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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 (Molinier et al., 2006), while maternal diet influences the epigenetic state of a transposable element in the *agouti* gene of A^{vy} mice, 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-BPa, 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 serotonin 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

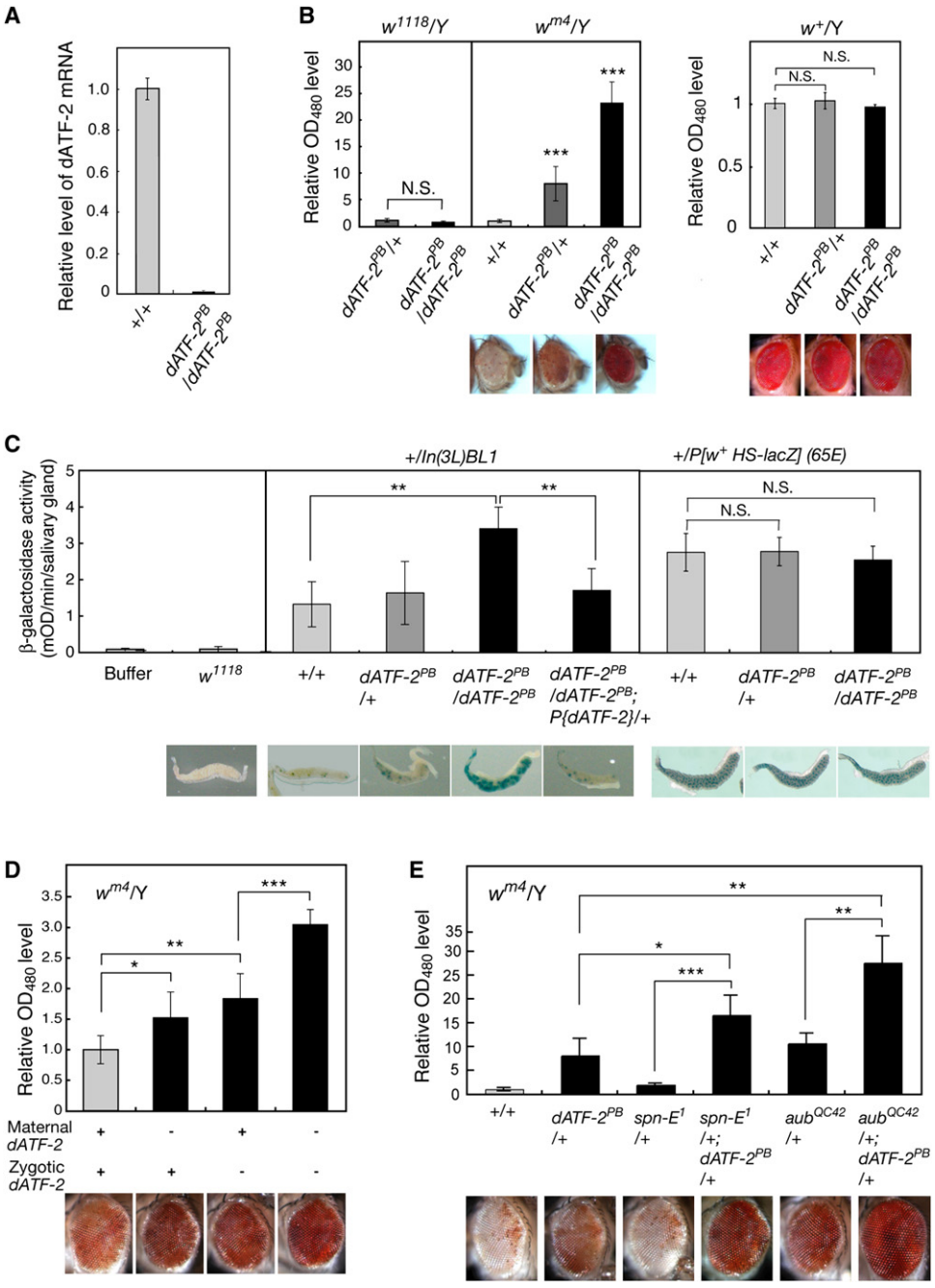

Figure 1. dATF-2 Is Required for Heterochromatin Formation

(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.

(Figure S1A available online). Therefore, *PB-c06407* was chosen as the *dATF-2* mutant and was renamed *dATF-2*^{PB}.

The effect of the *dATF-2* mutation on position effect variegation (PEV) was examined with the *In(1)w*^{m4} line (referred to hereafter as *w*^{m4}), in which a large inversion juxtaposes the *white* gene close to centromeric heterochromatin on the X chromosome (Tartof et al., 1984). The *w*^{m4} line, established by backcrossing with *w*^{1118} for six generations, was used in the present study. *dATF-2*^{PB} heterozygotes and homozygotes relieved *white* silencing in a dose-dependent manner, while *dATF-2* mutation did not affect the expression of *white* gene in the original eucromatin region (Figure 1B and Figure S1B). The *dATF-2* mutation also enhanced the expression of *CG32795*, which lies closer to heterochromatin than *white* in *w*^{m4}, but did not enhance the expression of *CG12498* (Figure S1B). For rescue experiments with transgenic flies expressing dATF-2, the *In(3L)BL1* line carrying an *hsp70-lacZ* transgene juxtaposed with pericentric heterochromatin of the third chromosome (Lu et al., 1996) was used instead of *w*^{m4} because the dATF-2 transgenic flies carried the *white* marker. *LacZ* silencing was relieved in *dATF-2*^{PB} homozygotes, while ectopic expression of dATF-2 prevented the relief of *lacZ* silencing (Figure 1C). *dATF-2* mutation did not affect the *hsp70-lacZ* expression located in the euchromatin (*P[W*$^{+}$ *HS-lacZ](65E)*).

The loss of either one or two copies of *dATF-2* also induced the derepression of *white* in lines 118E-15 and 118E-10, in which an *hsp70-w* transgene was inserted into the telomeric and centromeric regions of the fourth chromosome (Wallrath and Elgin, 1995), respectively (Figure S1D). Furthermore, the loss of two copies of *dATF-2* interfered slightly with *white* gene silencing in line 39C-12, in which the transgene was inserted in the banded region of the fourth chromosome. *dATF-2* mutation only slightly decreased the *hsp70-w* expression located in the euchromatin (39C-X), which could be due to the effect of the neighboring gene.

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 induced the derepression of *white* in *w*^{m4}, and the loss of both maternal and 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 Atf-1 contributes to heterochromatin formation 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 trasnposon 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 coprecipitated with HP1 in coimmunoprecipitation assays with S2 cell lysates (Figures S2A and S2B).

Immunostaining of polytene chromosomes indicated that both dATF-2 and HP1 colocalized at the heterochromatic chromocenter (Figure 2A). 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*^{m4} (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).

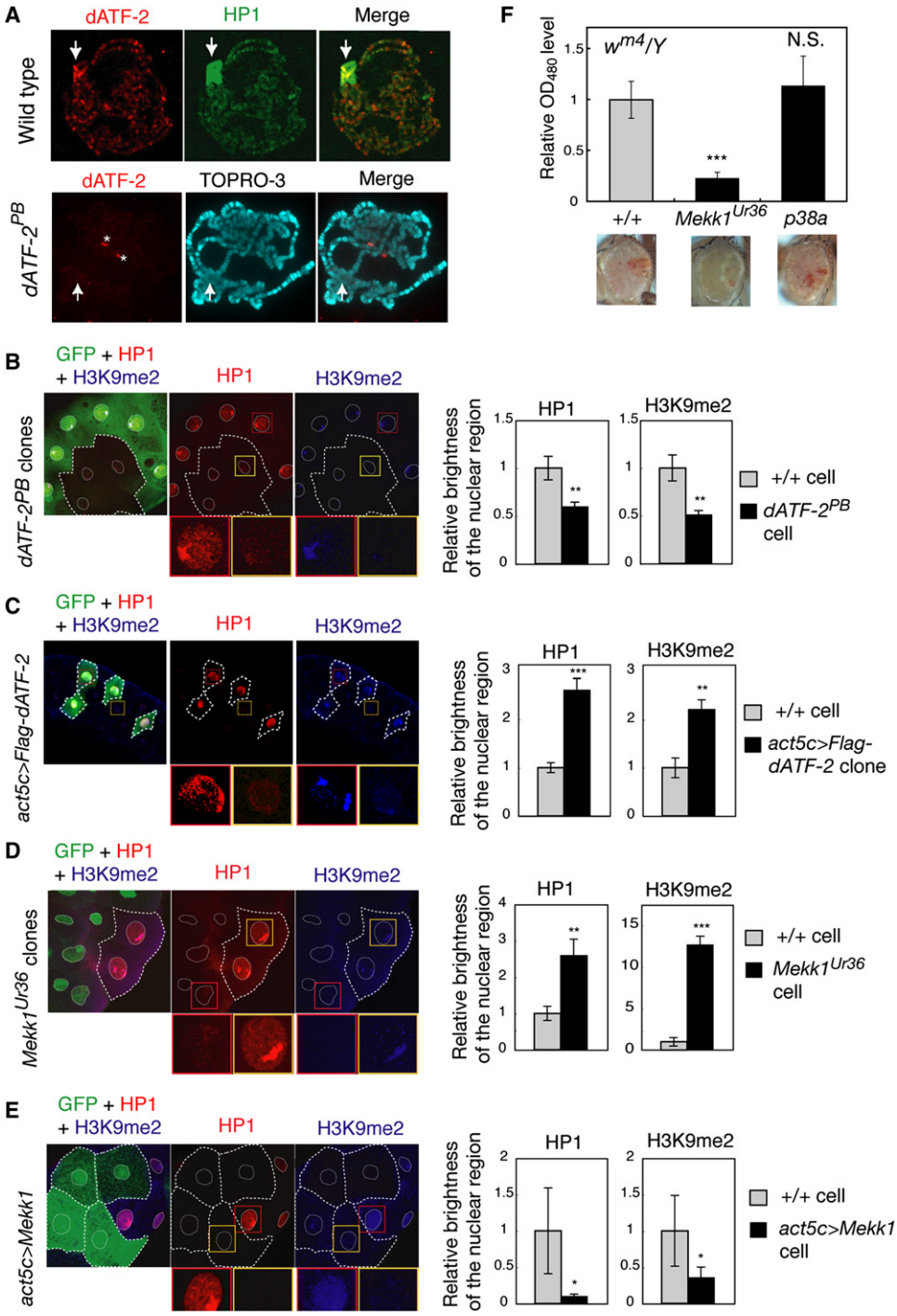

Figure 2. Effect of dATF-2 and Mekk1 on HP1 and H3K9me2 Signals in Heterochromatin

(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). ***$p < 0.001$; **$p < 0.01$.

(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 dATF-2 to these regions (Figure 3B) and the levels 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). As a control, we examined the binding of dATF-2 to *CheB38c*, located in the euchromatin region of the second chromosome (Figure 3A and Figure S3C). This gene encodes a chemoreceptor-like protein whose transcription is induced by osmotic stress (Sano et al., 2005). The binding of dATF-2 to this promoter region was enhanced by osmotic stress (Figure 3B), but the level of H3K9me2 bound to this region was not significant (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, re-ChIP assays were performed with S2 cells expressing Flag-tagged WT dATF-2 or an Ala mutant, in which Thr-59 and Thr-61, the p38 phosphorylation sites, were 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 heterochromatic 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 WT dATF-2-bound heterochromatin region but not the Ala mutant-bound heterochromatin region, supporting the notion that stress-induced disruption of heterochromatin is mediated by dATF-2 phosphorylation.

Heat Shock Stress during Early Embryogenesis Activates dATF-2 and Disrupts Heterochromatin

Various environmental stresses, such as HS, can activate p38 (Craig et al., 2004). The exposure of S2 cells to HS or osmotic stress induced the phosphorylation of dATF-2, while the reduction 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^{m4} 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^{m4} 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 to 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

(F) Mutation of *Mekk1* or *p38a* was combined with w^{m4} and eye pigment levels were measured. Averages with SD are shown (n = 6). ***p < 0.001; N.S., no significant difference.
See also Figure S2.

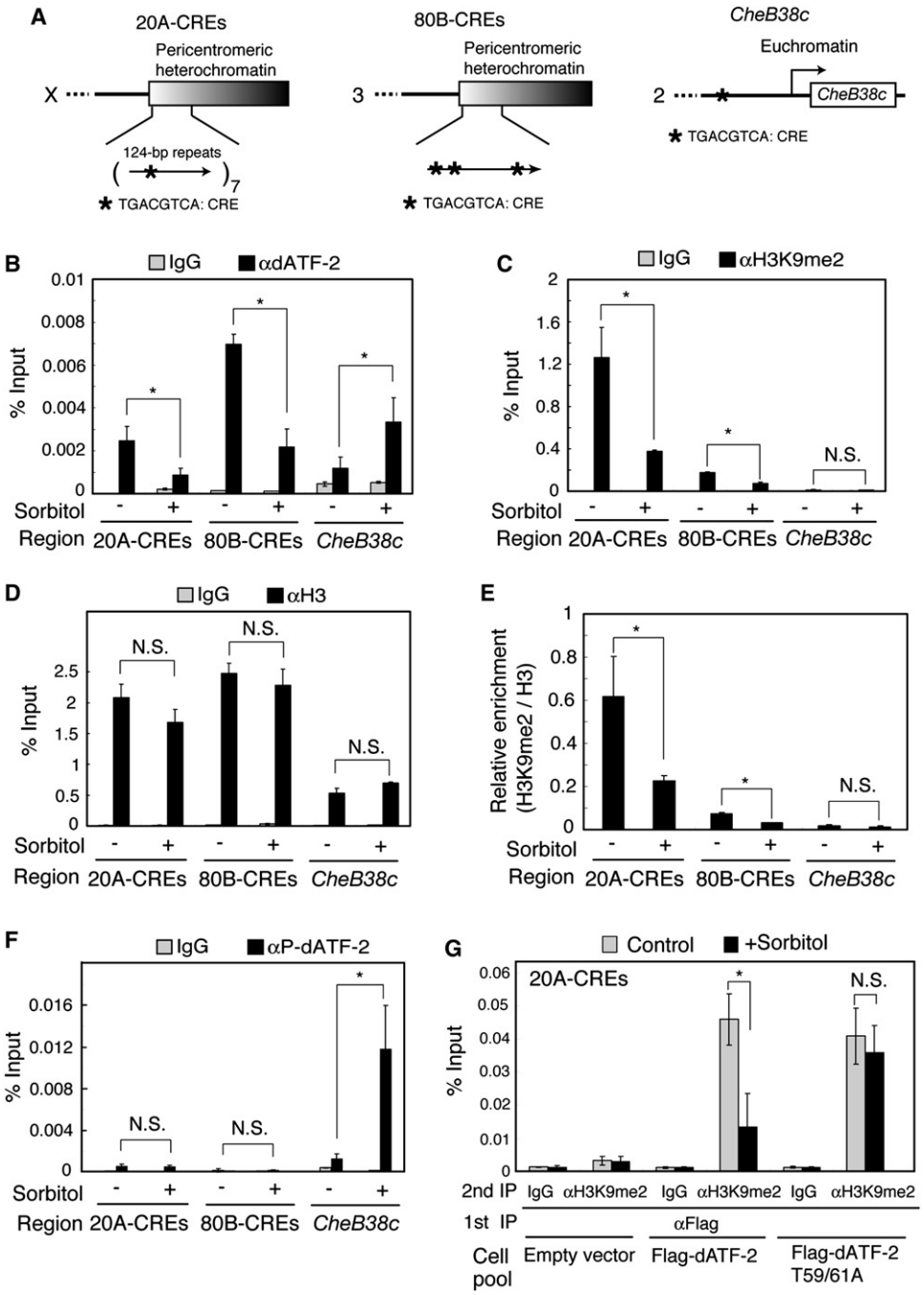

Figure 3. Stress-Induced dATF-2 Phosphorylation Causes the Release of dATF-2 from Heterochromatin, Leading to Heterochromatic Disruption

(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 H3K9m2/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-thir. 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^{m4} embryos (G1) to HS from 0 to 3 hr AEL and then mated HS-treated female flies with unstressed male w^{m4} 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^{m4} flies (G1) with unstressed female w^{m4} 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^{m4*} line, generated by repeating additional backcrossing to the w^{1118} line, and showing lower basal levels of *white* expression than the original w^{m4} line. When male w^{m4*} 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^{m4*} flies were mated with unstressed female w^{m4*} 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 $T(1;4)w^{m258\text{-}21}$ 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 $T(1;4)w^{m258\text{-}21}$ 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 $T(1;4)w^{m258\text{-}21}$ 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^{m4} flies over multiple generations (Figure 7). When male w^{m4} 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^{m4} 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^{m4} 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^{m4} 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^{m4} gene, and *white* expression levels were compared between the WT w^{m4} 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.

(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.

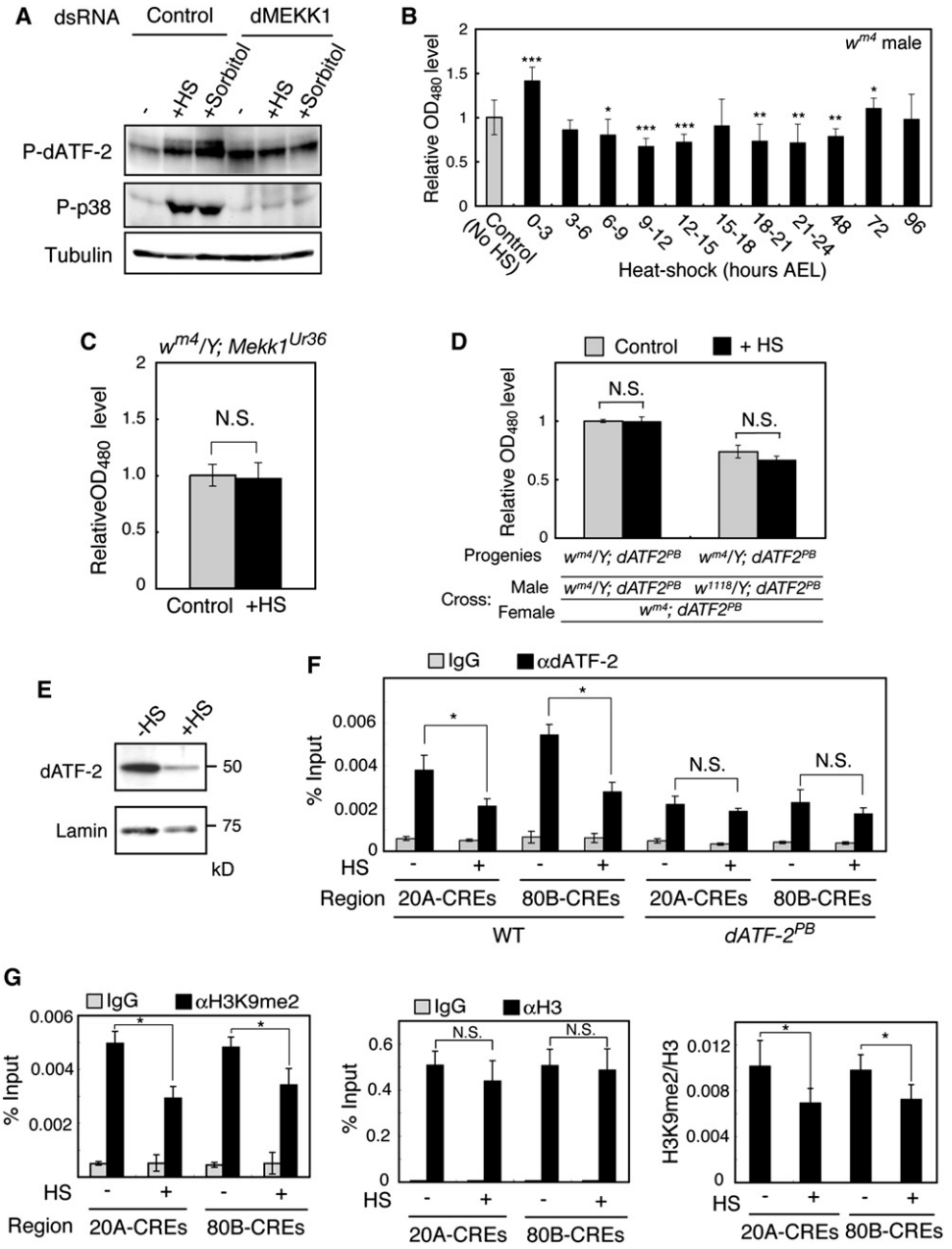

Figure 4. HS Induces dATF-2 Phosphorylation and Disrupts Heterochromatin

(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^{m4} 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^{m4} 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.

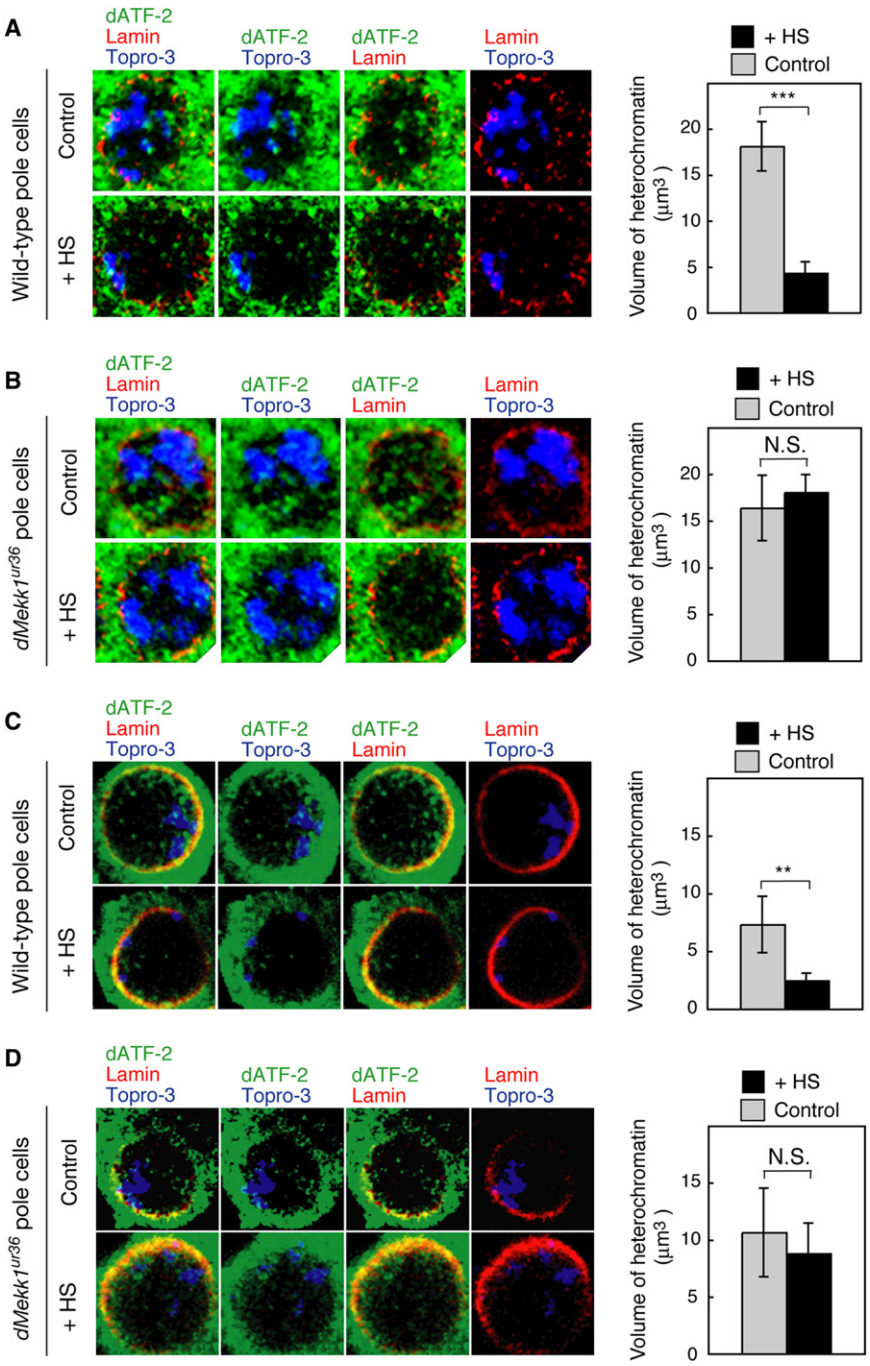

Figure 5. HS Heterochromatic Disruption Depends on Mekk1 in Somatic and Germ Cells

Wild-type (A and C) or *Mekk1* mutant (B and D) embryos were exposed to HS (37°C for 1 hr) from 0 to 3 hr AEL and stained at cycle 14 with anti-dATF-2 (green), lamin (red), and TOPRO-3 (for DNA, blue). Images (0.5 μm) of somatic (A and B) or pole (C and D) cells, obtained by laser confocal microscopy, are indicated, and merged images are shown in the far left panels. Most of the nuclear dATF-2 formed dot-like signals. Three-dimensional visualization of the signals is shown in Figure S5. The volume of heterochromatin is shown in the bar graph on the right. Data is the average of 30 cells ± SD. ***$p < 0.001$; **$p < 0.01$. See also Figure S5.

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 silence 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^{m4} 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^{m4} line, this was not statistically significant. On the other hand, the w^{m4*} line, generated by additional backcrossing to the w^{1118} line and showing lower basal levels of *white* expression than the original w^{m4} 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^{m4*} line may be more tightly suppressed and sensitive to stress than in the original w^{m4} line.

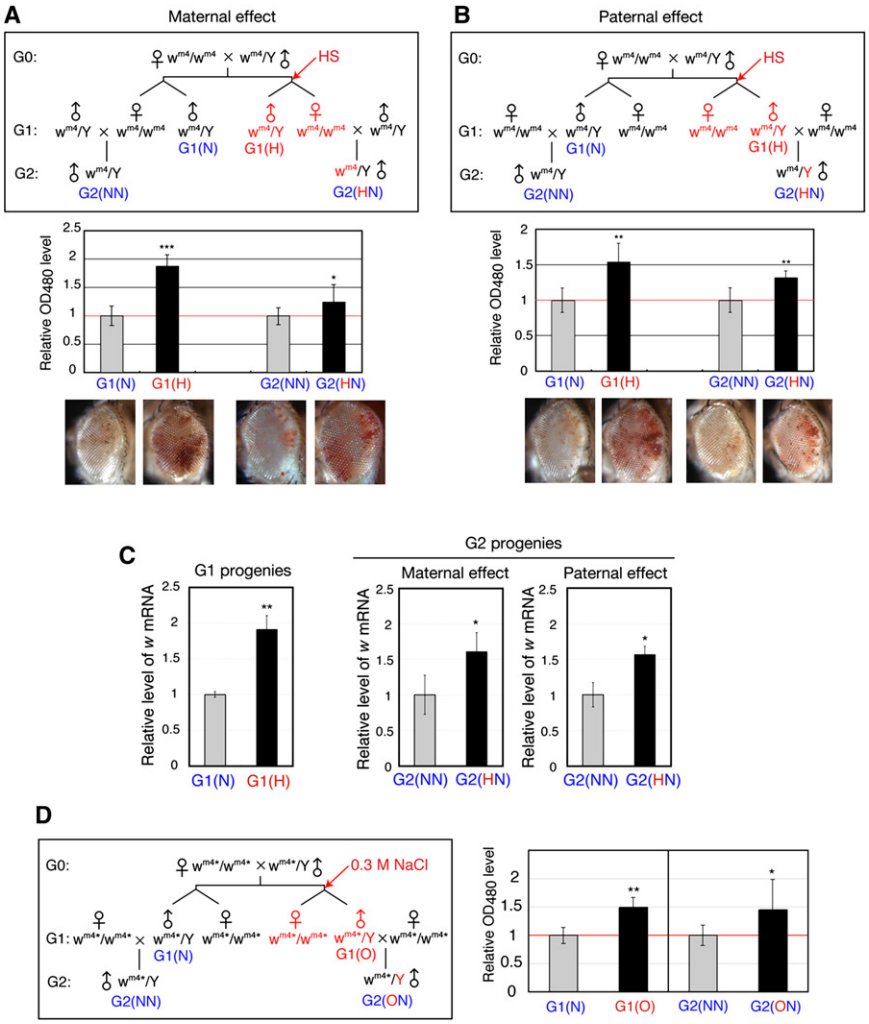

Figure 6. Non-Mendelian Transgenerational Transmission of Stress-Induced Disrupted Heterochromatin

(A and B) Male and female w^{m4} flies, carrying one or two w^{m4} genes, respectively, were mated (G0). Embryos were exposed to HS (37°C for 1 hr) from 0 to 3 hr AEL [G1(H)], or not exposed [G1(N)], and the amounts of eye pigment in 20 adult males were measured and presented in a bar graph with SD (n = 6). Females (A) or males (B) of G1(H) and G1(N) flies were then mated with unstressed male or female w^{m4} flies, and the amount of eye pigment in the G2 male progeny [G2(HN) and G2(NN)] was measured (n = 6). Note that the X chromosome harboring the *white* gene in the G2 progeny in (A) was derived from HS-exposed female flies, while that in (B) was derived from unexposed female flies. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Representative eye phenotypes are shown below.

(C) Flies were mated as described above, and the levels of *white* mRNA from 20 adult male flies were measured with qRT-PCR and are shown in the bar graph with SD (n = 3). **$p < 0.01$; *$p < 0.05$.

(D) Male and female w^{m4} flies were mated (G0). Eggs were laid on medium containing 0.3 M NaCl [G1(S)], or on control medium [G1(N)], and allowed to develop to adult flies on the same medium. Eye pigment levels in 20 adult males were measured and the data are presented as described above (n = 6). G1(S) and G1(N) males were then mated with unstressed female w^{m4} flies, and eggs were laid and allowed to develop to adult flies on the control medium. The amounts of eye pigment in G2 male progeny [G2(SN) and G2(NN)] were measured (n = 6). **$p < 0.01$; *$p < 0.05$.

See also Figure S6 and Table S2.

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).

We have observed localization of dATF-2 to heterochromatin by immunostaining polytene chromosomes and by performing ChIP assays. However, these data were obtained with anti-dATF-2 antibodies, which may recognize other proteins nonspecifically. Therefore, it is unclear whether dATF-2 localizes to heterochromatin via direct binding to CREs, interacts with heterochromatin via other proteins such as HP1, or indirectly regulates heterochromatin formation, for example by regulating the expression of certain factors involved in heterochromatin formation. In future work, we will clarify the mechanism by which dATF-2 regulates heterochromatin formation.

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,

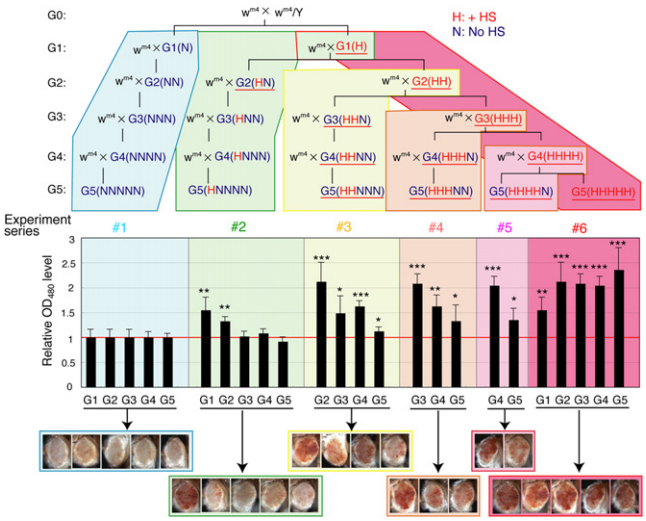

Figure 7. Multigenerational Transmission of Disrupted Heterochromatin Induced by HS

Male w^{m4} flies and female w^{m4} flies carrying two w^{m4} genes were mated (G0). In series #2 experiments, the embryos generated were exposed to HS (37°C for 1 hr) from 0 to 3 hr AEL, and the eye pigment levels in the adult males were measured [G1(H)]. G1(H) males were then mated with unstressed w^{m4} females, and eye pigment levels in adult male progeny [G2(HN)] were measured. Similar matings were repeated until the G5 generation, the eye pigment levels in the progeny [G3(HNN), G4(HNNN), and G5(HNNNN)] at each generation were measured, and the data are presented in bar graphs. In series #3 experiments, G1 and G2 embryos were exposed to HS, and in series #4 experiments, G1, G2, and G3 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 ± SD are shown (n = 6). ***p < 0.001; **p < 0.01; *p < 0.05. See also Figure S7 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, at the larval stage, 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 hetrochromatic 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^{1118} (wild-type), w^{1118}; *PBac{PB}dATF-2*^{c06467} (*dATF-2*^{PB} for short) (Thibault et al., 2004), *In(1)w*^{m4} (*w*^{m4} for short), *In(3L)BL1*, *spn-E*^{1}, *aub*^{QC42}, *p38a*^{1}, *dMekk1*^{Ur36}, *UAS-MEKK1*, *UAS-Flag-dATF2*, and *P{dATF-2}*. For normalization of the genetic background, *w*^{m4} and *dATF-2*^{PB} were backcrossed six times with w^{1118}.

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-heat-shocked 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-HP1 (C1A9, 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-Flp system, while GAL4-UAS-mediated overexpression clones were generated with the Flp-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 (C1A9, 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 (amino 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 proteinase inhibitors). Samples were centrifuged to separate the unbound chromatin (supernatant) from the chromatin fraction (pellet). The pelleted 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 (ADL67.10, 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 devitellinized with methanol, and then stained with anti-dATF-2 (raised against full-length dATF-2) (Sano et al., 2005) and anti-lamin (ADL67.10, DSHB) via standard techniques. Images were obtained with a confocal microscope. For calculation of the amount of dATF-2 foci on heterochromatin, image stacks (Z 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 transgenrational 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*^{m4} 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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