

Hsp90 and Physiological Stress Are Linked to Autonomous Transposon Mobility and Heritable Genetic Change in Nematodes

Calen P. Ryan^{1,2}, Jeremy C. Brownlie³, and Steve Whyard^{1,*}

¹Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

²Department of Anthropology, Northwestern University, Evanston, IL

³School of Biomolecular and Physical Sciences, Griffith University, Brisbane, Queensland, Australia

*Corresponding author: E-mail: Steve.Whyard@umanitoba.ca.

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Abstract

Transposable elements (TEs) have been recognized as potentially powerful drivers of genomic evolutionary change, but factors affecting their mobility and regulation remain poorly understood. Chaperones such as Hsp90 buffer environmental perturbations by regulating protein conformation, but are also part of the PIWI-interacting RNA pathway, which regulates genomic instability arising from mobile TEs in the germline. Stress-induced mutagenesis from TE movement could thus arise from functional trade-offs in the dual roles of Hsp90. We examined the functional constraints of Hsp90 and its role as a regulator of TE mobility by exposing nematodes (*Caenorhabditis elegans* and *Caenorhabditis briggsae*) to environmental stress, with and without RNAi-induced silencing of Hsp90. TE excision frequency increased with environmental stress intensity at multiple loci in several strains of each species. These effects were compounded by RNAi-induced knockdown of Hsp90. Mutation frequencies at the *unc-22* marker gene in the offspring of animals exposed to environmental stress and Hsp90 RNAi mirrored excision frequency in response to these treatments. Our results support a role for Hsp90 in the suppression of TE mobility, and demonstrate that the Hsp90 regulatory pathway can be overwhelmed with moderate environmental stress. By compromising genomic stability in germline cells, environmentally induced mutations arising from TE mobility and insertion can have permanent and heritable effects on both the phenotype and the genotype of subsequent generations.

Key words: Hsp90, transposable elements, piRNAs, genome stability, stress-induced mutagenesis, evolution.

Introduction

The capacity to buffer environmental and genetic perturbations is a fundamental property of all organisms (Waddington 1942; Pigliucci 1996). Stability in response to environmental and genetic disturbances requires numerous, highly responsive, and potentially overlapping mechanisms operating across multiple levels of biological organization (de Visser et al. 2003; Jarosz et al. 2010). At the cellular level, heat-shock proteins serve as molecular chaperones, helping fold other proteins into their appropriate conformations following environmental and physiological stress (Erlejman et al. 2014). Hsp90 is one such molecular chaperone, facilitating the proper functioning of many cellular proteins under normal conditions, as well as in response to hyperthermia, oxidative stress, and other environmental

extremes (Feder and Hofmann 1999; Sangster et al. 2004). Hsp90 interacts with a broad, yet select set of client regulatory proteins, including various kinases and transcription factors (Mayer and Bukau 1999; Young et al. 2001), and experimentally induced perturbations of this chaperone function have shown that Hsp90 also acts as a buffer of pre-existing, cryptic genetic variability (Sangster et al. 2004; Gangaraju et al. 2011). Overload of the cellular stress response by either Hsp90 inhibition or acute environmental stress increases *phenotypic* variation in both plants and animals (Queitsch et al. 2002; Jarosz and Lindquist 2010). However, there is also evidence that Hsp90 contributes to the buffering of *genetic* change, both through the regulation of DNA repair complexes involved in maintaining genetic fidelity, and through the suppression of

transposable element movements within the genome (Piacentini et al. 2014; Sekimoto et al. 2010).

Transposable elements (TEs) are mobile genetic elements found throughout most species' genomes, and their movements within the host genome are often associated with induced mutations; they can alter the expression of genes by introducing alternative promoters, splice variants, frame shifts, and sequence deletions or duplications (Kazazian 2004; Cowley and Oakey 2013). Although TE-induced mutations can have deleterious effects, particularly in germline DNA, TEs have also been important in generating adaptive genomic variability across a wide range of taxa (Kazazian 2004; Biémont and Vieira 2006). TEs are often categorized into two classes, based on their mechanism of transposition (Kidwell and Lisch 2001; Sela et al. 2010). Mobility among Class I TEs or retrotransposons is restricted to "copy and paste" mechanisms via RNA intermediates, whereas Class II or DNA transposon mobility involves an autonomous "cut and paste" mechanism through a DNA intermediate (Bonchev and Parisod 2013).

Transposon mobility can be regulated transcriptionally through histone modification and cytosine methylation (Yoder et al. 1997; Bestor 1998), as well as post-transcriptionally through the PIWI/piRNA pathway (Das et al. 2008). The PIWI/piRNA pathway involves a subfamily of Argonaute proteins, referred to as PIWI proteins, which act as catalysts in the gene silencing RNA-interference (RNAi) pathway, as well as a subclass of small RNAs that direct PIWI to its mRNA targets (PIWI-interacting RNAs or piRNAs). Along with a cochaperone known as *Shu*, the loading of pre-piRNAs onto the PIWI complex for TE suppression requires Hsp90 (Olivieri et al. 2012; Izumi et al. 2013) (fig. 1). In *Drosophila melanogaster*, transcription rates for several (Class I) retrotransposons were shown to increase in *hsp83^{scratch}* flies hypomorphic for the Hsp90 analog, as well as in the testes of males treated with an Hsp90 inhibitor, geldanamycin (Specchia et al. 2010). What remains unclear is the extent to which Hsp90 suppresses TEs across taxa, whether Hsp90 suppresses DNA (Class II) in addition to Class I transposon excision and insertion frequency, and how environmental stress within a naturally occurring range might constrain the capacity of Hsp90 to buffer genomic instability.

Here, we examine the effects of several moderate environmental stresses on the capacity of Hsp90 to regulate the transcription and excision of two groups of Class II DNA transposons. We used the model nematode species *Caenorhabditis elegans* and *C. briggsae*, where the mechanisms for TE silencing have been extensively studied (Sijen and Plasterk 2003; Bagijn et al. 2012) and sensitive assays for measuring TE excision frequencies have been successfully applied (Brownlie and Whyard 2004; Brownlie et al. 2005). To broaden our comparative analysis, we included multiple strains of each species, and excision frequencies at several different loci for each strain. To test if Hsp90 faces functional

constraints in buffering environmental and genetic instability, we subjected the nematodes to both heat and oxidative stresses, with and without RNAi-mediated knock-down of Hsp90 (*daf-21*). The stress-induced transposon excision and transposition events we observed in the progeny were exposure-dependent, and were amplified by RNAi-mediated Hsp90 knock-down. Our treatments, typically viewed as short-term physiological challenges, demonstrate that long-term, heritable genetic change may arise through TE-mediated activity following moderate environmental stress.

Materials and Methods

Nematode Strains and DNA Extractions

The *C. elegans* strains used in this study were N2 and AB2 (Egilmez et al. 1995) and the *C. briggsae* strains used in this study were transgenic lines of AF16 and DH1300 (supplementary fig. S1, Supplementary Material online). Genomic DNA was extracted from nematodes grown in liquid culture, at mixed developmental stages (Hope 1999), using the Genome 100 tip kit (Qiagen) according to the manufacturer's instructions. DNA was extracted from single worms using a previously described method (Williams et al. 1992).

Development of *sid-2* Transgenic Strains of *C. briggsae*

Transgenic lines of *C. briggsae* strains AF16 and DH1300 were produced using techniques established for *C. elegans* transformation (Evans 2006). The coding sequence of *Cel-sid-2* (Genbank NC_003281) and its 5' promoter and 3' sequences were amplified by PCR from *C. elegans* genomic DNA using Phusion DNA polymerase and the primers *Cel-sid2F* (5'-CCAACTTCGTAGAGCTGAGCAGC-3') and *Cel-sid2R* (5'-TTACTGCAAGCTGAGCTATTTTTTCG-3'). PCR products were gel-purified using the QIAquick Gel Extraction kit (Qiagen). The purified DNA (10 ng/μl) was independently injected into the gonad of adult worms, along with carrier pBS plasmid (150 ng/ml) and 10 ng/μl of the coinjection marker plasmid pWD47 (Jose et al. 2009), which carries the *Pmyo-2::DsRed* markers, to monitor progeny for visible phenotypes. Each injected animal was transferred singly to a culture plate. The *DsRed* phenotype was assayed in the F1 using a Leica MZ FL3 fluorescence stereomicroscope and those worms showing the phenotype were back-crossed to their respective parental stock for three generations before they were used for RNA interference assays. Individual worms (10) from each strain in the F4 were tested for the presence of the *sid-2* gene by extracting their DNA and using a PCR screen using the primers described above.

RNA Interference Knockdown of Hsp90

RNAi experiments were performed by feeding dsRNA-expressing *E. coli* to the two strains of *C. elegans* and the two strains of transgenic *C. briggsae* carrying the *Cel-sid-2* gene

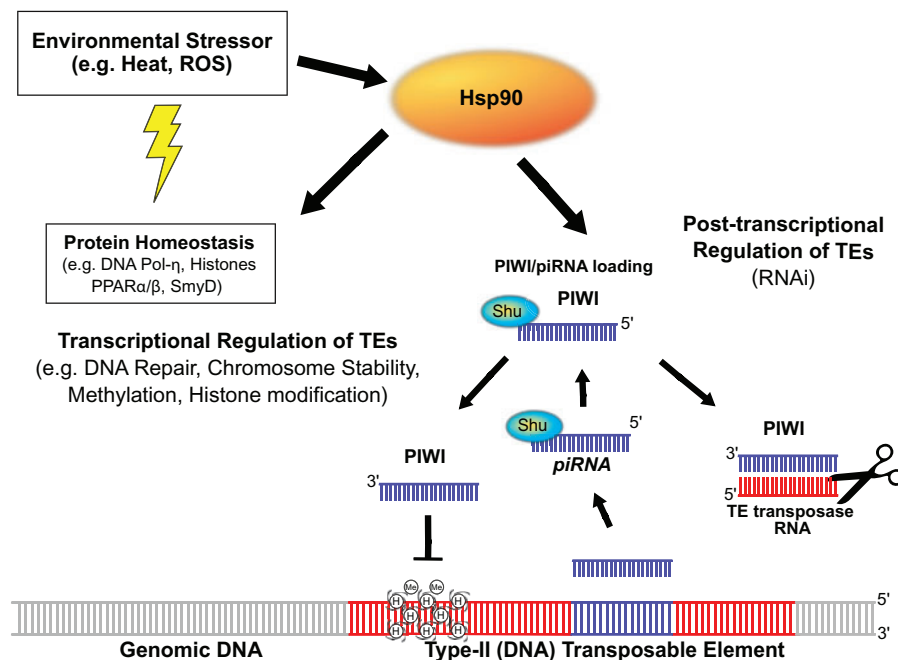


Fig. 1.—Pathway of the 90-kilodalton heat-shock protein (Hsp90) cellular stress response. Hsp90 expression increases in response to environmental stress, which perturbs protein form and function (protein homeostasis), including proteins involved in genomic DNA repair (e.g., DNA Pol- η), transcriptional activity (PPAR α/β), chromosome integrity, and epigenetic regulation (e.g., histone acetyltransferase, SmyD). Hsp90, along with the cochaperone *Shutdown* (*Shu*) is also necessary for loading of piwi-interacting RNAs (piRNAs) onto the PIWI protein complex. The PIWI/piRNA pathway can silence transposable elements (TEs) transcriptionally (left), through histone methylation (Me) or other modifications to histones (H), or post-transcriptionally (right), through RNA-interference-mediated targeting of TE transposase RNAs. Genomic DNA (gray), TE DNA/RNA (red), and piRNA (purple) are also shown.

(supplementary fig. S1, Supplementary Material online), according to standard procedures (Kamath et al. 2003). The Hsp90 (*daf-21*; WBGene00000915) gene of *C. elegans* was amplified by PCR from cDNA derived from RNA extracted from multi-staged *C. elegans* N2 strain using standard techniques, with the primers CeHsp90F (5'-AGTTCCTTCGGTTTTTCGGT-3') and CeHsp90R (5'-GCGAATGATTTCGAAACTAAAAA-3'). The primers used for PCR amplification of the Hsp90 gene in *C. briggsae* (Genbank XM_002637731) were as follows: CbrHsp90F, 5'-ATGTCCGAGAACGCTGAAAC-3' and CbrHsp90R, 5'-ATGAGACCAACCTGGTAGCG-3'. The PCR products were cloned into the PCR cloning vector pDrive (Qiagen) and then subsequently excised using the restriction enzymes *EcoRI/XhoI* and ligated into a similarly digested dsRNA expression vector pL4440. The plasmids were sequenced prior transforming *E. coli* HT115 cells, which were grown in LB containing ampicillin and 1 mM IPTG to induce expression of the dsRNA within the bacteria. A pL4440 plasmid lacking any insert served as a negative control. After 4 h, the bacteria were plated on NGM agar plates containing 50 μ g/ml ampicillin, 6 μ g/ml tetracycline, 1 mM IPTG, and 5 μ g/ml cholesterol. L1 larvae were grown on these plates at 20 °C and after 3 days, worms were collected for either RNA extractions to assess the extent of RNAi, or were subjected to different stresses, described below.

QRT-PCR Analysis of RNAi Treatments

To analyze the extent of *daf-21* (Hsp90) transcript reduction in worms fed *E. coli* expressing dsRNA, quantitative reverse transcriptase PCR (QRT-PCR) experiments were performed. Worms were washed off their plates with M9 medium, and bacteria were removed by several washing steps. RNA was isolated from groups of 20 worms using an RNeasy Mini Kit (Qiagen). The RNA was subjected to reverse transcription with random hexamer primers using ALV reverse transcriptase (Promega). QRT-PCR was carried out according to the manufacturer's instructions, using SYBR Green MasterMix (BioRad) in an iQ5 Real-Time PCR Detection System (BioRad). Primer pairs were designed to amplify a 200–250 bp segment of the target gene, using one primer per pair that spanned an exon-exon junction. The primer pairs for *C. elegans* were for *act-1* (Fwd: AATCCAAGA GAGGTATCCTTA, Rev: GATGGCGACAT ACATGGC T); *pgk-1* (Fwd: TTTGATCCGTGTTGACTTCAAT, Rev: GAAGAGAACATCTTTTTCAAGA); CeHsp90 (Fwd: AAG ATGAGGAGGCTGTCTGA, Rev: CATTGGACA AGCTTTGT AGA). The primer pairs for *C. briggsae* were for *Cbr-act-5* (Fwd: GCAACAGAGAAAAGATGACACAG, Rev: GATTCTTC TTACCTCATAGATTGG); *Cbr-pgk-1* (GAGTTGATGCTTCTGGG TCCAAGG, Rev: CCGATCGCAATACCTTGTCTGAAATAAG); and *Cbr-Hsp90* (Fwd: GAGTCCGTGCTCTTCTCTTCG, Rev: T

TCCGAGCTTAAGTTCTTCCG). The log 2-fold changes of the genes of interest upon heat shock were normalized relative to the *unc-60* (actin depolarizing factor) and *pgk-1* (phosphoglycerate kinase 1) genes and averaged for three separate biological experiments.

Heat Shocks and Oxidative Stress Treatments

The nematodes were subjected to sub-lethal heat shocks or oxidative stresses to assess impacts on transposon activity (supplementary fig. S1, Supplementary Material online). For the oxidative stress treatments, adult worms were transferred to 96-well plates (20 worms/well) containing M9 buffer with 0 mM (negative controls), 0.25 mM (low oxidative stress; H₂O₂-Low), or 1.5 mM (high oxidative stress; H₂O₂-High) H₂O₂ for 8 h incubations at 20 °C. Thereafter, the worms were washed with 4 successive washes of M9 buffer before being transferred to *E. coli*-coated NGM agar plates for 24 h to recover. For the heat shock treatments, the nematodes, in 96-well plates containing M9 buffer were subjected to 35 °C for 1 h (low heat shock; Heat-Low), five serial exposures of 35 °C for 1 h, 30 min at 20 °C (serial heat shock, Heat-Serial), or 39 °C for 2 h (high heat shock; Heat-High). Negative control worms were held at 20 °C for 5 h (No Stress). After the heat stresses, the worms were transferred to agar plates for 24 h as previously described. Thereafter, the worms were pooled in groups of 20 and their DNA was extracted as described above, to assess transposon mobility.

Quantitative PCR Assays

Excision frequencies for three different *Tc1* and three *CemaT1* loci in *C. elegans* and three different *Tcb1* and three *CbmaT1* in *C. briggsae* were determined using a quantitative PCR (QPCR) approach (supplementary fig. S1, Supplementary Material online). For each locus examined, three primers were designed: 1) a forward primer that flanked the transposon approximately 30–50 bp from the transposon ITR; 2) a reverse primer that spanned the transposon ITR sequence and the flanking sequence; and 3) a reverse primer that spanned the site of a putative excision footprint. The first pair of primers detected nonexcised TEs, whereas the first and third primers detected transposon excisions. QPCR reactions were performed in triplicate with 50 ng of genomic DNA (obtained from 100 pooled worms subjected to the same treatment) using SYBR Green Supermix (BioRad) on a BioRad iQ5 Real-Time PCR Detection System. A melt-curve analysis was performed on all samples to assess the purity of amplified products. PCR primers (see supplementary table S1, Supplementary Material online) were designed such that the PCR products of the excised and nonexcised transposons were of similar size (~80 bp) and GC% content. Efficiencies of the primer sets were calculated using the method of Pfaffl (2001), and were found to be essentially equivalent for all primer sets, ranging between 93% and 95%. The threshold cycle

values for all excised and nonexcised transposon loci were normalized relative to the internal standard 26S rRNA gene. From these calculations, the percentage of loci containing a transposon excision footprint was determined, as previously described (Brownlie and Whyard 2004; Brownlie et al. 2005).

Assessment of Mutation Frequency

The frequency of spontaneous mutations at the *unc-22* locus in one strain (N2) of *C. elegans* and two strains of (AF16 and DH1300) of *C. briggsae* was assessed using an assay similar to that described previously (Moerman and Baillie 1979) to screen for the generation of *unc-22* mutants in *C. elegans* (supplementary fig. S1, Supplementary Material online). Briefly, single worms were placed on (100 mm) NGM plates and left until the plates were crowded (10⁵ worms/plate). Worms were then washed off the plates in 5 ml of 1% nicotine solution (Sigma) and within 5 min. the animals were examined for the presence of any twitching individuals among the paralyzed worms. Although wild-type *C. elegans* worms become paralyzed when exposed to 1% nicotine, homozygous and heterozygous *unc-22* mutants will twitch. Plates with one or more twitching worms were scored as positive and those on which only wild type worms were observed were scored as negative. Any twitching worms were recovered and their progeny were tested to ensure they produced *unc-22* mutants. Three replicates of 20–30 plates for each combination of strain, stress treatment, and RNAi treatment were examined, with 2,717 plates examined for mutations overall. The proportion of plates not showing mutants (P_0) was used to calculate the spontaneous mutant frequency using the Poisson distribution ($P_0 = e^{-aN}$, where a is the mutant frequency and N is the population size/plate).

Ten lineages of *C. elegans* and ten lineages of *C. briggsae* with heat or oxidative stress-induced *unc-22* mutants were cultured and ~50 progeny worms were pooled and their DNA was extracted, as described above (supplementary fig. S1, Supplementary Material online). The DNA was subjected to PCR using 10 different pairs of primers that spanned almost the entire length of *unc-22*, excluding large introns (see supplementary table S2, Supplementary Material online). The PCR products were resolved on 0.8% agarose gels and any PCR products that were between 0.9 and 1.6 kb larger than expected were sequenced to determine whether a transposon sequence had inserted within *unc-22*. All sequenced insertions appeared to be homozygous.

Statistical Analyses

Excision frequency was examined for *CemaT1/CbmaT1* and *Tc1/Tcb1* transposons at three loci each in two strains in *C. elegans* (N2 and AB2) and two strains in *C. briggsae* (AF16 and DH1300). TE mobility and insertion frequency was measured at the *unc-22* gene for the N2 strain in *C. elegans* and both the AF16 and the DH1300 strains in *C. briggsae*. Excision

Table 1Hsp90 Transcript Knockdown Levels in *C. briggsae* Carrying *Cel-sid-2* and fed *E. coli* Expressing *daf-21* dsRNA^a

Strain	Transgenic F1	Transgenic F2	% RNAi Knockdown in F4 Transformants ^a
AF16	18/22	10/22	88 4
DH1300	7/25	4/25	84 3

^aThe extent of RNAi-mediated knockdown of *daf-21* transcripts was calculated relative to the expression levels of worms fed *E. coli* carrying the empty vector pL4440. The values represent the means and standard errors of 3 replicates of 10 pooled worms. The number of worms depicted are those expressing *DsRed* in the F1 and F2 worms, relative to the number of surviving injectants.

frequency was analyzed using ordinary least squares regression, and was logged to meet model assumptions. The effects of treatment and Hsp90 RNAi on mutation frequency were analyzed using a general linear model with quasibinomial family to account for overdispersion. All models were initially run with strain, locus, treatment, RNAi, and all two-way interactions between these parameters. To avoid over-parameterization, three- and four-way interactions were not included. Nonsignificant two-way interactions were sequentially removed until only significant ($P < 0.05$) interactions remained. Nonsignificant main effects were also removed, unless they contributed to a significant two-way interaction. Effects of treatment and RNAi (fold increases) on excision or mutation frequencies were derived from corresponding regression or generalized linear model estimates, and are relative to non-stressed control animals in the absence of RNAi. The reference strain and locus varied by species, and final models including all parameters and estimates are provided (see [supplementary tables S3–S5, Supplementary Material](#) online). Estimates of treatment effects in the results are for the range of all treatments except Heat-Low, which was not significantly different for any analysis.

Results

Knockdown of Hsp90 in *C. briggsae* Transformed with *Cel-Sid-2*

When certain strains of *C. elegans* are fed dsRNA, either as free oligonucleotides or expressed within transformed *E. coli*, RNAi is induced throughout most of the animal's tissues. This systemic RNAi is mediated by SID-2, a transmembrane protein in the worm intestine, and by SID-1, a related protein in most other tissues (Winston et al. 2002), both of which facilitate cellular uptake of the dsRNA. *C. briggsae* has a variant of *sid-2* that appears unable to take up dsRNAs. Winston et al. (2007) demonstrated that *C. briggsae* could be induced to take up ingested dsRNAs by expressing the *C. elegans sid-2* (*Cel-sid-2*) gene. Using standard transformation techniques, we similarly prepared two different strains of *C. briggsae* (AF16 and DH1300) that expressed the *Cel-sid-2* gene. Representative F4 worms from each lineage were PCR-screened for the presence of the transgene, and all individuals carried the *Cel-sid-2* gene. The worms were fed *E. coli* containing *Cbr-daf-21/L4440*, which expressed dsRNA that targets the Hsp90-

encoding gene, and qRT-PCR of the F3 worms confirmed that the gene transcript levels were reduced >80%, relative to negative control worms, in both strains (table 1).

CemaT1 and Tc1 excision frequency in *C. elegans*

The excision frequencies of two different transposons, *Tc1* and *CemaT1*, were examined in *C. elegans* exposed to different oxidative and heat stresses using a PCR-based assay developed previously (Brownlie and Whyard 2004). The assay compares the relative abundance of nonexcised transposons to excision footprints at a given locus. Three loci of each transposon were examined in two separate *C. elegans* strains, and the excision frequencies of these transposons in nonstressed, control worms were comparable to those observed in a previous study (Brownlie and Whyard 2004). For both *CemaT1* and *Tc1*, certain loci showed significantly higher excision frequency than others ($F_{2,358}=208.5$, $P < 0.001$ and $F_{2,345}=771.5$, $P < 0.001$, respectively; see [supplementary table S3, Supplementary Material](#) online), suggesting that individual transposon activity is influenced by the local genomic environment. For *Tc1*, excision frequencies were also significantly lower in the AB2 strain compared with the N2 strain ($F_{1,345}=48.2$, $P < 0.001$). Despite loci- and strain-specific differences in transposon activity, stress treatment had an overall significant effect on both *CemaT1* and *Tc1* excision frequencies ($F_{5,358}=303.3$, $P < 0.001$ and $F_{5,345}=361.5$, $P < 0.001$, respectively; fig. 2, [supplementary fig. S2, Supplementary Material](#) online). The two oxidative stress treatments and more extreme heat stresses (a series of 1 h 37°C shocks or a 2h 39°C shock) were associated with 2- to 7-fold increases in excision frequencies for both *CemaT1* and *Tc1* ($P < 0.001$ for all). Low-heat stress (a brief 1 h heat shock at 37°C) did not significantly affect excision frequencies for either *CemaT1* or *Tc1* ($P > 0.65$ for both; see [supplementary table S3, Supplementary Material](#) online). QRT-PCR analyses confirmed that all stress treatments caused significant ($P < 0.05$), approximately 2-fold increases in levels of *Hsp90* mRNA in both *C. elegans* strains (N2, AB2) relative to no treatment controls (see [supplementary table S6, Supplementary Material](#) online).

RNAi-mediated knockdown of Hsp90 increased excision frequencies of the *CemaT1* transposon overall ($F_{1,358}=856.2$, $P < 0.001$; fig. 2), and amplified the effect of stress treatment for all (RNAi × Treatment: $P < 0.001$ for all) except the Heat-Low treatment (Heat-Low: $P=0.931$; see

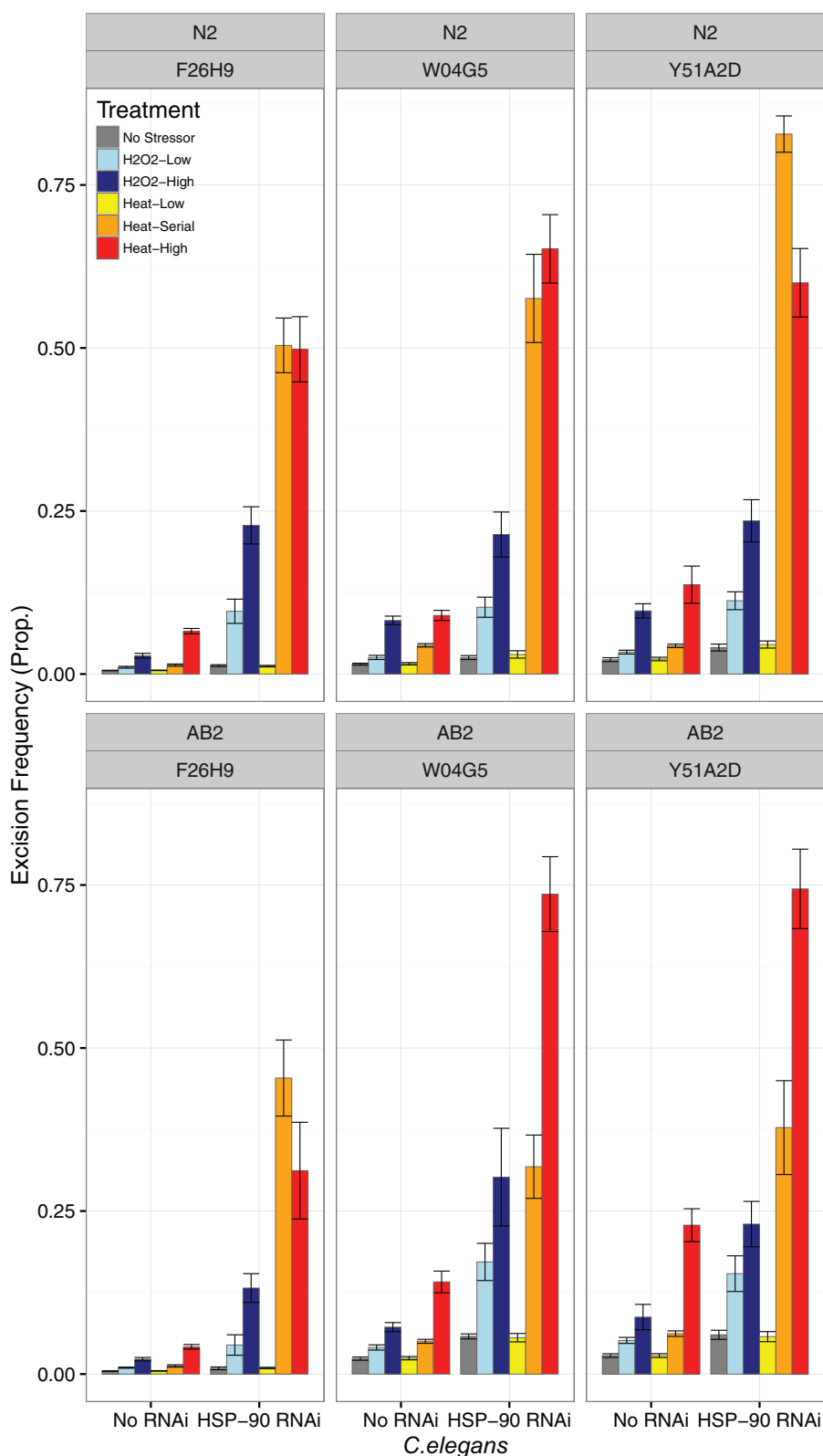


Fig. 2.—Excision frequency for *CemaT1* transposons at three loci (F26H9, W04G5, Y51A2D) for two strains of *C. elegans* (N2, AB2) in response to five different conditions of environmental stress, plus no stress controls. Excision frequency given as the proportion of excision footprints to the number of nonexcised transposons at a given locus. Bars show proportions of excision for Low- and High-oxidative stress, Low (35 °C for 1 h), Serial (five serial exposures of 35 °C for 1 h, 30 min at 20 °C) and High (39 °C for 2 h) heat exposures, and No Stress controls.

supplementary table S3, Supplementary Material online). Hsp90 RNAi also increased the excision frequency for *Tc1* overall ($F_{1,345}=35.95$, $P < 0.001$; supplementary fig. S2, Supplementary Material online), but only amplified the effects of stress treatment on excision frequency for H₂O₂-High and Heat-High treatments ($P < 0.05$ for both). For *Tc1*, RNAi-induced knockdown of Hsp90 had no effect on excision frequency for No Stress controls ($P = 0.303$; see supplementary table S3, Supplementary Material online), indicating that environmental stress is required to induce the effect of RNAi. QRT-PCR analyses confirmed that RNAi was effective, even under stress conditions; control, unstressed worms and stress-treated worms alike showed more than 70% knockdown of Hsp90 transcripts (see supplementary table S7, Supplementary Material online). The combined effect of the environmental stress treatment and RNAi-mediated knockdown of Hsp90 increased excision frequency between 2- and 9-fold for *Tc1* and between 7- and 41-fold for *CemaT1*.

CbmaT1 and Tcb1 Excision Frequencies in *C. briggsae*

The genome of *C. briggsae* has autonomous transposons related to *Tc1* and *CemaT1*, *Tcb1*, and *CbmaT1*, which have been previously shown to be active (Brownlie et al. 2005). As in *C. elegans*, there were significant differences in excision frequency for *CbmaT1* and *Tcb1* between loci ($F_{2,272}=541.37$, $P < 0.001$ and $F_{2,271}=1095.25$, $P < 0.001$, respectively; see supplementary table S4, Supplementary Material online). Excision frequencies at CB015K23 and CB046A04 were lower than at the CBRG21D19 locus for *CbmaT1* ($P < 0.02$ for both), and C007801047.Contig1 and C012001013.Contig3 were significantly lower than the C009001188.Contig2 locus for *Tcb1* ($P < 0.001$ for both). Compared with the AF16 strain, there was an increase in excision frequency for the DH1300 strain for *CbmaT1* ($F_{1,272}=51.52$, $P < 0.001$), and significant locus by strain interactions for both *CbmaT1* and *Tcb1* ($F_{2,272}=300.54$, $P < 0.001$ and $F_{2,271}=33.80$, $P < 0.001$, respectively; see supplementary table S4, Supplementary Material online). Stress treatment in general affected excision frequencies for both *CbmaT1* and *Tcb1* ($F_{2,272}=305.10$, $P < 0.001$ and $F_{2,271}=118.05$, $P < 0.001$, respectively; supplementary figs. S3 and S4, Supplementary Material online). More specifically, all stress treatments except the Heat-Low treatment increased excision frequency of *CbmaT1* and *Tcb1* ($P < 0.001$ for all except Heat-Low; see supplementary table S4, Supplementary Material online). QRT-PCR analyses confirmed that all stress treatments caused significant ($P < 0.05$), approximately 2-fold increases in levels of *Hsp90* mRNA in both *C. briggsae* strains (AF16, DH1300) relative to no treatment controls (see supplementary table S6, Supplementary Material online).

RNA-mediated knockdown of Hsp90 increased overall excision frequencies of *CbmaT1* ($F_{1,272}=63.84$, $P < 0.001$;

supplementary fig. S3, Supplementary Material online) and *Tcb1* ($F_{1,272}=26.80$, $P < 0.001$; supplementary fig. S4, Supplementary Material online). However, as with the *Tc1* transposon, Hsp90 RNAi had no independent effect on excision frequency for No Stress controls for either *CbmaT1* or *Tcb1* ($P = 0.290$ and $P = 0.437$, respectively; see supplementary table S4, Supplementary Material online), indicating that environmental stress is necessary to induce the effect of RNAi. For both *C. briggsae* transposons, RNAi amplified the effect of stress treatment overall (RNAi \times Treatment: $F_{5,272}=6.65$, $P < 0.001$ and $F_{5,271}=2.65$, $P = 0.023$). However, this effect was limited to Heat-High and H₂O₂-High treatments, where excision frequencies were significantly higher for both *CbmaT1* and *Tcb1* when combined with RNAi-induced knockdown of Hsp90 than for these treatments alone ($P < 0.02$ for all; see supplementary table S4, Supplementary Material online). QRT-PCR analyses confirmed that RNAi was effective, even under stress conditions; control, unstressed worms and stress-treated worms alike showed more than 70% knockdown of Hsp90 transcripts (see supplementary table S7, Supplementary Material online). The combined effects of stress treatment and RNAi increased excision frequencies from 2- to 5-fold in *Tcb1*, and 2- to 9-fold in *CbmaT1*.

unc-22 Mutation Frequency

Spontaneous *unc-22* mutations in *C. elegans* and *C. briggsae* appear to be rare events, based on the frequencies observed in nonstressed, non-RNAi treated worms, but the frequencies observed in this study are comparable to those reported in other studies (Moerman and Waterston 1984; Brownlie et al. 2005). In *C. briggsae*, where we examined two strains, we observed mutation frequencies that mirrored excision frequency, with higher overall mutation frequency in the DH1300 compared with AF16 strain ($\chi^2_1=20.87$, $P < 0.001$; fig. 3). Similar to the observed excision frequencies in *C. elegans* and *C. briggsae*, mutation frequency in *unc-22* generally increased in response to RNAi ($\chi^2_1=143.23$, $P < 0.001$ and $\chi^2_1=156.70$, $P < 0.001$), and stress treatment ($\chi^2_5=497.35$, $P < 0.001$ and $\chi^2_5=836.28$, $P < 0.001$, respectively; fig. 3). In the absence of RNAi, mutation frequency increased for all but the Heat-Low and H₂O₂ treatments in *C. elegans* ($P < 0.002$ for Heat-High, Heat-Serial, and H₂O₂-High), whereas in *C. briggsae* all but the Heat-Low treatment increased mutation frequency ($P < 0.006$ for all except Heat-Low).

In the absence of stress treatment, RNAi-induced knockdown of Hsp90 did not significantly increase mutation frequency in either *C. elegans* or *C. briggsae* ($P = 0.604$ and $P = 0.721$ for No Stress controls). However, for both species, the effect of treatment was amplified by RNAi-induced knockdown of Hsp90 (RNAi \times Treatment interaction: $\chi^2_5=33.52$, $P < 0.001$ and $\chi^2_5=33.18$, $P < 0.001$ for *C. elegans* and *C. briggsae*, respectively). RNAi increased mutation frequency in response to stress treatments for all except the Heat-Low

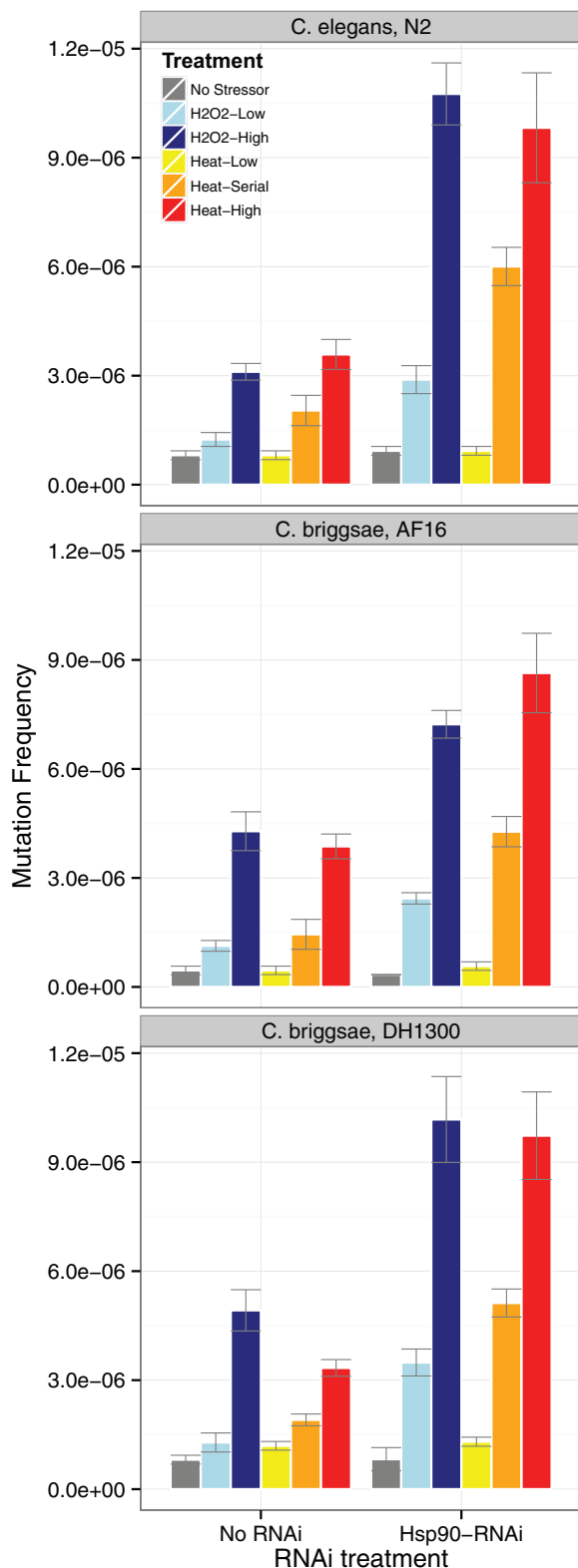


Fig. 3.—Proportion of plates with one or more mutants at the *unc-22* gene following exposure to one of the five environmental stresses or No Stress controls, with and without RNAi-induced knockdown of Hsp90. Experiments were carried out with one strain of *C. elegans* (N2) and

treatment in both *C. elegans* ($P < 0.005$ for all except H2O2-Low, where $P = 0.050$), and *C. briggsae* ($P < 0.003$ for all). The combined effect of stress treatment and RNAi-induced knockdown of Hsp90 increased *unc-22* mutation frequency by 3- to 20-fold for *C. elegans*, and 5- to 23-fold for *C. briggsae*. Incidence rate estimates for all parameters are provided in [supplementary table S7, Supplementary Material](#) online.

The mutant phenotypes observed in the worms were indistinguishable from *C. elegans unc-22* mutants, with uncoordinated movements and persistent body twitching when exposed to nicotine. A minimum of five putative *unc-22* worms were isolated from any positive (mutant) plate, and all showed segregation of mutant phenotypes in their progeny, indicating that the mutations were heritable. A total of 8 *Tc1* and 3 *CemaT1* insertions were detected in the induced putative *unc-22* mutant progeny of the oxidative and heat stressed worms (fig. 4). In *C. briggsae*, 4 *Tcb1*, and 2 *CbmaT1* insertions were detected (fig. 4). Both transposons target TA dinucleotides to insert, and consequently, there were some loci that independently had either transposon integrated. There were not enough insertions observed to confirm that some of these insertion sites are transposon insertion hotspots, although several insertion sites also showed insertion of the same transposon in independently derived worms, which suggests that some TA loci may be favored more than others.

Discussion

In this study, we demonstrate that moderate environmental stress can increase the excision and insertion frequency of two Class II DNA transposons, and that these effects can be amplified through RNAi-mediated knockdown of Hsp90. Our findings support a role for Hsp90 in the suppression of transposon mobility, and suggest a functional constraint on Hsp90 owing to its dual role in promoting protein homeostasis and genetic stability. Under conditions of stress, Hsp90 may prioritize cellular physiological functioning and protein homeostasis, but at the cost of failing to protect the genome from the mutagenic activity of transposable elements. The heritable nature of the TE-induced genetic changes that we observed provides additional support – and a molecular mechanism – for the suggestion that Hsp90 may serve as a capacitor for evolutionary change (Queitsch et al. 2002; Rutherford 2003; Rohner et al. 2013; Peuß et al. 2015).

Each of the environmental stress treatments used in this study—except the low, short-duration heat exposure—in-

Fig. 3.—Continued

two strains of *C. briggsae* (AF16 and DH1300). Mutants were detected by observing the “twitching” phenotype in response exposure to 1% nicotine solution, with proportions based on the average of three replicates comparing between 20 and 30 plates per replicate (2717 plates in total).

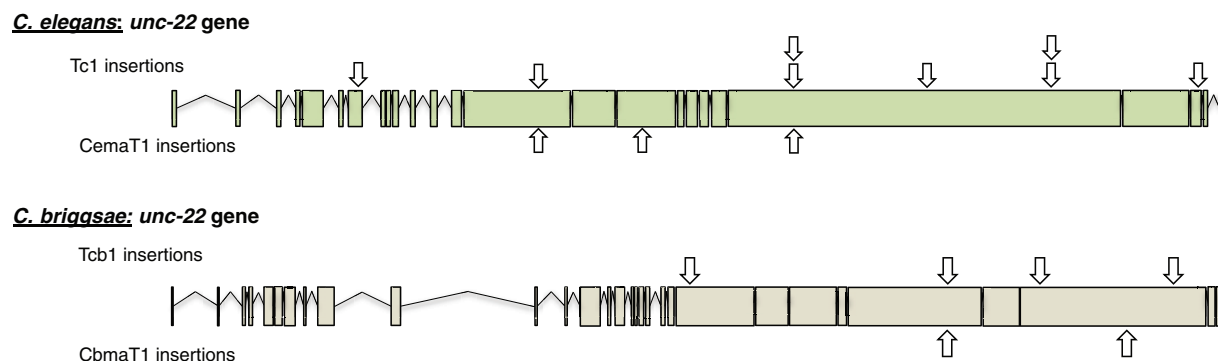


Fig. 4.—Transposable element (TE) insertion sites within the *unc-22* gene in *Caenorhabditis elegans* (top) and *C. briggsae* (bottom) subjected to environmental stress. Arrows above the gene indicate insertions of Tc1 (*C. elegans*) or Tcb1 (*C. briggsae*) TEs; arrows below indicate CemaT1 and CbmaT1 insertions. Locations with stacked arrows are loci where multiple independent TE insertions were detected.

creased transposon excision frequency in an exposure-dependent manner. Excision frequency increased in response to the stress treatments, despite concurrent up-regulation of *daf-21* (Hsp90 gene) transcription. The level of *daf-21* transcripts was enhanced in all stress treatments, including the low, short duration heat exposure, but the level of these transcripts did not increase significantly with the more intense stresses. As the number of cellular targets of Hsp90 increases during stress (Sangster et al. 2004; Jarosz and Lindquist 2010; Hartson and Matts 2012), the pool of available and newly synthesized Hsp90 was likely insufficient to suppress TE activity adequately. RNAi-mediated knock-down of *daf-21* transcripts also increased TE excision frequency in some, but not all, nonstressed nematodes, suggesting that Hsp90 may in some cases be limiting in both the presence and absence of stress.

The effects of heat/oxidative stress treatment and RNAi-mediated knockdown of Hsp90 on TE mobility could occur through several pathways, including either the disruption of the proteins associated with DNA maintenance, repair, or chromatin packaging and/or activation of the PIWI/piRNA pathway. We observed differences in TE excision frequency in response to stress treatment and RNAi-mediated knockdown of Hsp90 between both species and strains. More interestingly, we also observed differences in excision frequency across loci within each species and strain. These genomically localized differences in TE mobility suggest that suppression of TE activity is dependent on many factors, including DNA secondary structure, and the availability of Hsp90 and the proteins with which it interacts.

The suppression of TEs through the PIWI/piRNA pathway has been established in a number of species, including *C. elegans* (Ketting et al. 1999; Tabara et al. 1999; Das et al. 2008). Mutations in proteins in the PIWI/piRNA pathway have been associated with increases in TE transcription, although the specific mechanisms for TE suppression appear to differ across taxa (Mani and Juliano 2013). In contrast to *Drosophila*,

where piRNA biogenesis is thought to involve “ping-pong” amplification of piRNAs that then target TE transcripts for destruction, TE silencing in *C. elegans* seems to be mediated through piRNAs that facilitate the production of small-interfering RNAs that then target TE transcripts (Das et al. 2008). In *Drosophila*, the formation of the PIWI/piRNA complex appears to require the cochaperone *Shu*, as well as Hsp90 (Olivieri et al. 2012; Izumi et al. 2013; Izumi and Tomari 2014). Despite the differences in how piRNAs are produced and how they may mediate TE suppression in flies and nematodes (Gillan and Devaney 2014), our study provides evidence that Hsp90 may indeed share a similar role in nematodes to reduce TE activity. In *Drosophila*, where the PIWI/piRNA pathway in TE suppression has been investigated, *hsp83^{scratch}* mutants with reduced Hsp90 function exhibited an impaired ability to produce several piRNAs essential for the suppression of repetitive *Stellate* sequences and nearby transposons (Tritto et al. 2003; Specchia et al. 2010). The majority of these transposons were of the Class I (copy and paste) variety, including one *I* element-like insertion responsible for a mutation in the *noc* gene (Specchia et al. 2010).

In our study, we observed that RNAi-mediated knockdown of Hsp90 enhanced Class II (cut and paste) transposon activity. This increased TE activity was associated with increased TE-induced mutagenesis at the *unc-22* reporter gene. Our findings thus extend the work of Specchia et al. (2010) to the phylum Nematoda and to Class II (cut and paste) transposons, and demonstrate a direct link between Hsp90 and both TE excision and insertion frequencies. Importantly, we have shown that moderate environmental stress can have similar effects on transposon insertion frequency and mutagenesis as Hsp90 suppression using pharmacological means (i.e., geldanamycin) or using mutant strains. This supports the hypothesis that naturally occurring, stress-induced TE mobility and insertion mutagenesis could serve to radically reorganize host—or germline—genomes at precisely the time when environmental conditions threaten to overwhelm host’s capacity to buffer

them (McClintock 1984). Such rapid and widespread genomic reorganization in response to environmental or physiological extremes could have important implications for evolution and speciation (Zeh et al. 2009; Piacentini et al. 2014), particularly in light of the fact that all of the mutations observed in our stressed nematodes were heritable.

In nematodes the impacts of TE-activity can be heritable (Bégin and Schoen 2007), and tied to an increase in frequency of mildly deleterious mutations with effects on fecundity, longevity, and body size (Bégin and Schoen 2006). Poor genetic quality has also been associated with declines in phenotypic quality in *Drosophila* (Sharp and Agrawal 2012, 2016), which itself contributes to increased mutation rates over time (so-called “mutational meltdown”; Sharp and Agrawal 2012). Stress arising from mismatches with novel environments could similarly affect phenotypic quality and mutation rate, increasing standing genetic variation and providing the substrate for accelerated adaptation. Thus, depending on the relationship between genetic load, mutation rate, and fitness, stress-induced TE-activity could lead to either rapid extinction or adaptation to novel environments (Shaw and Baer 2011). Our study provides insights into several mechanisms by which stress could impact genetic change and population evolution. In addition to its role in protein homeostasis and the regulation of TE mobility, Hsp90 is considered an important facilitator of DNA repair, and numerous proteins associated with DNA double strand break repair machinery are client proteins of Hsp90 (Pennisi et al. 2015). Under conditions of stress, it is conceivable that Hsp90’s role in regulating genomic stability could be compromised at both the level of TE regulation and in managing general DNA repair. Both of the above pathways point to Hsp90 as a nexus for genetic change arising from physiological and environmental stress.

Bégin and Schoen (2006) observed that while transposition frequencies can vary in different strains of *C. elegans*, most transposon activity does not produce readily detectable phenotypes. The *unc-22* gene is one of the rare sites where a transposition-induced phenotype is easily observable, and our finding that multiple TEs inserted in the same locations within the *unc-22* gene in both nematode species suggests that transposon insertions are not entirely random and may be indicative of TE insertion “hot-spots” (Hackett et al. 2007). Although our experimental design limits our ability to comment on TE insertion patterns or frequency elsewhere in the genome, the number of such insertions no doubt far exceeds those detected in the *unc-22* reporter gene, providing a potentially important source of genetic variation. Moreover, a number of factors are thought to contribute to biases in TE insertion sites, including local DNA sequence and structure (Yant et al. 2005). Intriguingly, regions that are relatively highly expressed may also be biased towards TE or retroviral integration (Wu et al. 2003; Campos-Sanchez et al. 2014). Therefore, to the extent that expression levels increase TE

insertion probability, actively expressed genes responding to an environmental stressor may also provide biased targets for mutational change. Whether selection has favored TE insertion biases in transcriptionally active regions—or whether they arise incidental to the biochemical underpinnings of TE mobility, or from functional constraints such as those we have described for Hsp90—is not yet clear (Capy et al. 2000). However, Hsp90 expression levels appear to be restricted by the requirement for cellular buffering and protein homeostasis on one hand, and cytotoxic effects of Hsp90 accumulation on the other (Krebs and Loeschcke 1994; Feder and Hofmann 1999; Casanueva et al. 2012). By limiting peak Hsp90 expression levels, Hsp90 cytotoxicity would limit the buffering capacity of Hsp90 within the cell, giving rise to the functional constraints between protein homeostasis and genetic buffering proposed here.

We have shown that moderate environmental stress—and the reduction of Hsp90 using RNAi—has the capacity to increase the excision and insertion frequencies of two Class II DNA transposons at multiple loci in multiple strains of two different species of nematode. Differences in excision frequency between loci suggest a role for Hsp90 independent of protein buffering of DNA regulatory machinery. More empirical studies examining the role of Hsp90 and the PIWI/piRNA pathway in TE suppression are crucial to understanding the dynamic regulation of transposable elements as a dangerous, but potentially powerful force for evolutionary change.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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