The Tribolium castaneum cell line TcA: a new tool kit for cell biology

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The red flour beetle, Tribolium castaneum, is an agriculturally important insect pest that has been widely used as a model organism. Recently, an adherent cell line (BCIRL-TcA-CLG1 or TcA) was developed from late pupae of the red flour beetle. Next generation transcriptome sequencing of TcA cells demonstrated expression of a wide variety of genes associated with specialized functions in chitin metabolism, immune responses and cellular and systemic RNAi pathways. Accordingly, we evaluated the sensitivity of TcA cells to dsRNA to initiate an RNAi response. TcA cells were highly sensitive to minute amounts of dsRNA, with a minimum effective dose of 100 pg/mL resulting in significant suppression of gene expression. We have also developed a plasmid containing two TcA-specific promoters, the promoter from the 40S ribosomal protein subunit (TC006550) and a bi-directional heat shock promoter (TcHS70) from the intergenic space between heat shock proteins 68a and b. These promoters have been employed to provide high levels of either constitutive (TC006550) or inducible (TcHS70) gene expression of the reporter proteins. Our results show that the TcA cell line, with its sensitivity to RNAi and functional TcA-specific promoters, is an invaluable resource for studying basic molecular and physiological questions.
to be significantly enriched in the TcA Up5k (RPKM > 0.4686) group; 124 of which were found in the Up5k group of all four RNAseq databases (O in Fig. 2b). The enriched GO-terms for both the Up5k and Down0 had the highest similarity to that of the elytra RNAseq data, with the abdominal carcass and whole pupae comprising the second and third similar collection of enriched GO-terms. The similarity of the TcA enriched GO-terms with those of the elytra suggest that the TcA cell line may be of an epidermal origin (Fig. 2a, b, c).

The enriched GO-terms commonly found in all four RNAseq for the Up5k (O in Fig. 2b) generally included housekeeping genes, including those for ribosomal proteins (GO:0005840) and subunits (GO:0015934 and 0015935), as well as mitochondrial respiratory components (GO:0005739, 0005740, 0005743, 0005746, 0005747, 0005753, and 0005759). The commonly enriched GO-terms in Down0 groups of all RNAseq datasets included genes involved in sensory neural function (Fig. 2c, GO:0007600, 000718, 0007606, 0038023, 0004930, 0004888). Large numbers of enriched GO-terms for Up5k were shared between TcA, abdominal carcass, and elytra (N in Fig. 2b) or between TcA and elytra (G), while the similarity was relatively low between TcA and whole pupae (F). Among the GO-terms in the category N (common to TcA, abdominal carcass, and elytra in Fig. 2b), it is noteworthy to mention that many of the GO-terms are related to intracellular vesicle formation and activity (GO:0006897, 0006909, 0006911, and 0016192), as it was also the case with category G (common in TcA and elytra; GO:0030133, 0006888, and 0048193). In category A (uniquely enriched only in TcA), it is interesting to note that GO-terms related to negative regulators of cell death (GO:0043068 and 0043069) are among those in the Up5k group and may be partly responsible for the establishment of the immortalized, stable cell line.

We used immunohistochemistry to confirm the presence of proteins from genes that were highly expressed in the transcriptome. An antibody specific for a signaling peptide, calcitonin-like diuretic hormone, showed small cytoplasmic vesicles in every cell (Fig 3a), indicating a uniformity of expression among the TcA cells. However, an antibody specific for chitin deacetylase stained vesicles in only a subpopulation of TcA cells (Fig. 3b), suggesting some variability in gene expression between cells.

Expression of the genes involved in cuticle and chitin metabolism. Our transcriptome data showed that the TcA cell line highly expresses a set of genes associated with chitin metabolism and cuticle structure (Table S2). We also found a subset of cuticle proteins (Table S1), including CP6 (TC013135, RPKM 41.58) and Id-cp1v1 (TC000442, 7.537), that are highly expressed. In addition, chitinase 20, 21, and 11N were expressed at moderate levels. Other genes that are known to have critical functions in proper cuticle formation that were included in the Up5k group of TcA were Rtv (TC007364, RPKM 38.16), Obstructor A1 (TC011140, 10.91), tyrosine hydroxylase (TC002496, 6.665), laccase 1 (TC000821, 6.103), chitin deacetylase 1 (TC141100, 2.589), and KNK1 (TC010653, 2.219). Most notable is low, but significant levels of expressions of both chitin synthase 1 (42 reads, RPKM 0.06) and 2 (67 reads, 0.11) genes.

Although a number of genes involved in cuticle metabolism were found to be highly expressed, supporting the idea of TcA as an epidermal cell type, there were a large number of chitin metabolic genes that were captured in Down0. Indeed, category A, enriched only in TcA with no detectable expression (Down0, Fig. 2c), included many GO-terms for chitin catabolism and chitinase activities. Therefore, it seems TcA expresses a specific subset of cuticle proteins and chitinases at significant levels while it lacks expression of many other genes involved in cuticle metabolism, perhaps because the TcA cell line is a clonal descendent from a specific subtype of epidermal cells. It would be of interest in the future to determine if the TcA line can be used as a model as an insect epidermal cell type with the activity for chitin synthesis.

In addition to the genes involved in chitin metabolism discussed above, we also found that numerous genes putatively involved in insect immunity in T. castaneum are expressed in the TcA cell line. These are listed in Table S3 and entire expression data is in data set 1.

Expression of genes encoding candidate RNAi machinery. RNAi is very effective in T. castaneum during all life stages, and has been widely used to study candidate gene function. Previous studies have established the basic components of the RNAi pathway that are found in the T. castaneum genome. Based on the information from these studies, we examined the expression of genes encoding core
RNAi machinery (Table 1) and genes implicated in systemic RNAi (Table 2) in the TcA cell line. All genes encoding components of the cellular RNAi machinery were expressed in the TcA cell line (Table 1) including the three dicer family genes (dicer 1, dicer 2, and drosha), the genes for their associated dsRNA binding proteins (loquacious, R2D2 and C3PO, and pasha), all 5 argonaute genes (Ago 1, 2A, 2B, 3, and piwi), and the exonuclease, snipper. In addition, many of the candidate genes thought to be associated with the systemic RNAi response are expressed in the TcA cell line (Table 2). The apparent intact nature of the RNAi response system in the TcA cell line vastly increases the potential for this cell line to be used in highly informative investigations of cellular and molecular physiology.

Figure 2 | Enrichment tests for the TcA transcriptome. (a) Schematic diagram explaining the approach to perform the enrichment test comparing the transcriptome data of TcA to RNAseq data from abdominal carcass, elytra, and whole pupae. Enriched gene ontology (GO) terms for the top 5,000 genes and the genes with no transcript of each library were compared. (b) Venn diagram showing the numbers of common GO terms found for Up5k in each library with the representative categories of GO terms (c) Venn diagram showing the numbers of common GO terms found for Down0 in each library with the representative categories of GO terms.

Figure 3 | Immunocytochemistry of TcA showing positive reactions to anti-diuretic hormone antibody and anti-chitin deacetylase antibody. (a) immunoreactivity for calcitonin-like diuretic hormone (green), actin (phalloidin, red), and nuclei (DAPI, blue) are shown. (b) immunoreactivity for chitin deacetylase (green) and nuclei (DAPI, blue). Scale bar is 10 μm.

Table 1 | Expression of genes encoding core RNAi Machinery in the TcA cell line

| Description          | Name       | TC #      | RPKM         | Raw Reads |
|----------------------|------------|-----------|--------------|-----------|
| Dicer Family         | Dicer 1    | TC001750  | 0.2305       | 183       |
|                      | Dicer 2    | TC001108  | 3.455        | 2426      |
|                      | Drosha     | TC016208  | 0.8303       | 382       |
| dsRNA Binding        | R2D2       | TC008716  | 0.9222       | 128       |
|                      | C3PO       | TC007013  | 0.02626      | 46        |
|                      | loquacious | TC011666  | 0.4626       | 77        |
|                      | pasha      | TC015332  | 0.3076       | 75        |
| Argonautes           | Ago 1 [miRNA] | TC005857 | 4.813        | 2197      |
|                      | Ago 2a [siRNA] | TC011525 | 0.09176     | 34        |
|                      | Ago 2b [siRNA] | TC013762 | 7.539        | 2950      |
|                      | Piwi       | TC008711  | 8.248        | 3174      |
|                      | Ago 3      | TC008511  | 0.8715       | 337       |
| Exonuclease          | Snipper    | TC001174  | 0.3474       | 35        |
**Table 2 | Expression of genes associated with systemic RNAi in the TcA cell line**

| Description                          | Name                      | TC#          | RPKM   | Raw Reads |
|--------------------------------------|---------------------------|--------------|--------|-----------|
| Systemic RNAi – dsRNA uptake         | SiIA                      | TC011760     | 0      | 0         |
|                                      | SiIB                      | TC006161     | 0.04313| 14        |
|                                      | SiIC                      | TC015033     | 0.6015 | 200       |
| Systemic RNAi – dsRNA export         | Epsin-like (rsd-3)        | TC012168     | 1.877  | 439       |
|                                      | liquid facets (Epn-1)     | TC005393     | 1.174  | 261       |
| RNA-dependent RNA polymerase         | Elp-1 (RNA-dep RNA pol)   | TC015781     | 0.6645 | 337       |
| Eater                                | Odorant Receptor 138      | TC002053     | 0      | 0         |
|                                      | Eater XP _969372*         | TC030481     | 0.002727 | 1 |
|                                      |                           | TC015258     | 0      | 0         |
| Endosome Transport                   | Arf72A                    | TC008443     | 0.5325 | 38        |
|                                      | Rab 7                     | TC006036     | 3.107  | 219       |
| Endocytosis                          | AP 50                     | TC011923     | 6.401  | 1215      |
|                                      | Clathrin hc               | TC015014     | 7.213  | 5255      |
| Exocytosis                           | IdlCP                     | TC010866     | 0.5782 | 140       |
| Lyssosomal Transport                 | Light                     | TC015204     | 0.8023 | 290       |
| Rhodopsin-mediated signaling         | Nina C                    | TC014087     | 0.002047 | 1 |
| ATP Synthase/ATPase                  | Vha 16                    | TC011025     | 10.36  | 717       |
|                                      | VhaSFD                    | TC006281     | 5.631  | 1288      |
| Metabolism                           | Gmer                      | TC014956     | 0.9353 | 129       |
| Lipid Metabolism                     | P13K59F                   | TC000620     | 0.6661 | 269       |
|                                      | Saposin r                 | TC000449     | 16.11  | 5872      |
| Innate Immune Response/phagocytosis  | Odorant Receptor 138      | TC002053     | 0      | 0         |
|                                      | Eater XP _969372*         | TC030481     | 0.002727 | 1 |
|                                      | Sr-CI CG4099              | TC015258     | 0      | 0         |
|                                      | Sr-CII                    | TC015640     | 0.08079 | 19 |
| Oogenesis                            | Egghead                   | TC008154     | 0.4128 | 83        |
| Translation Regulation               | LOC662027 similar to CG5434-PA (Signal recognition particle protein 72) | TC012172 | 1.008 | 285 |
| GDP-fucose transmembrane transporter | Gfr CG9520                | TC012326     | 0.1036 | 15        |
| GDP-mannose 4,6-dehydratase          | Gmd CG8890                | TC004005     | 0.1879 | 29        |
| Peptidase                            | Unknown                   | TC002692     | 0.3452 | 70        |
|                                      |                           | TC007768     | 0.2769 | 51        |
| Signal Transduction                  | Unknown                   | TC004152     | 1.746  | 3029      |
| Ubiquitin Ligase                     | Unknown                   | TC016254     | 0.2672 | 105       |
| Peptidase                            | Unknown                   | TC009067     | 0.6178 | 78        |
|                                      |                           | TC012410     | 0.3755 | 138       |
|                                      |                           | TC014009     | 0.6331 | 49        |
|                                      |                           | TC004825     | 0.3245 | 86        |
|                                      | LOC658010 similar to kinase D-interacting substance 220 | TC007973 | 4.659 | 282 |

**TcA-specific promoters for reporter gene expressions.** Heterologous expression of a native, chimeric, or fluorescently-tagged protein in a cell line is a commonly used and extraordinarily useful tool. Use of the TcA for expression of target proteins depends on efficient promoters that constitutively or conditionally promote the expression of the exogenous gene. Our initial trials with promoters that are commonly used in insect cell lines were not successful at expressing either the fluorescent reporter protein, Tomato, or enhanced green fluorescent protein, EGFP. In a test of the common promoters frequently used in insect cell systems, the Actin 5 mon promoters (Invitrogen), and the alpha tubulin promoter of D. melanogaster (Fig. 4a and 5a). This sequence included the putative promoters for three genes encoding ribosomal subunits, TC014362, TC000476, and TC006550, and another highly similar hsp TC010172 after normalization to rps3 transcript levels and comparison to the no heat shock control (Fig. 4c). We subsequently used the 1910bp intergenic region between the coding genes for hsp68a and hsp68b as the TcHS70 promoter (Fig. 4a and 5a). This sequence included the putative TATA boxes, transcription start sites located in each end, and numerous heat shock sequence elements (Fig. 4b).

The TcHS70 contained multiple putative heat shock sequence elements (Fig. 3a and b). Initially, we tested the TcHS70 activity after a heat shock in the endogenous cell. Immediately after a heat shock of TcA cells at 40°C for 30 min, total RNA was extracted to investigate the transcriptional responses of the hsp genes. We found ~300 to 500-fold increases in mRNA levels for each hsp68a, hsp68b, and another highly similar hsp TC010172 after normalization to rps3 transcript levels and comparison to the no heat shock control (Fig. 4c).
imun expression and maturation time for nanoluciferase activity is ~3 hr after heatshock, and it reaches a plateau at ~8 hr following heat shock at 38 °C for 40 min (Fig. 5b and c). The cells with constitutive expression of EGFP were also found to have inductive expression of psOrange following heat shock, although induction of visible levels of psOrange appears to require a longer time for maturation than that of nanoluciferase. Occasionally, we found autofluorescence in the TcA that is similar to the EGFP wavelength. This autofluorescence was limited to large vacuolar structures appearing only in a small subpopulation of the cells (Fig. 5d).

In conclusion, based on expression of the reporter proteins, the TcHS70 is a highly inducible bidirectional promoter. Further, the Tc006550 promoter also provides a moderate level of constitutive reporter expression. The plasmid containing those promoters will be a valuable tool for expression of heterologous genes.

RNAi in the TcA cell line. Expression of many of the components of the RNAi machinery led us to investigate the sensitivity of this cell line to RNAi. Accordingly, we selected a relatively expressed chitin protein, CP6, as our target for silencing, and used the common control target, vermilion, as a negative control. dsRNA was synthesized (231 bp for CP6, 400 bp for vermilion) and mixed directly with culture media at various concentrations (1 fg/mL to 100 ng/mL) prior to applying to the TcA cells. qPCR quantification showed that the TcA cell line is highly sensitive to RNAi of CP6. Treatment with as little as 1 ng/mL resulted in >99% suppression of CP6 mRNA expression (Fig. 6a). Suppression of CP6 mRNA decreased in a dose-dependent manner as dsRNA concentration decreased below 1 ng/mL, with no significant reduction in CP6 expression occurring with 1 pg/mL dsRNA. The sensitivity to dsRNA of TcA cells is similar to that of the Tc81 cell line as measured with dsRNA targeting the med gene7, and more than 10,000 times greater than that for the S2 Drosophila cell line8.

We also tested our CP6 dsRNA in vivo by injecting dsRNA (2 or 200 ng/beetle) into T. castaneum early instar larvae to ascertain the effects of CP6 RNAi in vivo. No obvious phenotype was associated with silencing of CP6 in any developmental stage (late instar larvae, pupae, or adult), although the beetles injected with vermilion dsRNA showed the characteristic white eyes associated with this treatment. Analysis of CP6 mRNA showed that both 2 and 200 ng treatments per individual resulted in >99% suppression of expression at the organismal levels without noticeable phenotype in the observation until the early adult stage.

We also subsequently tested the efficiency of RNAi to silence the expression of heterologously expressed proteins, transiently transfected for heat shock inducible nanoluciferase (Fig. 6b). Silencing of nanoluciferase activity induced by heat shock was measured following treatment with dsNluc. Interestingly, significant suppression of nanoluciferase activity required a minimum of 1 ng/mL (~50% suppression, Fig. 6b), which is more than 10-fold greater than that required for suppression of CP6 expression. We suspect that this difference is likely caused by the rapid induction of extremely high levels of expression of the nanoluciferase following heat shock treatment.

High sensitivity of the TcA cells to dsRNA in the media requires efficient mechanisms for cellular uptake of primary dsRNA followed by dsRNA processing into small interference RNA (siRNA) which forms complexes with the target messenger RNA. Our results show that TcA expresses a large number of candidate molecular components of the RNAi pathway (Table 1) and shows high sensitivity to dsRNA, providing an excellent opportunity to explore novel, highly efficient RNAi mechanisms in the TcA cell.

Methods

Cell culture. TcA, an adherent cell line, was derived from BCIRL-TcA-CLG1 that was originally established from the late pupal T. castaneum. TcA cells were grown in ExCell 420 media (Sigma, St. Louis, MO.) supplemented with 10% insect cell culture-tested fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 4.5 μg/mL of bovine insulin at 27 °C and 60% relative humidity, although bovine insulin was found to be not necessary for normal cell growth in the later study (Park et al, unpublished data).

Transcriptome sequencing and analyses. Total RNA was harvested from confluent cells using a commercial kit (Qiagen, Valencia, CA.). RNA quality was tested using an Agilent Bioanalyzer prior to submission for sequencing. Illumina-based (GXII platform) rRNA-depleted transcriptome sequencing of a single RNA sample was carried out by Cofactor Genomics (St. Louis, MO.). Obtained sequences were single direction, 50 nt in the length, and filtered for the sequences with a q-score higher than 30 and longer than 30 nt by using the FastX toolkit implemented in DNA Galaxy9. Assembly of the sequences to the reference genome Tcasa3.0 was made in DNA Nexus (https://classic.dnanexus.com/home). The RPKM (read per kilobase per million
mapped reads) was used for identifying the top 5,000 highly expressed genes (Up5k) and the genes with undetectable transcript levels (Down0) (Fig. 1).

The transcriptome of TcA was evaluated by comparisons of enriched GO-terms to other tissue specific RNAseq data. Direct comparisons of RNAseq data for expression levels of each gene by RPKM in different sets of data were not possible because of varying sample preparation procedures and sequencing approaches in each RNA library. For example, normalization of our sequence data to another library introduced largely skewed raw RPKM values, and was thus not appropriate to use in conventional methods for comparing expression levels. As an alternative approach, the lists of genes for the Up5k and Down0 were used for a GO-term enrichment test against the official gene set (Tcas 3.0 OGS17,) in Blast2GO at the False Discovery Rate (FDR) of 0.05 (Fig. 2). Enriched GO-terms of each library representing RNAseq data from elytra of late pupal stage (elytra, the data provided by Dr. S. Muthukrishnan), abdominal carcass excluding the gut and the central nervous system in the early adult stage (abdominal carcass, data provided by Dr. T. Phillips), and whole pupal stage (whole pupae, data provided by Dr. G. Butcher) were compared for each Up5k and Down0.

Immunohistochemistry. Anti-DH37 (rabbit, 1:500), originally raised against the diuretic hormone 37 of Tenebrio molitor8, and anti-CDA1 (rabbit, 1:500) against T. castaneum chitin deacetylase 119 were used to label their respective targets in fixed TcA cells grown on a petri dish. A goat anti–rabbit IgG antibody conjugated with Alexa Fluor 488 (Life technologies) was used as the secondary antibody. Additionally, we used phalloidin conjugated with Alexa Fluor 594 (Life technologies) to stain actin and 4',6'-diamino-2-phenylindole (DAPI 300 nM, Sigma) to stain cell nuclei (Fig. 3).

Images were captured on an LSM 700 confocal microscope under a 40X objective (Zeiss).

Plasmid construct. pIZTV5-His (Invitrogen), the plasmid originally developed for lepidopteran insects, was used for constructions of other variants. Plasmids we constructed were for testing putative promoters in the TcA cell line for expression of fluorescent protein dTomato20 as the reporter. The promoters tested were: the CMV promoter from the pcDNA3.1 plasmid (Invitrogen), the OpIE1 and OpIE2 promoters from the pIZT vector, the Tribolium alpha tubulin promoter13, and the Tribolium heat shock promoter (TcHS70, in this study). The dTomato was amplified using the primers dTomatoF and dTomatoR (Table S1) and cloned into a pGEMT easy vector. Then, dTomato was introduced into the pIZTV5-His vector by using a NotI restriction site (pIZT-dTomato). Subsequently, the promoters were cloned into the pGEMT easy vector and then inserted into pIZT-dTomato replacing the OpIE2 promoter by digestion with the corresponding restriction enzymes and ligation.

Figure 5 | Heterologous expression of the reporter genes GFP::Zeocin R, nanoluciferase (Nluc) and photo-switchable Orange fluorescent protein.
(a) Construction of the heterologous expression plasmid using the pUC18 backbone with ampicillin resistance for expression of the reporter genes by the TC006550 (rpS40 protein) and TcHS70 promoters. (b) Induction of Nluc expression by different temperatures, durations, and numbers of heat shock. (c) Timecourse of Nluc activity in the media following heat shock (38°C for 30 min). (d) Green fluorescence from EGFP expression (green) and orange (red) fluorescence from psOrange over time after heat shock. The histogram was made by sampling more than 300 cells for each time point. The right panel shows an example of a confocal image for cells expressing both green and red fluorescence. Note that two cells on the bottom left of each photo are green, but not red, showing auto fluorescence of large vacuolar structures in those TcA cells.
For RNAi of nanoluciferase, TcA cells were seeded onto a 96-well plate (5 × 10^4 cells/mL) and left to grow for at least 24 h. The TcA cells were transfected with the expression plasmid (Fig. 5a), using FuGENE HD transfection reagent (Promega). dsRNA was synthesized using the Ampliscribe T7 Flash Transcription Kit (EpigenTech Biotechnologies, Madison, WI) from cDNA templates using gene-specific primers (Table S1) with a T7 nucleotide sequence attached to the 5' end of the primer sequences of CP6 (cuticle protein 6, or also named as CPR71), vermilion, and nanoluciferase. The dsRNA was purified using a commercial kit (MEGAClear, Life Technologies) and run on a 2% agarose gel to confirm the band size. dsRNA was quantified using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to the desired concentration with nuclease-free water.

For CP6 RNAi experiments in cell culture, TcA cells were seeded onto 24-well plates (5 × 10^4 cells/mL) and left to grow for at least 24 h. RNAi was achieved in TcA cells by adding dsRNA for CP6 or vermilion directly to the culture media. RNA was harvested after 96 h using TRIzol reagent (Life Technologies), and cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega). cDNA samples were then diluted 20-fold and CP6 expression was quantified by qPCR utilizing comparative Ct™ on a Biorad thermal cycler (Biorad) with ribosomal protein S3 (rpS3) serving as the internal loading control. Gene-specific primers for CP6 and rpS3 are listed in Table S1. The thermal cycling conditions were as follows: 95°C for 2 min, 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec. Melting curve analysis (55–95°C) and gel electrophoresis were applied to all reactions to ensure consistency, specificity, and amplicon size of the amplified product. All CP6 Ct values were first normalized to rpS3 in the same sample, and then secondarily to the vermillion treatment in order to produce a relative expression value. These experiments were replicated three times and the results are presented as the mean ± standard error.

For in vivo RNAi experiments of CP6, early last instar larvae were microinjected with dsCP6 (200 ng or 2 ng/individual) or vermillion (200 ng/individual) dsRNAs. Two weeks post-injection, adults were visually examined for any defects in development. Four individuals from each treatment group were pooled for total RNA isolation. cDNA was synthesized from total RNA (1 μg/rxn) using the ImProm-II™ Reverse Transcription System (Promega). RNAi efficiency was determined by qPCR as described above.

For RNAi of nanoluciferase, TcA cells were seeded onto a 96-well plate (5 × 10^4 cells/mL) and left to grow for at least 24 h. The TcA cells were transfected with the expression plasmid (Fig. 5a), using FuGENE HD transfection reagent (Promega). Subsequently, various concentrations of dsRNA for nanoluciferase were directly added to the culture media for final dsRNA concentrations ranging from 100 fg/mL to 10 ng/mL. About 48 h after the transfection, the cells were subject to heat shock (HS) at 38°C for 40 min. Approximately 18 h after the initial HS, the media was changed to new media and one more HS was made at 38°C for 40 min. Six hours after the HS, secretory nanoluciferase activity in the cell culture media was measured using the Nano-Glo luciferase assay system (Promega). dsVermilion (100 ng/mL) was used as a negative control.

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Author contributions
K.S., H.J., J.F., Y.P. performed the experiments. K.S., H.J., J.F., T.W.P., Y.P. provided and analyzed the data. K.S., Y.P., H.J., J.F. and R.W.B. wrote the paper. Y.P. and R.W.B. supervised the project.

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