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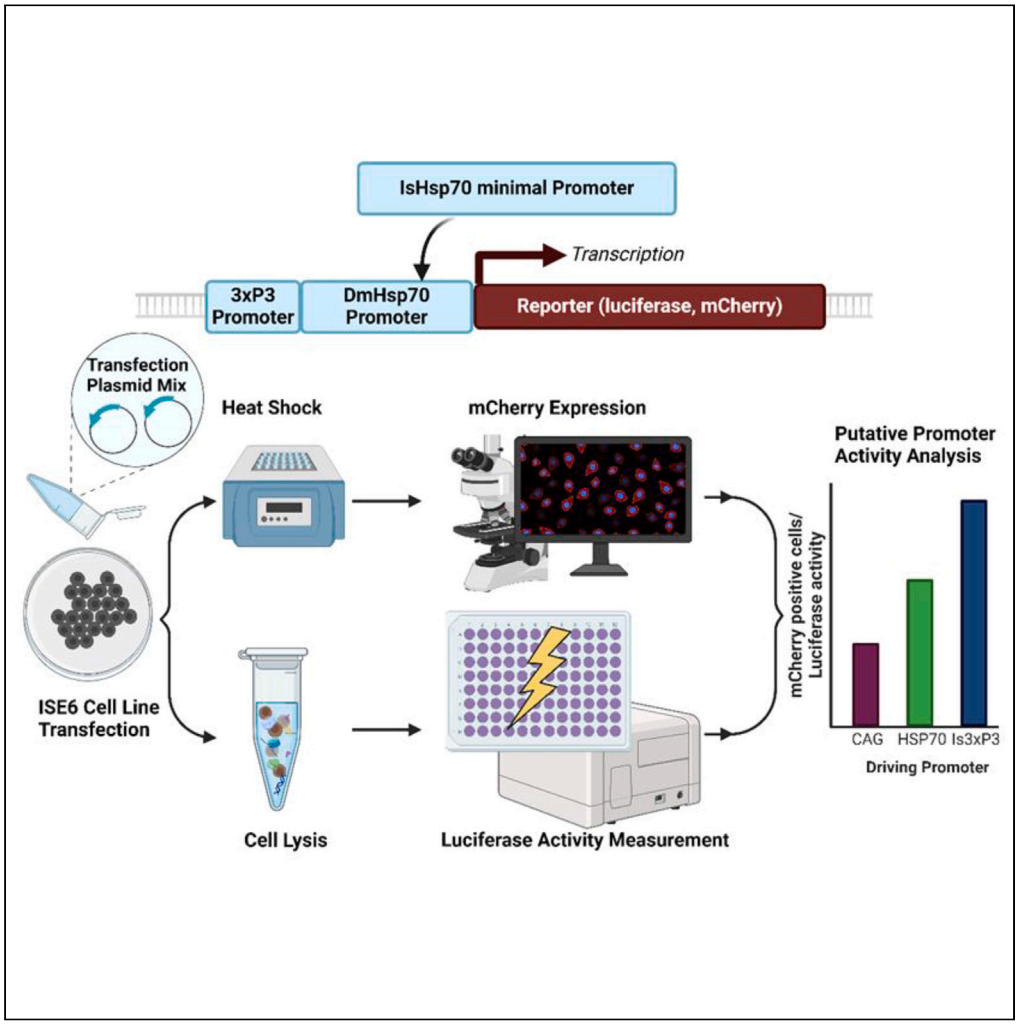

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Highlights

Two heat-inducible promoters functional in *Ixodes scapularis* cells

A tick-specific 3xP3 synthetic promoter was developed

Inducible HSP70 and tick-specific 3xP3 promoters developed

Temporal gene expression now possible

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Validation of heat-inducible *Ixodes scapularis* HSP70 and tick-specific 3xP3 promoters in ISE6 cells

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SUMMARY

Ixodes scapularis is an important vector of many pathogens, including the causative agent of Lyme disease. The gene function studies in _I. scapularis_ and other ticks are hampered by the lack of genetic tools, including an inducible promoter for temporal control over transgene-encoding protein or double-stranded RNA. We characterized an intergenic sequence upstream of a heat shock protein 70 (HSP70) gene that can drive _Renilla_ luciferase and mCherry expression in the _I. scapularis_ cell line ISE6 (IsHSP70). In another construct, we replaced the _Drosophila melanogaster_ minimal HSP70 promoter of the 3xP3 promoter with a minimal portion of IsHSP70 promoter and generated an _I. scapularis_-specific 3xP3 (Is3xP3) promoter. Both IsHSP70 and Is3xP3 have a heat-inducible expression of mCherry fluorescence in ISE6 cells with an approximately 10-fold increase in the percentage of fluorescent cells upon 2 h heat shock. These promoters described will be valuable tools for gene function studies.

INTRODUCTION

Ticks are obligate hematophagous parasites and are important vectors of a wide variety of pathogens.[1] Lyme disease (LD), caused by the spirochete *Borrelia burgdorferi* and vectored by the black-legged tick, *Ixodes scapularis*, is the most prevalent vector-borne disease in the United States. The Centers for Disease Control and Prevention estimates 476,000 cases of LD every year.[2] Despite their importance, our knowledge of the biology of ticks on a molecular level is limited. Advances in tick genomics and genetics have mainly been stymied by a lack of molecular tools for forward genetics. This is in contrast to insects for which numerous transgenic development and gene editing tools are available. CRISPR-Cas9 (CRISPR/CRISPR-associated protein 9) is revolutionizing genome editing in non-model organisms. We have made significant progress in optimizing tick embryo injections and developing CRISPR gene knockout strategies in ticks[3]; however, knockin through homologous-dependent repair depends on developing reporter constructs using specific promoters. Promoters from *Drosophila melanogaster* genes such as polyubiquitin,[4] Actin5C,[5] HSP70,[6] and the artificial 3xP3 promoter[7] have been successfully used to express marker genes through transposase or CRISPR-mediated knockin in a wide variety of insects and other organisms.[7–9] While these exogenous promoters have proven to be widely applicable, they are not universal, and our previous experiments showed that they were not functional in ticks.

Because of the unavailability of promoters and the lack of application of exogenous promoters, there is an urgent need to identify endogenous and non-endogenous promoters that may function across multiple tick species. Several tick endogenous promoters such as *Haemaphysalis longicornis* ferritin (HlFerritin), HlActin, *I. scapularis* ribosomal protein L4 (Isrpl4), *Rhipicephalus* (*boophilus*) *microplus* rpl4, Rm-EF-1α, Is-microsomal glutathione S-transferase, Is-ribosomal protein S24, and RmPyrethroid-metabolizing esterase gene[10–15] as well as non-endogenous promoters (human phosphoglycerate kinase, CAG [a synthetic promoter consisting of "C" (cytomegalovirus,CMV, early enhancer element), "A" (the promoter, the first exon, and the first intron of chicken beta-actin gene), and "G" (the splice acceptor of the rabbit beta-globin gene)], CMV, polyhedrin promoter, and cauliflower mosaic virus)[10,13–18] have been demonstrated to be functional in tick cell lines; however, only one (HlFerritin) is inducible (with ferrous sulfate). None of the endogenous tick promoters are available to other researchers. Identifying effective and consistent inducible promoter or enhancer sequences for use in driving transient transgene expression would broaden the types of experiments that could be performed using tick cell lines and permitting temporal control of gene expression. Therefore, we aimed to identify tick-specific promoters that could be beneficial for gene expression, RNAi, or knockin studies. Here, we

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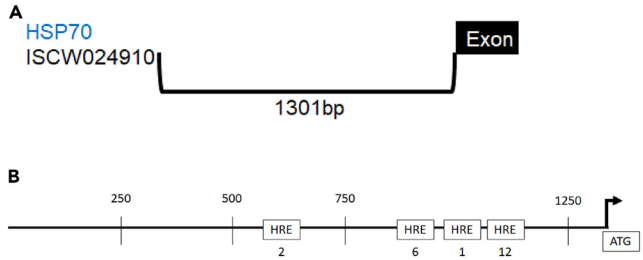

Figure 1. Gene map of the HSP70 (ISCW024910/ISCGN274230)

(A) The gene only has one exon. Upstream region including the entire 5′ UTR was cloned into the pGL-4.79 [Rluc] vector.

(B) Gene map of the cloned putative promoter region of HSP70 (1,301 base pairs in size) with heat response element locations marked. Numbers below HRE locations indicate the numbers present at that location. (HRE, heat response element).

demonstrate functional and heat-inducible *I. scapularis* heat shock protein 70 (IsHSP70) promoter and an artificial *I. scapularis*-specific 3xP3 promoter under the control of a minimal IsHSP70 core promoter (Is3xP3).

RESULTS

HSP70 expression

The HSP70 gene is expressed ubiquitously in tick tissues and eggs. Our reverse-transcription PCR data showed expression in salivary glands, synganglion, ovary, and eggs. Midgut tissue did not have any expression; however, that is likely due to the poor quality of cDNA because the housekeeping tubulin gene (+ve control) also did not amplify in midgut cDNA (Figure S1, related to Figure 1).

HSP70 promoter

Following a previously published strategy,[19–21] we identified the HSP70 genes in the fruit fly, *Drosophila melanogaster*, and mosquito, *Aedes aegypti*, that are ubiquitously expressed across tissues and life stages (Uniprot.org). Using blastp, we identified a putative ortholog of HSP70: ISCW024910 in the *I. scapularis Wikel* genome with greater than 70% amino acid sequence identity and consisted of a single exon. We confirmed this sequence with our highly contiguous genome assembly.[22] We located an intergenic sequence 5′ from the coding region of these genes and confirmed potential promoter function by bioinformatics analysis (data not shown)[23,24] (Figure 1). We also confirmed the heat response element sequences (IsHSP70) and arthropod initiation factor motifs[25] (Table 1 and Figure 1).

The sequence upstream of the predicted IsHSP70 gene was searched to identify putative heat regulatory element (HRE) sequences (sequence motifs listed in Table 1).[20,26] HREs are regions where heat shock factors bind and activate HSP70 transcription and regulate the HSP70 gene, and thus, an organism's response to heat stress is directly affected by HREs. These HRE sequence motifs are conserved across many species and if clustered, can act cooperatively.[27,28] Based on the conserved HRE sequence motifs, we identified 21 potential HREs in a single general area 200–700 bp 5′ from the start of the HSP70 coding region in *I. scapularis* (Figure 1). We synthesized the upstream sequence incorporating the entire 5′ UTR up to the start codon (1,301 bp) and including the added restriction enzyme cloning sites, it resulted in a 1,315 bp fragment.

To test the promoter function, we transfected the HSP70 endogenous promoter construct with *Renilla* luciferase into pGL-Rluc into ISE6 cells, an *I. scapularis* embryonic cell line. The cells were lysed 9 days after transfection and analyzed for luciferase activity. The initial results indicate that HSP70 is active (Figure S2, and Table 1, related to Figure 2). These experiments were conducted only once, and samples were read at four time points.

3xP3 promoter

The 3xP3 promoter is a synthetic promoter that contains three Pax6 transcriptional activator homodimer-binding sites. Multiple experiments with 3xP3-driven fluorescent constructs in ISE6 cells failed to show any fluorescence (data not shown), suggesting it is not functional in tick

Table 1. Heat response element motif types and number in *Ixodes scapularis* HSP70 gene

HRE motif type	Number of HREs in IsHSP70
2P tail-tail (nTTCn-nGAAn-nnnnn)	8
2P head-head (nGAAn-nTTCn-nnnnn)	4
Step/gap type (nTTCn-nnnnn-nTTCn)	4
4P type (nTTCnnGAAnnTTCnnGAAn)	1
3P type (nTTCnnGAAnnTTCn)	3
Gap type (nTTCnnGAAnnnnnnnGAAn)	1

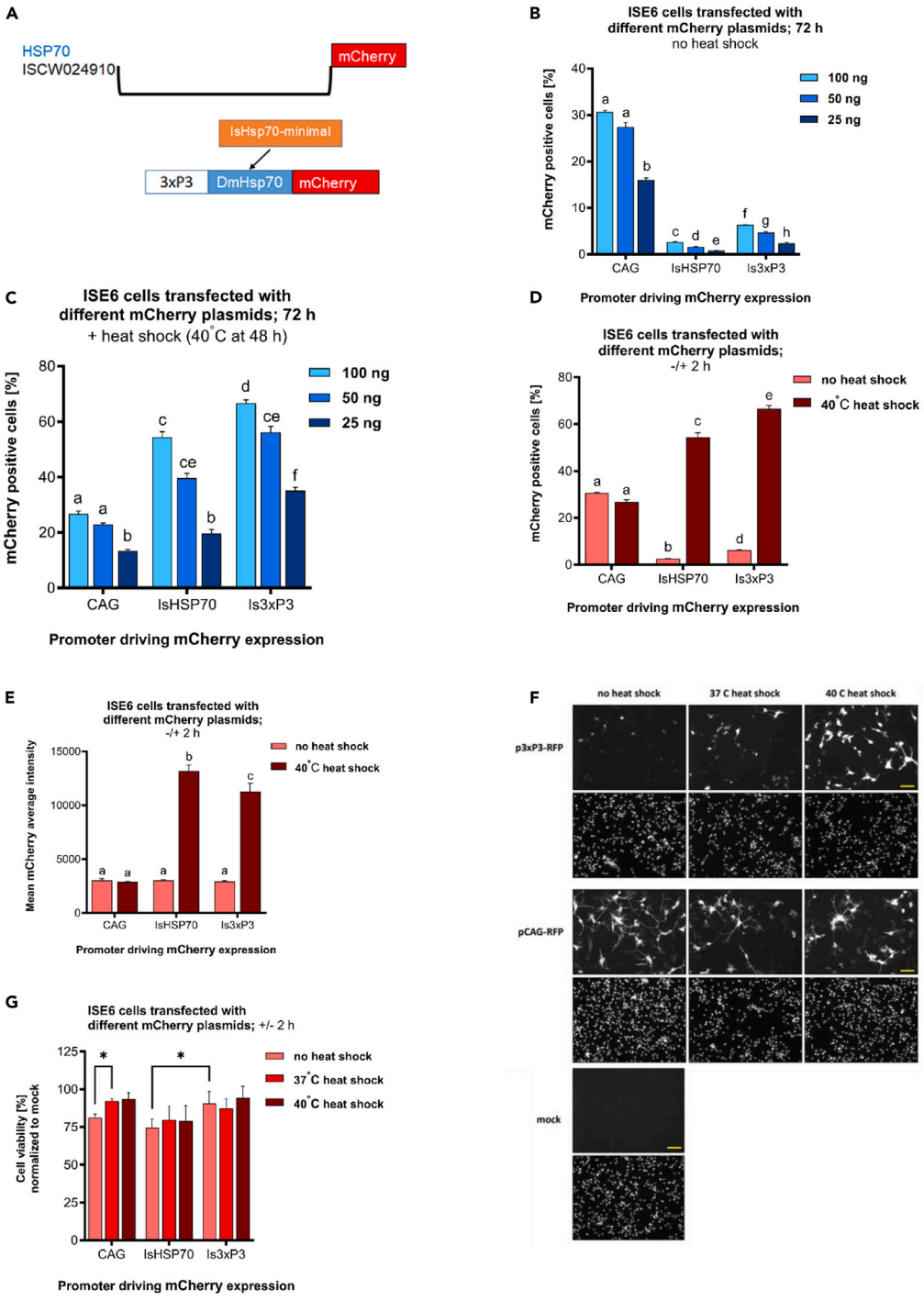

Figure 2. Is3xP3 map and promoter expression

(A) A gene map of swapped minimal *Drosophila* HSP70 (123 bp) with the *I. scapularis* HSP70 (453 bp).

(B) ISE6 cells transfected with 25, 50, or 100 ng of CAG-mCherry, IsHSP70-mcherry, and Is3xP3-mCherry constructs. Cells were fixed and imaged 72 h post-transfection.

(C) ISE6 cells transfected with 25, 50, or 100 ng of CAG-mCherry, IsHSP70-mCherry, and Is3xP3-mCherry constructs. Transfected cells were subjected to a 2 h heat shock (40°C) at 48 h post-transfection before being fixed 24 h later (72 h post-transfection) and imaged.

(D) Percentage of fluorescent-positive ISE6 cells transfected with 50 ng of CAG-, IsHSP70-, and Is3xP3-mCherry constructs. Cells were cultured at 32°C (non-heat shock) or subjected for 2 h to 40°C (heat shock) at 48 h post-transfection. Cells were then fixed and imaged 24 h later (72 h post-transfection).

Figure 2. *Continued*

(E) Mean fluorescent intensity of ISE6 cells transfected in D.

(F) ISE6 cells were transfected with Is3xP3-mCherry and CAG-mCherry, subjected at 48 h post-transfection to a 2 h heat shock at either 37°C or 40°C and imaged 24 h later (72 h post-transfection). Top panel: images showing RFP expression in transfected cells, Bottom panel: cell nuclei stained with Hoechst. Images were taken with a 20× objective of a Nikon Eclipse TE300 microscope. Non-heat shock-treated cells transfected similarly were used as control. Cells treated with transfection reagents without the plasmids are shown as mock. Scale bar, 25 μM.

(G) Cell viability based on the number of nuclei of ISE6 cells transfected in F. Data are represented as mean ± SE, $n = 3$. Bars with different letters above them are significantly different from each other. The alphabet or asterisk above bars represents statistical significance $p < 0.05$. ANOVA table with F (DFn, DFd) and p values and Tukey's multiple comparison test data are provided in Table S2.

cells. The 3xP3 used in these prior experiments contained a *Drosophila* HSP70 minimal promoter.[7,29] We swapped the minimal *Drosophila* HSP70 (123 bp) with part of the IsHSP70 (453 bp) from our confirmed functional construct (Figure 2A).

To test whether 3xP3 was functional, we transfected *I. scapularis*-specific 3xP3 (Is3xP3)-mCherry, IsHSP70-mCherry, and CAG-mCherry (positive control) at increasing concentrations (25, 50, and 100 ng/well) into ISE6 cells. We observed fluorescence comparable to the HSP70 promoter, with approximately 5% fluorescent-positive cells. However, the fluorescent signal was still lower compared to the CAG-mCherry construct (Figure 2B). We then conducted heat shock experiments with IsHSP70-mCherry and Is3xP3-mCherry by similarly transfecting ISE6 cells with increasing concentrations of plasmid DNA (25, 50, or 100 ng/well) followed by a 2 h heat shock at 40°C after 48 h. While the control cells cultured continuously at 32°C (non-heat shock conditions) were less than 5% fluorescent positive (Figure 2B), cells that were subjected to a heat shock showed an approximately 10-fold increase in fluorescent-positive cells with both IsHSP70 and Is3xP3 (Figure 2C).

Using an intermediate concentration of plasmid DNA (50 ng/well), we tested if the changes in the percentage of fluorescent cells were accompanied by an increase in fluorescent intensity. An increase of approximately 10-fold in the percentage of fluorescent-positive cells (Figure 2D), which was accompanied by an approximately 4-fold increase in average fluorescence intensity, was recorded (Figure 2E). Cell viability based on the number of cells/well was unaffected when comparing heat-shocked and non-heat-shocked cells both qualitatively (Figure 2F) and quantitatively (Figure 2G). We also electroporated Is3xP3-mCherry into tick larvae. However, we only observed one larva with fluorescent expression in tissues that appeared to be the upper alimentary canal (Figure S3, related to Figure 2).

DISCUSSION

Here, we have shown two heat-inducible promoters functional in ISE6 cells: *I. scapularis* endogenous HSP70 (IsHSP70) and an *I. scapularis*-specific artificial 3xP3 promoter (Is3xP3). Under non-heat shock conditions, IsHSP70 and Is3xP3 yield low percentages of fluorescent-positive cells (~5%). In contrast, under heat shock conditions such as 40°C, the percentage of fluorescent-positive cells increases significantly by approximately 10-fold. These data suggest that nearly 10 times the basal percentage of cells are successfully transfected with both promoter constructs but do not sufficiently express mCherry protein to be detected. Our data also demonstrate that IsHSP70 and Is3xP3 have low basal activity under non-heat shock conditions, which readily increases during a heat shock response.

The artificial 3xP3 promoter, tested originally in *D. melanogaster*, contains three binding sites for Pax6/eyeless homodimers upstream to a TATA box. The Pax6 is an evolutionarily highly conserved system described as the master regulator of eye development throughout the animal kingdom,[30] which is consistent with the broad function of 3xP3[7,31] as a promoter for fluorescent protein genes. The 3xP3 promoter has been successfully used as an adult eye and ocelli marker for transgenesis in *Drosophila*, houseflies, beetles, butterflies, mosquitoes, as well as flatworms.[8,29,32–36] Although the artificial 3xP3 promoter is widely used, it is not universal and is non-functional in organisms such as the tephritid fly[37] and has potentially weaker functionality in horn flies.[38] Similarly, our previous work showed that 3xP3 is non-functional in ticks. Therefore, we hypothesized that replacing the *Drosophila* HSP70 minimal promoter in 3xP3 with an endogenous tick IsHSP70 might overcome the functionality issues. The *Drosophila* HSP70 minimal promoter is 123 bp from the TATA box to the start codon and does not include any HRE sites, whereas the *I. scapularis* HSP70 minimal promoter is 453 bp and contains several HREs, and this difference might have resulted in a functional promoter. Although *I. scapularis* is an eyeless tick, Pax6 (which binds to 3xP3) is expressed in ISE6 cells, *I. scapularis* ticks,[1] as well as ticks such as *Rhipicephalus sanguineus* (XM_037656705.1/XP_037512638),[39] *H. longicornis*, and *Dermacentor silvarum* (XM_037709500I/XP_037565428) with fully formed eyes.[40]

In addition to eyes, 3xP3 has been shown to promote expression in the larval nervous system, which has been highly useful in identifying silk moth transformants.[41] In mosquito *A. aegypti*, the 3xP3 promoter expresses in neural tissues and anal glands/digestive tract in addition to eyes.[33,42] In one *D. suzukii* transgenic line, 3xP3 is expressed in the abdominal region.[43] In the marine crustacean, *Parhyale hawaiensis*, expression is not in the eyes but in cells at the posterior of the brain.[36,44] Therefore, Is3xP3 could drive the expression of transgenes in other tissues such as neural or digestive tract tissue. The experiments to test Is3xP3 expression in tick tissues are underway. It has been noted that tissue expression with 3xP3 is potentially different between injected G0 individuals and later transgenic G1 individuals,[45] which may be the case for ticks as well. Heat-inducible promoters are useful in controlling gene expression with applications including transgenesis and genome editing by allowing researchers to have temporal control of transposable elements and Cas9 in CRISPR expression systems. We expect that the potential localized tissue expression of Is3xP3, similar to insect-based 3xP3, would be helpful for transgenic tick applications.

Limitations of the study

The Renilla luciferase assay was conducted once with four technical replicates that were read at different time points. However, this assay was performed with a large volume of cells. Cells were seeded into 12.5 cm^2 flasks at 2 × 106 cells/mL, much higher than the 96-well plate assay (5×10^5 cells/mL). The Renilla luciferase and mCherry promoter constructs are the same; the only difference is the reporter.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Transfection
  - Larval electroporation
  - RNA extraction, cDNA synthesis, and RT-PCR
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110468.

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AUTHOR CONTRIBUTIONS

M.G.-N. and M.P. conceptualized the study and designed the experiments M.G.-N. supervised the research. M.P., R.C., O.G.-C., and S.A. performed the molecular cloning. H.-H.H. conducted the cell culture experiments including the heat shock treatments and fluorescence analysis. M.P. was responsible for the bioinformatics analysis and promoter characterization. T.J.K. conducted the luciferase assays. M.P., M.G.-N., and H.-H.H. interpreted data and S.A. conducted statistical analysis. M.P. and H.-H.H. drafted the manuscript with input from all authors. M.G.-N. and M.P. wrote the final draft. All authors reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Renilla Luciferase Assay System	Promega	E2810
Experimental models: Cell lines		
ISE6 cells	BEI Resources/Uli Munderloh	NR-12234
Experimental models: Organisms/strains		
Ixodes scapularis ticks	National Tick Research and Education Resource, Oklahoma State University	N/A
Recombinant DNA		
Primers for RT-PCR of tick tissue	IDT	N/A
Software and algorithms		
GraphPad Prism version 10.0.2 (232)	Graphpad	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Monika Gulia-Nuss (mgulianuss@unr.edu).

Materials availability

Plasmids generated in this study have been deposited to Addgene [Plasmids pGL 4.79-mCherry (ID# 220137), IsHSP70-mCherry (ID# 220092), and Is3xP3-mCherry (ID# 220091)].

Data and code availability

- **Data**: No sequencing data were obtained. The promoter constructs have been deposited to Addgene. Microscopy data reported in this paper will be shared by the lead contact upon request.
- **Code:** No computational codes were developed.
- **Additional information**: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines

ISE6 cells (Ixodes scapularis; sex: unspecified; embryonic origin) were obtained from Prof. Ulrike G. Munderloh (University of Minnesota, MN) and cultured in Leibovitz's L-15C-300 medium (pH = 7.25) supplemented with 5% fetal bovine serum (FBS), 5% tryptose phosphate broth (TPB) and 0.1% bovine lipoprotein cholesterol (BLC) concentrate at ambient atmosphere and 32°C.[46,47] Cells were tested negative for contamination with mycoplasma.

Ixodes scapularis *larvae*

Ticks were reared in our laboratory in an incubator at 95% relative humidity (R.H.) and 20°C. Ticks were blood-fed on New Zealand white rabbits. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Reno (IACUC # 21-01-1118).

METHOD DETAILS

Ixodes scapularis *HSP 70 promoter constructs*

IsHSP70- Renilla luciferase construct

We synthesized a 1,301 bp sequence fragment (GeneUniversal) upstream of an *I. scapularis* HSP70 (ISCW024910/ISCGN274230) (Figure 1A) including the entire 5′ untranslated region of the gene (UTR) with restriction enzyme sites added to the 5′ and 3′ end (HSP70, Acc65I-SacI, and

BglII), PCR (primers listed on Table 1) amplified with Taq polymerase PCR and cloned them into the pGEM-T-easy vector. Putative promoter sequences were digested and ligated into pGL-4.79 [Rluc] (Promega), a *Renilla* luciferase reporter construct lacking a promoter.

Renilla luciferase was swapped from pGL-4.79 [Rluc] and its associated 3′ SV40 polyadenlyation sequence with EGFP (with its SV40 polyadenlyation sequence) through restriction enzyme cloning (gift from Dr. Shengzhang Dong, Johns Hopkins University).

IsHSP70- mCherry construct

mCherry was subcloned from a mCherry plant expression plasmid (gift from Dr. Jeffrey Harper, University of Nevada, Reno), using primers containing a BglII site and Kozak sequence for the 5′ primer and a MfeI site for the 3′ primer. The restriction sites were used to insert mCherry and remove EGFP from the promoterless pGL4.79-EGFP construct we generated earlier. We retained the SV40 sequence from EGFP (GeneUniversal). pGL-4.79 vectors contain double SfiI sites that flank the multiple cloning sites (the site of promoter insertion). IsHSP70 sequences were subcloned from their current Rluc constructs into pGL-4.79 mCherry (GeneUniversal).

Ixodes scapularis-specific 3xP3 promoter construct

The sequences of 3xP3 with the three Pax6 binding regions and the *Drosophila melanogaster* HSP70 minimal promoter[7,48]) were determined. The *Drosophila* minimal promoter (TATA box to start codon, 123 bp) from 3xP3[7] was replaced with the minimal promoter from our IsHSP70 construct (TATA box to start codon, 453 bp) (GeneUniversal).

Transfection

ISE6 cell plasmid transfection

ISE6 cells were seeded into 96-well plates at 0.1 mL/well of $5x10^5$ cells/mL and incubated at 32°C overnight. The following day, plasmid DNA was diluted into 10 μL Opti-MEM and mixed with 10 μL Opti-MEM containing Lipofectamine MessengerMAX transfection reagent (ThermoFisher Scientific: LMRNA001) at a 1:1 ratio of plasmid DNA [μg] to transfection reagent [μL]. The transfection mix (20 μL) was incubated for 15 min at R.T. and added to wells containing 30 μL medium, followed by spin-transfection for 1 h at 1,000 *g* at 32°C. After 6 h, the transfection mix was replaced with 100 μL fresh L-15C-300 medium. Cells were incubated for 48 h before being exposed to a 2 h heat shock at 37°C or 40°C. At 72 h post-transfection (24 h post heat shock), cells were fixed by adding 100 μL of 7% formaldehyde to each well. Nuclei were stained with Hoechst 33342 (ThermoFisher Scientific: 62249) at 1 μg/mL. Images were acquired with a fluorescence microscope and analyzed using ImageXpress Micro XLS (Molecular Devices, Sunnyvale, CA). The mCherry fluorescence upon plasmid transfection was detected using a BioTek Cytation 7 Cell Imaging Multimode Reader.

Luciferase activity

ISE6 cells were transfected with *Renilla* luciferase constructs using Lipofectamine 3000 (Thermo Fisher Scientific Inc, USA). ISE6 cells were seeded into 12.5 cm^2 flasks at 2 X 10^6 cells/mL and incubated at 32°C overnight. Prior to transfection, cell layers were rinsed with Dulbecco's PBS and held in 1 mL of serum-free L15Cd (SFL15Cd). Lipofectamine 3000 (7.5 μL) in 125 μL SFL15Cd was mixed with 125 μL SFL15Cd containing P3000 (10 μL) and 5 μg of plasmid DNA. Plasmid, in 250 μL complete Lipofectamine 3000 mixture, was incubated at room temperature for 20 min and then added dropwise onto cell layers. Cell layers were gently rocked for 1 h at 32°C, and 2 mL of complete growth medium was added. Transfected cultures were incubated at 32°C for 7–9 days and evaluated for luciferase activity. Cell layers were washed with Dulbecco's PBS and incubated in 500 μL luciferase assay lysis buffer for 15 min. Lysates were stored at −20°C until evaluated. Lysates were examined for luciferase activity using a commercial kit assay (Promega Cat.#E2810). Lysates (20 μL) were inoculated into wells of a 96 well plate, and *Renilla* Luciferase Assay Reagent (100 μL/well) was added. Fluorescence was measured at 10 s at Relative Luciferase Units (RLU) using a luminometer microplate reader (Biotek synergy H1 hybrid plate reader with Gen5v. Software, Biotek, Winooski, VT, USA).

Larval electroporation

Ixodes scapularis larvae were placed in an electroporation buffer (5mM KCl, 0.1mM sodium phosphate at pH 6.8) containing 300 ng/μL of Is3xP3-mCherry plasmid DNA or electroporation buffer solution by itself. The larvae were transferred into 1mM Cuvette (Genesee Scientific) and electroporated for a total of 2 pulses, separated by 5 min on ice using a Gene Pulser Xcell electroporator (Biorad). The pulse condition settings were 35V, 25uF, and infinite resistance. Live larvae were imaged 2 days post-electroporation on a Keyence BZ700 microscope with mCherry fluorescence filters.

RNA extraction, cDNA synthesis, and RT-PCR

HSP70 ((ISCW024910/ISCGN274230) expression in eggs and unfed female tissues: salivary glands, synganglion, midgut, and ovaries was carried out with RT-PCR. Total RNA was isolated from a pool of samples (6 tissues; ~500 eggs) using TRIzol reagent according to the manufacturer's protocols (Invitrogen, Waltham, MA, USA). The total RNA quantity was measured with a Nanodrop spectrophotometer. Five μg of total RNA was used for DNase treatment (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. DNase-treated RNA samples were re-purified with TRIzol. Purity was determined by 260/280 and 260/230 ratios, with acceptable values in the ~2.0 and 2.0–2.2 range, respectively (all samples collected met purity standards). For cDNA synthesis, 1 μg of DNase-treated RNA was used, with iScript reverse transcription supermix (BioRad, Hercules, CA, USA). cDNA was diluted 10X in RNase/DNase-free water before using it as a template in RT-PCR

experiments. In each 10 μL RT-PCR reaction, 1 μL cDNA was used. RT-PCR was performed on a BioRad thermocycler and HSP70-specific primers: Hsp70-661: 5′CTCGTCACCTACTTTGCCGA3′ and Hsp70-1403R: 5′CCGTTTGCGTCCAAATCGAA3′. Primers were also designed for a tubulin gene: IscapB-TubFwd: 5′TGAATGACCTGGTGTCCGAG and IscapB-TubRev: 5′GCAAAGCTGTTCAAGCCTCT3′ which was used as a housekeeping control. RT-PCR conditions were: initial denaturation at 95°C for 5 min, 95°C for 30 s, 55°C for 1min, 72°C for 1 min, repeated for 34 cycles, and a final extension at 72°C for 10 min 10 μL PCR product was separated by electrophoresis on a 1.5% agarose gel along with a DNA ladder (Apex DNA Ladder II; Genesee, San Diego, CA, USA) and visualized by using ethidium bromide-free dye (Amresco, Solon, OH, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism version 10.0.2 (232) was used for analysis. two-way ANOVA followed by Tukey-Kramer multiple comparison test (95% confidence interval) with mean ± S.E., and $p < 0.05$ was considered statistically significant. Sample size (n) for each figure represents the number of replicates. The statistical data are presented in Table S1 (related to Figure 2).