Inheritance of Stress-Induced, ATF-2-Dependent Epigenetic Change

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SUMMARY

Atf1, the fission yeast homolog of activation transcription factor-2 (ATF-2), contributes to heterochromatin formation. However, the role of ATF-2 in chromatin assembly in higher organisms remains unknown. This study reveals that Drosophila ATF-2 (dATF-2) is required for heterochromatin assembly, whereas the stress-induced phosphorylation of dATF-2, via Mekk1-p38, disrupts heterochromatin. The dATF-2 protein colocalized with HP1, not only on heterochromatin but also at specific loci in euchromatin. Heat shock or osmotic stress induced phosphorylation of dATF-2 and resulted in its release from heterochromatin. This heterochromatic disruption was an epigenetic event that was transmitted to the next generation in a non-Mendelian fashion. When embryos were exposed to heat stress over multiple generations, the defective chromatin state was maintained over multiple successive generations, though it gradually returned to the normal state. The results suggest a mechanism by which the effects of stress are inherited epigenetically via the regulation of a tight chromatin structure.

INTRODUCTION

Living organisms are constantly exposed to various stresses. Evidence has suggested that stress elicits a transgenerational modification of the genome without a corresponding change in DNA sequence, a process known as epigenetic change. Ultraviolet (UV) light stress leads to increased homologous recombination in Arabidopsis thaliana, even in unstressed generations (Molnier et al., 2006), while maternal diet influences the epigenetic state of a transposable element in the agouti gene of Aplysia, as well as the phenotypes of the F1 offspring (Waterland and Jirtle, 2003). It has also been shown that increased pup grooming and nursing by rat mothers alters the epigenetic status of a glucocorticoid receptor gene and the behavioral phenotype of the offspring (Weaver et al., 2004). Furthermore, it was recently shown that paternal high-fat diet or low-protein diet exposure induced altered gene expression in offspring (Ng et al., 2010; Carone et al., 2010); however, the mechanism of inheritance of stress-induced epigenetic change is unknown.

Heterochromatin, which is enriched in histone H3 lysine-9 (H3K9) methylation and heterochromatin protein 1 (HP1), controls gene silencing epigenetically. Recent studies have demonstrated that, in addition to the RNA interference (RNAi) machinery (Volpe et al., 2002), Atf1, the fission yeast homolog of activation transcription factor-2 (ATF-2), functions in heterochromatin nucleation (Jia et al., 2004). ATF-2 is a member of the ATF/CREB superfamily and has a b-ZIP-type DNA-binding domain and a transactivation domain containing phosphorylation sites for stress-activated protein kinases (SAPKs) such as p38 (Maekawa et al., 1989; Hai et al., 1989). ATF-2 forms a homo- or heterodimer with c-Jun and binds to the cyclic AMP response element (CRE). In response to various stresses, SAPKs phosphorylate ATF-2 and enhance its transactivating capacity (Gupta et al., 1995). In addition to ATF-2, the mammalian ATF-2 subfamily contains two other members, ATF-7 and CRE-BP, while Drosophila has only one homolog of ATF-2 (dATF-2) (Sano et al., 2005). Both ATF-7 and dATF-2 are phosphorylated by p38, but not by JNK, suggesting that dATF-2 is functionally related to ATF-7. ATF-7 binds to the mAM component of the ESET complex (Wang et al., 2003), a histone H3K9 methyltransferase, and silences the transcription of the serotonon receptor 5b (Htr5b) gene by recruiting ESET (Maekawa et al., 2010). These results suggest that ATF-2 family members may contribute to the epigenetic silencing of certain euchromatic target genes by forming a heterochromatin-like structure in the absence of stress, while they induce gene expression in response to stress. However, the role of ATF-2 in heterochromatin formation in higher organisms remains unknown.

In this study, we show that dATF-2 is involved in heterochromatin formation and that stress-induced activation of dATF-2 disrupts heterochromatin. Furthermore, the effect of stress-induced heterochromatin disruption can be inherited by subsequent generations.

RESULTS

The dATF-2 Mutation Abrogates Heterochromatin Formation

One of the insertion lines of the piggyBac (PB) transposon (Thibault et al., 2004), PB-c06407, has an insertion close to the dATF-2 locus, 69 bp upstream of the translation initiation codon of dATF-2. Levels of dATF-2 messenger RNA (mRNA) were severely reduced in PB-c06407 flies compared to the wild-type (WT) (Figure 1A), and the dATF-2 protein band was barely detectable on a western blot of extracts from PB-c06407 embryos.
Figure 1. dATF-2 Is Required for Heterochromatin Formation

(A) The dATF-2 mRNA levels of the dATF-2 mutant flies relative to those of the wild-type are shown as the average of three measurements ± standard deviation (SD). The ribosomal protein L32 mRNA was used as the internal control.

(B) Top: Levels of eye pigment were measured, and the averages are shown ± SD (n = 6). ***p < 0.001; N.S., not significant. Bottom: Representative eye phenotypes of the genotypes indicated.

(C) Rescue experiments. Top: β-galactosidase activity obtained with salivary gland extract or control buffer was measured, and the averages are shown ± SD (n = 6). **p < 0.01; N.S., not significant. Bottom: X-Gal staining of representative salivary glands.

(D) w^m4 flies carrying the maternal and/or zygotic mutation of dATF-2 were generated, and the eye pigment levels were measured. Averages with SD are shown (n = 6). ***p < 0.001; **p < 0.01; *p < 0.05.

(E) Mutations of RNAi machinery components and dATF-2 were combined with w^m4 and eye pigment levels were measured. See also Figure S1 and Table S1.
Heterochromatin formation can be divided into two stages, establishment and maintenance (Hall et al., 2002), and yeast Atf1 is responsible for the establishment and maintenance of heterochromatin (Jia et al., 2004). Drosophila heterochromatin is established early in development, with contributions from some maternal proteins. The loss of either maternal or zygotic dATF-2 had an additive effect (Figure 1D), which suggests that dATF-2 is involved in both the establishment and maintenance of heterochromatin.

Yeast Atf1 contributes to heterochromatin independently of the RNAi machinery (Jia et al., 2004). When the mutation of spindle-E (spn-E), which encode a putative DEAD-box helicase (Aravin et al., 2001), was combined with the dATF-2 mutation, an additive effect was observed (Figure 1E). In the heterozygotes of aubergine (aub), which encode an Argonaute protein (Kennerdell et al., 2002), white silencing was relieved slightly as reported (Kavi and Birchler, 2009) and this effect was enhanced in the transheterozygotes of aub and dATF-2. Thus, the results suggested that dATF-2 and the RNAi machinery contribute independently to heterochromatin formation. Mutations in spn-E and aub are known to induce a derepression of traspson elements, and exhibit an abnormal morphology of egg shell structures (abnormal number of dorsal appendage) and a reduced hatch rate (Khurana and Theurkauf, 2010). However, the dATF-2 mutants exhibited no such phenotypes (Figures S1E and S1F), which suggests that dATF-2 is not involved in the transposon silencing.

**HP1 and dATF-2 Colocalize on Heterochromatin and at Specific Loci in Euchromatin**

Yeast Atf1 interacts with Swi6, the yeast homolog of HP1 (Jia et al., 2004). dATF-2 also co-immunoprecipitated with HP1 in communoprecipitation assays with S2 cell lysates (Figures S2A and S2B). Immunostaining of polytene chromosomes indicated that both dATF-2 and HP1 colocalized at the heterochromatic chromosome (Figure 1A). Signals from both proteins were also detected at specific loci in euchromatin, including 6B-C and 93A (Figure S2C), suggesting that the transcription of specific genes at these loci is silenced by dATF-2. In contrast, dATF-2, but not HP1, was detected at the 1E locus, suggesting that genes at this locus are activated by dATF-2.

The dATF-2 mutant cell clones in salivary glands exhibited weaker HP1 and H3K9me2 signals compared to WT cells (Figure 2B), whereas the dATF-2-overexpressing cell clones showed stronger HP1, H3K9me2, and H3K9me3 signals (Figure 2C and Figure S2D). These results further supported a requirement for dATF-2 in heterochromatin formation.

**The Mekk1-p38-dATF-2 Pathway Negatively Regulates Heterochromatin Formation**

Cells are constantly exposed to internal stresses, such as free radicals, generated as normal products of cell metabolism. To test whether background levels of stress affect dATF-2-dependent heterochromatin formation via p38 and its upstream kinase, Mekk1, we generated the Mekk1 mutant and Mekk1-overexpressing clones in salivary glands. The Mekk1 mutant cells showed strong HP1 and H3K9me2 signals (Figure 2D), whereas the Mekk1-overexpressing clones exhibited weaker HP1, H3K9me2, and H3K9me3 signals (Figure 2E and Figure S2E). Furthermore, Mekk1 heterozygotes showed enhanced white silencing in w^T^ (Figure 2F). The loss of one copy of p38a did not affect white silencing, possibly due to the existence of two p38 genes with similar functions (p38a and p38b) (Han et al., 1998). These results suggested that Mekk1 negatively regulates dATF-2-dependent heterochromatin formation.

**dATF-2 Localizes to Heterochromatin and Is Released from Heterochromatin in Response to Stress**

A search of the Drosophila genome sequence indicated that consensus CREs and half-CREs are frequently present in both the euchromatin and heterochromatin of all chromosomes (data not shown). The dATF-2 binding to the two regions containing multiple CREs was examined. One of the regions (20A-CREs), located in the pericentromeric heterochromatin of the X chromosome, involves seven tandem repeats of a 124 bp sequence, each of which contains one consensus CRE (Figure 3A and Figure S3A). The second region (80B-CREs), located in the pericentromeric heterochromatin of the third chromosome, contains three consensus CREs (Figure 3A and Figure S3B). Chromatin immunoprecipitation (ChIP) assays with S2 cells revealed that dATF-2 localized to both these regions (Figure 3B), which were enriched in H3K9me2 (Figure 3C).
Figure 2. Effect of dATF-2 and Mekk1 on HP1 and H3K9me2 Signals in Heterochromatin

(A) Polytene chromosomes from WT (top) or dATF-2 mutant (bottom) flies were stained with anti-dATF-2 (red) and anti-HP1 (green) antibodies or with the nuclear counter-stain TOPRO-3 (blue), and merged images are shown in the far right panel. An arrow indicates the chromocenter. The asterisk indicates a nonspecific signal.

(B and C) Clones of dATF-2 mutant cells (B) or Flag-dATF-2–overexpressing cells (C) marked by the absence (B) or presence (C) of GFP (green) in the salivary gland are superimposed with HP1 (red) and H3K9me2 (blue) staining. Clones of cells are surrounded by dotted lines. Merged images are shown in the far left panels. A typical staining pattern of a single cell surrounded by a red or yellow square is shown below at higher magnification. Quantitation of immunostaining signals is shown on the right by bar graphs with SD (n = 4 in B and C, respectively). ***p < 0.001; **p < 0.01; *p < 0.05.

(D and E) Clones of Mekk1 mutant cells (D) or Mekk1-overexpressing cells (E) were analyzed as described above. n = 4 (D) or 5 (E). ***p < 0.001; **p < 0.01; *p < 0.05.
Exposure of S2 cells to osmotic stress, which induced the phosphorylation of dATF-2 (Figure S3D), reduced the localization of H3K9me2 (Figure 3C). Osmotic stress did not affect the binding of histone H3 to these regions (Figure 3D), and thus the ratio of H3K9me2/H3 was reduced (Figure 3E). Osmotic stress did not affect the binding of H3K9me2 (Figure 3C). We also performed ChIP assays using anti-dATF-2 antibodies for the heterochromatin regions lacking CREs. Some signals were detected, but they were not reduced by osmotic stress (Figure S3E), which suggests that dATF-2 might localize to these regions via interaction with other factors such as HP1. Alternatively, these signals may have resulted from nonspecific binding by anti-dATF-2 antibodies because dATF-2 mutant embryos gave rise to weaker but significant signals compared to the wild-type, which were also not reduced by heat shock (HS) (Figure 4E). ChIP assays with anti-phospho-dATF-2 (anti-P-dATF-2) indicated that the amount of P-dATF-2 bound to the two putative binding site regions in heterochromatin was low and was not affected by osmotic stress, while P-dATF-2 binding to CheB38c was enhanced by osmotic stress (Figure 3F).

To investigate whether the observed decrease in the level of heterochromatin-bound H3K9me2 in response to osmotic stress was mediated by the phosphorylation of dATF-2, we performed similar experiments with S2 cells expressing Flag-tagged WT dATF-2 or an Ala mutant, which was replaced by Ala. Western blotting confirmed the expression levels of Flag-dATF-2 in each cell pool (Figure S3F). Chromatin was first immunoprecipitated with an anti-Flag antibody, and then released by the Flag peptide and reimmunoprecipitated with the anti-H3K9me2 antibodies. Significant levels of H3K9me2 were detected at the two heterochromatin binding site regions (20A-CREs and 80B-CREs) that bound Flag-dATF-2 WT or Flag-dATF-2 T59/61A (Figure 3G and Figure S3G). Osmotic stress reduced the levels of H3K9me2 at heterochromatic sites associated with WT dATF-2, but not those associated with the Ala mutant (Figure 3G and Figure S3G). Thus, the results suggested that osmotic stress disrupted the interaction of dATF-2 with H3K9me2. However, the exposure of S2 cells to osmotic stress induced the phosphorylation of dATF-2, while the phosphorylation of Mekk1 by double-stranded RNA reduced the levels of phospho-p38 and abrogated the phosphorylation of dATF-2 (Figure 4A). We next examined whether HS-induced activation of dATF-2 affects heterochromatin formation in w^{wm} flies. Embryos were exposed to HS (37°C for 1 hr) at various times after egg laying (AEL), and white gene silencing was examined in adult flies. From 0 to 3 hr AEL, HS partially, but significantly, relieved white silencing in male and female embryos (Figure 4B and Figure S4A). HS from 0 to 3 hr AEL was not lethal to the embryos (Figure S4B), which suggests that HS does not selectively kill embryos carrying the specific subset of genetic polymorphisms that affect heterochromatin. These results suggested that dATF-2 activation during early development, when heterochromatin is established, disrupted heterochromatin formation. HS also partially affected white silencing at other times during development.

We focused on the effects of HS from 0 to 3 hr AEL because its effect on PEV was the most profound during this stage. The expression of Mekk1 and p38 in early embryos has been previously reported (Inoue et al., 2001; Sano et al., 2005). When Mekk1 mutant flies were similarly exposed to HS, there was no effect on PEV in w^{wm} flies (Figure 4C), suggesting that Mekk1 is required for this phenomenon. When the dATF-2 homozygous mutants were exposed to HS, the high levels of white expression did not increase further (Figure 4D), which suggests that dATF-2 was necessary for the effect of HS on PEV.

When similar experiments were performed with heterozygotes of the RNAi machinery mutant, aub, it was found that HS between 0 and 3 hr AEL further enhanced the degree of heterochromatin disruption (Figure S4C). Similar heterochromatin disruption by HS at 0 to 3 hr AEL was also observed in the heterozygotes of two other RNAi machinery mutants, spn and piwi (Figure S4D). The results showed that a combination of dATF-2 and the RNAi machinery mutations abolished or decreased the effect of HS (Figure S4E), suggesting that heterochromatin disruption by HS requires dATF-2 but not the RNAi machinery.

**HS Induces the Release of dATF-2 from Heterochromatin**

Western blotting revealed a decrease in the amount of dATF-2 in the nuclear pellet (which may correspond to the chromatin-bound form) in response to HS between 0 and 3 hr AEL (Figure 4E). The P-dATF-2 levels in the non-chromatin-bound fraction increased immediately upon HS and then rapidly decreased (Figure S4F), which suggests that P-dATF-2 is released from the heterochromatin and rapidly degraded. In ChIP assays, HS reduced dATF-2 binding to the two heterochromatic regions, 20A-CREs and 80B-CREs (Figure 4F) and also reduced H3K9me2 levels in these regions (Figure 4G). These results suggested that HS induced the phosphorylation of dATF-2, which resulted in its release from the heterochromatin.

To study the effect of HS on heterochromatin, we exposed embryos to HS (37°C for 1 hr) from 0 to 3 hr AEL, immunostained them with anti-dATF-2 at cycle 14, and analyzed them by laser
Figure 3. Stress-Induced dATF-2 Phosphorylation Causes the Release of dATF-2 from Heterochromatin, Leading to Heterochromatic Disruption

(A) Consensus CREs in pericentromeric heterochromatin of the X (left) and third chromosomes (middle) and in the euchromatic promoter region of the CheB38c gene on the second chromosome (right).

(B) ChIP assays were performed using S2 cells untreated (—) or treated with 0.5 M sorbitol for 20 min (+). Anti-dATF-2 antibodies were used with control IgG. Immunoprecipitated DNA was amplified by real-time PCR with primers that covered the CREs in the three loci shown in (A). The amount of amplified DNA relative to the input DNA is shown, and each bar represents the mean ± SD (n = 3). *p < 0.05.

(C–E) ChIP assays were performed using anti-H3K9me2 (C), anti-H3 (D), or control IgG. The ratio of H3K9me2/H3 is shown in (E). *p < 0.05; N.S., no significant difference.

(F) ChIP assays were performed with anti-P-dATF-2 or control IgG. Note that the level of P-dATF-2 binding to the heterochromatin is very low, while its binding to the Cheb38c gene is enhanced by osmotic stress. *p < 0.05; N.S., no significant difference.
confocal microscopy. Three-dimensional images of somatic cells were obtained to visualize an individual cell in its entirety. In the absence of HS treatment, dot-like dATF-2 signals were observed, and approximately 54 dot-like dATF-2 signals per somatic cell nucleus were associated with heterochromatin, which was visible as TOPRO-3-positive staining at the apical pole of blastoderm nuclei (Figure 5A and Figure S5C). Note that the dot-like signals in the nucleus, but not the cytoplasmic signals, were lost in the dATF-2 mutant, which suggests that the cytoplasmic signals are background of antibodies (Figures S5A and S5B). When embryos were exposed to HS, the size of the heterochromatin signals was reduced and the number of dATF-2 signals on heterochromatin decreased to about one-third. In the Mekk1 mutant embryos, HS had no effect on the size of the heterochromatin signals or on the number of dATF-2 signals on heterochromatin (Figures 5B and Figure S5D). Similar results were obtained with primordial germ cells (pole cells) (Figures 5C and 5D and Figures S5E and S5F).

**Transgenerational Inheritance of Stress-Induced Epigenetic Change via dATF-2**

The HS-induced disruption of heterochromatin in germ cells suggested that this change could be transmitted to progeny. To test this, we exposed w<sup>md</sup> embryos (G1) to HS from 0 to 3 hr AEL and then mated HS-treated female flies with unstressed male w<sup>md</sup> flies. In the resulting male progeny [G2(HN)], white derepression was observed, as demonstrated by eye pigment levels and white mRNA levels (Figures 6A and 6C). These results indicated that the defective heterochromatin was transmitted to the next generation. To test the inheritance from males, we similarly mated HS-treated male w<sup>md</sup> flies (G1) with unstressed female w<sup>md</sup> flies. In the resulting male progeny [G2(HN)], white derepression was again observed (Figures 6B and 6C). However, in this case, the X chromosome harboring the white gene in the G2 male progeny was derived from the unstressed female (Figure 6B), suggesting that HS-disrupted heterochromatin was transmitted in a non-Mendelian fashion. This is reminiscent of paramutation, which occurs by transcommunication between chromosomes (Figure S6A) (Chandler, 2007).

When white expression was compared in individual flies after HS treatment, a high degree of white derepression was observed in four out of 20 flies, which did not represent the average derepression in all individuals (Figure S6B, left). Similar observations were also recorded in the G2 progeny from the stress-exposed G1 flies (Figure S6B, right).

The effect of osmotic stress on subsequent generations was also tested. Eggs that were laid by flies on a diet containing 0.3 M NaCl were allowed to develop into adult flies on the same diet. Under these conditions, flies were exposed continuously to osmotic stress from the larval to adult stage. This experiment used the w<sup>md</sup> line, generated by repeating additional backcrossing to the w<sup>1118</sup> line, and showing lower basal levels of white expression than the original w<sup>md</sup> line. When male w<sup>md</sup> flies were fed on a high salt-containing diet [G1(S)], white derepression was observed in these flies (Figure 6D), and when the osmotic stress-exposed male w<sup>md</sup> flies were mated with unstressed female w<sup>md</sup> flies, the resulting male progeny [G2(SN)] also exhibited white derepression (Figure 6D). Thus, disrupted heterochromatin, induced by both HS and osmotic stress, was transmitted to the next generation.

To test the inheritance of defective heterochromatin induced by HS, we used T(1;4)wm<sup>258-21</sup> flies in which part of the X chromosome is brought near the pericentromeric heterochromatin of the fourth chromosome, resulting in Notch gene silencing and a Notch wing phenotype (Figure S6C) (Reuter et al., 1982). When female T(1;4)wm<sup>258-21</sup> flies (G1) were exposed to HS from 0 to 3 hr AEL, the frequency of the Notch wing phenotype decreased from 11% to 5% (Figure S6D). Furthermore, this decrease in frequency was evident in the G2 progeny generated by mating HS-exposed G1 T(1;4)wm<sup>258-21</sup> females and WT males. These results further supported the notion that defective heterochromatin induced by HS is transmitted to the next generation.

**Multigenerational Transmission of Disrupted Heterochromatin Induced by HS**

The inheritance of the HS effect was tested in w<sup>md</sup> flies over multiple generations (Figure 7). When male w<sup>md</sup> embryos were exposed to HS from 0 to 3 hr AEL every generation for five generations (#6 experiment), white gene silencing was weakened in the progeny of all five generations; however, the degree of change was saturated by the G2 generation. When male w<sup>md</sup> embryos were exposed to HS only at the G1 generation (#2 experiment), white silencing was decreased in the G1 and G2 progeny but not in successive generations (G3 to G5). When male w<sup>md</sup> embryos were exposed to HS at the G1 and G2 generations (#3 experiment), white silencing was weakened in the progeny of the next three generations (G3 to G5), although the degree of silencing in the G5 progeny was lower than in the G3 and G4 progeny. Thus, exposure to stress over multiple generations stabilizes the degree of epigenetic change and prolongs the period of inheritance.

We also tested whether the derepression of w<sup>md</sup> due to the loss of one copy of dATF-2 was inherited. WT or dATF-2 heterozygous male flies were mated with WT female flies carrying two copies of the w<sup>md</sup> gene, and white expression levels were compared between the WT w<sup>md</sup> progeny generated by each mating. WT male and female progeny from the dATF-2 heterozygous males exhibited increased white expression compared to the WT progeny from WT male flies (Figure S7A). Similar results were obtained when WT or dATF-2 heterozygous female flies were mated with WT male flies (data not shown). These results indicated that heterochromatin, disrupted by a decrease in dATF-2, was inherited by the next generation.

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(G) Native re-ChIP assays were performed with S2 cell pools harboring the expression vector for Flag-dATF-2, the Flag-dATF-2 mutant (T59/61A, in which two phosphorylation sites were mutated to Ala), or the empty vector control. The first immunoprecipitation was performed with anti-Flag, and immunocomplexes were eluted with the Flag peptide, followed by a second immunoprecipitation with anti-H3K9me2 or control IgG. Immunoprecipitated DNA was quantified as described above. *p < 0.05; N.S., no significant difference.

See also Figure S3 and Table S3.
Figure 4. HS Induces dATF-2 Phosphorylation and Disrupts Heterochromatin

(A) S2 cell pools harboring the Flag-dATF-2 expression vector were transfected with the control or Mekk1 double-stranded RNA and exposed to HS (37°C for 1 hr). Lysates were used for western blotting with anti-P-dATF-2, anti-P-p38, or anti-tubulin antibodies.

(B) Male w^{mnd} flies were exposed to HS (37°C for 1 hr) at the indicated times after egg laying (AEL), and the amounts of eye pigment were measured. Control flies were not exposed to HS. Averages ± SD are shown (n = 12). ***p < 0.001; **p < 0.01; *p < 0.05.

(C) Male w^{mnd} flies harboring the Mekk1 mutation were treated eye pigment levels were measured (n = 6).

(D) Flies with the indicated genotype were mated, and the embryos were heat shocked (37°C for 1 hr) between 0 and 3 hr AEL. Eye pigment levels were measured (n = 6).

(E) WT w^{1118} embryos were exposed to HS (37°C for 1 hr) between 0 and 3 hr AEL and proteins were extracted with RIPA buffer. The pellet fraction (chromatin) was then extracted with 2% SDS-containing buffer, and the lysates were used for western blotting with anti-dATF-2 antibodies or anti-lamin.

(F) ChIP assays were performed with the wild-type or dATF-2 mutant embryos untreated (–) or heat shocked (37°C for 1 hr) between 0 and 3 hr AEL. Anti-dATF-2 antibodies were used with control IgG. Immunoprecipitated DNA was amplified, and the amount of amplified DNA relative to the input DNA is shown (n = 3). *p < 0.05.

(G) ChIP assays were performed with embryos with anti-H3K9me2 (left) or anti-H3 (middle), or control IgG. The ratio of H3K9m2/H3 is shown on the right. *p < 0.05; N.S., no significant difference.

See also Figure S4.
To identify the dATF-2 target genes for which upregulation by HS is transmitted to the next generation, we used a microarray to compare the expression pattern between wild-type larvae (G2) generated from male flies (G1) exposed to HS from 0 to 3 hr AEL and the control flies without HS treatment. The upregulation of 97 genes in the G2 larvae derived from HS-treated G1 flies was observed ($p < 0.05$, more than 2-fold), while 88 out of 97 genes were not upregulated in the dATF-2 mutant background (Figure S7B). Furthermore, 20 out of the 88 genes were also upregulated in the dATF-2 mutant larvae compared to the wild-type, which suggests that their expression is directly silenced by dATF-2. These genes are involved in various biological functions, such as metabolism and development, and show signals for H3K9me3 (Table S4). These results suggest that the HS-induced and dATF-2-mediated upregulation of these genes is transmitted to the next generation.

**DISCUSSION**

**Reliability of the PEV Assay System**

The dATF-2 mutation induced the derepression of white expression in four white gene PEV reporters. The degree of upregulation of white by HS was relatively small; however, $w^{1178}$ is known to exhibit stable white gene expression at low basal levels in multiple white PEV reporters (Lloyd et al., 2003). Furthermore, to eliminate the effect of the genetic background, we always compared the sibling progeny of crosses, and in some cases we also measured white mRNA levels. Although there was a tendency toward the upregulation of white expression in response to osmotic stress in the standard $w^{nd}$ line, this was not statistically significant. On the other hand, the $w^{nd}$ line, generated by additional backcrossing to the $w^{1178}$ line and showing lower basal levels of white expression than the original $w^{nd}$ line, displayed clear suppression of PEV by osmotic stress, allowing its inheritance to be observed. Since outcrossing is thought to eliminate the suppressor(s) of PEV (Lloyd et al., 2003), white expression in the $w^{nd}$ line may be more tightly suppressed and sensitive to stress than in the original $w^{nd}$ line.
Role of dATF-2 in Stress-Induced Heterochromatic Disruption

Osmotic stress and HS, both of which induce dATF-2 phosphorylation, resulted in the release of dATF-2 from heterochromatin and the production of disrupted heterochromatin. The levels of H3K9me2 associated with the heterochromatic regions bound by WT dATF-2 were decreased by osmotic stress, whereas the levels in regions bound by the dATF-2 Ala mutant (in which the dp38 phosphorylation sites were mutated) were not. These results suggested that osmotic stress disrupted heterochromatin via dATF-2 phosphorylation by dp38. Since ectopic expression of the dATF-2 Ala mutant caused lethality in flies (data not shown), it was not possible to use transgenic flies expressing this mutant to examine whether dATF-2 mediates the HS-induced disruption of heterochromatin. Instead, it was demonstrated that HS did not increase white expression in a dATF-2 mutant background (Figure 4D) and that HS did not increase white expression in dATF-2 heterozygotes compared to the WT background (compare Figures S4D and S4E).

Timing and Strength of Stress Required to Disrupt Heterochromatin

Years ago, it was reported that an increase in temperature suppressed PEV (Gowen and Gay, 1933) and that the period of sensitivity for such an increase is during the first few hours of embryogenesis (Spofford, 1976); however, the mechanism is still unknown. Our data indicated that HS during early embryogenesis, but not at later stages, derepressed white expression.
pH, and eye pigment levels in adult male progeny \( \text{G}_2(\text{HN}) \) were measured. Similar matings were repeated until the \( \text{G}_5 \) generation, the eye pigment levels in the \( \text{G}_3(\text{HNN}), \text{G}_4(\text{HNNN}), \text{and G}_5(\text{HNNNN}) \) at each generation were measured, and the data are presented in bar graphs. In series \#3 experiments, \( \text{G}_1 \) and \( \text{G}_2 \) embryos were exposed to HS, and in series \#4 experiments, \( \text{G}_1, \text{G}_2, \text{and G}_3 \) embryos were exposed to HS. In series \#5 experiments, embryos were exposed to HS at every generation, whereas no embryos were exposed to HS in series \#1 experiments (control). In all experiments, the amount of eye pigment from 20 adult males was measured, and the averages \pm SD are shown (\( n = 6 \)). **p < 0.001; *p < 0.01; *p < 0.05. See also Figure 57 and Table S4.

consistent with these early reports. During early embryogenesis, the chromosomes undergo morphological changes and distinct darkly staining regions, thought to be heterochromatin, appear, suggesting that this stage is critical for heterochromatin formation. These results imply that the formation of heterochromatin during early embryogenesis is more sensitive to stress than at a later stage of development. On the other hand, in the case of osmotic stress produced by feeding flies on a high salt-containing diet, flies are only exposed to the stress later in development, once they start to feed. In spite of this, osmotic stress also relieved white silencing. Since osmotic stress induced the phosphorylation of dATF-2 more efficiently than HS (Figure 4A), it is possible that the stress that produces a greater activation of dATF-2 may also induce heterochromatic disruption at later stages of development.

**Inheritance of Stress-Induced, dATF-2-Dependent Heterochromatic Disruption**

Our data indicated that HS-induced derepression of white expression can be inherited by the next generation. In addition, the heterochromatin status was altered by osmotic stress as well as by HS, both of which induced the phosphorylation of dATF-2, caused the release of dATF-2 from heterochromatin, and were inherited by the next generation. Furthermore, the disrupted heterochromatin in dATF-2 heterozygotes was also inherited by the next generation. These results supported the hypothesis that the disrupted state of heterochromatin caused by the stress-induced phosphorylation of dATF-2 can be inherited.

While new epigenetic state induced by HS was transmitted to the next generation, it was not inherited by successive generations. HS stress over multiple generations caused the inheritance of defective heterochromatin over multiple successive generations, but it gradually returned to the normal state, which suggests that the HS-induced new epigenetic state is unstable. There is probably an adaptive mechanism meant to maintain the heterochromatic state during epigenetic reprogramming in germ cells. Such a maintenance system might be responsible for the rescue of the partly disrupted heterochromatin state induced by stress. However, when this system is not sufficient to recover the heavily disrupted heterochromatic state, trans-generational effects may be observed.

This study showed that HS-induced white derepression was inherited maternally and paternally in a non-Mendelian fashion. Interestingly, the inheritance of a disrupted heterochromatic state due to mutations in the RNAi machinery has not been reported, in contrast to that of stress-induced, dATF-2-dependent epigenetic change. One possibility is that the RNAi machinery is required for the inheritance of epigenetic change, which would not be observed in RNAi machinery mutants. It is worth noting that an RNA-dependent RNA polymerase, required for RNAi in fission yeast, is needed for paramutation in plants (Alleman et al., 2006).

Developmental anomalies caused by HS-induced gene mutations are well recognized. In addition, HS-induced defects in protein homeostasis can result in the appearance of new phenotypes. In this situation, the impairment of HSP90 function by an increased number of HS-induced denatured proteins can unmask phenotypically cryptic genetic variations (Rutherford and Lindquist, 1998).

However, the dATF-2-dependent epigenetic change induced by HS can be discriminated clearly from these events. Both HS-induced gene mutation and the HSP90-dependent appearance of new phenotypes occur at low frequencies, usually only a few percent. In contrast, the stress-induced, dATF-2-dependent epigenetic change described in this study has high penetrance. This phenomenon has not received much attention so far, partly due to a lack of clear phenotypes, such as morphological defects, and also due to transmission to a limited number of generations.

HS treatment induced a large increase in white derepression in four out of 20 flies, but this did not represent the average derepression in all individuals. A similar frequency of flies exhibiting white derepression was observed in the G2 generation of progeny from stress-exposed G1 flies, suggesting that there is a threshold level of stress required for heterochromatic disruption. It is possible that the degree of dATF-2 phosphorylation could vary among individual flies and that a certain level of dATF-2 phosphorylation may be required to disrupt heterochromatin. A similar frequency was observed for the osmotic stress-induced white gene derepression; however, the frequency of heterochromatic disruption could also vary depending on the site of heterochromatin affected, which may have different levels of heterochromatin marks, such as H3K9me2.
Is ATF-2 Involved in the Epigenetic Silencing of Target Genes in Euchromatin?

The HS-induced and dATF-2-dependent upregulation of some genes was transmitted to the next generation (Figure S7B). The ATF-2 family of transcription factors regulates the transcription of various genes that control cellular proliferation, apoptosis, metabolism and behavior (Okamura et al., 2007; Maekawa et al., 2007; Shimizu et al., 2008; Maekawa et al., 2010), and it is possible that stress-induced epigenetic changes in some of these genes could result in the development of various diseases.

EXPERIMENTAL PROCEDURES

Fly Stocks

All flies were maintained at 25°C on standard medium. The strains used in this study were w 118 (wild-type), w 118; F8Bac [PS] dATF-2-2 m [dATF-2-2 m for short] (Thibault et al., 2004), ln[f]/w 118 (w 118 for short), In(3L)Bal1, spn-E 1, Aub 1242 p38Δ1, dMek1Δ396, UAS-MEKK1, UAS-Flag-dATF2, and P(dATF-2).

For normalization of the genetic background, w 118 and dATF-2-2 m were back-crossed six times with w 118.

PEV and Pigment Quantification

For elimination of the genetic background effect, sibling progeny of crosses were always compared. Eye pigmentation was measured with 20 flies per assay as described by Rabinow et al. (1991). For evaluation of the sensitivity of HS on PEV during development, fly siblings were exposed to HS (37°C for 1 hr) at various developmental stages or reared at 25°C as non-osmotic stress controls. Since HS from 0 to 3 hr AEL efficiently abrogated PEV, HS during this period was used for the evaluation of the effect of HS on PEV. So that the effect of osmotic stress could be investigated, F1 flies were reared on media containing 0.3 M NaCl and compared to their siblings raised on normal media.

Polytene Chromosome Staining

Salivary glands from third-instar larvae were fixed with formaldehyde, and polytene chromosomes were squashed and frozen. Chromosomes were incubated with anti-H3K9me2 (C14, DSHB) and anti-dATF-2, and incubated with FITC- and Cy3-conjugated secondary antibodies. DNA was counterstained with TOPRO-3 for confocal microscopy or DAPI for fluorescence microscopy. Images were obtained with a confocal microscope or a fluorescence microscope.

Clonal Analysis of Salivary Glands

Somatic mutant clones were generated using the FRT-Flip system, while GAL4-UAS-mediated overexpression clones were generated with the Flip-out technique. Salivary glands from third-instar larvae were fixed and stained via standard techniques, and the following primary antibodies were used: anti-H3K9me2 (Upstate), and anti-HP1 (C14, DSHB). Images were obtained with a confocal microscope.

Chromatin Immunoprecipitation

S2 cells were left untreated or were treated with 0.5 M sorbitol for 20 min (for dATF-2) or 1 hr (for H3K9me2 and histone H3). Embryos were untreated or exposed to HS (37°C, 1 hr) for 0 to 3 hr AEL. ChiP assays were performed with modifications of standard techniques as described in the Extended Experimental Procedures. The antibodies used were anti-H3K9me2 (Abcam), anti-histone H3 (Abcam), anti-dATF-2C raised against the GST-dATF-2 (aminoc acids 152 to 381) fusion protein; a phosphorylated dATF-2 (P-dATF-2)–specific antibody; and mouse or rabbit normal IgG as a control. Purified DNA samples were subjected to quantitative real-time PCR (q-PCR) with the primers shown in Table S3.

Native Re-ChiP assays with S2 cell pools expressing Flag-dATF-2 and the Flag-dATF-2 T59/61A mutant were performed by modification of the reported protocol (Wagschal et al., 2007) as described in the Extended Experimental Procedures. Anti-Flag (M2, Sigma) was used for the first immunoprecipitation, and the complexes were eluted with Flag peptide, followed by a second immunoprecipitation with anti-H3K9me2 (Abcam) or control IgG.

Western Blotting

So that the effect of HS on chromatin-bound dATF-2 levels could be examined, embryos (0 to 3 hr AEL) were either untreated or exposed to HS (37°C for 1 hr) and were homogenized with RIPA buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and protease inhibitors). Samples were centrifuged to separate the unbound chromatin (supernatant) from the chromatin fraction (pellet). The pellet fraction was suspended in SDS-sample buffer containing 2% SDS, boiled, and used for western blotting with anti-dATF-2 (raised against full-length dATF-2) (Sano et al., 2005) and anti-lamin (ADL76, DSHB).

Immunohistochemistry of Embryos

Embryos (1 to 4 hr AEL) were treated either with or without HS (37°C for 1 hr), dechorionated, fixed with paraformaldehyde and dehydrated with methanol, and then stained with anti-dATF-2 (raised against full-length dATF-2) (Sano et al., 2005) and anti-lamin (ADL76, DSHB) via standard techniques. Images were obtained with a confocal microscope. For calculation of the amount of dATF-2 foci on heterochromatin, image stacks (2 series) were taken at 0.6 µm intervals from the apex to the bottom of the blastoderm embryo and were reconstructed with Imaris 6.3 (Bitplane) software.

Transgenerational Effect of Stress on PEV

So that the transgenerational effect of HS on PEV could be investigated, male or female flies that had been exposed to HS from 0 to 3 AEL were crossed with unstressed w 118 female or male flies. In each cross, 80 females and 20 males per vial were used. With 20 male progeny, red eye pigment OD 480 levels were measured and compared with those of their non-stressed siblings. Similar HS treatments, crossing and measurement of red eye pigment levels were performed to examine the effect of HS over five generations. For examination of the effect of osmotic stress, flies were reared on media containing 0.3 M NaCl, and crossing and measurement of red eye pigment was performed as described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and four tables and can be found with this article online at doi:10.1016/j.cell.2011.05.029.

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EXTENDED EXPERIMENTAL PROCEDURES

Fly Stocks
All flies were maintained at 25°C on standard medium. The strains used in this study were w1118 (wild-type), w1118; PBac(PB)dATF-2OS646S (dATF-2P8 for short) (Thibault et al., 2004), In(l)w54 (w54 for short) (Tartof et al., 1984), T(1;4)w258-21 (w258-21 for short) (Reuter et al., 1982), y wPc23, Phsps26-p, hs70-w, 39C-12, 118E-10, 118E-15, 39C-X (Wallrath and Elgin, 1995), In(3L)BL1 (Lu et al., 1996), P(w+) HS-lacZ(65E) (Lu et al., 1996), snp-E1 (Aravin et al., 2004), abuOC42 (Kennerdell et al., 2002), p38a1 (Craig et al., 2004), dMekk1Urd3, UAS-MEKK1 (Inoue et al., 2004), and UAS-Flag-dATF2 and P(dATF-2) (Okamura et al., 2007). To normalize the genetic background, w54d and dATF-2P8 were backcrossed six times with w1118.

Quantitative RT-PCR
Total RNA was isolated from 20 fly heads by homogenization in 500 µl TRIZOL reagent (Invitrogen), followed by DNasel treatment. To measure the mRNA levels of dATF-2 and white (Figure 1A and Figure S1B), real-time quantitative RT-PCR was performed using the SuperScript II Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) and an Applied Biosystems ABI Prism 7000 Sequence Detection System. The PCR primers used are shown in Table S1. To evaluate the white gene expression of w54d flies either with or without heat shock (HS), a Quantitect Probe RT-PCR Kit (QIAGEN) was used with the primers indicated in Table S2.

Position Effect Variegation and Pigment Quantification
To eliminate the effect of the genetic background, sibling progeny of crosses were always compared. To examine the effect of the interaction between dATF-2 and the RNAi pathway on heterochromatin formation, w54d and w54d; dATF-2P8 females were crossed with either snp-E1/TM3 or abuOC42/CyO males and the red eye pigment levels were measured in the progeny that did not contain balancer chromosomes.

Eye pigmentation was measured as described by Rabinow et al. (1991). Briefly, flies of each genotype were frozen in liquid nitrogen and stored at ~80°C until use. The frozen flies were decapitated by vigorous shaking and vortexing, and 20 heads were homogenized with a pellet mixer in 500 µl methanol/0.1% HCl, followed by overnight extraction at 4°C. After centrifugation, the absorbance of the supernatant was measured at 480 nm. Newly enclosed flies (two days old) were used in the experiment.

β-Galactosidase Assay in Rescue Experiments
A pair of salivary glands was dissected from wandering third instar larvae and ground up in 50 µl extraction buffer (50 mM sodium phosphate, 1 mM MgCl2, pH 7.2, and proteinase inhibitors). The extract was centrifuged for 20 min at 15000 rpm, and 35 µl was used for the assay in 200 µl assay buffer containing 1 mM chlorophenol red β-D-galactopyranoside (CPRG). Reactions were incubated at 37°C and the OD at 562 nm was measured at 0, 1.5 and 2 hr after extract addition. The enzyme activity was calculated as the mOD562 variation per min and per pair of salivary glands. To test for background activity, w1118 larval salivary glands were used. X-gal staining was performed to compare the lacZ activity of salivary glands histochemically. The glands from wandering third instar larvae were dissected in phosphate buffered saline (PBS) and fixed in PBS containing 1% glutaraldehyde for 10 min. After washing twice with PBS containing 0.3% Triton X-100, the salivary glands were immersed in X-gal staining solution (0.05% X-Gal, 3.1 mM K4Fe(CN)6, 3.1 mM K3Fe(CN)6, 1 mM MgCl2, 150 mM NaCl, 10 mM Na2HPO4/NaH2PO4, pH 7.2, 0.3% Triton X-100) and incubated at 37°C. The staining reaction was stopped by washing the glands with PBS. As the control, the P[w+ HS-lacZ(65E)] line carrying an hsp70-lacZ gene in the euchromatin of the third chromosome at position 65E (Lu et al., 1996) was used.

Effect of Maternal and Zygotic dATF-2 Mutation on PEV
To investigate the effect of maternal and zygotic mutation of dATF-2 on heterochromatin (Figure 1D), w54d; dATF-2P8/T(2;3)SM6a-TM6B (for maternal progeny) and w54d; dATF-2P8/dATF-2P8 (for maternal progeny) females were crossed with either w1118/Y; +/- (for zygotic progeny) or w1118, dATF-2P8/dATF-2P8 (for zygotic progeny) males. Then, the eye pigmentation was measured for the following male progeny: w54d/Y: dATF-2P8/+ (maternal/zygotic), w54d/Y: dATF-2P8/dATF-2P8 (maternal/zygotic), and w54d/Y: dATF-2P8/dATF-2P8 (maternal/zygotic).

Western Blotting
To examine the dATF-2 levels in dATF-2 mutants (Figure S1A), 200 w1118 and dATF-2P8 early embryos (0 to 3 hr after egg laying [AEL]) were homogenized with 200 µl of RIPA buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, and proteinase inhibitors). After incubation for 20 min on ice, samples were centrifuged to separate the unbound chromatin (supernatant) and the bound chromatin (pellet) fractions. The pellet fraction was suspended in SDS sample buffer containing 2% SDS, boiled for 10 min, and used for Western blotting with anti-dATF-2, which was raised against the Pseudomonas exotoxin (PE)-full-length dATF-2 fusion protein (Sano et al., 2005) and anti-lamin (ADL67.10, DSHB).

To analyze the role of the Mekk1-p38 pathway in the stress-induced phosphorylation of dATF-2, RNAi method was used. The Mekk1 double-stranded RNA (dsRNA) was generated using the MEGAscript kit (Ambion) according to manufacturer’s instructions. The sequences of primers are as follows: 5’ TAATACGACTCATATAGGGACGTGCCAGAGACCGTCCGACCCA 3’ and 5’
TAATCGACTCACTATAGGGCTGAGGCGAGGTGTG 3’. For transfection of dsRNA in S2 cells, S2 cells harboring the Flag-dATF-2 expression vector were plated in serum-free medium and incubated with dsRNA at room temperature for 30 min before serum-containing medium was added. Four days after the addition of dsRNA, cells were treated with 0.5 M sorbitol for 20 min or exposed to HS (37°C, 30 min), and then the cell lysates were prepared and analyzed by Western blotting with anti-P-dATF-2, anti-P-p38 (Cell signaling #4831) and anti-β-tubulin.

To examine the effect of HS on the dATF-2 levels bound to chromatin (Figure 4E), 200 w 1118 embryos (0 to 3 hr AEL) were exposed to HS (37°C for 1 hr) or left untreated. The chromatin-bound fractions were prepared and used for Western blotting as described above. To analyze the effect of HS on the P-dATF-2 levels in the non-chromatin-bound fraction (Figure 4F), 200 UAS-Flag-dATF2 embryos were treated, and the supernatant fraction was used for Western blotting as described above.

**Polytene Chromosome Staining**

Salivary glands were dissected from w 1118 or dATF-2 2PB wandering third instar larvae in PBS with 0.1% Triton X-100. The glands were fixed in fixative I (3.7% formaldehyde and 1% Triton X-100 in PBS) for 30 s and transferred to fixative II (45% acetic acid and 4% formaldehyde) for 3 min. Polytene chromosomes were squashed and immediately frozen in liquid nitrogen. The chromosomes were incubated for 1 hr in blocking solution (10% skim milk, 3% BSA, 0.2% NP-40, 0.2% Tween-20 in PBS), and then overnight at 4°C with mouse anti-HP1 monoclonal antibody C1A9 (DSHB, 1:100) and rabbit anti-dATF-2 (1:50) raised against the PE-full-length dATF-2 fusion protein (Sano et al., 2005). After washing with PBS and washing buffer (300 mM NaCl, 0.2% NP-40, 0.2% Tween-20 in PBS), the chromosomes were incubated with FITC-conjugated goat anti-mouse IgG (1:200) and Cy3-conjugated goat anti-rabbit IgG (1:200) for 2 hr at room temperature. After washing, DNA was counterstained in PBS with 1 μM TOPRO-3 for the confocal microscope, or 0.1 μg/ml DAPI for the fluorescence microscope. Imaging was performed with a Zeiss fluorescence microscope (LSM510) or an OLYMPUS fluorescence microscope (BX60) equipped with a cooled CCD camera (CoolSnap, Photometrics).

**Clonal Analysis in Salivary Glands**

Somatic mutant clones were generated using the FRT-Flp system (Xu and Rubin, 1993). To generate the mutant clones, hs-FLP; P(neoFRT)42D P(Ubi-GFP(S65T)nls)2R/CyO females were crossed with either dATF-2 2PB or Mekk1 136/0 males, and the embryos (5 to 24 hr AEL) were heat shocked in a water bath at 37°C for 1 hr. GAL4-UAS–mediated overexpression clones were generated using the Flp-out technique (Pignon and Zipursky, 1997). To generate the overexpression clones, hs-FLP; act-FRT-yr-FRT-GAL4, UAS-GFP females were crossed with either UAS-Flag-dATF-2 or UAS-Mekk1 males, and the clones were exposed to heat shock for 1 hr at 37°C between 48 to 72 hr AEL. Salivary glands from Drosophila third instar larvae were fixed and stained using standard techniques. The primary antibodies used in this study were as follows: rabbit anti-H3K9me2 (1:50, Upstate), rabbit anti-H3K9me3 (1:50, Upstate), and mouse anti-HP1 C1A9 (1:50). FITC- and Cy3-conjugated secondary antibodies were used. All images were obtained with a Zeiss LSM 510 confocal microscope and the average brightness of each signal in the nuclear region was measured using Adobe Photoshop and normalized to the average brightness at the outer region of the tissue.

**Co-immunoprecipitation**

S2 cells were maintained in Schneider’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and 10 mg/ml of dATF-2 expression vector were plated on 10-cm dishes to achieve 10% confluence and three dishes were used to prepare each sample. After 48 hr, the cells were either untreated or treated with 0.5 M sorbitol for 20 min (for dATF-2) or 1 hr (for H3K9me2 and histone H3). The cells were then fixed for 15 min in 1% formaldehyde/PBS and were immediately quenched with glycine. The cells were lysed with 2 ml of SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS and protease inhibitors) on ice for 10 min. The samples were then sonicated on ice to obtain fragmented DNA with an average size of approximately 1 kb. The samples were diluted (1:10) with IP buffer (0.5% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and protease inhibitors) and incubated with the following genomic sequences of Drosophila melanogaster were obtained from the FLYBASE site (http://flybase.bio.indiana.edu/). A whole genome search of full CRE (TGACGTCA) and half CRE (TGACGCGTCA) was performed using Genome Enhancer (http://opengenomics.org/).

**Estimation of CRE Site Frequency**

Chromatin Immunoprecipitation (ChIP) using S2 Cells

S2 cells were plated on 10-cm dishes to achieve 10% confluence and three dishes were used to prepare each sample. After 48 hr, the cells were either untreated or treated with 0.5 M sorbitol for 20 min (for dATF-2) or 1 hr (for H3K9me2 and histone H3). The cells were then fixed for 15 min in 1% formaldehyde/PBS and were immediately quenched with glycine. The cells were lysed with 2 ml of SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS and protease inhibitors) on ice for 10 min. The samples were then sonicated on ice to obtain fragmented DNA with an average size of approximately 1 kb. The samples were diluted (1:10) with IP buffer (0.5% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and protease inhibitors) and incubated with the following
antibodies: mouse anti-H3K9me2 (Abcam), anti-histone H3 (Abcam), rabbit anti-dATF-2C raised against the GST-dATF-2 fusion protein (amino acids 152 to 381), or phosphorylated dATF-2 (P-dATF-2)-specific antibody. The co-immunoprecipitation experiments with HP1 demonstrated that the anti-dATF-2C antibodies were able to immunoprecipitate dATF-2 (Figures S2A and S2B). For controls, mouse (Santa Cruz) or rabbit (Jackson ImmunoResearch) normal IgG were also used. After overnight incubation, the immunocomplexes were precipitated by adding protein A or protein G Dynabeads (Invitrogen), and then washed five times with Wash buffer I (0.5% Triton X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and protease inhibitors), once with Wash buffer II (0.5% Triton X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and protease inhibitors), once with Wash buffer III (0.25 M LiCl, 0.5% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 250 mM LiCl and protease inhibitors), and twice with TE buffer. For ChIP assays using anti-H3K9me2 or anti-histone H3 antibodies, the DNA samples were eluted with 200 μl of freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃), while for dATF-2 or phosphorylated dATF-2 ChIP assays, the DNA samples were eluted with 200 μl of freshly prepared elution buffer (20 mM DTT, 500 mM NaCl). The samples were then incubated for 6 hr at 65 °C to reverse cross-links. DNA samples were purified and resuspended in 100 μl of TE using the QIAquick PCR purification kit (QIAGEN). A 5-μl portion of the eluate was subjected to quantitative real-time PCR (q-PCR) using the primers shown in Table S3.

Native Re-ChIP Assay using S2 Cell Pools
To establish the S2 cell pools expressing Flag-dATF-2 and the Flag-dATF-2 T59/61A mutant, S2 cells were grown to about 50% confluence in 10-cm dishes and then transfected with 10 μg of pact5C-Flag-dATF-2, pact5C-Flag-dATF-2 T59/61A expression plasmid or empty vector using Cellfectin according to the supplier’s instructions (Invitrogen). Twenty-four hours after transfection, the medium was replaced with fresh medium containing 2 mg/ml G418 (Invitrogen) to select for G418-resistant cells. After two to three weeks of selection, pools of G418-resistant cells were generated, and expression of Flag-dATF-2 was confirmed using Western blotting.

The native Re-ChIP assay was performed based on a previously reported native ChIP protocol (Wagschal et al., 2007). Briefly, isolated nuclei from S2 cell pools with or without sorbitol treatment were digested with Micrococcal nuclease (MNase, NEB) to obtain chromatin of mono- to penta-nucleosome-sized units. The chromatin was then incubated with 5 μg of anti-Flag antibody (M2; Sigma). After overnight incubation, the immunocomplexes were precipitated with protein G-Dynabeads and washed using the same method as for the ChIP assay described above. Complexes from the first ChIP assays were eluted in buffer containing 10 mM DTT, 500 μg/ml Flag peptide, and 250 mM NaCl for 3 hr at 37° C. Eluates were diluted 1:30 in Re-ChIP buffer (20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 2 mM EDTA and 150 mM NaCl) and re-precipitated with anti-H3K9me2 or control IgG. After washing, the samples were eluted with buffer containing 1% SDS and 0.1 M NaHCO₃. DNA recovered from the second immunoprecipitation was then subjected to qPCR analysis as described above.

Effect of HS on PEV
To evaluate the sensitivity of HS (37° C, 1 h) on heterochromatin formation during development, fly siblings were exposed to HS at various developmental stages and the results indicated that HS at early embryonic stages (0 to 3 AEL) efficiently disrupted heterochromatin (Figure 4B and Figure S4A). Therefore, HS from 0 to 3 hr AEL was used to evaluate the HS effect on heterochromatin. Untreated fly siblings were reared at 25° C and used as non-heat shocked controls.

ChIP using Embryos
To collect a large number of embryos, the large-scale fly facility described by Kunert and Brehm (2008) was used. The embryos were collected between 0 to 3 hr AEL and stored at 4° C until enough had been collected. Embryos were then exposed to heat shock stress (37° C for 1 h) or were left untreated and dechorinated immediately with 50% bleach for 2 min at room temperature. Dechorinated embryos were fixed for 15 min in 10 ml of crosslinking solution (1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, 1.8% formaldehyde, 50 mM HEPES, pH 8.0) in the presence of 30 ml of n-heptane. Crosslinked embryos were washed once with 30 ml of Stop solution (125 mM glycine, 0.1% Triton X-100 in PBS) and twice with 25 ml of PBT (0.1% Triton X-100 in PBS). Crosslinked embryos (1 g) were homogenized with a Wheaton Dounce Tissue Grinder (loose pestle) in 15 ml of cold PBT containing protease inhibitors. The vitelline membrane and large debris were removed by centrifugation for 1 min at 400 g, and cells were pelleted by centrifugation for 15 min at 1100 g. The cell pellet was resuspended in 10 ml of ice-cold Cell Lysis buffer (85 mM KCl, 0.5% IGEPAL CA-630, 5 mM HEPES, pH 8.0) containing protease inhibitors and homogenized using a Wheaton Dounce Tissue Grinder (tight pestle). Then, the homogenate was centrifuged at 2000 g for 4 min at 4° C. The nuclear pellet was resuspended in 2 ml of Nuclear Lysis buffer (10 mM EDTA, 0.5% N-lauroylsarcosine, 50 mM HEPES, pH 8.0) containing protease inhibitors. The samples were sonicated on ice to obtain fragmented DNA with an average size of approximately 1 kb. For immunoprecipitation, the samples were diluted in IP buffer (1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 140 mM NaCl, and protease inhibitors), and incubated with antibodies pre-bound to magnetic protein A or G Dynabeads (Invitrogen). After overnight incubation, the beads were washed five times with Wash buffer I (1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 140 mM NaCl and protease inhibitors) and once with Wash buffer II (1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 500 mM NaCl and protease inhibitors). The beads were washed with Wash buffer III (0.5% IGEPAL CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 250 mM LiCl and protease inhibitors) and twice with TE. The DNA
samples were eluted with 300 μl of freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃) for 15 min. The samples were transferred to new tubes, mixed with 12 μl of 5 M NaCl, and incubated at 65°C for 6 hr for reverse cross-linking. DNA samples were purified and resuspended in 50 μl of TE using the Qiaquick PCR purification kit (Qiagen). A 5-μl aliquot of the eluate was subjected to qRT-PCR as described above.

Immunohistochemistry of Embryos

Embryos were collected between 1 and 4 hr AEL, and were heat shocked at 37°C for 1 hr or left untreated. The dechorionated embryos were fixed with 4% paraformaldehyde in PEM buffer (0.1 M PIPES, pH 6.9, 1 mM EGTA, and 1 mM MgCl₂) for 20 min and devitellinized by vigorous shaking with methanol. After fixation, the embryos were stained with rabbit anti-dATF-2 antibody (1:100) raised against the PE-full-length dATF-2 fusion protein (Sano et al., 2005) and mouse anti-lamin (ADL67.10, 1:100, DSHB) using standard techniques. The embryos were mounted using ProLong Gold antifade reagent (Invitrogen). All images were obtained with a Zeiss LSM 510 confocal microscope. During the blastoderm stage, heterochromatin was mainly located at the apical pole of nuclei and showed an intense DNA-specific dye stain compared with the euchromatic regions (Rudolph et al., 2007). Therefore, the gain parameter was adjusted to detect the bright TO-PRO-3 signal of the apical heterochromatic region using non-heat stressed WT embryos and fixed for all acquisitions. To compare the control and heat stressed embryos, identical laser powers and acquisition settings were also used for all other parameters. For calculating the amount of dATF-2 foci on heterochromatin, image stacks (z-series) were taken at 0.6-μm intervals from apical to bottom of blastoderm embryo. The stacks of images were reconstructed by using Imaris 6.3 (Bitplane) software. The number of dATF-2 dot-like signals that overlapped with heterochromatic regions was scored. Thirty cells from 3-4 independent embryos were analyzed for the experiment.

Effect of Osmotic Stress on PEV

To investigate the effect of osmotic stress, F1 flies were reared on media containing 0.3 M NaCl and compared to their siblings raised on normal media. When exposed to osmotic stress, the w^md strain showed increased expression of the white gene compared to siblings raised on normal media. However, it was not possible to obtain results that were statistically significantly different (data not shown). Therefore, another w^md strain (w^md*), generated by additional backcrossing with w^1118, was used. The w^md* strain showed very strong silencing of the white gene, and w^md* flies that were exposed to osmotic stress showed increased expression of the white gene compared to siblings from normal media.

Examination of the Transgenerational Effect of Stress on PEV

To investigate the maternal effect of HS on PEV, female flies that were exposed to HS between 0 and 3 AEL were crossed with normal w^md males. For the paternal effect, male flies that were exposed to HS between 0 and 3 AEL were crossed with normal w^md females. In each cross, 80 females and 20 males per vial were used. Using 20 male progeny, the OD₄₈₀ levels of red eye pigment were measured and compared with those of their non-stressed siblings. Similar HS treatments, the crossing and measurement of the amount of red eye pigment, were performed to examine the effect of HS over five generations. To examine the effect of osmotic stress, flies were reared on media containing 0.3 M NaCl, and the crossing and measurement of the amount of red eye pigment was performed similarly.

Examination of the Inheritance of the Effect of losing One Copy of dATF-2 on PEV

To examine the inheritance of disrupted heterochromatin in dATF-2 heterozygotes, the following crosses were performed: w^md/ w^md; +/- X w^1118/Y; +/-T(2;3)SM6a-TM6B and w^md/w^md; +/- crossed with w^1118/Y; dATF-2^{Pθ}/T(2;3)SM6a-TM6B for the maternal effect, and w^md/w^md; +/- T(2;3)SM6a-TM6B crossed with w^1118/Y; +/- and w^md/w^md; dATF-2^{Pθ}/T(2;3)SM6a-TM6B X w^1118/Y; +/- for the paternal effect. In each cross 15 females and 15 males were used per vial, and were transferred every two days at 25°C. Then, the red eye pigment levels of the w^md/Y; +/- T(2;3)SM6a-TM6B and w^md/w^1118; +/-T(2;3)SM6a-TM6B progeny derived from each cross were measured.

Microarray Gene Expression Analysis

Total RNA was extracted from three independent collections of each third instar male larva genotype by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the RNaseasy kit (Qiagen, Hilden Germany) kit, following the manufacturers’ instructions. Double-stranded cDNA was synthesized from 10 μg total RNA using the SuperScript Double Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) and labeled with the Bioarray HighYield RNA transcript labeling kit (Enzo, Farmingdale, NY, USA). Biotin-labeled cRNA was then cleaned up using the Rneasy kit, which was followed by the cRNA fragmentation. Labeled probes (20 μg) were hybridized to Affymetrix Drosophila Genome 2.0 arrays and processed by a GeneChip Fluidics Station 450. Data were acquired by a GeneChip Scanner 3000 7G and processed/normalized by Affymetrix GeneChip Operating Software. Genes were identified as present when all three replicates had present (P) assignment (p < 0.05). A Student’s t test analysis was performed on the data from the three biological replicates. The signals of H3K9me3 and H3K4me3 on the identified genes at the larval stage were examined using the data from the modENCODE project (Accession Nos.: ESX00000443 and ESX00000424).
SUPPLEMENTAL REFERENCES


Figure S1. Decreased dATF-2 Protein Levels in the dATF-2 Mutant and Effect of dATF-2 Mutation on Heterochromatin at Other Loci, Related to Figure 1

(A) dATF-2 protein levels in the dATF-2PB mutant. Proteins were extracted from the chromatin pellet fraction of wild-type and dATF-2PB embryos and used for Western blotting with the anti-dATF-2 antibody that was raised against the PE-full-length dATF-2 fusion protein (Sano et al., 2005), or with an anti-lamin antibody as a control.

(B) The dATF-2 mutation causes derepression of the white gene in wm4 flies, but not in wild-type flies. Two genes next to the white gene are shown schematically. Total RNA was prepared from the flies with the indicated genotype, and mRNA levels were measured. The mRNA levels of three genes of dATF-2 mutant flies relative to those of the wild-type are shown as the averages of three measurements with SD. The ribosomal protein L32 mRNA was used as the internal control. ***p < 0.001; N.S., no significant difference. The CG32795 mRNA levels in the wm4 line were enhanced by the dATF-2 mutation; however, it has been reported that CG32795 is not repressed by heterochromatin in two wm4 lines (Vogel et al., 2009). One possible reason for this discrepancy could be differences in the genetic backgrounds. In the present study, the wild-type and the dATF-2 mutant had similar genetic backgrounds because backcrossing was repeated. However, in the study by Vogel, HP1 binding and mRNA levels in wm4 and Oregon-R-S were compared, and it was unclear whether or not these flies had similar genetic backgrounds. In fact, they noticed a small increase (1.2-fold) in HP1 binding ratios on chromosome 4, which could have been due to differences in the genetic backgrounds.

(C) The dATF-2 mutation also disrupts heterochromatin on other chromosomes. The effect of the dATF-2 mutation on white gene expression was examined using the 118E-15 and 118E-10 lines, in which a hsp70-white transgene is inserted at the telomeric and centromeric regions of the fourth chromosome, respectively, and 39-C12 lines in which the transgene was inserted in the banded region of the fourth chromosome. As the control, similar experiments were performed using...
the 39C-X line, in which a hsp70-white transgene is inserted in euchromatin at cytological region 2D on the X chromosome. Eye pigment levels were measured, and the results are shown as described in Figure 1B. n = 6, ***p < 0.001; *p < 0.05; N.S., not significant.

(D) The dATF-2 mutation does not affect egg hatch rate. Hatch rates were measured in seven experiments, in which 40-170 embryos were examined, and are shown as the averages with SD.

(E) The dATF-2 mutant does not exhibit the abnormal morphology of egg shell structures. The number of eggs with a normal (two) or abnormal (one or 0) number of dorsal appendages was counted.
Figure S2. Colocalization of dATF-2 and HP1 at Specific Loci in Euchromatin, and Effect of dATF-2 and Mekk1 on the H3K9me3 Signals, Related to Figure 2

(A and B) Coimmunoprecipitation of dATF-2 and HP1. (A) S2 cells were transfected with the Flag-dATF-2 expression vector, and whole cell lysates were precipitated with anti-dATF-2C raised against the GST-dATF-2 fusion protein (amino acids 152 to 381) or control IgG. The immunocomplexes were subjected to Western blotting with anti-HP1 or anti-Flag. (B) Whole cell lysates of S2 cells were precipitated with anti-dATF-2C or control IgG, and Western blotting was performed using anti-HP1. Note that immunoprecipitated dATF-2 could not be detected because it was masked by the IgG signal.

(C) Colocalization of dATF-2 and HP1 at specific loci in euchromatin. Polytene chromosomes of wild-type flies were stained with anti-dATF-2 (red), raised against the PE-full-length dATF-2 fusion protein, and anti-HP1 antibodies (green). DNA was stained with DAPI (blue). Merged images are shown in the bottom panels. Arrows indicate the colocalization of dATF-2 and HP1, while dotted arrows show the localization of dATF-2 alone.

(D) Overexpression of dATF-2 enhances histone H3K9me3 signals on heterochromatin. Clones of cells overexpressing dATF-2 and marked by the presence of GFP (green) in the salivary gland were stained with H3K9me3 (red) and TOPRO-3 (blue). Clones of cells are surrounded by dotted lines. Merged images are shown in the panels on the left. Quantitation of immunostaining signals is shown on the right in bar graphs with SD (n = 4). *p < 0.05.

(E) Overexpression of Mekk1 reduces histone H3K9me3 signals on heterochromatin. Clones of cells overexpressing dMekk1 and marked by the presence of GFP (green) were analyzed as described above. n = 3, and *p < 0.05.
Figure S3. Consensus CREs in Heterochromatin and dATF-2 Phosphorylation-Dependent Heterochromatin Disruption by Osmotic Stress, Related to Figure 3

(A–C) Three loci harboring consensus CREs are shown. (A) In the region close to the natural transposons in pericentromeric heterochromatin on the X chromosome, seven tandem repeats of a 124-bp unit, which contains a consensus CRE (red), are present. (B) Three consensus CREs (red) are present in the region close to the natural transposons in pericentromeric heterochromatin on the third chromosome. (C) One CRE (red box) is present in the promoter region of the CheB38c gene, which occurs in euchromatin on the second chromosome. This gene encodes a chemoreceptor-like protein and its transcription is induced by osmotic stress.

(D) Osmotic stress induces dATF-2 phosphorylation. S2 cell pool expressing Flag-dATF-2 were treated with 0.5 M sorbitol for 20 min or left untreated (control). Whole cell lysates were prepared and used for Western blotting with anti-P-dATF-2.

(E) Osmotic stress does not reduce the dATF-2 binding to the region lacking CRE. ChIP assays using S2 cells and anti-dATF-2 antibodies were performed as described in Figure 3B, except for the use of the primers covering the region lacking CRE. No-CRE1 and No-CRE2 regions are located in the pericentromeric heterochromatin of the X chromosome, 35 kb and 32 kb away from the centromere compared to the 20A region, respectively. Each bar represents the mean ± SD (n = 3). *p < 0.05.
(F) Generation of S2 cell pools harboring the expression vector for Flag-dATF-2 or the Flag-dATF-2 mutant (T59/61A), in which two p38 phosphorylation sites were mutated to Ala or the control empty vector. S2 cells were transfected with each expression vector, together with a marker vector expressing the neomycin-resistance gene, and were selected on G418-containing medium. Whole cell lysates of the cell pools obtained were prepared and used for Western blotting with anti-Flag antibodies.

(G) dATF-2 mediates osmotic stress-induced heterochromatin disruption. Native re-ChIP assays were performed as described in Figure 3G, and immunoprecipitated DNA was amplified by real-time PCR using primers that covered the CREs in the 80B-CREs or CheB38c regions. Each bar represents the mean ± SD (n=3). *p < 0.05; N.S., no significant difference.
Figure S4. Effect of HS on Heterochromatin in Female Flies and in Mutant Flies on Components of the RNAi Machinery, Related to Figure 4

(A) HS disrupts heterochromatin in female w<sup>1118</sup> flies. Female w<sup>1118</sup> flies were exposed to HS, and amounts of eye pigment were measured as described in Figure 4B.

(B) HS (37°C for 1 h) from 0 to 3 hr AEL does not kill embryos. Viability was calculated as the number of adult flies per the number of embryos, and the averages of five independent experiments are shown ± SD. One hundred embryos were examined in each experiment. N.S., not significant.

(C) HS during early development disrupts heterochromatin in a mutant of a component of the RNAi machinery. Male w<sup>1118</sup> flies harboring the aub mutation were exposed to HS, and the amounts of eye pigment were measured as described in Figure 4B.

(D) HS disrupts heterochromatin in the mutants of other components of the RNAi machinery. Male w<sup>1118</sup> flies harboring spnE, piwi or aub mutations were exposed to HS (37°C for 1 h) from 0 to 3 hr AEL, and the amounts of eye pigment were measured as described in Figure 4B.

(E) The loss of one copy of dATF-2 relieved the HS-induced disruption of heterochromatin in an RNAi machinery mutant background. Male w<sup>1118</sup> flies with the indicated genotype were exposed to HS (37°C for 1 h) from 0 to 3 hr AEL, and the amounts of eye pigment were measured as described in Figure 4B.
Release of P-dATF-2 from the chromatin in response to HS in embryos. The embryos carrying UAS-Flag-dATF-2 without specific driver to express Gal4 activator, in which Flag-dATF-2 was expressed at low levels from the hsp70 basal promoter, were used. Embryos were exposed to HS (37°C for 1 h) between 0 and 3 hr AEL and proteins were extracted with RIPA buffer as described in Figure 4E. The supernatant fraction was prepared and used for Western blotting with anti-P-dATF-2, anti-Flag, anti-P-p38, and anti-β-tubulin antibodies.
Figure S5. HS Disruption of Heterochromatin Depends on Mekk1 in Somatic and Germ Cells, Related to Figure 5

(A and B) Wild-type or dATF-2 mutant embryos were stained at cycle 14 with anti-dATF-2 (green) and anti-lamin (red). Images of somatic (A) or pole (B) cells obtained by laser confocal microscopy are indicated.

(C–F) Three-dimensional cell images obtained using Imaris 3D reconstruction software are shown. dATF-2 (green), lamin (red), and TOPRO-3 (for DNA, blue). The number of nuclear dATF-2 dot-like signals that overlapped with heterochromatin is shown in the bar graph on the right. Data is the average of 30 cells ± SD.

***p < 0.001.
Figure S6. Non-Mendelian Transgenerational Transmission of Disrupted Heterochromatin Induced by HS, Related to Figure 6

(A) Model for the paramutation-like inheritance of HS-induced heterochromatin disruption. Paramutation is an epigenetic phenomenon that has been extensively studied in plants, whereby combining two specific alleles results in a heritable change in the expression of one of the alleles. Paramutation-like interactions can occur between homologous pairings at allelic or non-allelic positions. The X chromosome, harboring the white gene of G2(HN)\(^{wm4}\) males in Figure 6B, was derived from an unstressed G1 female, so the centromeric heterochromatin on this X chromosome had not been exposed to HS. On the other hand, the Y chromosome and either of the autochromosomes of G2(HN) males were derived from HS-exposed G1 males, so that the centromeric heterochromatin on these chromosomes may be partly disrupted. Physical pairing between the heterochromatic regions of X chromosomes and other chromosomes, or the trans-action of some molecules, such as siRNA, may induce partial disruption of the heterochromatin on the X chromosome.

(B) Variation of disrupted heterochromatin induced by HS between individual flies. Flies were generated as described in Figure 6B, and the eyes of individual flies are shown. Note that some eyes, indicated by arrows, exhibit increased eye pigment.

(C) Use of the heterochromatin-dependent Notch phenotype to demonstrate the inheritance of disrupted heterochromatin induced by HS. (Left panel) The position of the Notch gene in T(1;4)\(^{wm258-21}\) is shown schematically. Part of the X chromosome is brought near the pericentromeric heterochromatin of the fourth chromosome, leading to the silencing of Notch. (Right panel) Notch wing phenotype of T(1;4)\(^{wm258-21}\) is shown.

(D) Inheritance of disrupted heterochromatin induced by HS using the heterochromatin-dependent Notch phenotype. Female T(1;4)\(^{wm258-21}\) flies were crossed with wild-type males (G0). Embryos generated were exposed to HS (38°C for 1 h) from 0 to 3 hr AEL, or were left untreated, and the number of adult male flies exhibiting the Notch wing phenotype was counted. G1 females were then mated with unstressed wild-type male flies, and the number of adult male flies exhibiting the Notch wing phenotype was counted.

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Figure S7. Inheritance of Disrupted Heterochromatin Induced by the Loss of One Copy of dATF-2 and Identification of dATF-2 Target Genes, Related to Figure 7  

(A) Wild-type (left) or dATF-2 heterozygous male flies (right) were mated with w\textsuperscript{me4} female flies. The amount of eye pigment in 20 wild-type male or female progeny (1/C24) was measured and presented in a bar graph with SD (n = 6). **p < 0.01; *p < 0.05. Note that the w\textsuperscript{me4} expression levels in the wild-type progeny from the dATF-2 heterozygotes are higher than those generated from wild-type flies.

(B) Wild-type (upper left) or dATF-2 mutant G1 embryos (upper right) were exposed to HS (37°C for 1 h) from 0 to 3 hr AEL, or left untreated, and males of G1 flies were then mated with unstressed female flies. Total RNA was prepared from the generated G2 3rd larvae and used for microarray analysis. By comparison between #1 and #2, the upregulation of 97 genes in #1 was observed (p < 0.05, > 2-fold). By comparison between #3 and #4, the upregulation of 42 genes in #3 was observed (p < 0.05, > 2-fold). Most of the genes (88 out of 97), which were upregulated in #1 compared to #2 (on the wild-type background), were not upregulated in #3 compared to #4 (on the dATF-2 mutant background). Furthermore, comparison between #4 and #2 indicated that 218 genes were upregulated in #4 compared to #2, which suggests that these genes are silenced by dATF-2 in the absence of stress. Among these 218 genes, 20 genes (A) were upregulated in #1 compared to #2, which suggests that these genes are directly regulated by dATF-2. These results suggest that HS-induced upregulation of these 20 genes, which are silenced by dATF-2, are transmitted to the next generation. These 20 genes are involved in various biological functions, such as metabolism and development (Table S4).