An RNA aptamer perturbs heat shock transcription factor activity in Drosophila melanogaster

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ABSTRACT

Heat shock transcription factor (HSF1) is a conserved master regulator that orchestrates the protection of normal cells from stress. However, HSF1 also protects abnormal cells and is required for carcinogenesis. Here, we generate an highly specific RNA aptamer (iaRNAHSF1) that binds Drosophila HSF1 and inhibits HSF1 binding to DNA. In Drosophila animals, iaRNAHSF1 reduces normal Hsp83 levels and promotes developmental abnormalities, mimicking the spectrum of phenotypes that occur when Hsp83 activity is reduced. The HSF1 aptamer also effectively suppresses the abnormal growth phenotypes induced by constitutively active forms of the EGF receptor and Raf oncoproteins. Our results indicate that HSF1 contributes toward the morphological development of animal traits by controlling the expression of molecular chaperones under normal growth conditions. Additionally, our study demonstrates the utility of the RNA aptamer technology as a promising chemical genetic approach to investigate biological mechanisms, including cancer and for identifying effective drug targets in vivo.

INTRODUCTION

HSF1 is a highly conserved transcription factor that responds to a variety of signals to regulate the expression of a broad spectrum of target genes (1,2). HSF1 activity in Drosophila and Saccharomyces cerevisiae is encoded by a single HSF1 gene; while in mammals and plants multiple isoforms exist that appear to have specialized functions (3–6). In response to thermal exposure, HSF1 is responsible for activating the heat shock (HS) response, a highly conserved mechanism among different kingdoms (7). During this response, HSF1 activates the expression of a specific set of HS genes, resulting in the accumulation of proteins possessing chaperoning activities that allow organisms to cope with cellular damage induced by thermal stress. Additionally, HSF1 activity has been shown to be important during certain cell and developmental processes in various organisms. In S. cerevisiae, HSF1 is essential for cell viability and for vegetative growth (8). Unlike yeast, animals do not require HSF1 activity for general cell growth; rather HSF1 is required during specific developmental stages. For instance, in Drosophila, HSF1 activity is required for early larval development and during oogenesis (9); while in Caenorhabditis elegans, HSF1 has been shown to be required for maintenance of longevity (10,11). Mammals have evolved multiple HSFs with specialized functions which appear to play multiple regulatory roles during development that extend beyond the HS/stress response (2–5).

Although mouse HSF1 activity is not required for animal viability, it does protect cells from cellular insults (4,12,13) and, interestingly, also has been implicated in cancer as a ‘non-classical oncogene’ (14). Importantly, HSF1 activity promotes tumor formation and participates in the maintenance of the transformed phenotype of cancer cells without affecting the viability of normal cells (14). Therefore, HSF1 can function to promote cell survival even under conditions that could potentially become deleterious to cells, such as development of the transformed state.

To further understand HSF1 function during animal development and its role in tumor maintenance, we used RNA aptamer technology as a chemical–genetic approach to inhibit HSF1 activity in Drosophila melanogaster. Aptamers are single-stranded RNA molecules that can bind with high affinity to specific molecular surfaces through ionic, hydrophobic and hydrogen bond interactions. They are isolated from combinatorial libraries containing $\sim 1 \times 10^{15}$ different RNA molecules through

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an iterative process of selection and amplification called systematic evolution of ligands by exponential enrichment (SELEX) (15, 16). The large sequence complexity associated with such a starting library enhances the probability of isolating specific and high-affinity aptamer RNAs to various types of molecular targets, ranging from single small molecules (17) to distinct functional domains on a protein (18). Aptamers have also been used previously in therapies (19,20) and in basic research (21,22), demonstrating the broad utility of these molecular inhibitors. Aptamers can not only be selected to bind and inhibit distinct molecular surfaces with high specificity, they can also be expressed in vivo under tight genetic control (23) and assert their effect within specific cells, tissues or at specific developmental stages without eliciting an immune response in the targeted organism (24).

Herein, we report the design, construction and validation of a potent inhibitory aptamer RNA molecule for HSF1 (iaRNAHSF1). This iaRNAHSF1 contains two HSF1 binding domains engineered from a previously isolated RNA aptamer that targets the highly conserved HSF1 DNA binding domain-linker region (25). In Drosophila, we demonstrate that this iaRNAHSF1 is highly specific to HSF1 and can interfere with the HSF1 trans-activation function under both non-induced and HS conditions in vivo. Because of the broad implication of increased Hsp levels in diseases, such as human cancer (14,26–29), we examined the effect of iaRNAHSF1 under conditions that model cellular transformation in flies. In Drosophila, HSF1 inhibition by iaRNAHSF1 suppresses the abnormal phenotypes that are induced by the expression of gain-of-function mutants of the epidermal growth factor receptor (EGFR-ER mutant) and Raf oncogenes, and the effects of iaRNAHSF1 expression are similar to the usage of Hsp83 loss-of-function mutants or treatment of flies with the Hsp83 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), a frequently used anticancer agent in humans (30).

MATERIALS AND METHODS

Oligonucleotides and other reagents

A single iaRNAHSF1 unit was constructed in two parts by extending 50 pmol of each of the following primer sets (I and II; III and IV) in 100 μl using a single round PCR reaction:

(I) 5'-CGCTCGAGTACGTTGCGATCGCATAGCA AAATAGTTGAAGCCGGCTTCGCAGAT,  
(II) 5'-GCCCCGAAATTCAGGATGATAGCAAGGG AGTGGATTTCCAAGAAGACTCGTTCAACTT,  
(III) 5'-GGCCGGACATCTGCTCGGCGACGG CATGTCAAATAAGTAGCTGACGCAGTCT TTGGAGCTCGTACGTCG,  
(IV) 5'-GGCCGGACGTGGTCTGTCGACGGACGACT ATCAGTACGCAAACACATCGTACAGCT CGAGGCTCAGAACACTCG.

Each half of the molecule was purified by running the extended products on high-resolution 8% native gel and extracted from the gel matrix as visualized by EtBr staining. Then each template was restricted with EcoR1 (Invitrogen), ligated together, and cloned into pSTBlue-blunt cloning vector (Invitrogen): pSTBlue.iaRNAHSF1X1 is a coding sequence that contains two individual (AptHSF1-I) gene upstream of a self-cleaving hammer-head ribozyme.

Construction of synthetic genes

Repetitive head-to-tail iaRNAHSF1 genes were created by sub-cloning iaRNAHSF1X1 into a Gateway donor vector (pDONR221.iaRNAHSF1X1) by lifting the iaRNAHSF1X1 sequence from pSTBlue.iaRNAHSF1X1 using primers containing the AttB1F and AttB2R Gateway cloning sequences (Invitrogen): 5'-AAG TTT GTA CAA AAA AGC AGG CTT CGG ATC CAG AAT TCG TGA TC and 5'-GGG GAC CAC TTT GTA CAA GAA AGC. Because each iaRNAHSF1 unit is flanked by the complementary asymmetric Xho1 and SalI restriction sites at the 5’- and 3’-ends, respectively, we can use the general Gateway cloning strategy to select for correctly ligated tandem iaRNAHSF1 repeats (Supplementary Methods S1). In this method, a single iaRNAHSF1 unit is first lifted from pDONR221.iaRNAHSF1X1 via PCR and the resulting amplicon is cut with either SalI or Xho1 before the cut products are combined and ligated together. Using this scheme, only those products that are in proper head-to-tail orientation contain the required Gateway AttB sites in the 5’- and 3’-ends (AttB1F.iaRNAHSF1X2. AttB2R) needed for creation of an Gateway compatible Drosophila transformation expression vector, pUAS.iaRNAHSF1X2. Using the polymer of two as template and repeating the polymerization strategy creates a polymer of four, p(UAS.iaRNAHSF1X4, w+). Overall, geometric progression of polymeric length is achieved in each subsequent round of polymerization.

Drosophila strains

Parental iaRNAHSF1 animals were created by injecting Drosophila w1118 embryos with p{UAS.iaRNAHSF1X8, w+} and p{UAS.iaRNAHSF1X16, w+} transformation vectors and screening the progeny of F1 females for animals that contain the mini-white gene when crossed to a double-balanced CSX fly line containing CyO(2); TM6(3); Xasta(2,3). Sites of p-element insertions were determined genetically by continuous backcrossing to the CSX stock, resulting in homozygous fly lines that contain aptamer genes in various chromosomes: (i) UAS.iaRNAHSF1X8(X), (ii) UAS.iaRNAHSF1X16(X), (iii) UAS.iaRNAHSF1X8,16(X), (iv) UAS.iaRNAHSF1X8(II), (v) UAS.iaRNAHSF1X16(II), (vi) UAS.iaRNAHSF1X8,16(II), (vii) UAS.iaRNAHSF1X8,16(III). To express iaRNAHSF1, we crossed homozygote UAS.iaRNAHSF1 parents with various Gal4 sources purchased from Drosophila Stock Center (Bloomington): 6983 (Salivary Gland Gal4), 5138 (UBiquitous tubulin Gal4). Systemic iaRNAHSF1 expressing animals were created by isolating F1 females from aptamer parents in the second chromosome (UAS.iaRNAHSF1 and UAS.iaRNAHSF18,16) mated to 5138 animals.
Heterozygote F1 males were then mated to CSX females, and the resulting F2 animals were then used to generate both aptamer and Gal4 proteins (UAS.iaRNAHSF1/CyO; Tub.Gal4/Sb) were isolated and isogenized to create true breeding aptamer expressing lines. Other Bloomington stocks used in this study include: 5693 (Hsp83[^1]), 3628F: 5′-CTGCGGCTTGGGAATTACTGA, Hsp26+580F 5′-CAAGGTTCCCGATGGCTACA, Hsp26+667R 5′-CTGCGGCTTGGGAATTACTGA.

All statistical analyses in this study were calculated using Student’s t-test.

**Immunofluorescent assays of polytene chromosomes**

Salivary glands were dissected from third stage instar larvae in 0.5× Grace’s medium. Chromosomes were spread, fixed onto slides and immunostained using antibodies targeting HSF1, GAGA factor (GAF) as described previously in Schwartz et al. (31).

**Morphological studies**

Aptamer expressing animals were scored for phenotypic abnormalities using a dissecting microscope. Here, the abnormal *Drosophila* traits were quantified by screening a population of aptamer expressing animals (>500 flies) and determining the number of animals with abnormal traits in the total population. Pictures were taken using an 8.0 Mb Nikon digital camera mounted onto the microscope. Quantification of morphological abnormalities was calculated by quantifying abnormal size or area using the ImageJ software.

**Cell culture**

iaRNA[^1]xs was subcloned into Gateway pDEST48 (Invitrogen) and stable *Drosophila* S2 cells were selected by maintaining cells in 6 µg/ml Blasticidin reagent. iaRNA[^1] was induced using 0.5 mM CuSO4, iaRNA[^1] half-life (t1/2) determination was performed by treating cells with 0.5 mM CuSO4 for 24 h before adding 1 µg/ml alpha-amanitin, a potent RNA Pol II inhibitor. Upon the addition of amanitin, cells were collected and the total RNA samples were isolated using the Trizol reagent and protocol. Total iaRNA[^1] values were calculated by comparing their relative levels to 18S RNA levels at specific time points following amanitin treatment.

**RNAi treatment**

Approximately 1 × 10⁶ *Drosophila* S2 cells were incubated with 10 µg dsRNA targeting HSF1 and Hsp83 for 5 days using genes containing T7 promoter targeting each sequence amplified from *Drosophila* genomic DNA:

T7Hsp83+378F: 5′-TAATACGACTCACTATAGGGTT CCATGATCGGCCTGACATTGTTGTTTGGTGTAATGCT, T7Hsp83+1048R: 5′-TAATACGACTCACTATAGGGTT CCATGATCGGCCTGACATTGTTGTTTGGTGTAATGCT, T7Hsf1 F: 5′-GAATTATAATGAGCTACTAGTGGTGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
RESULTS

Design, construction and validation of the iaRNAHSF1 expression system

Previously, we isolated an RNA aptamer that binds the DNA binding domain of Drosophila HSF1 with an apparent dissociation constant ($K_d$) of 20–40 nM (25). Because HSF1 is multimeric, we used this aptamer to construct a divalent version that we demonstrate has a several fold higher affinity, $K_d \sim 8$ nM (Figure 1A–C). This improved avidity of iaRNAHSF1 is sufficient to prevent HSF1 from binding to its natural binding sites on the Hsp70 and Hsp83 promoters in vitro (Figure 1D and E). As shown here, increasing the concentrations of HSF1 results in the formation of various protein–DNA complexes as visualized by the altered electrophoretic motility (Figure 1D, lanes 2–4). The long HS element (HSE) of Hsp83 can bind multiple HSF1 trimers, and the weaker bands of intermediate mobility likely represent binding of non-saturating amounts HSF1 trimers to this HSE. These weaker bands and the major shifted band are all effectively inhibited by increasing amounts of iaRNAHSF1 (Figure 1D, compare lanes 5–8 versus 9–12).

To test the effect of iaRNAHSF1 on HSF1 function in animals, we generated an aptamer expression system designed to rapidly produce high levels of nuclear localized dimeric iaRNAHSF1 in desired cell types. The approach was a systematic step-wise variation of that used by Shi et al. (23). The dimeric iaRNAHSF1 was joined to a hammerhead ribozyme and this unit was duplicated and the product reduplicated by a forced-Gateway cloning strategy to generate up to 16 repetitive head-to-tail repeats under the control of a Gal4-activated promoter (Figure 2A and Supplementary Methods S1). The resulting expression system allows high-level expression of dimeric aptamer RNAs, because each repeating aptamer coding unit within a given polymeric template is flanked by a self-cleaving hammer-head ribozyme. Upon transcription of the polymeric template RNA in a tissue expressing the Gal4 transcription activator, the hammer-head ribozymes undergo self-cleavage resulting in the release of multiple free functional iaRNAs from every transcription cycle (23) (Supplementary Figure S1A). This self-cleavage ensures that the aptamer RNAs are not polyadenylated and not substrates for export; therefore, they should remain localized within the nucleus. Additionally, the self-ligation activity of the released form of the hammerhead creates covalently closed circles that are thought to stabilize and protect the RNA aptamer from degradation (23).

To test the stability of iaRNAHSF1 in living cells, we analyzed the rate of iaRNAHSF1 decay in stable Drosophila cells at specific times following alpha-amanitin treatment. In these experiments, the iaRNAHSF1 exhibited an in vivo half life of $\sim 2$ h (Supplementary Figure S1B).

Finally, to test the production of iaRNAHSF1 in whole Drosophila animals, we crossed flies with the Gal4-regulated polymeric aptamer gene with a line expressing a tubulin-promoter-driven Gal4 gene. The iaRNAHSF1 level is elevated $\sim 150$-fold over parental strains that lack Gal4 protein (Supplementary Figure S1C) demonstrating that the aptamer gene is regulated by Gal4. Collectively, our results reveal that this engineered polymeric dimeric aptamer construct has an improved apparent affinity for HSF1, is stable under cellular conditions, and can be effectively induced to high levels in vivo.

iaRNAHSF1 is a potent HSF1 antagonist under non-heat shock conditions

To determine the in vivo efficacy of the iaRNAHSF1 as a HSF1 antagonist, we measured its effect on known HSF1 gene targets under both non-heat shock (NHS) and HS conditions. We focused initially on the Hsp83 gene locus (63B), which is the ortholog of mammalian Hsp90, because it is expressed under non-stress inducing conditions, and HSF1 is significantly enriched at this locus compared to other loci (32,33). We investigated the effects of iaRNAHSF1 expression at three different levels: (i) HSF1 binding to the Hsp83 chromosomal locus, (ii) Hsp83 mRNA levels and (iii) traits in animals. Antibody staining for HSF1 on Drosophila salivary gland chromosomes confirms that under normal growth conditions HSF1 preferentially binds to the Hsp83 gene locus (63B) [Figure 2B, compare HSF1 signal (red) relative to GAF control (green)]. However, upon iaRNAHSF1 expression, the HSF1 levels at the 63B locus is significantly reduced by $\sim 50\%$ ($P = 0.035$) (Figure 2C, left).

Previous reports have implicated the enhancer elements and upstream regulatory sequences present in the promoter regions of the Hsp83 gene as being critical for Hsp83 gene expression (34); however the Hsp83 upstream region also has a tandem array of nine binding sites for the HSF DBD. Because, there is currently no direct evidence indicating whether HSF1 has a role in regulating the basal expression of Hsp83, we tested whether HSF1 inhibition compromises Hsp83 expression levels by quantifying the levels of Hsp83 mRNA in iaRNAHSF1 expressing and wild-type (Gal4 parental) animals not exposed to thermal stress (NHS). In these and the following RT–qPCR experiments, we determine and compare HS mRNA levels by normalizing their values to a housekeeping gene whose level does not vary relative to total RNA in response to HSF1 aptamer expression, HSF1 RNAi treatment, or temperature. Here, animals that express iaRNAHSF1 contain an $\sim 50\%$ reduction of Hsp83 mRNAs compared to control animals (Figure 2C, right) ($P = 0.008$), indicating that under non-HS conditions HSF1 activity is required for the expression of Hsp83. We confirmed this result using another approach, which involved RNAi-depletion of HSF1 from Drosophila S2 cells. In these experiments, HSF1 knockdown resembled the effects of iaRNAHSF1 expression and similarly reduced the expression levels of Hsp83 and Hsp70 transcripts (Supplementary Figure S1D), thus further demonstrating that normal HSF1 activity is required for the full expression of HS mRNAs under normal growth conditions.

Adult animals that constitutively express iaRNAHSF1 display phenotypic abnormalities in the abdominal segments, wing shape and morphology, bristles and eye
protrusions in specific genetic backgrounds at high frequencies (Figure 2D). Intriguingly, the aptamer-induced phenotypes closely resemble the abnormalities that occur when Hsp83 activity is reduced; although the developmental defects occur at much greater frequencies (35,36). This increased penetrance is best illustrated by the notched wing phenotype observed in >90% of iaRNAHSF1 expressing animals; while this same phenotype is only present in 5–20% of the wild-type animals that have been raised in media containing the Hsp83 inhibitor (17-AAG) during the first two generations, and in <1% of Hsp83e6D antimorphic mutants. The observed increased penetrance of abnormal traits that occurs among aptamer expressing animals might be a result of inbreeding of fly populations that have such abnormal traits, as has been shown in previous studies of Hsp83 mutant animals or feeding animals with 17-AAG (36). Additionally, or alternatively, we cannot rule out HSF1 having some contribution to basal expression of other HS proteins that are known to contribute to Hsp83's

Figure 1. Biochemical characterization of an inhibitory aptamer that targets HSF1, iaRNAHSF1. (A) Lowest energy diagram predicting the secondary structure of the dimeric HSF aptamer (iaRNAHSF1) using M-fold (upper case = HSF1 aptamer; lower case = self-cleaving hammer head ribozyme). (B) Electrophoretic motility shift assay (EMSA) using radiolabeled iaRNAHSF1 (1 nM) and increasing amounts of dHSF1 protein shows that the aptamer RNA binds to its target avidly. (C) Quantification of independent EMSA assays reveals the apparent affinity of the iaRNAHSF1 ~Kd = 8 nM (n = 5, error indicates %SEM). (D) iaRNAHSF1 competes with HSF1 DNA binding at native promoters in vitro. HSF1 EMSA using limiting amounts of Hsp83 promoter DNA (1 nM (−449 +114)(lanes 2–12), and increasing molar (M) concentrations of cold ‘non-radiolabeled’ iaRNAHSF1 (lanes 9–12) or yeast tRNA (lanes 5–8). (E) Quantification of competition experiments by filter binding assays using Hsp83 (−449 +114) or Hsp70 (−200 +64) promoter DNA. Data normalized to highest ytRNA signal (Hsp70 %SEM n = 3, Hsp83 %SEM n = 6).
chaperone functions. Taken together, our findings demonstrate that under NHS conditions, iaRNAHSF1 expression can compromise HSF1 binding to its native binding sites, such as the Hsp83 locus (63B), resulting in decreased Hsp83 transcript levels and giving rise to animals with phenotypes that resemble loss of function Hsp83 mutants (Figure 2).

**iaRNA**<sub>HSF1</sub> is a potent HSF1 antagonist under HS conditions

It is well documented that under HS conditions, HSF1 undergoes homo-trimerization and binds with high affinity to the HS elements (HSE) of HS promoters (33,37). HSF1 binding to promoters results in the recruitment of various components of the transcription machinery (38), dramatic changes in chromatin architecture and nucleosome disruptions over the entire gene locus (39), and ~200-fold increase in expression of major HS genes. Although iaRNA<sub>HSF1</sub> is predicted to have more difficulty competing against the DNA binding capacity of HS-activated HSF1 homotrimers, we do observe a modest but reproducible inhibitory effect. Figure 3A shows that fly lines that contain 48 dimeric aptamer repeats (three 16-mer arrays inserts crossed into a single line) have high-level iaRNAHSF1 expression that is sufficient enough to compromise HSF1 binding to the Hsp70 locus under NHS conditions in vivo (observed signals normalized to GAF intensities at 63A). (D) HSF1 inhibition by iaRNA<sub>HSF1</sub> results in adults that resemble Hsp83 loss-of-function mutants, and in animals that display abnormal animal morphology within abdominal segments, wings and bristle structures at high frequencies ($n > 500$ animals).

![Figure 2](image-url)

**Figure 2.** iaRNA<sub>HSF1</sub> is a potent HSF1 antagonist under NHS conditions. (A) Design of iaRNA expression system in vivo. Diagram of a polymeric template of 16 iaRNA<sub>HSF1</sub> gene units and their corresponding transcripts (i) corresponds to the first processed iaRNA<sub>HSF1</sub> which lacks a hammerhead, (ii) correspond to the middle iaRNA<sub>HSF1</sub> repeats that contain the ‘self-cleaving’ hammer head ribozymes and (iii) corresponds to the final processed hammer head ribozyme that does not have the iaRNA<sub>HSF1</sub>. (B) Constitutive iaRNA<sub>HSF1</sub> expression results in decreased HSF1 binding to Hsp83 gene (63B locus) during non-induced (NHS) conditions (note: the average diameter of a polytene chromosome is 4 μm). (C) Quantification of the relative intensities shown in panel B among WT ($n = 29$) and iaRNA<sub>HSF1</sub> expressing animals ($n = 29$) (left), and quantification of Hsp83 mRNAs in WT ($n = 6$) and iaRNA<sub>HSF1</sub> ($n = 6$) expressing animals (right) shows that constitutive iaRNA<sub>HSF1</sub> expression inhibits HSF1 binding to the Hsp83 locus under NHS conditions in vivo (observed signals normalized to GAF intensities at 63A). (D) HSF1 inhibition by iaRNA<sub>HSF1</sub> results in adult animals that resemble Hsp83 loss-of-function mutants, and in animals that display abnormal animal morphology with abdominal segments, wings and bristle structures at high frequencies ($n > 500$ animals).
Functional specificity of the in vivo iaRNAHSF1–HSF1 interaction

Overexpression of either iaRNAHSF1 or HSF1 results in increased lethality and an increased frequency of specific morphological phenotypes. To assess the specificity of iaRNAHSF1 for HSF1, we reasoned that overexpression of both molecules within the same animal should ameliorate the aberrant phenotypes of each. This genetic approach is analogous to factor titration or add-back experiments in biochemical assays, where the inhibition of a protein by an RNA aptamer is reversed by the addition of excess protein (22, 23, 25).

First, we assessed if HSF1 overexpression could suppress the abnormalities induced by iaRNA HSF1 overexpression. Here, systemic iaRNAHSF1 expression results in lethality that occurs with increasing iaRNAHSF1 gene dosage (Figure 4A, compare animals that express 8, 24 and 48 iaRNAHSF1 repeats). We reasoned that the observed lethality that occurs among animals expressing high levels of iaRNAHSF1 is likely due to the fact that HSF1 is an essential gene for Drosophila development (9). This iaRNAHSF1-induced effect is effectively suppressed upon HSF1 co-expression (Figure 4A, compare gray and blue column). Additionally, we took advantage of the abnormal wing (notching) defect that occurs with a high frequency in iaRNAHSF1 expressing animals to further determine the specificity of iaRNAHSF1 to HSF1. We choose to focus on the abnormal (notch) wing phenotype because it occurred most frequently in the aptamer expressing population, and as with any genetic suppression analysis, it provided us with an easily observable phenotype that was quick to score. Figure 4B shows that the notched wing defect occurs in iaRNAHSF1 expressing animals and is absent in any of the parental stocks (Figure 4B, compare parental controls). Moreover, this abnormality is not affected by GFP overexpression, but it is effectively rescued upon overexpression of either HSF1, or Hsp83, a major product of HSF1 activity in non-stressed cells (Figure 4B).

Second (a complementary test), we assayed if iaRNAHSF1 expression could suppress the abnormalities induced by HSF1 overexpression. We find that tissue-specific HSF1 overexpression results in abnormally small salivary glands (Figure 4C, compare right and left panel). This abnormality is effectively suppressed when iaRNAHSF1 co-expressed with overexpressed HSF1 (Figure 4C, middle panel). Quantification of the salivary gland length among WT animals and animals that either overexpress HSF1 alone or with iaRNAHSF1 shows that iaRNAHSF1 co-expression restores the salivary gland morphology to nearly WT size (Figure 4D). Furthermore, we find that the high frequencies of lethality in flies with high-level systemic HSF1 overexpression (Figure 4A) is effectively suppressed by iaRNAHSF1 co-expression, resulting in viable and fertile animals (Figure 4A). Lastly, we decided to express the HSF1 aptamer (iaRNAHSF1) and compare its effects with a control aptamer RNA (Rev) that lacks the sequence specificity to target HSF1 protein in stably selected Drosophila S2 cells. Placing each aptamer gene under the copper-inducible promoter results in tight chemical control and high amounts of aptamer levels upon addition of low amounts of CuSO4 (Supplementary Figure S2A). In this system, we observe decreased HSF1 levels at the Hsp70 promoter upon iaRNAHSF1, but not control RNA (Rev) expression (Supplementary Figure S2B). Moreover, iaRNAHSF1 expression inhibits CuSO4 induction of Hsp70 while expression of the control RNA sequence does not (Supplementary Figure S2C).
Taken together, we conclude that iaRNA^HSF1 does not produce its phenotypes non-specifically, but rather acts on the intended target, HSF1, thereby inhibiting expression of Hsp83, which is HSF1’s primary target of binding and regulation under non-induced conditions.

**iaRNA^HSF1 expression attenuates phenotypes of hyperactive mutations in the MAPK signaling pathway**

Hsp83 is known to modulate the MAPK signaling pathway, a well-conserved and important regulatory pathway that is frequently overactivated in human cancers (40). In *Drosophila*, gain-of-function mutations within the MAPK pathway do not result in tumor formation; rather, hyper-activation of the MAPK pathway results in an altered cell fate specification and abnormal tissue morphology. Components of this pathway, such as the EGFR and Raf oncogenes, depend on normal levels of Hsp83 activity for their proper folding, localization or kinase activity (1,41). Thus, the inhibitory potential of the aptamer on the MAPK pathway can be analyzed in vivo by comparing the effects of iaRNA HSF1 expression in animals that also harbor gain-of-function mutations of the *Drosophila* *EGFR* (ellipse) and *Raf* (*RafBT98*) oncogenes. Here, tissues that have decreased MAPK signaling activity should more closely resemble the tissues of animals that do not harbor the gain-of-function mutants.

Expression of iaRNAHSF1 inhibits HSF1 and this, in turn, decreases Hsp83 levels. Because Hsp83 is needed for MAP kinase pathway function, we sought to determine if iaRNA^HSF1 expression might suppresses the abnormalities induced by gain-of-function mutations of the *Drosophila* *EGFR*^{ellipse} and *Raf*^{BT98} oncogenes. Heterozygote animals that express *EGFR*^{ellipse} have been previously shown to contain abnormal wing veins morphology (42). Indeed, this abnormality is effectively
suppressed when HSF1 is inhibited by iaRNAHSF1, or when Hsp83 activity is compromised by expression of the Hsp83e6D antimorphic mutant or when EGFRellipse hemizygote flies are treated with the Hsp90 inhibitor 17-AAG (Figure 5A).

Similarly, heterozygote animals that express a gain-of-function RafBT98 protein have been shown to contain multiple cells within each ommatidium resulting in flies with a distinct rough eye morphology (Figure 5B). Moreover, it has been previously demonstrated that RafBT98 mutants require normal Hsp83 activity to exert this eye specific defect (41). In agreement with the previous findings, we find that the rough eye phenotype that is induced by RafBT98 expression can be reversed by reducing Hsp83 activity through the co-expression of the Hsp83e6D antimorphic mutant, or by treating RafBT98 animals with 17-AAG (Figure 5B). Consistent with our findings that HSF1 controls the expression of Hsp83, we find that HSF1 inhibition by the aptamer also results in the strong suppression of the rough eye phenotype that is caused by increased RafBT98 signaling activity (Figure 5B). The affected surface area of the eye with a rough eye phenotype in each of these overactive MAPK signaling genetic backgrounds can be effectively attenuated by iaRNAHSF1 expression or direct Hsp83 inhibition (Figure 5C).

DISCUSSION

iaRNAHSF1 is a novel HSF1 DNA binding domain inhibitor in vitro and in vivo

In this study, we describe the in vivo utility of an aptamer that targets the highly conserved HSF1 DNA binding domain. We engineered a potent inhibitor of trimeric HSF1 by constructing a dimeric molecule derived from two copies of a previously selected RNA aptamer, which had a modest $K_d$ of $20-30$ nM. The dimeric aptamer (iaRNAHSF1) binds HSF1 with an improved affinity of $K_d$ of $8$ nM. By creating a genetically controlled expression system, which contains polymers of a dimeric aptamer fused to a self-cleaving ribozyme, we demonstrate that we can express iaRNAHSF1 at high levels in whole animals. In cells, this RNA molecule displays an in vivo half-life of 2–4 h, is adequate to produce the phenotypes in animals seen here. Moreover, it can be a useful inhibitor particularly in basic studies of the HS response that are often performed within the first few hours following stress induction. We do, however, acknowledge that the effectiveness of the aptamer could benefit from other modifications that further limit exonuclease degradation.

It is well-documented that HS stress can affect the monomer–oligomer equilibrium status of HSF1 (43). Within seconds following a heat stress, HSF1 shifts from a monomer to a homotrimer state, binding stably and cooperatively to HS gene promoters (37). This can be visualized in vivo using real-time imaging techniques; under normal growth conditions, where monomeric HSF1 displays rapid off-rates with its target genes, but is stably associated with target loci after the cells have been exposed to heat stress (33). Here, we show that our HSF1 aptamer can prevent HSF1 binding to HS loci and its ability to induce gene expression under both normal and stress conditions in vivo. While the aptamer is effective in inhibiting the modest HSF1 DNA binding activity in NHS cells, it only partially inhibits strong HSF1 binding...
in HS cells (Figure 3). We note that aptamer expressing animals show normal survival after heat stress, presumably because the levels of chaperone expression is sufficient to overcome the proteotoxic effects of heat. However, high-level expression of this aptamer in yeast cells results in strong growth defects at elevated temperatures (44).

Whenever a ligand such as an aptamer is used in vivo to study and manipulate the function of a protein, it is important to know whether the intended target is specifically recognized by the ligand/drug. However, testing the binding of iaRNAHSF1 to every protein in a cell is not feasible. Instead, we use functional assays to demonstrate that effect of iaRNAHSF1 is specific to HSF1. The abnormalities that arise from the expression of iaRNAHSF1 in Drosophila are effectively suppressed by HSF1 co-expression and not a control protein like GFP, suggesting that the aptamer is exerting its effects by targeting HSF125. Conversely, the abnormalities observed in Drosophila induced by HSF1 overexpression are also effectively suppressed by iaRNAHSF1 overexpression. Moreover, in Drosophila S2 cells iaRNAHSF1 expression effectively attenuates HSF1 activity while expression of a control aptamer sequence (Rev) does not. Collectively, our analysis provides further supporting evidence for the specific nature of the aptamer–HSF1 interaction in vivo.

HSF1 regulates the activity of the Hsp83 (Hsp90) buffering system that promotes adaptation to stress

In contrast to mammals, where the Hsp83 locus (Hsp90) is not controlled by HSF1 but rather by other HSF isoforms (45), here we show that Drosophila, which has a single of HSF gene, requires HSF1 for the proper expression of Hsp83 during development. The constitutive level of Hsp83 protein is impressive, reaching concentrations of 1–2% of the total protein content in vivo (45–47). This HSF1 involvement in constitutive expression of Hsp83 was first suggested by the fact that the Hsp83 locus (cytological site 63B) shows the highest HSF1 occupancy over any site on Drosophila chromosomes. We also find that either iaRNAHSF1 or HSF1 RNAi expression in Drosophila reduces the levels of HS transcripts, and in particular, the constitutive levels of Hsp83. Hsp83 has a general role in biological processes such as spermatogenesis, protein trafficking, signal transduction, cytoskeletal organization and cell survival pathways (41,48–52). Given the fact that Hsp83 exerts its chaperone functions in concert with other HS proteins; it is, therefore, likely that decreasing various HS mRNAs levels with iaRNAHSF1 expression attenuates HSF1 activity resulting in animals that have phenotypes of previously reported Hsp83 mutants (35), albeit at much higher frequencies than seen previously in Hsp83 hemizygotes. Moreover, we find that iaRNAHSF1 expression effectively attenuates the abnormal activities of Hsp83 client proteins, EGFR and Raf oncoproteins. Collectively, our data suggest that in Drosophila the Hsp83 gene is a primary target of HSF1 regulation during normal conditions, and is highly responsive to HSF1 inhibition during animal development.

This study builds upon a previous Hsp83-directed chaperone ‘buffering’ model (35,36), and our data supports the hypothesis that the master regulator HSF1 is critical for constitutive expression of molecular chaperones. In particular, HSF1 inhibition results in an altered chaperone-driven buffering system that promotes animal trait variation and the signaling activities of cancer causing mutations (Supplementary Figure S3). Herein, we provide an in vivo approach aimed at understanding HSF1 function during animal development and its putative role for early drug target validation. Because the HSF1 DNA-Linker domain is highly conserved among eukaryotes (53), it is likely that this novel HSF1 inhibitor (iaRNAHSF1), or derivatives thereof will prove to be a useful reagent(s) that will further aid in unraveling the functions of related HS transcription factors in other model organisms or of HSF1-dependent diseases such as cancer.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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