Efficient expression of genes in the Drosophila germline using a UAS-promoter free of interference by Hsp70 piRNAs.

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ABSTRACT

Controlling the expression of genes using a binary system involving the yeast GAL4 transcription factor has been a mainstay of Drosophila developmental genetics for nearly thirty years. However, most existing GAL4 expression constructs only function effectively in somatic cells, but not in germ cells during oogenesis, for unknown reasons. A special UAS promoter, UASp was created that does express during oogenesis, but the need to use different constructs for somatic and female germline cells has remained a significant technical limitation. Here we show that the expression problem of UASSt and many other Drosophila molecular tools in germline cells is caused by their core Hsp70 promoter sequences, which are targeted in female germ cells by Hsp70-directed piRNAs generated from endogenous Hsp70 gene sequences. In a genetic background lacking genomic Hsp70 genes and associated piRNAs, UASSt-based constructs function effectively during oogenesis. By reducing Hsp70 sequences targeted by piRNAs, we created UASz, which functions better than UASp in the germline and like UASSt in somatic cells.

INTRODUCTION

Drosophila is an extremely powerful model organism for studies of animal development and disease because of its low maintenance costs, rapid generation time, and expansive collection of tools to genetically modify its cells. One particularly useful tool is the Gal4/UAS two-component activation system, in which the Gal4 transcriptional activator protein recognizes an upstream activator sequence (UAS) to induce the expression of any gene of interest (Fischer et al. 1988; Brand and Perrimon 1993). By controlling the activity of Gal4 with tissue-specific or inducible promoters or the Gal80 inhibitor protein, one can manipulate genes in specific cells.
or times of development, visualize cell types, probe cell function, or follow cell lineages. One of
the most useful applications of these techniques has been to carry out genetic screens by
expressing RNAi in targeted tissues or cultured cells (Dietzl et al. 2007; Ni et al. 2008).

The original pUASt vector from Brand and Perrimon (1993), which contains an Hsp70-
derived core promoter and SV40 terminator, has undergone several optimizations to improve its
expression (Fig 1A). Popular versions, such as the Valium10 or 20 vector used by the
Drosophila Transgenic RNAi project (TRiP) (Ni et al. 2009; 2011) and the pMF3 vector used by
the Vienna Drosophila Research Center (VDRC) GD collection (Dietzl et al. 2007) added a ftz
intron, and the Janelia Gal4 enhancer project used derivatives of pJFRC81, which added a
myosin IV intron (IVS), synthetic 5’UTR sequence (syn21) and viral p10 terminator to boost
expression levels across all Drosophila cell types (Figure 1A) (Pfeiffer et al. 2012). However,
these modifications did not correct UASt’s major problem- that it drives woefully poor
expression in the female germline compared to somatic tissues. Consequently, genetic
manipulation in this important tissue has often relied on a special GAL4-activated promoter,
UASp, produced by fusing 17 copies of the UAS activator to a germline compatible promoter
derived from the P-element, a transposon naturally active in the female germline (Figure 1B)
(Rørth 1998). Although UASp expression is qualitatively higher than UASt in the female
germline, it is generally known to be lower in somatic tissues.

The lack of a UAS construct that is widely useful in all Drosophila tissues has remained
an obstacle to providing optimum genetic tools to the research community. Transgenic RNAi
collections were first constructed using UASt and screening of genes for germline functions has
relied on increasing the effectiveness of RNAi by co-expressing Dcr2 or expressing short hairpin
RNAi from UASp promoters (Ni et al. 2011; Yan et al. 2014; Sanchez et al. 2016). A
significant obstacle to obtaining a widely effective GAL4 vector has been the lack of understanding of the reason UASt functions poorly in germ cells, and the paucity of accurate comparisons between the UASp and UASt promoters in the absence of other significant variables.

**MATERIALS AND METHODS**

**Drosophila strains:**

*Mef2-Gal4* (BL26882) w[ ]; Kr[If-1]/CyO, P{w+ GAL4-Mef2.R}2, P{w+ UAS-mCD8.mRFP}2

*Tub-Gal4* (BL5138) y[1] w[ ]; P{w+ tubP-GAL4}LL7/TM3, Sb[1] Ser[1]

*FLP/phiC31int* (BL33216) P{hsFLP}12, y[1] w[ ]; M{vas-int.B}ZH-2A; S[1]/CyO;

*Pri[1]/TM6B, Tb[1]*

*Hsp70Δ* (BL8841) w[1118]; Df(3R)Hsp70A, Df(3R)Hsp70B

*Nanos-Gal4VP16* (BL4937) w[1118] ; P{nos-GAL4::VP16-nos.UTR}MVXI

*Vasa-Gal4* was obtained from Zhao Zhang’s lab: y[ ] w[ ]; P{w+ vas-GAL4.2.6} (Zhao et al. 2013)

*UASt-Rhi-RNAi* (VDRC313156 from Julius Brennecke stocks)

*UASt-w-RNAi* (VDRC313772 from Julius Brennecke stocks)

**New stocks created for this study:** Bestgene Inc. introduced pMRtGFP and pMRpGFP into yw flies using a P-transposase helper plasmid and we isolated GFP+ insertions by crossing the F0 to a *Mef2-Gal4* background and scoring for GFP+ muscles. We introduced UAStGFP or UASpGFP into MI04106 and other MiMIC lines using a cross strategy outlined in (Nagarkar-Jaiswal et al. 2015). Rainbow transgenics introduced pJFRC81 (UAStGFP-attB), pUASpGFP-
attB, and pUASzGFP-attB into attP40 using an X-chromosome encoded phiC31 integrase source and we isolated multiple w+ phiC31 minus insert lines by standard fly genetics.

Vectors created for this study: Genescript synthesized pMRtGFP. We created pMRpGFP by replacing the Nhel-BglII UASr promoter in pMRtGFP with a SpeI-BglII UASp promoter from Valium22. We created pUASpGFP-attB by replacing the PstI-BglII UASr promoter in pJFRC81 with the PstI-BglII UASp promoter from Valium22. We created UASzGFP-attB by replacing the 259 bp Nhel-BglII fragment of pJFRC81 containing the 203 bp Hsp70 promoter with annealed oligos encoding 63 bp from the 5’ end of the same promoter.

Top oligo: 5’
CTAGCGACGTCGAGCGCCGGAGTATAAATAGAGGCGCTTCGTCTACGGAGCGACAA
TTCAATTCAAAACAAGCAAA 3’

Bottom oligo: 5’
GATCTTTGCTTGTTTGAATTGAATTGTCGCTCCGTAGACGAAGCGCCTCTATTTTATAC
TCCGGCGCTCGACGTCG 3’

We created UASz by replacing the NotI-syn21-GFP-XbaI fragment in UASzGFP with annealed oligos encoding NotI-sny21-BamHI-Xhol-KpnI-Spel-XbaI

Top oligo:
GGCCGCAACTTAAAAAAAAAAATCAAAGGATCCCTCGAGGGTACCCTTAGTT

Bottom oligo:
CTAGAACTAGTGGTACCCTCGAGGGATCCCTTTGATTTTTTTTTTTAAAGTTGC

We created UASz1.1 by replacing the KpnI-EcoRI p10 terminator in UASz with a PCR amplified p10 terminator containing Kpn1-XbaI-EcoRI and ApoI tails.
F primer: 5’

CATGGTACCCTCTCTAGAGTGTGAATTCTGGCATGAATCGTTTTTAAAATAACAAATCAATTGTATTATAAT

R primer: 5’

GGAAATTTTCGAAATCGCTATCCAAGGCCAGCT

We created UASz1.2 by destroying the NheI and EcoRI sites in UASz1.1 by cloning annealed oligos into the NheI-EcoRI backbone.

Top oligo:

CTAGGAGCGCCGAGTATAAATAGAGGCCTTCGTCTACGGAGCGACAATTCAATTCAAAACAAGCAAGATCTGGCCTCGAGCT

Bottom oligo:

AATTACTCGAGGCAGATCTTGGCTTTTAATTGAATTGTCGCTCCGTAGACGAAGCGCCTCTATTTATACTCCGGCGCTC

To create UASzMiR, we cloned a BglII-XhoI fragment containing the MiR1 cassette and ftz intron from Walium22 into the BglII-XhoI backbone of UASz1.2.

**Tissue Preparation Imaging and Quantitation:** For all experiments, we crossed UAS-GFP or UAS-GFP *Hsp70Δ* males to control (*yw*), *Tub-Gal4/TM3*, homozygous *Vasa-Gal4*, or homozygous *Vasa-Gal4 Hsp70Δ* females. For whole larvae imaging, we picked wandering 3rd instar larvae of various genotypes, aligned them on the same glass slide, and placed them the freezer for 30 minutes prior to imaging. For adult ovary or larval tissue imaging, we fixed dissected tissue with 4% paraformaldehyde for 13 minutes (whole ovary) or 20 minutes (larval tissue) and stained with DAPI in PBS + 0.1% Triton X-100. We imaged GFP fluorescence of semi-frozen whole 3rd instar larvae or whole ovaries mounted in 50% glycerol on a Leica Stereoscope equipped with mercury arc light source, GFP filters, and CCD camera. We imaged
GFP fluorescence in larval imaginal discs, salivary glands, and epidermis, and manually separated ovarioles mounted in 50% glycerol using a custom-built spinning disc confocal with 20x 0.8 NA lens. For each genotype and tissue type, we acquired a single plane image from at least 4 individuals using Metamorph software and the same laser power, CCD camera gain, and exposure time between equivalent samples. We measured average pixel intensity in 14 bit images of the GFP channel using Image J. We acquired representative images of single planes through single ovarioles for Figure 2 on a Leica Sp8 scanning confocal with 63x 1.4 NA lens and PMT (for DAPI) and HiD (GFP) detectors using identical settings between samples.

**UASt piRNA analysis:** We clipped and aligned sequenced small RNA libraries from (Mohn et al. 2014) (SRR1187947:control germline knockdown and SRR1187948:rhino germline knockdown) to D. melanogaster Genome Release 6 (Hoskins et al. 2015) or UAStGFP using the Bowtie2 aligner with no filtering for repetitive mappers (Langmead and Salzberg 2012). We visualized piRNA read depth to UAStGFP or both Hsp70 clusters using the Interactive Genome Browser (Robinson et al. 2011).

**DATA AVAILABILITY**

Fly strains and vectors are available upon request. pUASz1.0 and pUASzMiR, and sequences, are available from the Drosophila Genomics Resource Center, as items 1431 and 1432.

**RESULTS AND DISCUSSION**

**Difference between UASp and UASt:** To study the difference between the UASp and UASt promoters, we first created UAStGFP and UASpGFP constructs controlled for other variables between the original UASt and UASp, such as UTR components, introns, terminators, and genomic insertion site. Both constructs were based on pJFRC81 and only varied at the
promoter and 5’ UTR of the transcript (Fig 1, red letters). We made these vectors compatible with PhiC31-catalyzed recombination-mediated cassette exchange with MiMIC transposons containing these constructs (pMRtGFP and pMRpGFP), allowing us to integrate UAS-GFPs into many common sites throughout the genome (Venken et al. 2011). Using a previously established protocol (Nagarkar-Jaiswal et al. 2015), we recombined both UAS-GFPs into several MiMICS, including MI04106, which resides in a region enriched for ubiquitously expressed genes and active chromatin marks (Filion et al. 2010; Kharchenko et al. 2011) referred to as “the gooseneck” by Calvin Bridges for its long stretch of low density in salivary gland polytene chromosome preps (Bridges 1935). Consistent with previous reports, UASt drove significantly stronger expression than UASp in all somatic tissues examined while UASp drove significantly stronger expression in the female germline (Fig 2A,B).

**Hsp70 piRNAs repress UASt:** We next investigated the reason for the extremely weak UASt expression in the female germline. Several lines of evidence implicated piRNA-directed silencing as a mechanism limiting UASt expression. *Drosophila* piRNAs are ovary and testis-enriched, 23-29 nucleotide (nt) RNAs that complex with Argonaut family proteins and silence transposons through homologous base-pairing-directed mRNA cleavage and heterochromatin formation (Siomi et al. 2011). Some of the most successful UASt-based genetic screens in the female germline knocked down piRNA biogenesis genes (Czech et al. 2013; Handler et al. 2013). If piRNAs were silencing UASt, then UASt-RNAi against a piRNA biogenesis gene would boost UASt expression leading to maximal knockdown. Where might these UASt-piRNAs originate from? Previously, Olovnikov et al (2013) and Mohn et al (2015) characterized an abundance of germline-specific piRNAs mapping to both *Hsp70* gene clusters. Because UASt contains the *Hsp70* promoter and 5’UTR, we hypothesized that germline piRNAs against
Hsp70 may be targeting UASt. When we searched for UASt sequences in the piRNAs identified by Mohn et. al (2015), we identified abundant piRNAs perfectly homologous to UASt (Fig 2E pink bars, and Fig 2F). Similar to UASt silencing, these UASt piRNAs are restricted to the female germline because germline-specific knockdown of rhino, a gene required for Hsp70 piRNA production eliminates UASt piRNAs from whole ovaries (Fig 2E) (Mohn et al. 2014).

To directly test whether Hsp70 piRNAs silence UASt, we tested UASt expression in Hsp70Δ flies (Gong and Golic 2004), which completely lack all genetic loci producing piRNAs homologous to UASt (Fig 2E, grey boxes deleted). Despite missing all copies of the inducible Hsp70 gene family and related piRNAs, Hsp70Δ flies have no significant defects in viability or egg production in the absence of heat stress (Gong and Golic 2006). However, Hsp70Δ flies showed greatly enhanced UAStGFP expression. Furthermore, UAStGFP expression was significantly stronger than UASpGFP, which was unaffected by Hsp70Δ (Fig 2D). Repression of full UAStGFP expression in germ cells requires normal piRNA production, since UAStGFP expression was also boosted by germline knockdown of rhino, which is required for the production of Hsp70 and many other germline-specific piRNAs (Fig 2C). These results argue strongly that UASt is normally silenced by Hsp70 piRNAs and that UASt is a stronger expression vector than UASp in cells lacking Hsp70 piRNAs.

**Construction of UASz:** We next attempted to create a new version of the UAS expression vector that works well in both the soma and female germline. We hypothesized that eliminating the part of UASt targeted by piRNAs would boost UASt expression by the same amount as eliminating the piRNAs themselves. Hsp70 piRNAs are homologous to 247 nt of the UASt promoter and 5’UTR. While we could make enough substitutions along this stretch to prevent all possible 23 nt piRNAs from binding, we were afraid this approach might impair
important promoter sequences. Instead, we hypothesized that Hsp70 piRNAs might recognize UASl RNA to initiate piRNA silencing. To prevent Hsp70 piRNAs from recognizing UASl RNA, we trimmed down the UASl 5’UTR to be shorter than a single piRNA, from 213 nt to 19 nt (Fig 1A, Fig 2E). We named this UTR-shortened UASl variant “UASz,” because we optimistically hoped it would be the last one anyone would make.

**Comparison of UAS vectors:** To compare the relative expression levels of our UASz to UASp and UASl, we created all three variants in the same GFP vector backbone (pJFRC81) with a single attB site. We used phiC31 integrase to introduce these UAS-GFP variants into a commonly used genomic site, attP40, and recombined all three inserts with Hsp70Δ to determine the influence of Hsp70 piRNAs on their expression. When combined with Tub-Gal4, a somatic Gal4 driver, UASz was expressed at least 4 times higher than UASp in all somatic tissues tested and was equivalent or greater than UASl in some somatic tissues like the larval epidermis and salivary gland (Fig 3A,C,E). However, UASz was expressed at about 40% of UASl in discs, suggesting some elements of the UASl 5’UTR may boost expression in some tissues (Fig 3C,E). To measure germline expression, we crossed the three UAS-GFPs to vasa-Gal4, which is evenly expressed up to stage 6 of oogenesis. In the germline, UASz was expressed about 4 times higher than UASp at all stages, while UASl was expressed at much lower levels than UASp, except in region 1 of the gerarium (Fig 3B,D-F) where piRNA silencing is weaker (Dufourt et al. 2014). We conclude that UASz is a superior expression vector to UASp in all tissues, and is equivalent to UASl in many, but not all, somatic tissues.

Finally, we wanted to test if UASz is still targeted by Hsp70 piRNAs because it contains 63 nt of Hsp70 sequence and about 10% of the putative piRNAs targeting UASl (Fig 2F). We crossed UASzGFP into the Hsp70Δ background and compared UASzGFP levels with or without
Hsp70 piRNAs. We observed no enhancement of UASzGFP when Hsp70 piRNAs were removed (Fig 3B,D,F). Therefore, Hsp70 piRNAs likely target the UASt but not UASz 5′UTR, consistent with the model that piRNAs must initially recognize RNA but not DNA.

Is UASz the final, fully optimized iteration of a UAS vector? Probably not. UAS without Hsp70 piRNAs induces about twice the expression of UASz in the ovary (Fig 3B,D,F). This twofold advantage of UASt over UASz in the germline or imaginal discs lacking Hsp70 piRNAs is similar to the twofold advantage of UASt over the UAS fused to the Drosophila Synthetic Core Promoter (Pfeiffer et al. 2010). Perhaps adding back some sequences within the first 203 nt of the Hsp70 5′UTR while avoiding piRNA recognition may improve UASz. However, the current iteration of UASz remains an unequivocal upgrade over UASp for all applications and UASz should be preferred over UASt if both germline and soma studies are planned from a single vector. Alternatively, one could boost germline expression of an existing UAS construct by crossing it into the Hsp70Δ background.

Current UAS-RNAi collections are heavily biased toward UAS-RNAi-based constructs. To date, the VDRC and DRSC/TRiP RNAi projects used UAS-RNAi to target 12,539 and 8,876 genes, respectively. Germline screens for developmental phenotypes using UAS-RNAi were enriched for phenotypes in germline region 1 (Yan et al. 2014; Sanchez et al. 2016), where piRNA silencing is weakest (Dufourt et al. 2014) and UAS shows maximum expression (Fig 3D arrow). Perhaps these screens were depleted for developmental defects in later germline stages because of poor UAS-RNAi expression in these stages. Although UASp-RNAi from the Valium22 vector (Fig 1B) increased the efficiency of obtaining phenotypes in a germline screen, only 1,596 genes are currently targeted by this collection (Yan et al. 2014). Additionally, when screening somatic cells, Ni et al. (2011) recommend UAS-RNAi because UASp-RNAi gave
incomplete knockdowns. Our results revealed that UASp is equally weak in the germline as somatic tissues when compared to UASz (Fig 3E). Therefore, UASp-RNAi may also generate incomplete knockdowns in the germline. To increase germline RNAi expression, we propose using a UASz-based RNAi expression vector, such as UASzMiR (Sup Figure 1), which is compatible with previously generated shRNA oligo cloning (Ni et al. 2011).

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FIGURE LEGENDS

Figure 1: Components of common UAS constructs used by the fly community. (A) Cartoon depicting a Drosophila Hsp70 gene relative to sequences in UASt-based vectors. In pUASt, multiple copies of optimized Gal4 binding sites (5xUAS) replace heat-inducible enhancers (Heat Shock Elements, HSEs) in a fragment of Hsp70 containing the transcription start site (TSS) and 5’UTR. In derivatives of UASt, a multiple cloning site (MCS), RNAi constructs, GFP coding sequence, synthetic UTR elements (syn21), and introns (ftz or myosin IV, IVS) replace 39 bp of Hsp70 5’UTR and Hsp70 coding sequence (CDS). Viral-derived SV40 or p10 sequences terminate transcription and contribute to the 3’UTR. For this study, we created a derivative of pJFRC81 with a truncated 5’UTR (pUASzGFP-attB) and a derivative compatible with MiMIC RMCE (pMRtGFP). (B) Cartoon depicting two common UASp vectors containing the K10 terminator and Drosophila P-element promoter, TSS and 5’UTR in place of the SV40 terminator and Hsp70 sequences. We created two new UASp vectors, pUASpGFPattB and pMRpGFP, based on pJFRC81 and pMRtGFP to directly compare the effect of P-element and Hsp70 sequences on transgene expression. Vector names colored red are used in this study.
Figure 2: Expression from UAS\textit{t} is greater than UAS\textit{p} in cells lacking \textit{Hsp70} piRNAs. (A-D) pMRtGFP (UAS\textit{t}GFP) and pMRpGFP (UAS\textit{p}GFP) integrated into the same MiMIC site (Mi04106) and crossed to either a control without Gal4 to visualize UAS-GFP leakiness, Tub-Gal4 for somatic UAS-GFP expression, or Vasa-Gal4 or Nanos-Gal4 for germline UAS-GFP expression. Each panel is a single inverted GFP fluorescence image genotypes mounted side by side to compare expression levels. Scale bar is 1 mm. (A) Wandering third instar larvae. (B-D) Adult ovaries. (C) Nanos-Gal4 driving germline UAS\textit{t}GFP expression in addition to w-RNAi (control) or Rhino-RNAi. (D) Germline UAS-GFP expression in the presence or absence of \textit{Hsp70} genes and piRNAs. Image in (B) is longer exposure than (D) to show minimal induction of UAS\textit{t}GFP when vasa-Gal4 added in presence of \textit{Hsp70} piRNAs. (E,F) Genome browser view of whole-ovary-derived piRNAs from MTD-Gal4>UAS\textit{t}-wRNAi (control) or MTD-Gal4>UAS\textit{t}-rhinoRNAi (Mohn et al. 2014) aligned to the Drosophila genome release 6 (E) or our UAS\textit{t}GFP construct (F). (E) \textit{Hsp70} genes colored in green. piRNA read depth in black. piRNA read depth also mapping to \textit{UAS}\textit{t} in magenta. Grey shaded area represents the DNA deleted in the \textit{Hsp70}\Delta background. (F) piRNAs from MTD-Gal4>UAS\textit{t}-wRNAi (Mohn et al. 2015) aligned to our UAS\textit{t}GFP construct to show the mapping position of previously described piRNAs that could theoretically silence the transgenes used in this study. The green shaded area shows the 184 bp of UAS\textit{t} deleted to make UAS\textit{z}.

Figure 3: Expression level of UAS\textit{z} relative to current UAS variants. UAS\textit{t}GFP, UAS\textit{p}GFP, and UAS\textit{z}GFP integrated into a single genomic site, attP40, crossed to control (no \textit{Gal4}) or the indicated \textit{Gal4} driver in either wild type or \textit{Hsp70}\Delta background. Inverted GFP fluorescence
images of wandering 3rd instar larvae (A), whole adult ovaries (B), or 3rd instar larval wing discs and salivary glands (C). (D) paired images showing single ovarioles of the indicated genotype imaged in one channel for GFP fluorescence (bottom) and another for DAPI (DNA, top). Arrows indicate germline region 1, where piRNA silencing is reduced. Scale bars are 1mm for (A,B) 0.1mm for (C,D). (E) average GFP fluorescence intensity from UASτGFP and UASζGFP relative to UASpGFP in the indicated tissue expressing Tub-Gal4 (soma) or Vasa-gal4 (germline). Error bars indicate standard deviation from the mean from at least 4 samples. (F) Average GFP pixel intensity in germ cells of the indicated stage and genotype. WD = wing disc, SG = salivary gland, LE = larval epidermis, GSC/CB = germline stem cell or cystoblast, R1, R2A, or R2B = germline region 1, 2A, or 2B, St_ = nurse cells of indicated stage number.
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Figure 1

A

D. mel. Hsp70Ba

pUASp

VDRC RNAi KK

VDRC RNAi GD

TRiP RNAi V10/20

pJFRC81 (UAStGFP)

pMRtGFP

pUASzGFP-attB

B

pUASp

TRiP RNAi V22

pUASpGFP-attB

pMRpGFP

10XUAS

10XUAS

10XUAS

14XUAS

203 bp 5'UTR

203 bp 5'UTR

203 bp 5'UTR

242 bp 5'UTR

19 bp 5'UTR

HSEs

MCS

RNAi

RNAi

IVS-syn21

GFP

p10

MCS

SV40

ftz intron

SV40

10XUAS

10XUAS

10XUAS

P-promoter / UTR

P-promoter / UTR

P-promoter / UTR

TSS

TSS
Figure 2

A  UASGFP  UASpGFP
    Tub-Gal4  Control
    Vasa-Gal4

B  UASGFP  UASpGFP
    Control

C  Nanos-Gal4  UASGFP
    UAS-rhinoRNAi

D  UASGFP  UASpGFP
    Vasa-Gal4
    Hsp70Δ

E  wRNAi  rhinoRNAi
    Hsp70A cluster

F  10XUAS  hsp70  IVS-sy  GFP
    TSS  p10
Figure 3

A) UAS-GFP, UASp-GFP, UASz-GFP

B) Vasa-Gal4, UAS-GFP, UAS-GFP

C) wing disc, salivary gland

D) Vasa-Gal4, Vasa-Gal4 hsp70Δ

E) Fold increase relative to UASp

F) GFP pixel intensity (AU)