Drosophila melanogaster dHCF Interacts with both PcG and TrxG Epigenetic Regulators

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Abstract

Repression and activation of gene transcription involves multiprotein complexes that modify chromatin structure. The integration of these complexes at regulatory sites can be assisted by co-factors that link them to DNA-bound transcriptional regulators. In humans, one such co-factor is the herpes simplex virus host-cell factor 1 (HCF-1), which is implicated in both activation and repression of transcription. We show here that disruption of the gene encoding the Drosophila melanogaster homolog of HCF-1, dHCF, leads to a pleiotropic phenotype involving lethality, sterility, small size, apoptosis, and morphological defects. In Drosophila, repressed and activated transcriptional states of cell fate-determining genes are maintained throughout development by Polycomb Group (PcG) and Trithorax Group (TrxG) genes, respectively. dHCF mutant flies display morphological phenotypes typical of TrxG mutants and dHCF interacts genetically with both PcG and TrxG genes. Thus, dHCF inactivation enhances the mutant phenotypes of the Pc PcG as well as brm and mor TrxG genes, suggesting that dHCF possesses Enhancer of TrxG and PcG (ETP) properties. Additionally, dHCF interacts with the previously established ETP gene skd. These pleiotropic phenotypes are consistent with broad roles for dHCF in both activation and repression of transcription during fly development.

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Introduction

Much of the early foundations of our understanding of genetic and epigenetic regulation of animal development originates from studies of the fruit fly Drosophila melanogaster. These studies revealed groups of genes with shared developmental functions. Two such well-known groups are the Polycomb group (PcG) and the Trithorax group (TrxG) whose members are generally involved in long-term maintenance of expression patterns of cell fate-determining genes, such as homeotic genes, during fly development (reviewed in [1]). PcG and TrxG proteins act primarily by controlling chromatin states through their incorporation into protein complexes possessing chromatin-modifying enzymatic activities. Consistent with the central role that PcG and TrxG proteins play during development, their function and corresponding protein complexes have been well conserved during evolution (reviewed in [2]).

In Drosophila, PcG-related protein complexes are associated with repression of gene transcription by mechanisms that include (i) direct modification of histones, (ii) recruitment of histone variants, and (iii) regulating ATP-dependent chromatin remodeling ([3] and reviewed in [4]). In contrast, TrxG-related protein complexes, while using similar mechanisms, generally support active gene transcription (reviewed in [2]). Genetically linking PcG and TrxG gene activities is a less well-characterized class of genes called Enhancer of Trithorax and Polycomb (ETP) that may act as cofactors of specific PcG and/or TrxG complexes in the activation and repression of subsets of cell-type and developmental stage-specific genes (reviewed in [1] and [5]). Here, we present a genetic analysis of the Drosophila homolog of the gene encoding the human herpes simplex virus (HSV) host-cell factor-1 (HCF-1) protein and show that it enhances phenotypes associated with PcG and TrxG mutants, thus displaying ETP properties.

Human HCF-1 is associated with the activation and repression of gene expression (reviewed in [6,7,8]). It possesses known enzymatic nor DNA-binding activities, but rather is brought to specific promoters by association with DNA-sequence-specific transcription factors such as Sp1, GABP, YY1, Ronin/THAP11, and E2F1 and E2F4 [8,9,10,11,12,13]. In turn, HCF-1 associates with and promotes the recruitment of chromatin-modifying activities such as Set1/Ash2 [14] and Mixed Lineage Leukemia (MLL)/Ash2 [15] Trx-related histone methyltransferases, MOF acetyltransferase [16] and Sin3A histone deacetylase [14]. HCF-1 appears to integrate DNA-sequence-specific transcription factors with specific combinations of chromatin modifying activities to both activate and repress transcription (see [8]).

Properties of HCF-1 have been highly conserved amongst animals. For example, the Drosophila homologue, dHCF, shares (i) a Kelch domain often responsible for transcription factor interaction, (ii) regions biased for basic (Basic) or acidic (Acidic) amino acids, (iii) fibronectin type 3 repeats, and (iv) a nuclear localization signal [17,18]. In addition, although by different enzymes – O-GlcNAc transferase and taspase1, respectively [19,20] – both HCF-1 and dHCF proteins undergo a process of proteolytic maturation to produce a heterodimeric complex of HCFN and HCFC subunits [17]. The conservation between
human and Drosophila homologues goes beyond a structural similarity because both proteins have been shown to interact with common transcription factors [6,17], and chromatin modifiers [14,21]. This conservation between human and Drosophila HCF proteins as well as the rich genetic resources for studying epigenetic regulation afforded by the fly, led us to study the function of the dHCF gene in Drosophila.

**Results**

To study fly dHCF function, we undertook a multifaceted investigation of the Drosophila dHCF gene involving analyses of (i) dHCF expression, (ii) dHCF genetic disruption, and (iii) genetic dHCF interaction with known epigenetic regulators. The structures of the dHCF gene and encoded protein are shown in Figure 1A.

**Drosophila dHCF is broadly expressed throughout of development**

Figure 1B shows an immunoblot analysis of the dHCFN subunit at different embryo (lane 1) and larval (lanes 2-6) stages of wild-type flies. The dHCFN and dHCFc subunits (Fig. S1) were present at all stages, including adult (data not shown). Furthermore, immunostaining of embryos (Fig. 1D), imaginal discs (Fig. 1E and F) and ovaries (Fig. 1G) also revealed broad dHCF expression, with the dHCF protein localizing in the nucleus (see Fig. 1D insert for an example). The robust specificity of the affinity purified dHCFN antibody for dHCF protein in immunofluorescence is shown in Figure S2. The broad pattern of dHCF expression suggests that the dHCF protein can have important roles throughout development. Pre-synaptic embryos and developing egg chambers (Fig. 1G) contain extensive levels of dHCF, which suggests that the protein and/or mRNA are maternally contributed to the embryo.

**Genetic disruption of dHCF by homologous recombination**

The Drosophila dHCF gene is located on the highly heterochromatic and relatively poorly studied chromosome 4. Because there were no described dHCF-mutant alleles, we used ends-out homologous recombination [22] to generate the dHCFHR1 knock-out allele (see Fig. S3), in which dHCF promoter sequences and exons 1 through 7 were replaced with the mini-white gene (Fig. 1A, bottom). Precise replacement was verified by PCR and sequence analysis (data not shown) and Southern blot analyses (Fig. S3C). Consistent with disruption of the dHCF gene, transcription of dHCF mRNA was not detected in homozygous dHCFHR1 third-instar larvae, whereas the neighboring PMCA gene was apparently unaffected (Fig. 1C, compare lane 3 with lane 1). Suggesting maternal contribution of the dHCF protein or mRNA, analysis of dHCF subunit levels (Fig. 1B, lanes 7–12 and Fig. S1) in homozygous dHCFHR1 offspring revealed a gradual loss of both dHCF subunits over the course of embryogenesis and larval development. Consistent with this extinction, larval imaginal discs stained negatively with dHCF antibodies in immunofluorescence assays (Fig. 1H). We used the dHCFHR1 knock-out allele for the remainder of this study by generating dHCFHR1 homozygous mutant individuals from dHCFHR1/P[Act-GFP]yAc-135 or dHCFHR1/+/yAc parents. Because dHCFHR1 flies were extensively backcrossed onto Df(1)w67c23, yAc flies, we used the latter as wild-type controls which are referred to as such.

**Loss of dHCF function results in lethality and sterility**

Homozygous dHCFHR1 animals survived until the pupal stage, where they exhibited an approximately 50% lethality, indicating that maternally derived dHCF reservoirs became limiting during pupal development. Of those surviving to adulthood, 30% of males and 100% of females were sterile.

Homozygous dHCFHR1 females, while sterile, did lay some eggs, which were often fragile, smaller, and possessed misshapen anterior termini as well as dorsal appendages, as illustrated in Figure 2A. Consistent with this female sterility phenotype, the ovaries of homozygous dHCFHR1 females were generally smaller and underdeveloped. The expressivity of this phenotype was variable and Figure 2 B and C illustrate an extreme example of this defect. We stained these ovaries with DAPI to examine the egg chambers in more detail and observed the punctuated pattern indicative of egg chamber degeneration around stage 8 of oogenesis, as illustrated in Figure 2D and E, suggesting that dHCF is essential for proper oogenesis.

**dHCF disruption leads to decreased size**

In addition to lethality and sterility, we also observed that homozygous dHCFHR1 pupae and adults were consistently smaller than wild-type. Figure 3 shows side-by-side images of wild-type (Fig. 3A) and homozygous dHCFHR1 adult males (Fig. 3B) and, similarly, wings (Fig. 3D and E) and male pupae (Fig. 3F). To quantify the size differences we compared pupal volume (Fig. 3G) and wing size (Fig. 3H). dHCFHR1 homozygous pupae and adult wings were on average 30% and 28% smaller than wild-type, respectively.

We also analyzed the small-size wing phenotype using UAS-dHCF-RNAi transgenic flies [23]. When dHCF was broadly down-regulated in tub-GAL4/UAS-dHCF-RNAi flies, the dHCFHR1 wing-size defect was fully phenocopied (Fig. 3I, left), as were other dHCFHR1 phenotypes described below (e.g., loss of humeral bristles, extra wing vein) that were not quantified. We targeted dHCF knockdown to the posterior wing compartment in en-GAL4/UAS-dHCF-RNAi flies and to the anterior wing compartment in c-GAL4/UAS-dHCF-RNAi flies, and measured the size of each wing compartment separately. As shown in Figure 3I (middle and right), we observed a reduction in size of the corresponding compartment with little effect on the other.

To demonstrate the specificity of these growth phenotypes on the loss of dHCF function, we generated and characterized wild-type and dHCFHR1 flies with a full-length dHCF transgene (UAS-Fl-dHCF) under the control of either actin-GAL4 or tubulin-GAL4. We found that Act-GAL4/UAS-Fl-dHCF; dHCFHR1 flies grown at 18°C were of normal body (Fig. 3C) and wing (Fig. 3H) size. This 18°C phenotypic rescue suggests that the size defect observed in homozygous dHCFHR1 flies occurs as a consequence of loss of dHCF function. Enhanced expression of the dHCF transgene in Act-GAL4/UAS-Fl-dHCF or Act-GAL4/UAS-Fl-dHCF; dHCFHR1 animals at 25°C resulted in male lethality and developmental abnormalities, including unextended or misshapen wings and absence of movement and oviposition in females. Additionally, overexpression of the dHCF transgene in tub-GAL4/UAS-Fl-dHCF or tub-GAL4/UAS-Fl-dHCF; dHCFHR1 animals at either 18°C or 25°C resulted in a developmental delay and larval lethality, suggesting that ubiquitous dHCF over-expression can be disruptive to fly development (data not shown).

**dHCFHR1 wings display reduced cell size and corresponding imaginal discs display increased apoptosis**

The wing-size reduction of homozygous dHCFHR1 flies could result from a reduction in cell size and/or in cell number. To determine the cause, we used the density of trichomes as a measure of wing-cell size in wild-type and dHCFHR1 mutant wings. In the wings of homozygous dHCFHR1 flies the trichome density increased by 18% compared to wild-type, indicating that the
mutant wing cells themselves are approximately 15% smaller than wild-type as shown in Figure 3J. This change in cell size does not account for the overall 30% decrease in mutant wing size, suggesting that the cell number is also affected.

We considered two possible explanations for a decrease in cell number in dHCFHR1 wings: a reduction in cell proliferation or an increase in programmed cell death or apoptosis. To identify potential defects in cell proliferation, we performed FACS analysis on dissociated and propidium iodide-stained cells from wing discs of wild-type and homozygous dHCFHR1 larvae. No differences were observed (data not shown). In addition, in situ measurement of S-phase cells by BrdU incorporation and M-phase cells by histone H3 phosphoserine 10 immunolabeling did not reveal significant changes between wild-type and homozygous dHCFHR1 larvae (data not shown). In contrast, we did observe increased levels of apoptosis as indicated by an increase in the number of cells staining positively for activated caspase 3 as shown in Figure 3K and L. Acridine orange incorporation in live wing discs yielded similar results (data not shown). These results suggest that, in fly-wing development, the absence of dHCF protein does not
The **dHCFHR1** allele interacts with mutant alleles of the *brm* and *mor* TrxG genes

Having observed TrxG phenotypes in homozygous **dHCFHR1** flies, we asked whether the **dHCFHR1** allele might display interactions with mutant TrxG alleles. We generated flies homozygous for **dHCFHR1** and heterozygous for mutant alleles of four TrxG genes: *brm* and *mor*, which encode components of the SWI/SNF BRM complex, and *tre* and *Ash1*, which encode histone H3 methyltransferases (reviewed in [2]). Although we did not observe modification of TrxG homoetic phenotypes (data not shown), *brm*/+, **dHCFHR1**; *brm*/+; **dHCFHR1** and *mor*/+; **dHCFHR1** flies often presented grossly misshapen metathoracic and, to a lesser extent, mesothoracic legs (see Figure 5A-C and Table 1). In addition, the leg position was shifted to a more dorsal position. Importantly, this phenotype was not observed in either one of **dHCFHR1**; *brm*+/+, *brm*/+, or *mor*/+ flies (Table 1). This result shows that the **dHCF** gene not only displays TrxG phenotypes when mutated but that it also interacts with TrxG genes encoding for the SWI/SNF nucleosome remodeling complex BRM.

The **dHCFHR1** allele interacts with mutant PcG alleles

Because TrxG mutants are frequently defined by their ability to suppress PcG phenotypes, we examined whether the **dHCFHR1** mutation could suppress mutant phenotypes of the PcG founder gene. *Pc* mutants can display a variety of homeotic transformations including the appearance of sex combs in meta and mesothoracic legs in males, and, mostly in females, transformation of antenna-to-leg [29], abdominal segment A4 to A5 transformation, and reduction of the sternopleural region, which reflects a mesothoracic segment (T2) to prothoracic segment (T1) transformation [30] (see Figure 5D and E). Neither *Pc*+/+; **dHCFHR1** nor *Pc*+/+; **dHCFHR1** flies showed suppression or enhancement of extra sex combs or transformation of antenna to leg compared to *Pc*+/+ and *Pc*+/+ respectively (data not shown). Contrary to expectation, for sternopleural transformation, the **dHCFHR1** allele did not suppress the *Pc* phenotypes but instead the phenotype was observed at a much higher frequency in *Pc*+/+; **dHCFHR1** and *Pc*+/+; **dHCFHR1** flies than in either *Pc*+/+ and *Pc*+/+ flies, as quantified in Table 1. Figure 5 shows lateral views of sternopleurals of a wild-type fly (Fig. 5D) as well as representative examples of *Pc*+/+ (Fig. 5E) and *Pc*+/+; **dHCFHR1** (Fig. 5F) flies displaying a sternopleural transformation. To illustrate the T2 to T1 sternopleural transformation more clearly, we prepared scanning electron microscopy (SEM) high resolution images of wild-type and transformed *Pc*+/+; **dHCFHR1** sternopleurals, are shown in Figure 5G and H. These images reveal the reduction in size of the sternopleura (see region between broken lines). In addition, these *Pc*+/+; **dHCFHR1** flies also displayed defects in the first thoracic spiracle, which is a phenotype characteristic of double *Pc* and *Autocorpus* (Aut) (Autocorpus) mutants [31] (compare the first thoracic spiracle marked by the star in wild-type Fig. 5G and mutant Fig. 5H flies). Thus, the **dHCFHR1** allele, while displaying TrxG phenotypes, can unexpectedly also enhance rather than suppress a PcG phenotype. Thus, the **dHCF** gene displays both TrxG and PcG characteristics, a phenotype shared with the ETP group of genes.

We complemented the aforementioned study with an analysis of the effects of PcG mutants on **dHCFHR1** phenotypes. For this analysis, we selected mutants of subunits of the PcG complexes PRC1 (*Pc* alleles *Pc* and *Pc*3) and PRC2 (*Enhancer of zeste* allele *E(z) su301*). These mutations in the heterozygous state with **dHCFHR1** did not modify the penetrance and/or expressivity of the **dHCFHR1** adult size or humeral bristle phenotypes. The *Pc* and *Pc* alleles also did not modify the penetrance and/or expressivity of the sex comb size phenotype (data not shown). In contrast, as shown in Table 1, the *Pc* and *Pc* alleles suppressed the extra vein phenotype whereas *E(z) su301* had no effect. We also noted that approximately 3 to 4% of **dHCFHR1** males carrying the *Pc* or *Pc* alleles significantly affect cell proliferation but does lead to increased apoptosis, which could contribute to the reduced size of **dHCFHR1** wings.

**dHCF**-loss-of-function flies display TrxG-like and other developmental phenotypes

In addition to viability, sterility and size defects, homozygous **dHCFHR1** flies exhibited a series of phenotypes that are hallmarks for loss of TrxG function [24,25,26] including the homoeotic phenotypes loss of humeral bristles (Fig. 4A and B), and sex-comb-tooth reduction phenotype loss of humeral bristles (Fig. 4A and B), and sex-comb-tooth reduction. The sex-comb-tooth reduction was from an average of 11.1 teeth in wild-type males to an average of 8.9 teeth in homozygous **dHCFHR1** males (Fig. 4C and D). As shown in Figure 4G, the penetrance of these phenotypes was incomplete. These results indicate that the pleiotropic TrxG-like and developmental phenotypes observed in **dHCFHR1** flies result from the loss of **dHCF** function.

**Figure 2. dHCF is essential for proper oogenesis.** (A) Eggs laid by wild-type and homozygous **dHCF** females. (B–C) Ovaries dissected from 4 day old (B) wild-type and (C) homozygous **dHCF** female flies. (D–E) DAPI staining of fixed egg chambers from ovaries of 4 day old (D) wild-type and (E) homozygous **dHCF** female flies.

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alleles had defects in genitalia rotation (Table 1). In summary, there is a complex interaction with PcG mutations, in particular Pc itself.

The dHCFHR1 allele interacts with mutant alleles of the skuld ETP gene

We also examined the interaction between the dHCFHR1 allele and mutant alleles of the ETP group genes skuld (skd), Trithorax like (Trl) and Additional sex combs (Asx). The skd gene product is a component of an accessory subcomplex of the Mediator [32] and the Trl gene product is the GAGA DNA-binding transcription factor [33,34] and the Asx gene product interacts with the histone deubiquitinase Calyspo to form the PcG complex PR-DUB. No interaction was observed between dHCF and Trl while a small percentage of AsxXp23/+; dHCFHR1 flies had genitalia rotation defects (see Table 1). As shown in Figure 5, skd2/+; dHCFHR1 flies had ectopic bristles in the scutellum (Fig. 5I and J and Table 1) and genitalia rotation defects in males (Fig. 5K and L and Table 1), phenotypes that were not present in the single mutants. In addition, skd2/+; dHCFHR1 flies showed increased expressivity of the wing-vein phenotype observed in homozygous dHCFHR1 flies, as the extra-vein is considerably longer compared to dHCF single mutants (see Fig. 5M and N). These phenotypes suggest that the dHCF and skd / Asx genes, together, are important in suppressing vein and bristle formation as well as in genitalia development.

Discussion

We have studied the role of dHCF in Drosophila development. By generating a targeted loss-of-function allele of the dHCF gene, we show that dHCF is an essential gene that when mutated has a pleiotropic phenotype, suggesting that dHCF is involved in a range of developmental pathways. The major phenotypes discerned include (i) female sterility, (ii) small size, and (iii) morphogenic
**dHCF is an essential gene in Drosophila**

Prior to this study, the only organism in which the HCF gene has been genetically disrupted, also by deletion, is in the worm *C. elegans*, where it is called *hcf-1* [36,37]. The *hcf-1* mutant worms are viable at normal temperatures although they display fertility defects, cold-sensitive lethality, and increased longevity. The sterility and lethality phenotypes are more evident in the *Drosophila dHCF* mutant, perhaps owing to a more complex developmental program in this organism. In both worms [36,37,38,39] and flies (this study), however, the *dHCF* mutant phenotypes are pleiotropic, suggesting that in both organisms HCF proteins are involved in a multitude of different developmental pathways. Together, these genetic results suggest that certain aspects of the developmental role of HCF proteins are shared, whereas others differ amongst species. Thus, as a developmental regulator, HCF proteins have probably assumed different gene regulatory roles during evolution.

**HCF proteins can contribute to growth using diverse mechanisms**

Organism size is the product of cell growth, proliferation, and death. In human cells, HCF-1 has been implicated in cell proliferation and programmed cell death or apoptosis. For cell proliferation, it promotes the G1–S transition by association with and activation of the transcription factor E2F1 [8]. HCF-1 promotes apoptosis also via its interaction with E2F2 when the latter is disregulated [40]. In *Drosophila*, dHCF is also known to associate with E2F1 as well as the repressive E2F2 protein [8]. We have not detected, however, cell-cycle defects in imaginal discs lacking dHCF and loss of dHCF promotes apoptosis rather than inhibit apoptosis as in human cells [40]. Thus, HCF proteins indeed appear to possess different regulatory roles in humans and flies. As in *Drosophila* dE2F2 inhibits p53-independent radiation-induced apoptosis [41], perhaps dHCF inhibits apoptosis via dE2F2 as opposed to promote apoptosis in humans via E2F1. Whichever the case, these observations suggest that HCF proteins are implicated in different aspects of cell growth, proliferation and death in different species.

*Drosophila* dHCF apparently plays a role in cell-size determination as *dHCF* mutant flies show a reduction in cell size. Recently, Furrer et al. [42] have reported that the oncoprotein Myc and dHCF interact and that this interaction is important for the transcriptional activation and growth promoting functions of Myc. Indeed, Myc is a transcriptional factor that plays an important role in growth regulation [43]. The reduced cell size phenotype of *dHCF* mutant flies may be due to impaired function of Myc in the absence of dHCF proteins.

**dHCF interacts with TrxG, PcG, and ETP genes, positive and negative regulators of gene expression**

The genetic analysis of *dHCF* loss of function presented here reveals pleiotropic collaboration with (i) the TrxG *bmy* and *mor* genes for proper morphogenetic regulation of leg development, (ii)
the PcG Ptc gene for proper specification of thoracic segment identity, and (iii) the ETP skd gene for bristle, vein and genitalia development. Consistent with these genetic interactions, dHCF can physically associate with the epigenetic TrxG protein Ash2 [21]. These findings in Drosophila are paralleled in human cells where HCF-1 associates with both TrxG – Set1 and MLL [human
Table 1. Genetic interaction between dHCF and TrxG, PcG and ETP genes.

| Phenotype                                      | Genotype             | Penetration | n   |
|------------------------------------------------|----------------------|-------------|-----|
| Leg defects (1)                                | dHCF	extsuperscript{FR1} | 0%          | 50  |
|                                                | brm	extsuperscript{1}+/+ | 0%          | 50  |
|                                                | brm	extsuperscript{1}+/dHCF	extsuperscript{FR1} | 20%         | 50  |
|                                                | brm	extsuperscript{20}+/+ | 0%          | 50  |
|                                                | brm	extsuperscript{20}+/dHCF	extsuperscript{FR1} | 10%         | 50  |
|                                                | mor	extsuperscript{1}+/+ | 0%          | 50  |
|                                                | mor	extsuperscript{1}+/dHCF	extsuperscript{FR1} | 10%         | 50  |
| T2 to T1 transformation (2)                    | dHCF	extsuperscript{FR1} | 0%          | 64  |
|                                                | Pc	extsuperscript{1}+/+ | 3%          | 72  |
|                                                | Pc	extsuperscript{1}+/dHCF	extsuperscript{FR1} | 60%         | 65  |
|                                                | Pc	extsuperscript{2}+/+ | 3%          | 68  |
|                                                | Pc	extsuperscript{2}+/dHCF	extsuperscript{FR1} | 40%         | 52  |
| Genitalia rotation defects (2)                 | dHCF	extsuperscript{FR1}/dHCF	extsuperscript{FR1} | 0%          | 100 |
|                                                | Pc	extsuperscript{1}+/+ | 100%        |     |
|                                                | Pc	extsuperscript{2}+/+ | 100%        |     |
|                                                | Pc	extsuperscript{2}+/dHCF	extsuperscript{FR1} | 4%          | 104 |
|                                                | Axa	extsuperscript{F23}+/+ | 0%         | 100 |
|                                                | Axa	extsuperscript{F23}+/dHCF	extsuperscript{FR1} | 2%          | 92  |
|                                                | skd	extsuperscript{1}+/+ | 0%          | 100 |
|                                                | skd	extsuperscript{1}+/dHCF	extsuperscript{FR1} | 30%         | 50  |
| Ectopic vein (2)                               | dHCF	extsuperscript{FR1} | 50%         | 64  |
|                                                | Pc	extsuperscript{1}+/+ | 72%         |     |
|                                                | Pc	extsuperscript{1}+/dHCF	extsuperscript{FR1} | 5%          | 65  |
|                                                | Pc	extsuperscript{2}+/+ | 68%         |     |
|                                                | Pc	extsuperscript{2}+/dHCF	extsuperscript{FR1} | 0%          | 52  |
| Extra scutellar bristles (2)                   | dHCF	extsuperscript{FR1} | 0%          | 50  |
|                                                | skd	extsuperscript{1}+/+ | 0%          | 50  |
|                                                | skd	extsuperscript{1}+/dHCF	extsuperscript{FR1} | 30%         | 50  |

(1) This phenotype was quantified at 18°C and both eclosed and pharate adults were included. An observed increased mortality at the pharate state of dHCF	extsuperscript{FR1} mutant combinations was likely due to leg defects.

(2) dHCF	extsuperscript{FR1} mutant combinations displayed lethality phenotypes similar to dHCF	extsuperscript{FR1} mutants.

Trx) histone H3 lysine 4 methyltransferase complexes [14,15] – and PcG – BAP-1 deubiquitinase (human Calypso) [3,11,44,45] and YY1 (human Pho) [11] – related factors. Thus, in both human and Drosophila, HCF proteins apparently interact with transcriptional regulators possessing roles in both activated and repressed states of gene expression.

The products of the identified dHCF collaborating genes exist in the cell in the form of multiprotein complexes – Brm and Mor are part of the BRM SWI/SNF complex [46,47], Pc is a part of PRC1 [48], and skd [49] is a part of the Mediator – that possess transcriptional regulatory activities not previously associated with HCF proteins: the ATPase dependent chromatin remodeling activity of the BRM complex, the histone ubiquitination activity of PRC1, and the RNA polymerase II interaction activity of Mediator. These results portend more complex roles for HCF-1 in transcriptional regulation than currently appreciated and support the notion that HCF proteins are versatile integrators of gene regulatory information.

Materials and Methods

Fly stocks

Fly stocks were kept in standard corn/yeast media at 25°C unless otherwise indicated. The following alleles are described in flybase.org and available through the Bloomington Stock Center: Df(1)wu67c23, y^2 (utilized as wild-type throughout this study), c^O and P/UasGFP

P unc-13C (these two alleles were utilized throughout this study to maintain dHCF	extsuperscript{FR1} stocks and distinguish homozygotes from heterozygotes), tub-GAL4, en-GAL4, brm	extsuperscript{1}, Act-GAL4, brm	extsuperscript{20}, mor	extsuperscript{1}, Pc	extsuperscript{1}, Pc	extsuperscript{2}, skd	extsuperscript{1}, E(zg) nsa	extsuperscript{1}, Asx	extsuperscript{F23}, UAS-dHCF-RNai transgenic flies (46998 and 46999) were obtained from the Vienna Drosophila RNAi Center. Stocks carrying the UAS-dHCF RNAi transgene on chromosome 2 and 3 were used in RNAi experiments. ci-GAL4 flies were obtained from R.A. Holmgren's laboratory [50]. The generation of dHCF	extsuperscript{FR1} and of UAS-Fl-dHCF alleles is described in Materials and Methods S1. For RNAi experiments, UAS-dHCF-RNai; UAS-dHCF-RNai flies were crossed with the GAL4 carryover stocks to generate GAL4>UAS-dHCF-RNai flies. For the rescue experiments UAS-Fl-dHCF; dHCF	extsuperscript{FR1}/c^O flies were crossed with Act-GAL4 / CyO; dHCF	extsuperscript{FR1}/c^O flies to generate Act-GAL4>UAS-Fl-dHCF; dHCF	extsuperscript{FR1} flies.

Protein extracts for immunoblotting (IB)

Protein extracts were prepared by cold homogenization of embryos or larvae in protein extraction buffer (50 mM TrisHCl pH 8, 150 mM NaCl, 1 mM EDTA, 4 mM EGTA, 0.1% SDS, 0.1% Triton X-100, 2X complete protease inhibitor cocktail (Roche)), followed by 10 min incubation on ice. Protein extracts were boiled for 5 min. in 1X Laemmli buffer, separated in Tris-Glycine acrylamide gels, transferred onto nitrocellulose Hybond membranes and probed with antibodies using the LI-COR system. Membranes were scanned with an Odyssey infrared imager (LI-COR).

RNA extraction and Reverse Transcriptase (RT) PCR

RNA from third instar larvae was extracted using Trizol (Invitrogen) according to manufacturer specifications. cDNA was prepared by reverse transcription using the ImProm-IITM Reverse Transcription System (Promega) following manufacturer specifications. In addition, a negative control sample for genomic DNA amplification in the subsequent PCR reaction was prepared following the same protocol in the absence of reverse transcriptase. PCR amplification of cDNA or negative control sample was performed using the following primers for dHCF 5’-gattatatggtg-3’ and 5’-ctgcaacaggaaggtcagc-3’ and for PMCA 5’-aagggctcaggtcagcatt-3’ and 5’-aacatcgggagctctgca-3’. PCR-amplified DNA fragments were resolved in agarose gels and visualized with ethidium bromide.

Immunofluorescence (IF)

Embryos were dechorionated and fixed using standard methods [51]. Third instar wandering larvae were dissected in PBS to expose imaginal discs and fixed in (1:3) 8% paraformaldehyde: Brower buffer (0.15 M PIPES, 3 mM Mg SO4 1.5 mM EDTA, 1.5% NP-40 pH6.9) for 4h to overnight at 4°C. Ovaries were dissected from 3 day old well fed females in PBS and fixed in 3.7% formaldehyde in PBST for 20 min. Fixed tissue was washed in PBST (PBS-0.1% Tween 20) and rocked in blocking buffer (PBST with 1%BSA and 1% normal rabbit serum) for 1 h at RT to overnight at 4°C. Blocked tissue was incubated with primary antibodies in blocking buffer overnight at 4°C, washed in PBST, incubated with fluorescent secondary antibodies in blocking buffer for 1 hour at RT and washed in PBST before
being mounted in VECTASHIELD® mounting medium with DAPI (Vectorlabs) to counterstain DNA. Images were taken with a microscope Leica DM6000B or a microscope Zeiss AXIO Vert 200 M with a Zeiss LSM 510 Meta confocal system.

**Scanning electron microscopy (SEM)**

Three day old female flies were prepared for SEM by dehydryation through increasing concentrations of ethanol at room temperature, and then dried with critical point evaporation of liquid CO2 (Balzers CPD 030). The samples were then attached with carbon cement to aluminum stubs and then metal coated with a 5–10 nm gold and palladium layer using high vacuum evaporation (Cressington Scientific). The samples were then imaged at 20 keV in a field emission SEM (FEI Company, XLF30-PEG).

**Viability assays**

Embryos were collected and let hatch on fruit juice-agar plates and first instar larvae counted and transferred to fruit juice-agar plates with standard corn/yeast media to quantify larval viability or to standard corn/yeast media tubes to quantify pupal and adult viability. Viability of a given developmental phase was calculated as the ratio between the number the individuals exiting and the number of individuals entering that phase.

**Fertility assays**

Two day old virgin females (n = 40) or males (n = 40) of the appropriate genotype were individually mated with two wild-type males or virgin females, respectively. After two days, eggs were counted daily for a three day period. Individuals were considered sterile when they laid, in total, less than 5% of the number of eggs laid by wild-type females mated with wild-type males.

**Wing, wing cell and pupal size quantification**

Wings of male flies were dissected, mounted in Euparal and photographed in a microscope Leica DM6000B. Pictures taken with the 5X objective were used to determine the area using the Image J software. The fourth vein was used as border between the posterior and anterior compartments. Pictures of a specific area in the wing were taken with the 40X objective and used to determine manually using Image J the trichome number per surface unit or cell density. The relative cell size was calculated as the reverse of the cell density. Pupal volume was determined as in Layalle et al. using the formula $\frac{4}{3}\pi(L/2)(L/2)^2$ (L, length; l, diameter) [52].

**Antibodies**

The following primary antibodies were used: rabbit anti-dHCF$_N$ (affinity purified, IB 1:500, IF 1:50) [19], mouse anti-β-tubulin (Clone B-5-1-2, Sigma, IB 1:5000), mouse anti-activated-caspase 3 (Cell Signaling, IF 1:50), Alexa 680- and IRDye 800-conjugated secondary antibodies (Molecular Probes and Rockland Immunocchemicals, IB 1:10,000–20,000, Alexa 488- and Alexa 543-conjugated secondary antibodies (Molecular Probes, IF 1:500).

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**Supporting Information**

**Figure S1** Analysis of the dHCF$_C$ subunit during development in wild-type and dHCF$_{PR1}$ mutants. Protein extracts from wild-type and homozygous dHCF$_{PR1}$ embryos and larvae (indicated in hours after egg laying) were analyzed by immunoblotting with anti-dHCF$_C$ antibodies. The same blots were incubated with β-tubulin antibodies to control for protein loading. Star, non-specific band of unknown origin. (PDF)

**Figure S2** Specificity of RNAi inactivation of dHCF and of the dHCF$_N$ antibody for immunofluorescence. Wing imaginal disc of α-GAL4/ UAS-dHCF-RNai; UAS-dHCF-RNai / UAS-GFP third instar wandering larvae. (A) Immunostaining with dHCF$_N$ antibodies. (B) GFP fluorescence. (C) DAPI staining. Note specific loss of dHCF$_N$ immunofluorescence in GFP-positive cells. (PDF)

**Figure S3** dHCF gene deletion by ends out homologous recombination. (A) Schematic drawing illustrating the dHCF- gene structure. Represented are exons (white and black boxes), coding sequence (black boxes) and the main transcription initiation site (arrow). Restriction sites and probes used in the Southern blot analysis shown in (C) are shown under the line. Bx-BstX I, S-Sal I, N-Nde I, B-BamH I. (B) Targeting vector and structure of the dHCF genomic region after homologous recombination. Numbers indicate the position of the sequence with respect to the dHCF transcription-initiation site. Dotted boxes represent regions of identity between the targeting vector and dHCF gene locus. (C) Southern blot analysis of dHCF$_{PR1}$ recombinant flies: genomic DNA from wild-type and heterozygous dHCF$_{PR1}$/+ males was digested with the indicated enzymes and detected by Southern blotting using the indicated probes. (PDF)

**Materials and Methods S1** Supporting materials and methods. (DOC)

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**Author Contributions**

Conceived and designed the experiments: SRJ AB WH. Performed the experiments: SRJ. Analyzed the data: SRJ AB. Contributed reagents/materials/analysis tools: AB WH. Wrote the paper: SRJ WH. Reviewed the manuscript: AB.

**Supplementary References**

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