HHS Public Access

Author manuscript

Nat Genet. Author manuscript; available in PMC 2012 September 15.

Published in final edited form as:

Nat Genet. 2011 February ; 43(2): 153–158. doi:10.1038/ng.743.

Drosophila Piwi Functions in Hsp90-Mediated Suppression of Phenotypic Variation

Vamsi K. Gangaraju, **Hang Yin**[1], **Molly M. Weiner**, **Jianquan Wang**, **Xiao A. Huang**, and **Haifan Lin**[‡]

Yale Stem Cell Center and Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06509

Keywords

Piwi; Hop; Hsp90; canalization; phenotypic variation; *Drosophila*

Canalization, also known as developmental robustness, describes an organism's ability to produce the same phenotype despite genotypic variations and environmental influences[1,2]. In *Drosophila*, Hsp90, the Trithorax group proteins, and transposon silencing have been implicated in canalization[3,4]. Despite this, molecular mechanism underlying canalization remains elusive. Here, using an *Drosophila* eye-outgrowth assay sensitized by the dominant $Kr^{Irregular\ facets\text{-}1}$($Kr^{If\text{-}1}$), allele[3], we show that the piRNA pathway, but not siRNA or miRNA pathways, is involved in canalization. Furthermore, we isolated a protein complex composed of Hsp90, Piwi, and the Hsp70/Hsp90 Organizing Protein Homolog (Hop), and demonstrated the function of this complex in canalization. Our data indicate that Hsp90 and Hop regulate the piRNA pathway via Piwi to mediate canalization. Moreover, they point to epigenetic silencing of the expression of existing genetic variants and the suppression of transposon-induced new genetic variation as two major mechanisms underlying piRNA pathway-mediated canalization.

In both plants and animals, Hsp90 buffers against morphological changes induced either by genetic or non-genetic mechanisms, thereby promoting the robustness of the developmental programs that have been subjected to natural selection[5-8]. However, under certain conditions, such as environmental stress, Hsp90 becomes overwhelmed, loosens its grip on canalization, and fails to repress the expression of genotype variants that have accumulated during evolution. The expressed phenotypes quickly become independent of Hsp90 deficiency, can be inherited in later generations, and could be subject to natural selection[3,5]. In addition to Hsp90, maternally inherited epigenetic machineries also prevent expression of

‡Corresponding author: (Haifan.lin@yale.edu) .

[1]Present address: Regenerative Medicine Program, Sprott Centre for Stem Cell Research 501 Smyth Road, Ottawa ON K1H 8L6, Canada

AUTHOR CONTRIBUTIONS V.K.G and H.L designed the project and wrote the paper. H.Y made the initial observation that *piwi* mutations affect canalization. M.M.W showed that *Hop* mutations enhance eye outgrowth phenotype. J.W performed 2D gel electrophoresis and X.A.H assisted with column chromatography.

genotypic variants[3], indicating that faithful transmission of epigenetic marks across generations is also critical for canalization. Hence, examining the regulation of epigenetic inheritance should provide important insights into the molecular mechanisms underlying canalization. Piwi, a piRNA binding protein, is implicated in epigenetic regulation as both a maternal and a zygotic factor[9-14]. Hence, we reasoned that Piwi might mediate canalization through its epigenetic function.

To test our hypothesis, we used a dominant gain-of-function allele, Kr^{If-1}, that ectopically expresses *Krüppel*, a zinc-finger transcription factor, in the ventral region of the developing eye imaginal disc[15]. This ectopic expression misregulates homeotic genes in the eye disc and generates eye outgrowths, which, however, are normally repressed and present only in less than 0.1% of Kr^{If-1} progeny[3,15,16]. The loss-of-function mutations of *Hsp83* and the *trithorax* group of genes enhance the expression of this phenotype, implicating these factors in canalization[3]. We used this sensitized assay to examine if a reduction in maternal dose of Piwi also enhances the outgrowths. The genetic cross was setup as shown in Figure 1A. We observed that strong *piwi* alleles, $piwi^{-1}$ *and* $piwi^{-2}$, are dominant enhancers of the eye outgrowth phenotype induced by *Krüppel* ectopic expression (Figure 1B). When $piwi^{-1}$/+ *or* $piwi^{-2}$/+ female flies were crossed to Kr^{If-1}/+ males, the outgrowth phenotype was observed in approximately 7% progeny (n=146). The reciprocal cross (Kr^{If-1}/+ females x $piwi^{-1}$/+ or $piwi^{-2}$/+ males), however, produced no offspring with the outgrowth (n=122; data not shown), indicating that maternal Piwi mediates canalization in a dose-sensitive fashion.

If canalization is solely mediated by maternal Piwi, it should be independent of the genotype of the progeny. Instead we found that the expression of the outgrowth phenotype also depends on the presence of *piwi* mutation in the progeny, since only Kr^{If-1}/ $piwi^{-2}$, but not their Kr^{If-1}/+ siblings, express the phenotype (Figure 1B). These data indicate that zygotic Piwi also plays a role in canalization and that both $piwi^{-1}$ and $piwi^{-2}$ produce the same phenotype as the loss-of-function alleles of *Hsp83* and the *trithorax* group of genes[3].

To further link the outgrowth phenotype to the ectopic expression of genes underlying the outgrowth, we examined the expression of *wingless* in eye imaginal discs of the progeny of +/+ and $piwi^{-1}$/+ females. *wingless* is a target gene of maternal enhancers of Kr^{If-1}-induced eye outgrowth[3]. When $piwi^{-1}$/+ *or* $piwi^{-2}$/+ female flies were crossed to Kr^{If-1}/+ males, *wingless* became ectopically expressed in approximately 10% of the eye imaginal discs of the progeny (Supplementary Figure 1A). This indicates that the Piwi/piRNA pathway can affect nontransposon gene expression in a dose-sensitive manner to achieve canalization.

We next examined whether canalization is specific to *piwi*. Reduction in maternal dose of Aubergine, another Piwi subfamily protein involved in the piRNA pathway, resulted in ~16% of progeny with the eye outgrowth phenotype (Supplementary Figure 1B). However, reduction in maternal doses of Dicer-1 or Dicer-2, key proteins in the miRNA and siRNA pathways, respectively, did not result in any eye outgrowth phenotype (Supplementary Figure 1C). These findings indicate that canalization is mediated by the piRNA pathway, but not the miRNA or siRNA pathway.

It is argued that canalization is a general feature of complex gene networks and that perturbation in any node of these networks can lead to compromised canalization[17]. Hence, we examined whether Piwi and Hsp90 function in the same pathway or in parallel pathways that happen to produce similar phenotypes. We tested if over-expression of maternal Piwi suppresses the eye outgrowth phenotypes of *Hsp83* induced by geldanamycin, a chemical that specifically inhibits Hsp90 and induces eye outgrowths in $Kr^{If\text{-}1}$ flies[3]. To over-express maternal Piwi, we used a transgenic *myc-piwi* line (G38) wherein a fully functional *myc-piwi* gene was inserted into the second chromosome that contains endogenous *piwi*[13,18], thereby increasing the *piwi* copy number to four. We generated $Kr^{If\text{-}1}$/*myc-piwi* virgin females (which contain three copies of *piwi*), and crossed them to $Kr^{If\text{-}1}$/+ males to generate $Kr^{If\text{-}1}/Kr^{If\text{-}1}$ flies. In $Kr^{If\text{-}1}/Kr^{If\text{-}1}$ flies from females containing two copies of *piwi*, inhibition of Hsp90 by geldanamycin increased ectopic outgrowth by ~10 fold (Figure 1C; increase from 1.2±1.79% to 10.8±2.3%). However, in $Kr^{If\text{-}1}/Kr^{If\text{-}1}$ flies from females containing three copies of *piwi* ($Kr^{If\text{-}1}$/*myc-piwi*; ~50% increase in maternal Piwi), the ectopic outgrowth phenotype was rescued by ~52% (from 10.8±2.3% to 5.6±1.7%). These results indicate that *piwi* and *Hsp83* genetically interact in achieving canalization. This interaction could reflect that *Hsp83* and *piwi* act on different pathways with additive effect towards canalization. Alternatively, it could reflect that *piwi* and *Hsp83* function in the same pathway, with *piwi* downstream of *Hsp83* in regulating canalization (Figure 1D).

To explore molecular mechanism underlying the Piwi-mediated canalization, we fractionated cytoplasmic extracts of 0-12 hour (h) embryos using column chromatography (Figure 2A and Supplementary Figure 2). After the final column, Piwi migrated with an apparent molecular weight of ~150kDa (Figure 2C, upper panel). The peak fraction for Piwi (# 27) was resolved using gel electrophoresis. Co-migrating peptides were visualized using silver staining (Figure 2B), excised from the gel, and identified by mass spectrometry. In addition to Piwi that migrates at ~90 kDa, another abundant protein migrating at ~60kDa was identified as Hsp70/Hsp90 Organizing Protein Homolog (Hop, CG2720; Figure 2B). Western blotting of fractions from the Superdex 200 column showed that Piwi and Hop co-migrate during size exclusion chromatography (Figure 2C). The interaction was further confirmed by coimmunoprecipitation of Piwi with Hop from 0-12h embryonic extracts (Figure 2D). Hop contains three tetratricopeptide repeats (TPR1a, TPR2a and TPR2b) and a small DP repeat motif called DP2[19,20]. TPR1 domain of Hop binds to Hsp70 and TPR2a domain binds to Hsp90[21,22]. Furthermore, in support of our genetic experiments implicating Piwi as a client of the highly selective chaperone Hsp90 (Figure 1C), we found that Piwi and Hop together coimmunoprecipitate with Hsp90 (Figure 2D and E). These results indicate that Piwi, Hop, and Hsp90 likely exist in the same complex.

To seek for a second line of evidence for the Piwi-Hop-Hsp90 complex, we tried immunoprecipitating Piwi, but noted that antibody against first 200 amino acids of Piwi, failed to immunoprecipitate both Hop and Hsp90 (Figure 2D, see Piwi IP lane). This may be due to either of the two possibilities: a) Hop and Hsp90 hold Piwi in a conformation where its N-terminus is not accessible; b) the Piwi antibody recognition site is the same as Hop or Hsp90 binding. This possibility is supported by the fact that this antibody cannot co-immunoprecipitate proteins such as Heterochromatin Protein 1a that binds to residues 28-32

of Piwi[12]. Hence, to provide an independent line of evidence for the Piwi-Hop-Hsp90 complex, we co-expressed His-SUMO-Piwi, Myc-Hop, and HA-Hsp90 in the rabbit reticulocyte lysate system and conducted serial immunoprecipitation on the lysate (Figure 2F). After the second immunoprecipitation, we found that Piwi exists in the same pool as both Hop and Hsp90. This verified that Piwi, Hop, and Hsp90 exist in the same complex. The above findings, together, led us to hypothesize that Hsp90, Hop, and Piwi function in the same complex in which Hop mediates interaction between Piwi and Hsp90.

To test this hypothesis, we reasoned that a reduction in maternal dose of Hop would also compromise canalization. When we crossed Hop^{k00616}/+ virgin females with $Kr^{If\text{-}1}$/$Kr^{If\text{-}1}$males, we observed 5-6% of the male progeny with the eye outgrowth phenotype (Figure 3). This observation confirmed our hypothesis and demonstrated that Hop is also a dominant enhancer of the $Kr^{If\text{-}1}$ phenotype. The male-only outgrowth might be because some of the mutations required for the outgrowth are X-linked recessive and need hemi- or homo-zygosity to be expressed. As in the case of Piwi and Hsp90, the reciprocal cross with the Hop^{k00616} inherited from the father did not produce any outgrowth phenotype (data not shown). Hence, maternal Hop plays a critical role in canalization, likely by mediating interaction between Hsp90 and Piwi.

We then further examined if the outgrowth phenotype generated in $piwi^{-1}$ and Hop^{k00616} mutants can become independent of the *piwi* and *Hop* mutations and transmitted to the next generation (Figure 4A). Males with eye outgrowths were crossed with virgin Canton-S flies to separate $piwi^{-1}$ and Hop^{k00616} mutations from $Kr^{If\text{-}1}$. The resulting heterozygous $Kr^{If\text{-}1}$/+ progeny did not have any eye outgrowth. However, when we intercrossed these flies among themselves, the resulting $Kr^{If\text{-}1}/Kr^{If\text{-}1}$flies had the outgrowths. When the $Kr^{If\text{-}1}/Kr^{If\text{-}1}$flies with the outgrowths were selected and intercrossed among themselves in subsequent crosses, the percentage of both male and female flies with the outgrowths increased in each successive generation (Figure 4B). Consistent with the link between *wingless* expression and eye outgrowth phenotype, we observed higher *wingless* expression in the heads of F8 flies with the outgrowth phenotype (Figure 4C). This indicates that phenotypic variants and their corresponding gene expression patterns, once induced by *piwi* and *Hop* mutations, can be ‘fixed’ in a population and then stably inherited in subsequent generations under selection.

As Hsp90 and Piwi are in the same complex, yet over-expression of Piwi can rescue the deficiency of Hsp90 in canalization, Hsp90 and Piwi must function in the same pathway, with Piwi down stream of Hsp90. We therefore further examined how Hsp90 may regulate Piwi function. We first examined whether Hsp90 regulates Piwi expression and/or stability by comparing the Piwi levels in wildtype flies with and without geldanamycin treatment (Figure 5A), and further confirm these results in $Hsp83^{08445}/Hsp83^{08445}$ mutants (Figure 5B). As expected, the known Hsp90 client proteins Akt and B-Raf become unstable after geldanamycin treatment (Figure 5A). However, the Piwi protein levels do not change either with geldanamycin treatment or in $Hsp83^{08445}/Hsp83^{08445}$ mutants (Figure 5A and B). This indicates that Hsp90 does not regulate the expression and/or stability of Piwi.

However, Hsp90 regulates the posttranslational modification of Piwi. In wild type conditions, two-dimensional gel electrophoresis reveals three isoforms of Piwi with pI~10

(Figure 5C, first panel, isoforms 2, 3, and 4). These isoforms are likely due to different levels of phosphorylation because they have very similar molecular weights but different pI values. Upon inhibition of Hsp90 by geldanamycin, we noticed the appearance of a new isoform that is less negatively charged (Figure 5C, second panel, isoform 1 labeled by white arrow head). This indicates that that Hsp90 mediates post-translational modification of Piwi. This was further confirmed by comparing Piwi isoforms in ovary lysates from *Hsp83*^{08445}*/TM3* and *Hsp83*^{08445}*/Hsp83*^{08445} flies. In *Hsp83*^{08445}*/TM3* flies with a reduced level of Hsp90, we noted the four isoforms that we initially observed in geldanamycin-treated flies, Further reduction of Hsp90 levels in *Hsp83*^{08445}*/Hsp83*^{08445} flies resulted in complete absence of isoform 3 (Figure 5C, third panel, black arrow) and an appearance of a new isoform that migrates between isoforms 2 and 3, closer to isoform 2 (Figure 5C, fourth panel, black arrow head).

To test whether the posttranslational modification is indeed phosphorylation, we treated *Hsp83*^{08445}*/TM3* ovary lysate with calf intestinal phosphatase (CIP) and then subjecting the lysate to 2D gel analysis. After CIP treatment, we noticed complete absence of isoforms 3 and 4 (Figure 5C, bottom panel, circled area) and reduced intensity of isoforms 1 and 2. This confirms that the four isoforms are indeed phosphorylated forms of Piwi.

To further verify the phosphorylation of Piwi and determine the type of phosphorylation, we performed immunoprecipitation using anti-phospho-serine, anti-phospho-threonine, and anti-phospho-tyrosine antibodies, followed by western blotting analysis of the immuno-precipitates with anti-Piwi antibody. Piwi was immunoprecipitated by both anti-phopho-serine and anti-phospho-tyrosine antibodies, but not by the anti-phospho-threonine antibody (Figure 5D). Consistent with this, anti-phospho-serine and anti-phospho tyrosine antibodies failed to immunoprecipitate Piwi when the lysate was treated with CIP prior to immunoprecipitation (Figure 5E). These results indicate that Piwi is phosphorylated on serine and tyrosine residues.

To investigate whether the phosphorylation of serine and tyrosine residues in Piwi is dependent on Hsp90, we conducted the phospho-Piwi immunoprecipitation in wildtype and *Hsp83* mutant ovarian lysates. Both anti-phopho-serine and anti-phospho-tyrosine antibodies immunoprecipitated Piwi from wildtype but not from *Hsp83* mutant ovarian lysates (Figure 5F). These results indicate that Hsp90 is required for the phosphorylation of Piwi. A salient feature of Hsp90-mediated chaperoning, unlike that of Hsp70, is that it predominantly binds to metastable states of proteins instead of hydrophobic stretches[7]. It will be interesting to see in the future how Hsp90 binding to Piwi results in its phosphorylation and what affect this may have on the function of Piwi and canalization.

Recently, Specchia et al. suggested that Hsp90 prevents phenotypic variation by suppressing transposon-induced mutagenesis via piRNAs[4]. Having demonstrated that Hsp90 forms a complex with Piwi and regulates its phosphorylation, we set out to test whether this hypothesis is true. It has been observed that deficiency in the Hsp90 activity reduces piRNA expression, activates transposition, compromises multiple aspects of DNA damage repair, and increases CAG repeat instability, which ultimately produce genotype variations[4,23-26]. Consistent with Specchia *et al*, [23], we observed an increase in the RNA levels of transposons

upon geldanymycin treatment (Supplementary Figure 3), even though this treatment did not decrease the mRNA and protein levels of Piwi (Supplementary Figure 3B and C). Furthermore, this increase can be largely repressed by increasing the *piwi* copy number to four (Supplementary Figure 3A). These data further support that a mechanism through which Hsp90 achieves canalization is the suppression of new mutations via transposition and deficiency in DNA repair.

However, our experiments suggest that Hsp90, Hop and Piwi mediate canalization also through a non-genetic mechanism. First, we found that the eye outgrowth phenotype was observed only when either *piwi* or *Hop* mutations were in the mother. This is in contrast to the recent report that geldanaymycin treatment can de-repress transposons mainly in the male germline[4]. If eye outgrowths resulted from a genetic lesion, it should be independent of the parental source of contribution. Second, we have observed no increase in transposon RNA levels in the female germ line of $piwi^{-1}$/+ (Supplementary Figure 4A), i.e., one copy of *piwi* is sufficient to silence transposons, which is consistent with a previous report that *piwi* is haplo-sufficient to prevent new transpositions in the progeny[27]. However, *piwi* is haplo-insufficient to suppress eye outgrowths (Figure 1) as well as position effect variegation (Supplementary Figure 4B). Thus, the eye outgrowth phenotype observed in $Kr^{(If\text{-}1)}/piwi^{1}$ is unlikely due to new genetic mutations caused by transposons. Third, in $Kr^{If\text{-}1}/Kr^{If\text{-}1}$ files eight generations (F8) after *piwi* and *Hop* mutations were outcrossed, new mutations from the F1 flies, if any, should have been fixed. However, among these F8 flies, those with the outgrowth phenotype had approximately 50-60% more *Kr* mRNA and at least twice as much *wg* mRNA in their heads as compared to their siblings without the phenotype (Figure 4C). These statistically significant differences in *Kr* and *wg* expression among the same population of flies are more difficult to be explained by a stable genetic change by transposons. Last but not least, Piwi has been broadly implicated in epigenetic regulation, from long range physical interaction among Polycomb group response elements to Heterochromatin Protein 1-mediated epigenetic silencing[9-14]. Hence, we conclude that eye outgrowth phenotypes we observed in this study are due to defects in epigenetic silencing of normally non-expressed genotypes, so-called cryptic genotypes, by maternal Piwi rather than new transposon insertions.

The mechanism of canalization has been a subject of great debate. Rutherford and Lindquist's findings indicate that Hsp90 acts as a capacitor for phenotypic variation[5]; however, a complex gene network model generated by Bergman and Siegal predicts that a mutation in any one gene can result in expression of cryptic genotypes[17]. Yet another report argues that expression of cryptic genotypes is not caused by canalization and no particular mechanism is needed to prevent expression of the cryptic phenotypes [28]. Our finding of *piwi* and *Hop* mutations as enhancers for expression of cryptic genotypes validates the existence of a piRNA-pathway dependent mechanism for preventing phenotypic variation. Piwi is a piRNA-binding protein that is required for silencing of transposons[29] and epigenetic regulation[13,30]. Hence, post-translational regulation of Piwi by Hsp90 and Hop may allow Piwi both suppress the generation of new genotypes and epigenetically silence the expression of existing genetic variants (Figure 6). Both mechanisms can be 'fixed' and inherited in subsequent generations. Our study also shows that Piwi acts at two distinct

phases of fly development in mediating phenotypic capacitance. First, maternal Piwi plays a direct role in canalization and/or suppresses transposon-induced mutagenesis during embryogenesis. This allows the inheritance of correct epigenetic and genetic codes from parental cells to daughter cells, thereby ensuring the robustness of the developmental programs. Subsequently, zygotic Piwi is required for maintaining the inherited developmental programs during subsequent stages of development. This Piwi function likely represents the piRNA pathway, since Aubergine, a Piwi homolog also involved in the piRNA pathway, has similar function. Furthermore, canalization appears to involve only the piRNA pathway, but not miRNA or siRNA pathway, since Dicer 1 and 2 deficiency does not lead to increased eye outgrowth.

Then, what could be the roles for Hsp90 and Hop in Piwi-mediated canalization? As an essential component in canalization, Hsp90 likely ensures proper function of its clients involved in canalization, such as Piwi, by mediating their proper post-translational modification, such as phosphorylation, that is required for their molecular activities. A salient feature of Hsp90-mediated chaperoning, unlike Hsp70, is that Hsp90 predominantly binds to metastable states of proteins instead of hydrophobic stretches[7]. The Hsp90-bound metastable state of Piwi may be a necessary step for its phosphorylation at proper sites, which may then be required to form active complexes with piRNAs and/or epigenetic factors to promote epigenetic and transposon silencing, leading to canalization.

METHODS

Fly stocks and maintenance

All fly stocks were maintained at 25°C. *piwi*$^{-1}$, *piwi*$^{-2}$ alleles and *myc-piwi* transgene were generated by P-element insertions and are described elsewhere[18,31]. *b*^{1} *Kr*$^{If-1}$ (#4194), *w*^{1118}; *aub*^{QC42} *cn*^{1} *bw*^{1}/*CyO*, *P{ry*$^{+t7.2}$*=sevRas1.V12}FK1* (# 4968), *y*^{1} *w*^{67c23}; *P{w*$^{+mC}$*=lacW}Hop*^{k00616}/*CyO* (#10483), *P{ry*$^{+t7.2}$*=PZ}Hsp83*^{08445} *ry*^{506}/*TM3, ry*^{RK} *Sb*^{1} *Ser*^{1} (#11797) were obtained from Bloomington stock center. The numbers in parentheses represent the stock IDs at the Bloomington stock center. *yw eyFLP;FRT*^{82B} *Dcr-1*^{Q1147X}/*TM3* and *yw eyFLP;FRT*^{42D} *Dcr-2*^{L811fsX}/*Cyo* strains were gifts from Richard Carthew and are described elsewhere[32].

Pharmacological inhibition of Hsp83

Geldanamycin (Sigma) was first dissolved in 50% ethyl alcohol to make a 1000X stock (3.56mM). To make *Drosophila* medium containing geldanamycin, a scoop of Formula 4-24 (Carolina biological supply), was mixed with 15ml of water containing 3.56μM geldanamycin. After mixing food and water in a vial, it was left to solidify for a minute before adding flies. Alternatively, geldanamycin was dissolved in DMSO to make a stock of 10mM from where fly medium containing geldanamycin was prepared as described above. For inhibiting Hsp90 *in vivo* we allowed female flies to feed on geldanamycin containing medium for either 2 days (Supplementary Figure 4A) or 4 days (Figure 5A) prior to dissecting ovaries and lysate preparation.

Embryo collection and extract preparation

w^{1118} flies were grown at 25°C and 70-80% relative humidity in population cages and embryos were collected on grape juice agar plates at 12-hour intervals. Embryos were carefully transferred using a soft paint brush into mini embryo collection cages (Genesee Scientific) and washed well with tap water to remove traces of yeast. The embryos were then dechorionated by immersing in 50% bleach (Chlorox) for 2 min and rinsed with embryo wash buffer [0.7% NaCl (wt/vol); 0.02% Triton X-100 (vol/vol)] and tap water. They were then dried on a paper towel and stored at −80°C until further use.

All steps in extract preparation were performed at 4°C. Ten grams of frozen embryos were thawed on ice and carefully resuspended in 3 ml per gram of embryos of H (0.3) buffer (25 mM HEPES-NaOH, pH 7.8, 300 mM NaCl, 0.5 mM EGTA, 0.1 mM EDTA, 2 mM $MgCl_2$, 0.02% NP-40 and 20% glycerol) where the number in the parenthesis represents the final NaCl concentration in the buffer (0.3 M). Just before use, the buffer was supplemented with 1 tablet of Complete Mini-EDTA free protease inhibitor cocktail (Roche) per 10ml of the buffer, 1mM phenylmethanesulfonyl fluoride (Fluka), 2mM 2-mercaptoethanol (Sigma) and 0.5mM sodium metabisulfite. The resuspended embryos were transferred 1ml at a time into a Wheaton homogenizer and were lysed using 10 strokes of loose pestle A and 10 strokes of tight pestle B. The homogenate was then centrifuged at 14,000 rpm for 30 min using SW 40 Ti rotor and Optima L-XP series preparative ultra centrifuge (Beckman) to pellet the nuclei and debris. Supernatant was carefully collected and again centrifuged at 100,000g for 4h to further clarify the extract. Supernatant (cytoplasmic fraction) was collected and stored at −80°C until further use.

Biochemical purification and identification of cytoplasmic Piwi interacting peptides

All chromatographic steps were performed using ÄKTA purifier (GE Healthcare) at 4°C. After each step, Piwi was detected by western blotting. Cytoplasmic extract was diluted with buffer H(0) to bring down the final salt concentration of NaCl to 100mM. The extract was then fractionated on a DEAE Sepharose (GE Healthcare) column over a linear gradient of 100-1000mM NaCl. Piwi was detected in the flow through of the column showing that Piwi does not bind to this resin. Flow through was fractionated on 10ml Bio-Rex70 (Bio-Rad) column over a linear gradient of 100-1000mM NaCl. Fractions containing Piwi were again pooled; final concentration was brought down to 100mM and fractionated on HiPrep 16/10 Heparin FF column (GE Healthcare) over a linear gradient of 100-1000mM NaCl and 7ml fractions were collected. Fractions containing Piwi were pooled and concentrated by fractionation on 1ml HiTrap Heparin FF column. Three 1ml fractions containing Piwi were pooled and 500μl aliquots were fractionated using Superdex 200 size exclusion chromatography. Fraction number 27 was first resolved using 4-20% gradient polyacrylamide gel electrophoresis and stained with coomassie blue staining. Bands detected were excised and then identified by mass spectrometry. In addition, liquid sample was also analyzed in order to identify peptides that were missed by coomassie blue staining. Mass spectrometry analysis was performed using services provided by ProtTech, Inc located in Norristown, PA, USA.

Whole ovary lysate preparation

Flies, either wild type or with desired genotypes as shown in individual figures, were yeast fed for 36 h prior to ovary dissections. Ovaries were first dissected into ice cold phosphate buffered saline (PBS) and transferred into eppendorf tubes. PBS was exchanged with ovary lysis buffer (20 mM HEPES pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mM dithiothreitol and 5% glycerol) containing 1 tablet of complete mini EDTA free protease inhibitor cocktail (Roche) per 10 ml of lysate and 1mM PMSF. Lysis was performed manually using pestles designed for eppendorf tubes. Lysate was then cleared by spinning at 14,000rpm for 30min. Cleared lysate was carefully separated from the pellet and floating fat and stored at −80°C until use.

In vitro reconstitution of Piwi, Hop and Hsp90 complex

N-terminal His-Sumo tagged Piwi was generated by cloning Piwi CDS into pETSUMO vector(Invitrogen). N-terminal Myc and HA tagged versions of Hop and Hsp90 CDS respectively were generated by PCR and cloned into pCR2.1 (Invitrogen) vector to create pHop and pHsp90 plasmids. Equimolar concentrations of pETSUMO-Piwi, pHop and pHsp90 were added to TnT T7 coupled rabbit reticulocyte lysate system (Promega) and were co-expressed following manufacturer's protocol. Prior to Myc IP, co-expression lysate was diluted to 500µl with H(0.1) buffer. Myc-Hop was first immunoprecipitated using 50µl EZview Red Anti-cMyc Affinity gel (Sigma) suspension. After 3h incubation at 4°C, beads were washed thrice with 750µl of H(0.1) buffer. Myc-Hop was then eluted using 100µl of 100µg/ml c-Myc peptide (Sigma). The eluted sample was then diluted to 500µl using H(0.1) buffer and used as input for HA tag IP using EZ view Red Anti-HA affinity gel (Sigma). IP procedure was the same as for Myc IP. After the final wash, beads were resuspended in 50µl 2X SDS sample loading buffer (62.5 mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue) and proceeded with standard western blotting protocol.

Immunoprecipitation and western blotting

For Figures 2D and 2E, cytoplasmic extracts from 0-12h embryos were prepared in buffer H (0.1) buffer as described in extract preparation section. A total of 1mg of total protein in a volume of 1ml was used for each immunoprecipitation reaction. The lysates were pre-cleared using Protein A/G PLUS-Agarose (Santa Cruz) for 1h at 4°C. Pre-cleared lysates were incubated with appropriate antibody over night at 4°C with gentle agitation. Twenty µl of beads were then added to the lysate-antibody mixture and incubated further for 3h at 4°C. Beads were then washed thrice with 1ml of H(0.1) buffer and finally analyzed by western blotting.

For IP using anti-phospho antibodies, 15 pairs of ovaries for each genotype were lysed in 50µl ovary lysis buffer. SDS was then added to a final concentration of 1% and the lysate was then boiled at 95°C for 10minutes to disrupt protein-protein interactions and also to inactivate any dephosphatases. The lysate was then centrifuged at 14000rpm for 5min and the supernatant was diluted 10X with H(0.1) buffer prior to immunoprecipitation.

For calf intestinal phosphatase (CIP) treatment, NEB buffer 3 (NEB) was added to the ovary lysate to 1X final concentration. CIP (NEB) was then added to a final concentration of 1unit/μg of protein and incubated at 37°C for 60min prior to immunoprecipitation.

2D gel electrophoresis

2D gel electrophoresis was performed following manufacturer's protocol provided for ReadyPrep™ 2-D Starter kit (Bio-Rad). For each experiment, 150μg of total ovary lysate was used. The samples were concentrated either by freeze drying or TCA precipitation and resuspended in 125μl of 2D rehydration buffer. The samples are then applied to pH 3-10 IPG strips provided in the kit (BioRad). After overnight rehydration step, the strips were electro focused using PROTEAN IEF Cell (BioRad). Following electro-focusing, second dimension gel electrophoresis was performed and Piwi was detected by western blotting.

Antibodies

Anti Piwi, Aub and Ago3 antibodies, all mouse monoclonal, were a kind gift from Haruhiko Siomi and were used at a concentration of 1:10, 1:5000 and 1:5000 respectively. Anti Hop antibody (rabbit) was provided by Dr. Michael Chinkers and was used at a dilution of 1:1000. Hsp90 antibodies were obtained either from Santa Cruz biotechnologies or Cell Signaling and were used as recommended. Immunoprecipitation of Hsp90 as shown in figure 2E was performed using antibody obtained from Santa Cruz biotechnologies. Mouse monoclonal antibody for anti-phospho-serine was obtained from Sigma and was used at a dilution of 1:100 for IP. Anti-phospho-threonine and anti-phospho-serine antibodies were obtained from Cell Signaling and were used at dilutions of 1:50 and 1:100 respectively for IP.

Total RNA extraction from ovaries and real time quantitative PCR

Ten pairs of ovaries were dissected into ice cold Buffer B from flies that have been fed on yeast for at least 36 h. For checking the effect of geldanamycin, flies were fed on geldanamycin containing medium for 2 days before dissecting the ovaries. Total RNA was then extracted using TRIzol™ (Invitrogen) and then cleaned up using RNeasy kit (Qiagen) following manufacturers' protocols. RNA was finally eluted in 30μl nuclease free water and quantified by Nanodrop. One μg of eluted RNA was used to generate cDNA in a 20μl reaction using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Prepared cDNA was diluted 5X in nuclease free water, 1μl of which was used for quantitative PCR using primer sets mentioned in Supplementary Table 1. Quantitative PCR was performed using iQ SYBR green super mix and CFX96 real time PCR detection system (both from Bio-Rad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Siomi Lab for Piwi, Aub, and Ago 3 antibodies; Richard Carthew and Young Sik-Lee for *FRT-dcr-1/2* flies and Michael Chinkers for the Hop antibody. We would also like to thank Li Liu and Vladimir Shteyn for

technical assistance; for piwi PEV assay (Supplementary Figure 3B), the Lin lab members for their valuable comments, and Prof. Arthur Horwich for stimulating discussion. This work is supported by the NIH Grant R01HD33760, the G. Harold and Leila Mathers Foundation, and the Connecticut Stem Cell Research Fund (06SCD01, 06SCE01, and 08SCD-Yale-004) to HL.

REFERENCES

1. Waddington CH. Canalization of development and the inheritance of acquired characters. Nature. 1942; 150:563–5.
2. Waddington CH. Canalization of development and genetic assimilation of acquired characters. Nature. 1959; 183:1654–5. [PubMed: 13666847]
3. Sollars V, et al. Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. Nat Genet. 2003; 33:70–4. [PubMed: 12483213]
4. Specchia V, et al. Hsp90 prevents phenotypic variation by suppressing the mutagenic activity of transposons. Nature.
5. Rutherford SL, Lindquist S. Hsp90 as a capacitor for morphological evolution. Nature. 1998; 396:336–42. [PubMed: 9845070]
6. Queitsch C, Sangster TA, Lindquist S. Hsp90 as a capacitor of phenotypic variation. Nature. 2002; 417:618–24. [PubMed: 12050657]
7. Sangster TA, Lindquist S, Queitsch C. Under cover: causes, effects and implications of Hsp90-mediated genetic capacitance. Bioessays. 2004; 26:348–62. [PubMed: 15057933]
8. Ruden DM, Garfinkel MD, Sollars VE, Lu X. Waddington's widget: Hsp90 and the inheritance of acquired characters. Semin Cell Dev Biol. 2003; 14:301–10. [PubMed: 14986860]
9. Pal-Bhadra M, Bhadra U, Birchler JA. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila. Mol Cell. 2002; 9:315–27. [PubMed: 11864605]
10. Pal-Bhadra M, et al. Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. Science. 2004; 303:669–72. [PubMed: 14752161]
11. Grimaud C, et al. RNAi components are required for nuclear clustering of Polycomb group response elements. Cell. 2006; 124:957–71. [PubMed: 16530043]
12. Brower-Toland B, et al. Drosophila PIWI associates with chromatin and interacts directly with HP1a. Genes Dev. 2007; 21:2300–11. [PubMed: 17875665]
13. Yin H, Lin H. An epigenetic activation role of Piwi and a Piwi-associated piRNA in Drosophila melanogaster. Nature. 2007; 450:304–8. [PubMed: 17952056]
14. Brennecke J, et al. An epigenetic role for maternally inherited piRNAs in transposon silencing. Science. 2008; 322:1387–92. [PubMed: 19039138]
15. Carrera P, et al. A modifier screen in the eye reveals control genes for Kruppel activity in the Drosophila embryo. Proc Natl Acad Sci U S A. 1998; 95:10779–84. [PubMed: 9724781]
16. Rutherford SL, Henikoff S. Quantitative epigenetics. Nat Genet. 2003; 33:6–8. [PubMed: 12509772]
17. Bergman A, Siegal ML. Evolutionary capacitance as a general feature of complex gene networks. Nature. 2003; 424:549–52. [PubMed: 12891357]
18. Cox DN, Chao A, Lin H. piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development. 2000; 127:503–14. [PubMed: 10631171]
19. Carrigan PE, Riggs DL, Chinkers M, Smith DF. Functional comparison of human and Drosophila Hop reveals novel role in steroid receptor maturation. J Biol Chem. 2005; 280:8906–11. [PubMed: 15632128]
20. Blatch GL, Lassle M. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays. 1999; 21:932–9. [PubMed: 10517866]
21. Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev. 1997; 18:306–60. [PubMed: 9183567]
22. Odunuga OO, Longshaw VM, Blatch GL. Hop: more than an Hsp70/Hsp90 adaptor protein. Bioessays. 2004; 26:1058–68. [PubMed: 15382137]

23. Mittelman D, Sykoudis K, Hersh M, Lin Y, Wilson JH. Hsp90 modulates CAG repeat instability in human cells. Cell Stress Chaperones. 15:753–9. [PubMed: 20373063]
24. Camphausen K, Tofilon PJ. Inhibition of Hsp90: a multitarget approach to radiosensitization. Clin Cancer Res. 2007; 13:4326–30. [PubMed: 17671112]
25. Dote H, Burgan WE, Camphausen K, Tofilon PJ. Inhibition of hsp90 compromises the DNA damage response to radiation. Cancer Res. 2006; 66:9211–20. [PubMed: 16982765]
26. Noguchi M, et al. Inhibition of homologous recombination repair in irradiated tumor cells pretreated with Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin. Biochem Biophys Res Commun. 2006; 351:658–63. [PubMed: 17083915]
27. Kalmykova AI, Klenov MS, Gvozdev VA. Argonaute protein PIWI controls mobilization of retrotransposons in the Drosophila male germline. Nucleic Acids Res. 2005; 33:2052–9. [PubMed: 15817569]
28. Hermisson J, Wagner GP. The population genetic theory of hidden variation and genetic robustness. Genetics. 2004; 168:2271–84. [PubMed: 15611191]
29. Lin H. piRNAs in the germ line. Science. 2007; 316:397. [PubMed: 17446387]
30. Lin H, Yin H. A novel epigenetic mechanism in Drosophila somatic cells mediated by Piwi and piRNAs. Cold Spring Harb Symp Quant Biol. 2008; 73:273–81. [PubMed: 19270080]
31. Cox DN, et al. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. 1998; 12:3715–27. [PubMed: 9851978]
32. Lee YS, et al. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell. 2004; 117:69–81. [PubMed: 15066283]

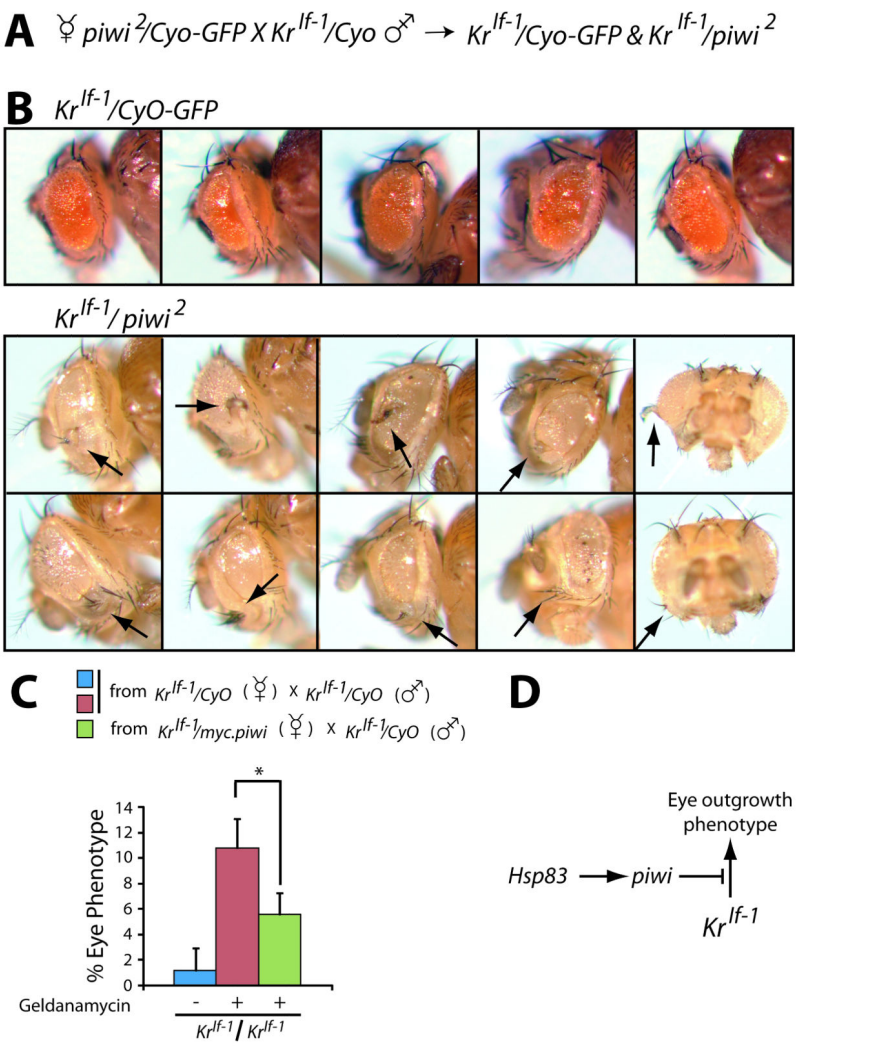

Figure 1. Maternal Piwi is an enhancer of ectopic outgrowth phenotype

A. Genetic cross to check if Piwi is an enhancer of the eye outgrowth phenotype caused by ectopic expression of *Krüppel* where CyO or CyO-GFP balancer chromosomes carries a $piwi^{+}$ allele. Reverse crosses with $piwi^{-1}$ and $piwi^{-2}$ males are not shown here. **B**. Light microscopic images of the adult fly eyes with various types of ectopic outgrowths (black arrows). Images of the eyes of control flies with wild type levels of Piwi are shown in the upper panel. **C**. Over-expression of maternal Piwi suppresses eye outgrowths when Hsp90 is inhibited. From each cross 50 flies with $Kr^{If\text{-}1}/Kr^{If\text{-}1}$ background were collected and scored for the phenotype. Experiment was repeated five times with five independent crosses. Average of five independent crosses with standard deviations (s.d.) are plotted. Unpaired *t* test was performed to calculate statistical significance. * represents a *p* value of 0.0254 **D.** Genetic linkage between Piwi and Hsp90 in mediating canalization.

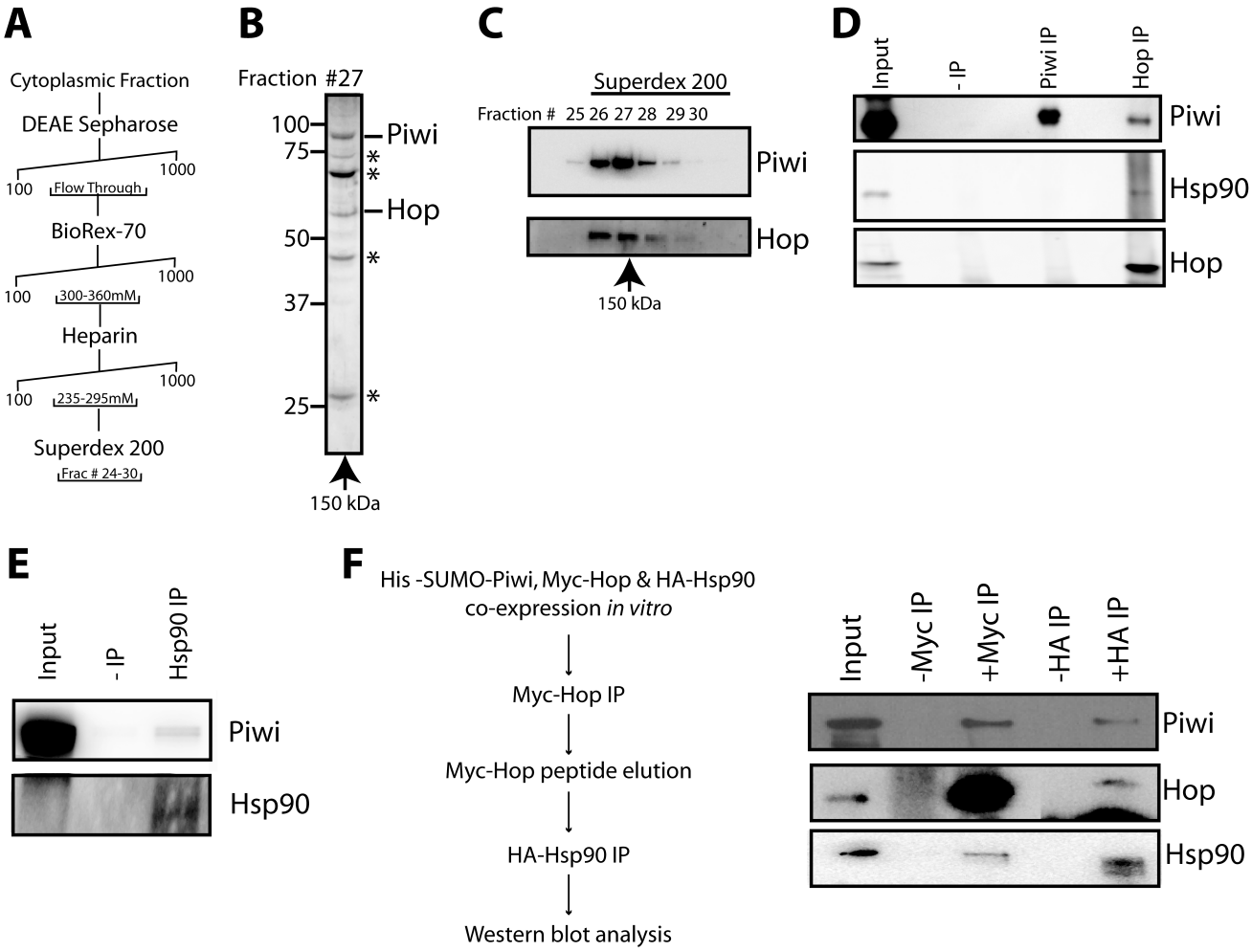

Figure 2. Biochemical isolation of Hop as an interactor of Piwi

A. Fractionation scheme for identifying peptides interacting with Piwi. **B**. Fraction #27 obtained from Superdex 200 chromatography was resolved on a 7.5% SDS polyacrylamide gel and stained with silver stain. Identities of individual bands were obtained by excising bands from a colloidal coomassie blue stained gel (not shown here) followed by mass spectrometry. Peptides identified but not relevant to this study are marked by (*). **C.** Western blotting analysis showing the co-migration of Piwi and Hop in Superdex 200 column. Fraction numbers are marked above and fraction corresponding to ~150kDa is marked below. **D**. Co-immunoprecipitation of Piwi and Hsp90 with Hop. Control reactions (-IP) did not contain Hop specific antibody. **E.** Co-immunoprecipitation of Piwi with Hsp90. Control reactions (-IP) did not contain Hsp90 specific antibody. **F.** Piwi, Hop and Hsp90 function in the same complex. Left panel shows the scheme of the serial immunoprecipitation experiment. Right panel shows western blotting analysis of Piwi, Hop and Hsp90 after the first and second immunoprecipitations. Control reactions (-Myc IP and –HA IP) contained protein A/G plus agarose beads.

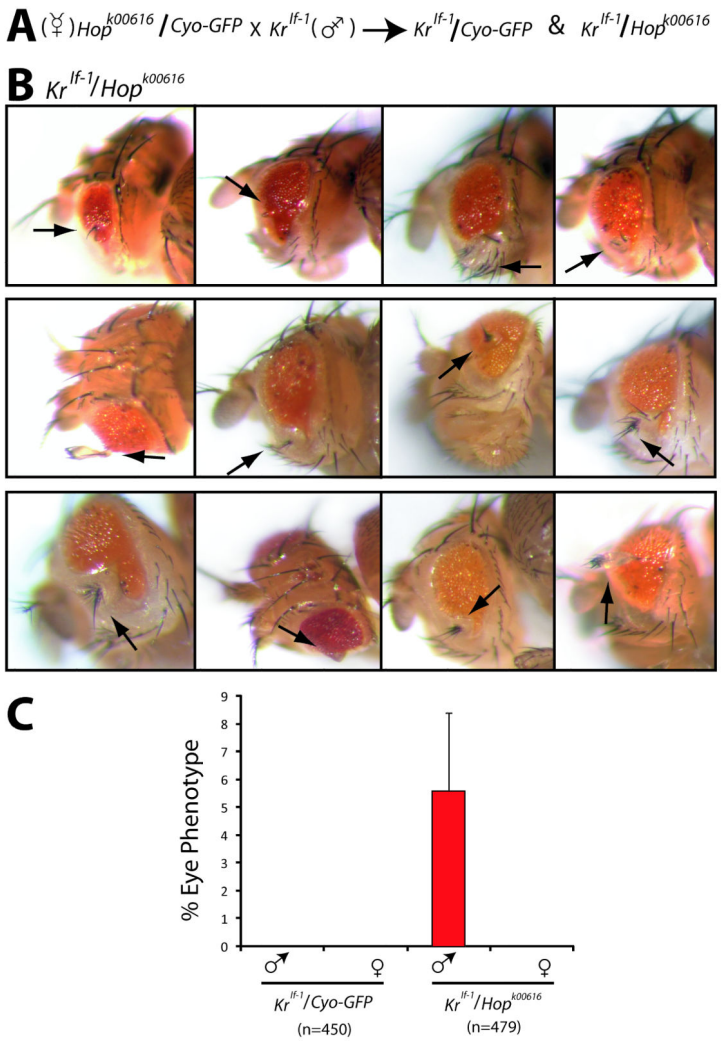

Figure 3. Hop is a maternal enhancer of the eye outgrowth phenotype

A. Genetic cross to test if Hop is a maternal enhancer of the outgrowths. Reverse cross with *Hop*^{k00616} males is not shown here. **B**. *Kr*$^{If\text{-}1}$/*Hop*^{k00616} fly eyes exhibiting the outgrowths (arrows). **C**. Quantification of the outgrowths observed in *Kr*$^{If\text{-}1}$/*Hop*^{k00616} flies. 479 flies were collected from three independent crosses and scored for the outgrowths. Average percentage of flies with the outgrowths and s.d are plotted. The error bar indicates s.d.

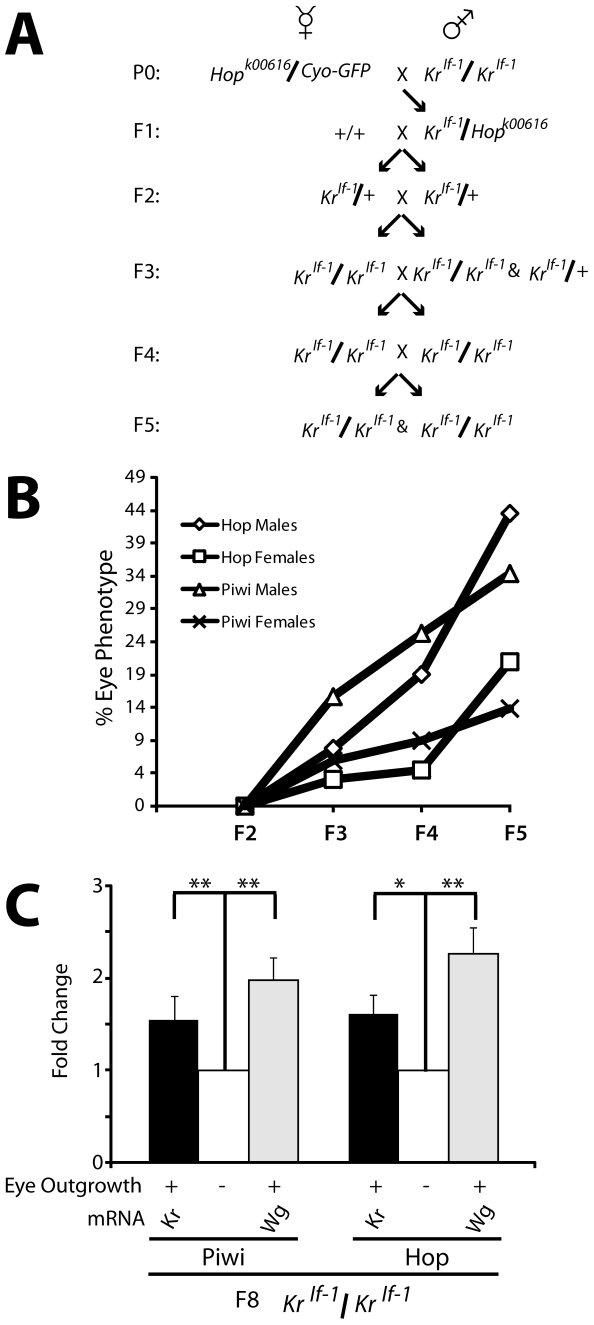

Figure 4. Germline transmission of Piwi- and Hop-induced mutations

A. Genetic crosses for *Hop*^{k00616} selection experiment. Similar cross was setup with *piwi*$^{-1}$. *Hop*^{k00616} and *piwi*$^{-1}$ mutations are present only in the F1 *Kr*$^{If-1}$ flies. A single male F1 fly with outgrowth was crossed with virgin wild type Canton S female flies to remove *Hop*^{k00616} and *piwi*$^{-1}$ mutations. From F4 generation, *Kr*$^{If-1}$/*Kr*$^{If-1}$ males and females containing the outgrowths were selected and inter crossed. **B**. Quantification of flies with the outgrowths in each generation. 100 flies/generation were scored and counts of male and female flies with the outgrowths are individually plotted. **C.** Over-expression of *wg* is

'fixed' over multiple generations. *Krüppel* and *wingless* mRNA expression in the heads of 5 F8 males and 5 F8 females with eye outgrowths were quantified by qPCR, with the same number of F8 flies without the eye outgrowths as controls. Average values of three independent experiments and s.d. are plotted. Statistical significance was calculated using a paired *t* test, with *p* values that are less than 0.05 and 0.001 indicated by * and **, respectively.

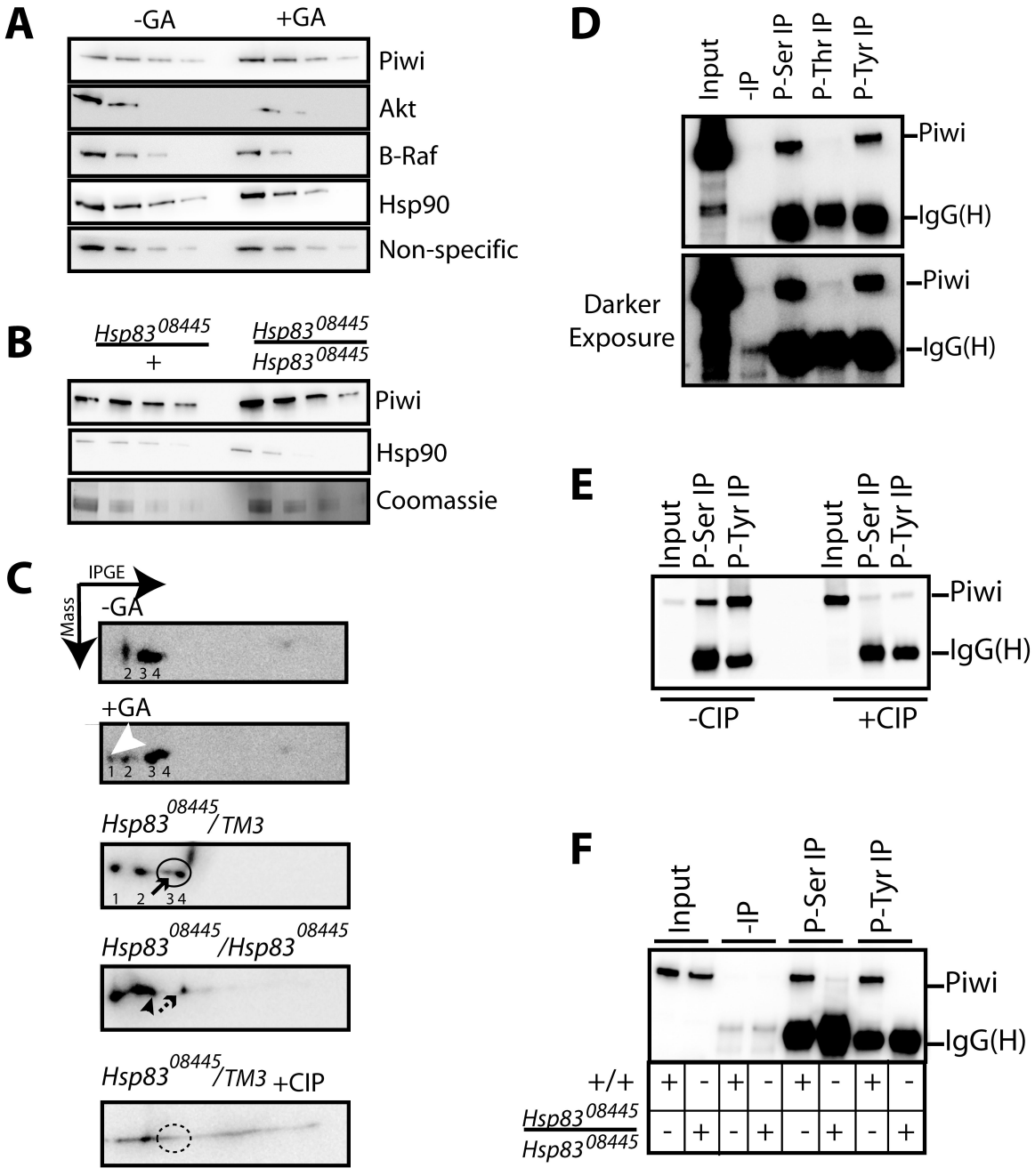

Figure 5. Hsp90-dependent phosphorylation of Piwi

A. Hsp90 inactivation by geldanamycin does not change Piwi protein levels. Two-fold serial dilutions of total ovary lysate were resolved by SDS polyacrylamide gel electrophoresis followed by western blotting using various antibodies as shown on the right side of the panel. Non-specific control for the amount of protein loaded in each lane is a signal from a cross reaction of B-Raf antibody to an abundant protein in the ovary lysate. **B.** Piwi levels do not change in *Hsp83* mutants. Two-fold serial dilutions of ovary lysates from either *Hsp83*^{08445}/*TM3* or *Hsp83*^{08445}/*Hsp83*^{08445} flies were resolved by SDS polyacrylamide gel

electrophoresis and analyzed by western blot. Notice a decrease in the levels in Hsp90 but not Piwi. As a measure of total protein in each lane, coomassie staining of the most abundant protein (~60 kDa) in the ovary lysate is shown. **C.** Hsp90-dependent phosphorylation of Piwi. Western blot analysis of second dimension SDS PAGE gel electrophoresis using anti-Piwi antibody is shown. Directions of the first and second dimensions are indicated on the upper left corner of the panel. Presence or absence of geldanamycin and various genotypes of flies used in this study are marked on top of each panel. CIP represents calf intestinal phosphatase. Different isoforms of Piwi are marked from '1' through '4' with '1' being the most positive isoform of Piwi. White arrow head in the second panel represents the most positive Piwi isoform that is enriched in the presence of geldanamycin. Black arrow in the third panel represents the isoform of Piwi that is depleted in $Hsp83^{08445}/Hsp83^{08445}$ mutants. Compare it with dashed black arrow in the fourth panel. Black arrow head in the fourth panel represents an isoform of Piwi that gets enriched in $Hsp83^{08445}/Hsp83^{08445}$ ovaries. Black circle in the third panel marks the area where two isoforms that are depleted upon CIP treatment in the fifth panel (dashed circle) **D.** Immunoprecipitation of Piwi with anti-phospho-serine, anti-phospho-threonine and anti-phospho-tyrosine antibodies from wild type ovarian lysate. Band representing Piwi is marked on the right. Levels of IgG(H) were used to monitor loading in each lane. Lower panel is a darker exposure of the upper one to show that absence of Piwi in phosphorthreonine IP is not due to less loading **E.** Immunoprecipitation of Piwi with anti-phospho-serine and anti-phospho-tyrosine antibodies in the presence or absence of CIP treatment. Notice that Piwi is mostly depleted from the immunoprecipitates upon CIP treatment, further confirming that Piwi is phosphorylated. **F.** Immunoprecipitation of Piwi with anti-phospho-serine and anti-phospho-tyrosine antibodies from wildtype and $Hsp83^{08445}/Hsp83^{08445}$ ovarian lysate. Notice depletion of Piwi from both anti-phospho-serine and anti-phospho-tyrosine IP from $Hsp83^{08445}/Hsp83^{08445}$ mutant ovarian lysate.

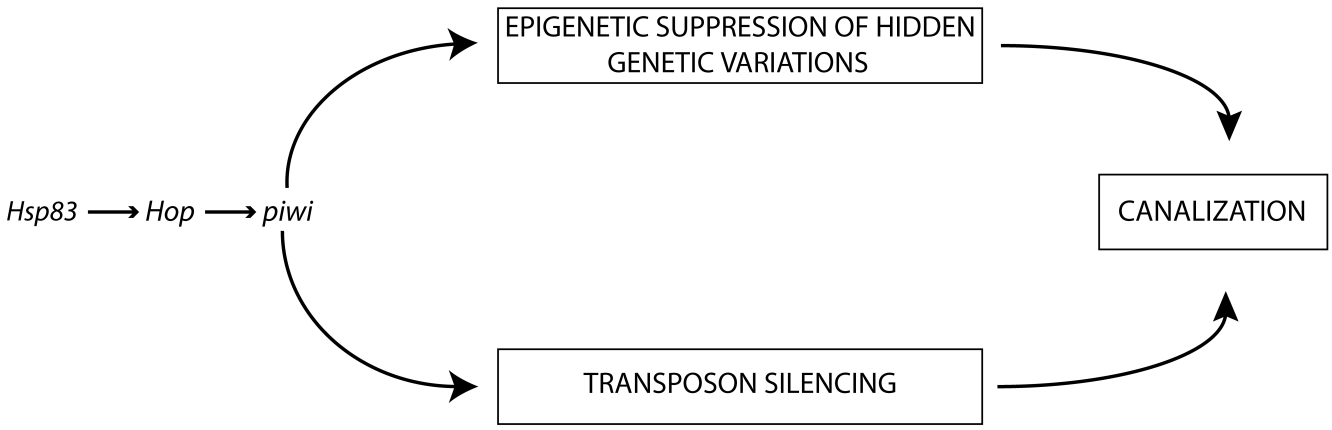

Figure 6. A schematic illustration for the role of the Hsp90-Hop-Piwi complex in canalization

See text for details.