turnover. Cells were heat shocked as above, after 2 hours at 37ºC cells were incubated in the absence (control), or presence of 800 μM of H_2O_2 for 2 hours, then harvested at the indicated timepoints, and analyzed by flow cytometry. **(D)** H_2O_2 inhibits the heat-induced luciferase reporter translation mediated by the Hsp70 3'UTR. Cells transfected with a pGL3/luc/*hsp70.1* 3'-UTR and control plasmids were treated by 650 μM H_2O_2 for 2 hours, then kept at 37ºC or heat shocked. At the indicated timepoints enzyme activities were determined, and expressed as a ratio. Values are means ± SD-s of 3 experiments.

Fig. 3. RNA interference mediates H_2O_2-induced inhibition of Hsp70 induction and acquired thermotolerance in COS-7 cells. (A) Effect of H_2O_2 treatment and anti-Dicer siRNA on Dicer protein level. Two days after transfecton by anti-Dicer or control siRNA, respectively, cells were treated by 800 μM H_2O_2 for 2 hours. Protein levels were analyzed by

Western blot. Image is a representative of 3 experiments. **(B)** Effect of Dicer siRNA and H_2O_2 on the Hsp70 3'UTR activation. Cells undergoing a 2-day co-transfection with a control/Dicer siRNA and the 3'UTR reporter plasmids were treated by 650 μM H_2O_2 for 2 hours, then heat shocked. 6 hours later enzyme activities were determined and expressed as a ratio. **(C)** Effect of Dicer siRNA and H_2O_2 on Hsp70 protein expression. Cells transfected with a control/Dicer siRNA were treated by μM H_2O_2 for 2 hours, then kept at 37ºC or heat shocked. 5 hours later Hsp70 levels were analyzed by flow cytometry. **(D)** Effect of Dicer siRNA and H_2O_2 on heat preconditioned thermotolerance. Cells transfected with a control/Dicer siRNA were treated by 800 μM H_2O_2 for 2 hours, then kept at 37ºC or heat shocked. Lethal heat stress and survival assay was performed as in Fig. 1A. Values are means ± SD-s of 3 experiments.

Fig. 4. A prior H_2O_2 treatment inhibits aquired thermotolerance in an HSF1-dependent manner in *C. elegans*. (A) Effect of preconditioning H_2O_2 treatments on oxidative tolerance. Oxidative stress was applied in liquid NGM for 1 hour at 20°C, 12-14 hours before a lethal oxidative challenge. Data are means ± S.D.-s of two separate experiments. **(B)** Intensity-normalized ratio image demonstrating a rapid rise of oxidized/reduced HyPer ratio in jrIs[Prpl-17::HyPer] worms in response to a 1-min challenge by 100 μM H_2O_2 in liquid NGM. Representative image from 5 independent experiments. **(C)** Effect of H_2O_2 (100 μM for 1 hour) on intrinsic and acquired thermotolerance induced by a preconditioning heat shock (heat shock, 30º for 2 hours). Lethal heat stress was employed 12 hours later. Only heat shock induces a significant difference in survival ($p<0.0001$). **(D)** No change in thermotolerance by a preconditioning heat shock and/or an H_2O_2 treatment in *hsf-1(sy441)* mutant worms ($p>0.1$). **(E)** H_2O_2 employed after the preconditioning heat shock does not abrogate acquired thermotolerance ($p>0.1$ compared to heat shock). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars)

Fig. 5. DCR-1 mediates the H_2O_2-induced inhibition of thermotolerance in *C. elegans*. Effect of H_2O_2 on intrinsic and acquired thermotolerance in worms fed by empty (EV, **A**), or *dcr-1(RNAi)* vectors (**B**), respectively. Treatments were as in Fig. 4. Note that the activatory effect of preconditioning heat shock was less pronounced on RNAi plates. In EV-fed worms (A) only heat shock, while in *dcr-1(RNAi)* fed worms (B) both heat shock as well as H_2O_2 + heat shock induced a significantly higher survival compared with controls ($p<0.001$). **(C)**

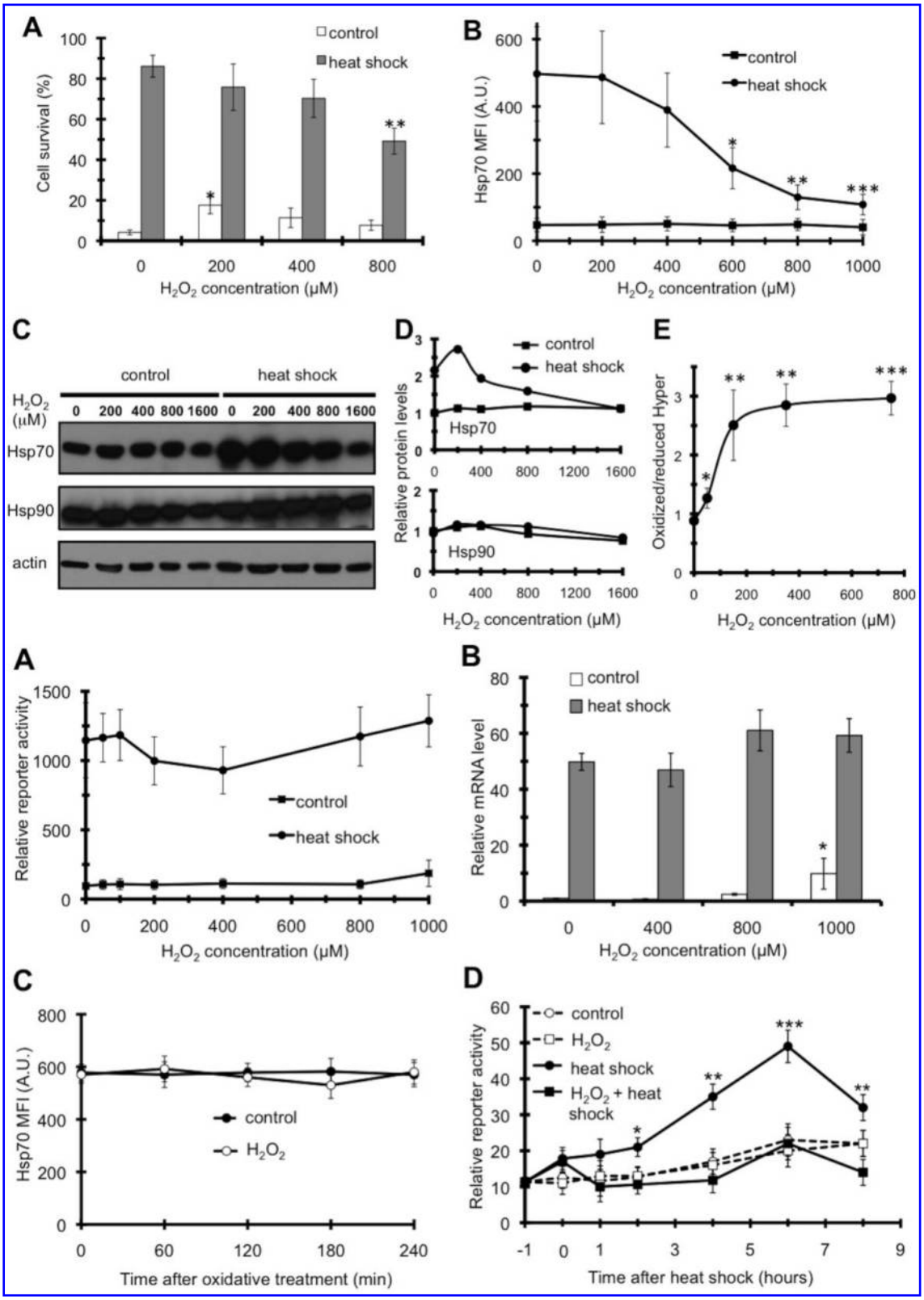
Both heat shock and H_2O_2+heat shock induces a significant increase in thermotolerance in *dcr-1(ok247);unc-32(e189)* nematodes ($p<0.0001$ *vs* control). Survival curves are representatives of 3 experiments yielding similar results. **(D)** H_2O_2 does not compromise RNA interference. Epifluorescence image demonstrating the increased expression of p*ajm*:GFP (harboring an anti-GFP hairpin siRNA in addition to the GFP sequence) in the GR1401 RNA interference reporter strain fed by *dcr-1(RNAi).* Arrows point to specific dots localized to epithelial seam cells. In contrast, H_2O_2 (100 μM for 1 hour) treatment followed by a 12- or 24-hour recovery did not inhibit GFP silencing. Please note the autoflurescence of oxidatively stressed worms. Representative image from 3 independent experiments. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

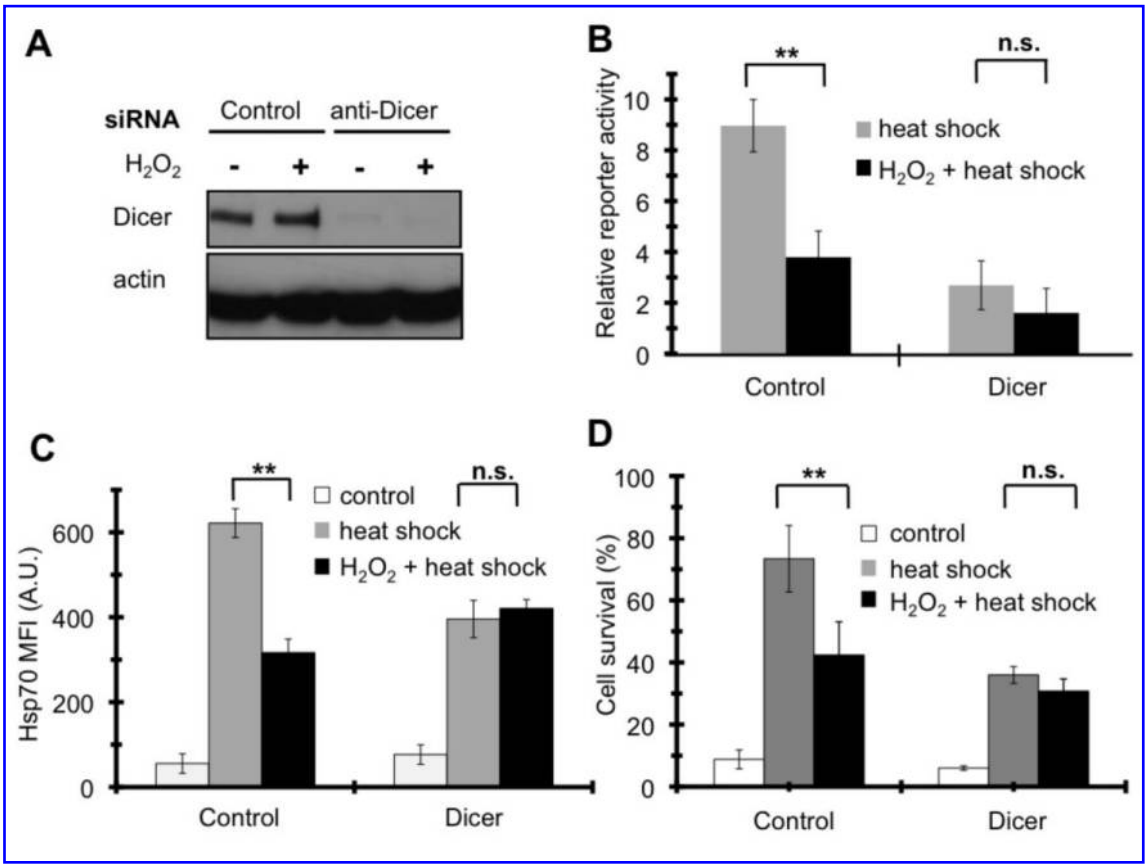
Fig. 6. Loss of RNA interference rescues thermotolerance in nematodes with genetic defects of H_2O_2 disposal. (A) Effect of *dcr-1(RNAi)* on thermotolerance of N2 and *ctl-2(ok1137)* worms. *ctl-2(ok1137)* worms exhibited significantly shorter survival ($p<0.001$), while other survivals were not significantly different ($p>0.2$), compared to N2 control. **(B)** *pash-1(RNAi)* phenocopies *dcr-1(RNAi)* by inducing a significant increase in thermotolerance of *ctl-2(ok1137)* ($p<0.0001$) compared to that of the EV control. **(C)** *pash-1(RNAi)* does not change thermotolerance of wildtype worms ($p>0.1$) compared to that of the EV control. **(D)** *pash-1(RNAi)* extends thermotolerance independently of *hsf-1* in *ctl-2(ok1137)* worms ($p<0.01$ *vs pash-1/hsf-1(RNAi)*). **(E)** Effect of *dcr-1(RNAi)* on thermotolerance of N2 and *prdx-2(gk169)* worms. *prdx-2(gk169)* worms fed by EV exhibited significantly shorter ($p<0.0001$), while those fed by *dcr-1(RNAi)* exhibited slightly longer survival ($p<0.05$) compared to N2 control. Survival curves are representatives of 3 independent experiments giving similar results.

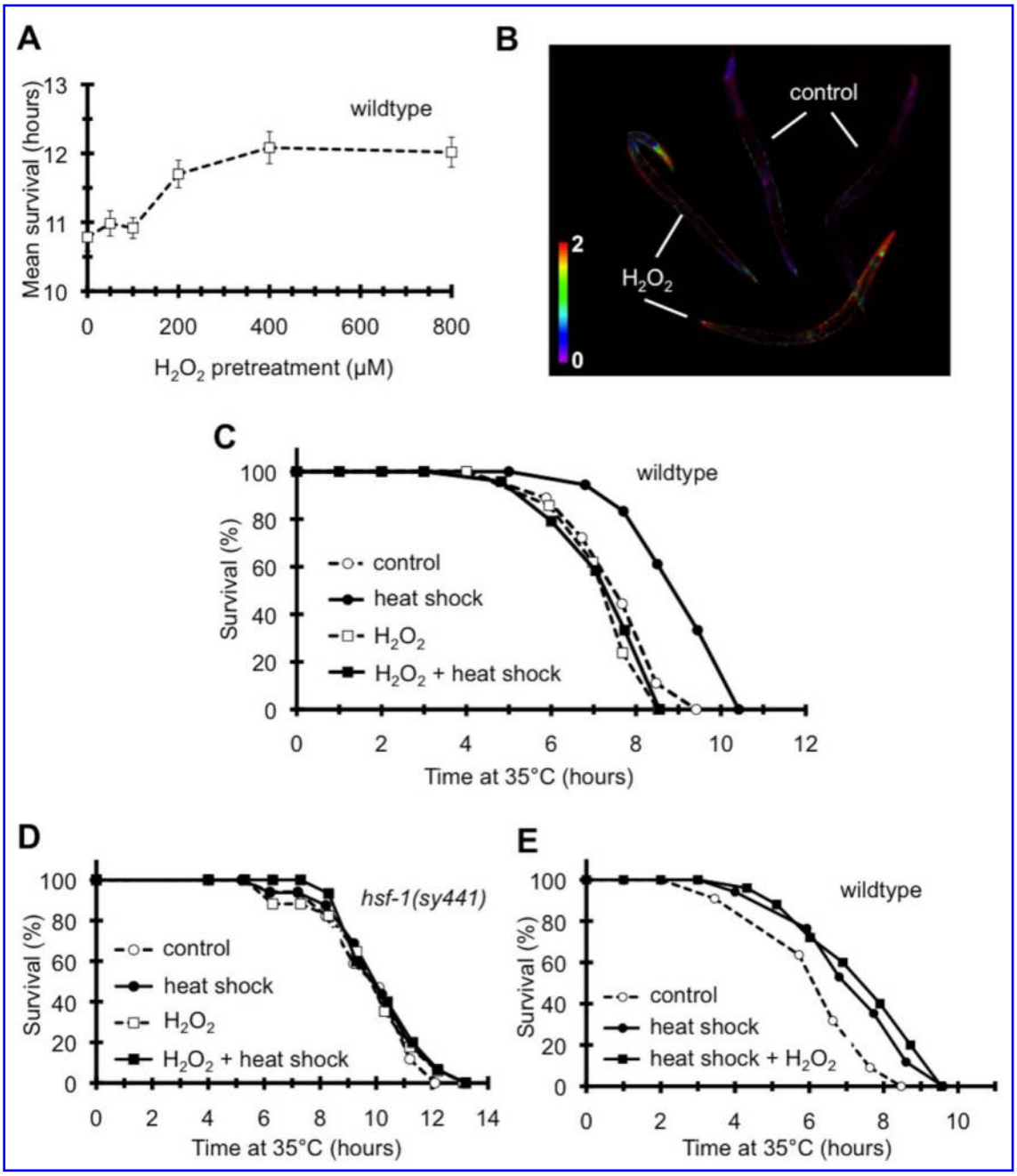
Fig. 7. Loss of RNA interference and the antioxidant N-acetylcysteine ameliorate age-associated decline of thermotolerance in *C. elegans*. Thermotolerance of 2-day **(A)** and 10-day **(B)** old nematodes treated by *dcr-1(RNAi)* and/or 5 mM NAC (from day 1 of adulthood). There was no significant difference in survival between treatments at day 2 ($p>0.1$). The 10-day old control (EV) exhibited a significantly shorter survival ($p<0.001$ *vs* 2-day EV). All *dcr-1(RNAi)* and/or 5 mM NAC induced a significant increase in survival ($p<0.001$ *vs* 10-day old EV) which approached ($p=0.028$ 10-day EV+NAC *vs* 2-day EV), and became non-significant ($p>0.05$ 10-day *dcr-1(RNAi)* and *dcr-1(RNAi)*+NAC *vs* 2-day EV) compared with

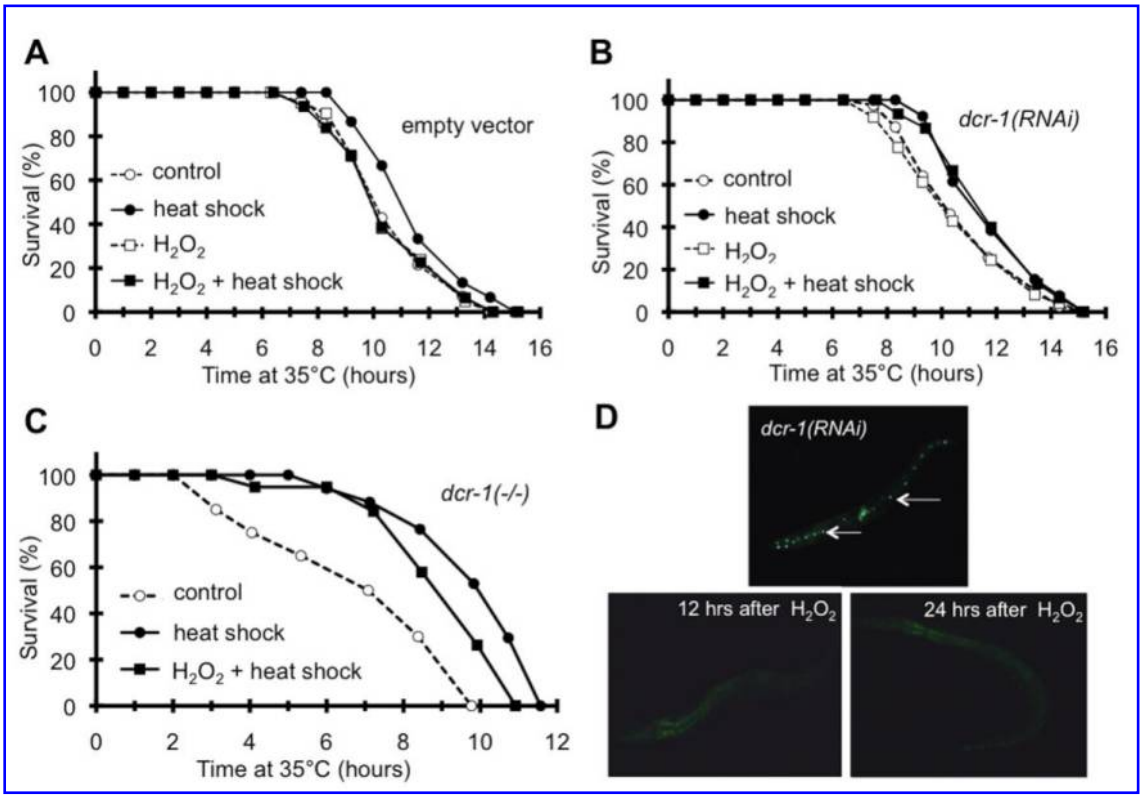
the survival of the 2-day old EV control. Survivals of 10-day *dcr-1(RNAi)* and *dcr-1(RNAi)*+NAC strains were not significantly different ($p>0.4$). **(C)** Mean thermotolerance of nematodes treated by *dcr-1(RNAi)* and/or 5 mM NAC as a function of age. Panels are representatives of 2 independent experiments yielding similar results.

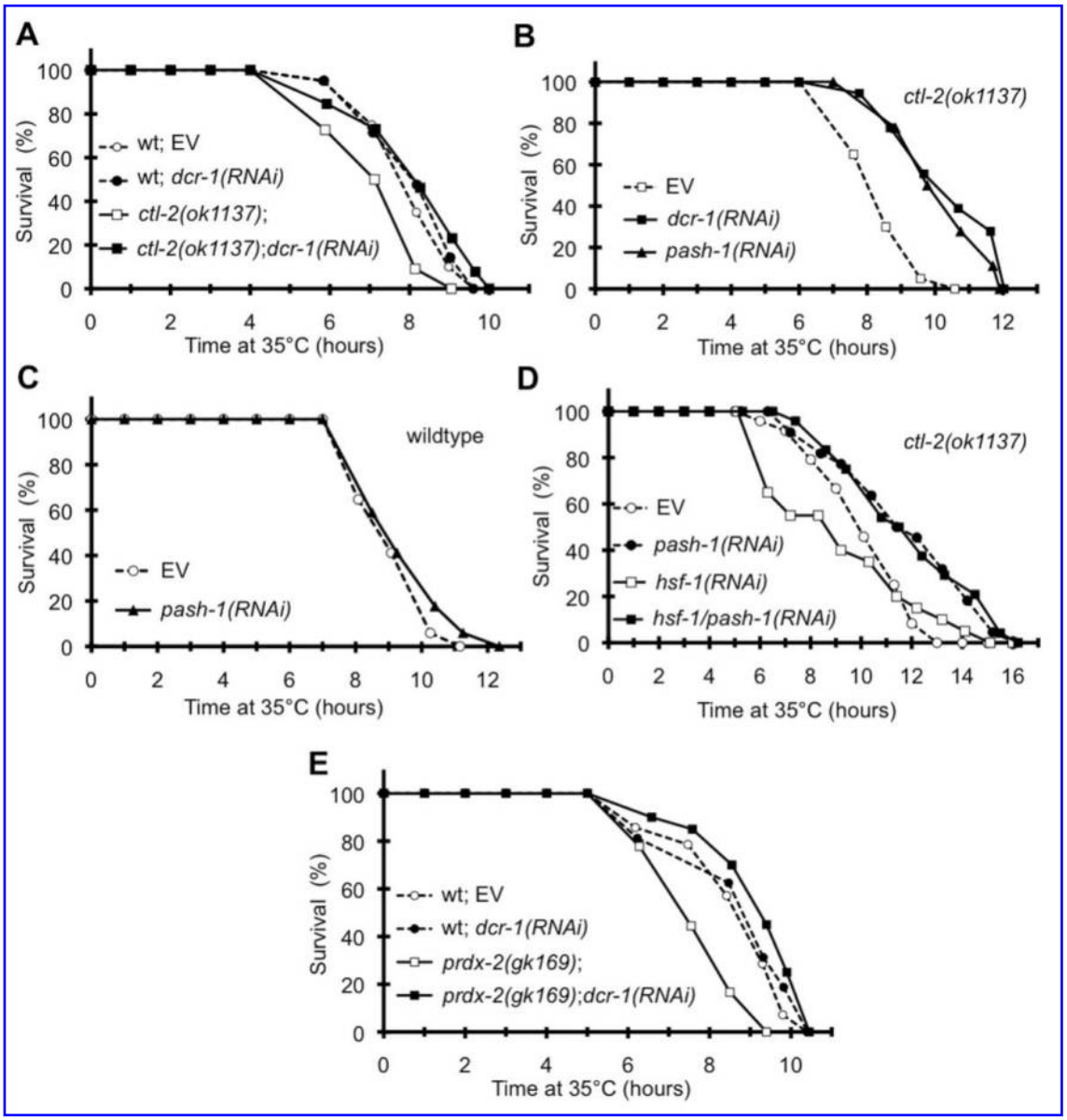
Fig. 8. Model for the role of RNA interference in the modulation of heat stress responses by oxidative stress. Oxidative stress may be induced by various sources, such as increased production/extrinsic factor (H_2O_2), decreased elimination (impaired defense, endogenous mutants) or a combination of the two (aging). RNA interference mediates an inhibitory action of oxidative stress and reduces heat resistance.

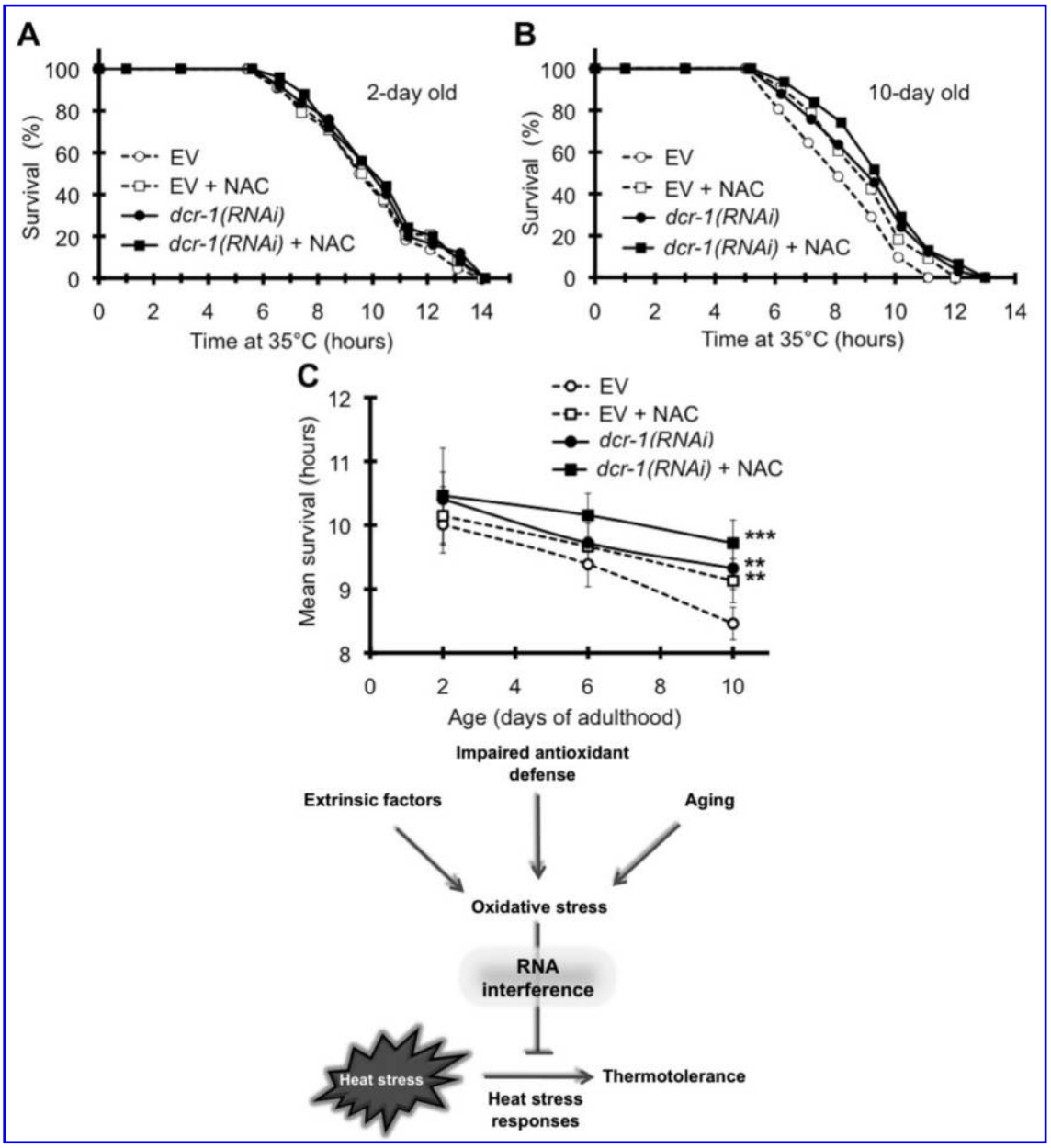












Supporting Information

ORIGINAL RESEARCH COMMUNICATION

RNA interference links oxidative stress to the inhibition of heat stress adaptation

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Running head: miRNA links oxidative and heat stresses

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This file includes: Supplemental Materials and Methods, Supplemental Figure Legend.

Supplemental Materials and Methods

Analysis of Hsp70 mRNA expression in nematodes mRNA was prepared using the GeneJET RNA Purification Kit (Fermentas). mRNA was reverse transcribed using the RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas). Quantitative PCR was performed in an ABI 7300 System by the Power SYBR Green PCR Master Mix (Applied Biosystems) using the primers: *hsp-70* (C12C8.1), forward: 5'-cgg tat tta tca aaa tgg aaa ggt t-3', reverse: 5'-tac gag cgg ctt gat ctt tt-3'; β-actin (*act-4* (4)). Relative amounts of *hsp-70* mRNA were determined using the Comparative CT Method for quantitation and normalized to actin mRNA levels.

Supplemental Figure Legend

Fig. S1. Effect of H_2O_2 and *dcr-1(RNAi)* on *hsp-70* mRNA expression in *C. elegans*. Treatments were as in Fig. 4. mRNA levels were determined 1-hour after treatments by quantitative RT-PCR and expressed relative to β-actin. Values are means ± SD-s of 3 experiments.