The Drosophila Huntington’s disease gene ortholog dhtt influences chromatin regulation during development

Kevin N. Dietz1, Luisa Di Stefano2, Robert C. Maher3, Hui Zhu1, Marcy E. Macdonald1, James F. Gusella1 and James A. Walker1,*

1Center for Human Genetic Research, Massachusetts General Hospital and Harvard Medical School, 185 Cambridge St., Boston, MA 02114, USA, 2Laboratoire de Biologie Cellulaire et Moléculaire du Contrôle de la Prolifération, UMR 5088, Université de Toulouse and Centre National de la Recherche Scientifique, 31062 Toulouse, France and 3Center for Cancer Research, Massachusetts General Hospital, Building 149, 13th Street, Charlestown, MA 02129, USA

Received July 7, 2014; Revised and Accepted August 26, 2014

Huntington’s disease is an autosomal dominant neurodegenerative disorder caused by a CAG expansion mutation in HTT, the gene encoding huntingtin. Evidence from both human genotype–phenotype relationships and mouse model systems suggests that the mutation acts by dysregulating some normal activity of huntingtin. Recent work in the mouse has revealed a role for huntingtin in epigenetic regulation during development. Here, we examine the role of the Drosophila huntingtin ortholog (dhtt) in chromatin regulation in the development of the fly. Although null dhtt mutants display no overt phenotype, we found that dhtt acts as a suppressor of position-effect variegation (PEV), suggesting that it influences chromatin organization. We demonstrate that dhtt affects heterochromatin spreading in a PEV model by modulating histone H3K9 methylation levels at the heterochromatin–euchromatin boundary. To gain mechanistic insights into how dhtt influences chromatin function, we conducted a candidate genetic screen using RNAi lines targeting known PEV modifier genes. We found that dhtt modifies phenotypes caused by knockdown of a number of key epigenetic regulators, including chromatin-associated proteins, histone demethylases (HDMs) and methyltransferases. Notably, dhtt strongly modifies phenotypes resulting from loss of the HDM dLsd1, in both the ovary and wing, and we demonstrate that dhtt appears to act as a facilitator of dLsd1 function in regulating global histone H3K4 methylation levels. These findings suggest that a fundamental aspect of huntingtin function in heterochromatin/euchromatin organization is evolutionarily conserved across phyla.

INTRODUCTION

Huntington’s disease (HD) is a dominantly inherited neurodegenerative disorder characterized by motor, cognitive and behavioral symptoms. It is caused by expansion of a polymorphic CAG trinucleotide repeat (to >35 CAGs) in the first exon of HTT. The length of the CAG repeat, which encodes a polyglutamine (polyQ) tract in the huntingtin protein, determines the rate of the disease process that leads to clinical diagnosis (1). Genetic studies support a fully dominant gain of function that may trigger the disease process through the structure, localization or activity of the full-length huntingtin protein (2).

Huntingtin is a large protein (>3000 amino acids) that is highly conserved in vertebrates, with discrete orthologs in lower animals, such as Drosophila and Dictyostelium, but not in yeast or plants. It is characterized by multiple HEAT (and HEAT-like) repeats (for Huntingtin, Elongation factor 3, the A subunit of protein phosphatase 2A, and TOR1) (3,4) that confer a continuous alpha-solenoid structure (5) (Vijayvargia and Seong, unpublished data) that is consistent with a role in scaffolding members of large complexes (5).

In contrast to vertebrates, where knockout of the murine and zebrafish HTT orthologs resulted in abnormal development and embryonic lethality (6–10), huntingtin is dispensable for...
viability in the lower organisms studied so far. A deficiency of the HTT ortholog in the slime mold Dictyostelium results in defects in actin cytoskeleton, chemotaxis, osmotic regulation and multicellular development (11). Drosophila lacking huntingtin develops without obvious defects; the only reported phenotypes in adult flies deficient in huntingtin are a reduced climbing ability in aged adults, slight reduction in longevity (12) and defects in mitotic spindle orientation in neuroblast precursors, similar to those seen in mice (13).

There is mounting evidence suggesting that aberrant post-translational modifications (PTMs) may be involved in HD pathogenesis (14). Alterations in histone acetylation in HD have been well documented and huntingtin has been found in various complexes containing histone deacetylases (HDACs) (15). Recently, huntingtin has been shown to play a role in epigenetic regulation during mouse development by facilitating the function of Polycomb group (Pc-G) protein repressive complex 2 (PRC2) (5), which catalyzes histone H3 lysine 27 (H3K27) trimethylation (16–18). Mouse huntingtin physically associates with two of the core PRC2 components, Ezh2 and Suz12 (5), the histone methyltransferase (HMTs) and nucleosome-binding protein, respectively (19). Through its interaction with these proteins, full-length huntingtin stimulates the trimethyltransferase activity of PRC2, leading to a global increase in histone H3K27 trimethylation levels in the formation of embryonic bodies and, in this way, may play a role in developmental heterochromatin formation and gene silencing (5). Another histone methylation mark, H3K4me3, which is found at transcriptional start sites, has recently been shown to undergo changes in both mouse HD models and HD patients (20).

We have capitalized on the power and flexibility of genetic approaches in Drosophila to test the hypothesis that huntingtin functions in regulating chromatin activity. Using a novel dhtt allele generated by homologous recombination, we have uncovered subtle phenotypes indicating that huntingtin influences chromatin during fly development. First, we found that huntingtin suppresses position-effect variegation (PEV) in Drosophila, suggesting that it alters the structure at heterochromatin/euchromatin boundaries. We then identified novel interactions with genes involved in chromatin function through a genetic screen to test whether dhtt enhances or suppresses phenotypes caused by shRNA knockdown of genes known to influence PEV. Notably, dhtt modifies defects caused by knockdown of genes encoding several histone demethylases (HDMs; dLsd1, lid, JHDM2 and Jarid2) and HMTs (gpp and esc). Finally, we determined that dhtt collaborates with dLsd1 to facilitate demethylation of histone H3K4 and, like dLsd1 mutants, dhtt affects heterochromatin spreading. Taken together, our results indicate that huntingtin regulates chromatin during Drosophila development by influencing histone methylation.

RESULTS
Huntingtin is well conserved across different Drosophila species
In contrast to the sea urchin and chordate huntingtins, Drosophila huntingtin (dhtt) does not contain a polyglutamine or polyproline repeat in the corresponding region within the amino terminus of the protein (4,21). Although dhtt appears to be dispensable for fly development and survival in the laboratory, an analysis of the genomes of 12 Drosophila species sequenced to date show that it is highly conserved, suggesting that huntingtin is essential for maintaining biological fitness, at least in the natural environment (12,22). Comparison of Drosophila huntingtin amino acid sequences shows conservation following the phylogenetic tree (Supplementary Material, Fig. S1) with the N- and C-terminal portions of huntingtin being most highly conserved among the 12 Drosophila species.

Generation of a new dhtt allele by homologous recombination
The previously reported dhtt knockout allele (dhtt<sup>ko</sup>) involved deletion of dhtt together with the adjacent gene, CG9990, which encodes an ABC transporter (12). Since deletion of CG9990 results in embryonic lethality, it was necessary to introduce a genomic rescue construct encoding this gene in order to examine the phenotype of dhtt<sup>ko</sup> homozygous flies (12). To generate a clean null allele of dhtt, we used homologous recombination in an ends-in strategy (23), with a targeting construct designed to result in duplication of a dhtt region beginning within exon 2 and ending in exon 8. An intervening insertion of a mini-white marker enabled screening by eye color for candidate lines having undergone successful homologous recombination. Characterization of the single line obtained showed that, instead of the expected duplication and insertion, one portion of the region to be duplicated suffered complete deletion of exons 3 and 4 and partial deletion of exon 5 (Fig. 1A). Analysis using qRT-PCR showed dramatically reduced levels of any dhtt transcription (Fig. 1B) and western blot analysis using anti-d htt (exons 18–20) serum failed to detect any full-length protein in embryo extracts from flies homozygous for the insertion (Fig. 1C and Supplementary Material, Fig. S2). This line, referred to henceforth as dhtt<sup>mo</sup>, represents a novel mutant allele of dhtt. Like the previously described dhtt<sup>ko</sup> line (d htt deletion with transgenic replacement of CG9990), d htt<sup>mo</sup> homozygous flies are viable and fertile with no obvious developmental defects. However, in contrast to the reported d htt<sup>ko</sup> line, we found no decrease in longevity when compared with wild-type flies (w<sup>1118</sup>, Fig. 1D).

Drosophila huntingtin is a suppressor of PEV
To investigate whether huntingtin has a role in the establishment or maintenance of chromatin during Drosophila development, we examined whether dhtt affects PEV. PEV is a mosaic or variegated phenotype that results from rearrangements that place euchromatic genes immediately adjacent to heterochromatic regions (24,25). The variegated phenotype results from the inactivation of genes by heterochromatin spreading from the breakpoint in some cells, but not in others and is exquisitely sensitive to the dosage of genetic modifiers. Many genetic modifiers of PEV [denoted Su(var) and E(var) mutations] in Drosophila have been described; molecular characterization has revealed these to include mutants of heterochromatin-associated proteins and enzymes involved in PTMs of histones (24,25). To test whether huntingtin plays a role in the balance between euchromatin and heterochromatin in flies, we introduced the dhtt<sup>mo</sup> mutation into two models with class I PEV rearrangements, which
cause aberrant expression of the endogenous genes Stubble (Sb) and yellow (y).

In the T(2;3)Stubble \textsuperscript{variegated} (Sbv) variegating system, the T(2;3)Sbv \textsuperscript{translocation juxtaposes the Sb mutation and the centric heterochromatin of the second chromosome, resulting in mosaic flies with both Sb and normal bristles (Fig. 2A). Activation of the dominant Sb allele results in short, stubble bristles. When T(2;3)Sbv was crossed to dhtt\textsuperscript{int} flies, we observed a significant increase in the frequency of Sb bristles (Fig. 2B and C). A deficiency uncovering dhtt (dhtt\textsuperscript{A}), as well as the previously described dhtt\textsuperscript{D} allele, gave similar results indicating that reduced dosage of dhtt results in suppression of variegation. Null alleles of the HDMs, dLsd1 and lid, respectively, provided suppressor and enhancer controls of Sb PEV ([26–29]; Fig. 2C).

The yellow (y) gene is required for dark pigmentation of the adult body cuticle, wing blades, thoracic and wing bristles, and other ectodermal structures (30). In flies bearing the inversion In(1)y\textsuperscript{3P}yellow, a sensitive measure of y expression in individual cells is provided by the triple row bristles, located on the anterior margin of the wing (Fig. 2D). We found that dhtt acts a dominant suppressor of PEV at the yellow locus, giving a 15% reduction in yellow bristles (Fig. 2E and F). A similar level of dominant
suppression was also noted with the dhttko allele and a null allele of dLsd1, as has been reported previously [(28); Fig. 2F].

**Drosophila huntingtin affects heterochromatin spreading in a PEV model**

Another commonly used PEV model is In(1)wm4, in which the white gene is relocated to X heterochromatin by a paracentric inversion (Fig. 3A). This inversion results in a salt-and-pepper mottling phenotype of the eye pigment, which is readily affected by PEV modifiers (25,31). For technical reasons, we were unable to assess the effect of dhtt in this PEV model; the insertion element in the dhttint mutant contains a mini-white gene, giving a uniform wild-type eye appearance that would obscure any visible modification of wm4 variegation. However, we were able to perform chromatin immunoprecipitation (ChIP) analysis to examine the spreading of the heterochromatic mark histone H3K9me2 along the locus of the In(1)wm4 inversion. Histone H3K9me2 is very abundant at heterochromatin loci and it has been postulated that variable spreading of this mark along the wm4 rearrangement influences the expression of the genes located in this inverted region (27). As reported
previously, reducing the dosage of dLsd1 resulted in a sharp decrease in the level of histone H3K9me2 at the white locus and at the adjacent CG12498 gene, as well as in the heterochromatic rDNA loci (26,27). Interestingly, dhtt depletion produced a similar decrease, suggesting that dhtt, like dLsd1, acts as a suppressor of variegation by modulating histone H3K9 methylation levels at euchromatin–heterochromatin boundaries (Fig. 3B).

Taken together, the results from the T(2;3)Sbv, In(1)wm4 and In(1)y3P models indicate that dhtt is a haplo-suppressor of PEV that is involved in the normal maintenance of chromatin structure in the developing fly.

A candidate genetic screen for dhtt modification of chromatin-modifying genes

We postulated that, as a suppressor of PEV, dhtt collaborates with chromatin-associated complex components to affect chromatin structure. To explore this possibility, we used UAS-shRNA targeting to test whether dhtt genetically interacts with previously reported suppressors or enhancers of PEV (25) (Supplementary Material, Table S1). We also included in our screen a small collection of RNAi lines targeting genes encoding components of complexes involved in chromatin regulation even though they have not been demonstrated to affect PEV, such as the PRC1, PRC2 and SIN3 complexes. The 152 RNAi lines targeting known PEV modifiers and components of chromatin-modifying complexes were first crossed to Gal4 driver lines to knockdown expression either ubiquitously (Act5C-Gal4), or more specifically in the developing wing (en-Gal4 and nub-Gal4) or eye (ey-Gal4 and GMR-Gal4), revealing a number of strong, highly penetrant phenotypes (Supplementary Material, Table S1, and Figs S5 and S6). In many instances, the ubiquitous Act5C-Gal4 driver resulted in lethality, while in other cases viable progeny displayed phenotypes recapitulating defects associated previously with loss-of-function alleles of these genes (Supplementary Material, Table S1).

Next, for the RNAi lines that resulted in clear, scorable phenotypes in the wings and eyes of adult flies (105 lines, totaling 82 unique genes), we examined whether these phenotypes could be dominantly modified by the dhtt<sup>int</sup> allele or using

Figure 3. Loss of dhtt influences spreading of H3K9me2 in the heterochromatic boundary of the w<sup>md</sup> inversion. (A) Schematic representation of the X chromosome in wild-type and w<sup>md</sup> flies. (B) dhtt<sup>int</sup> and dLsd1<sup>DN</sup> mutations result in a reduction of H3K9me2 levels at the white locus. ChIP was performed with antibodies recognizing H3K9me2 in wild-type, dhtt<sup>int</sup> and dLsd1<sup>DN</sup> mutant males. Target loci along the w<sup>md</sup> region and at the RpL32 locus were analyzed, and the values are presented as the percentages of the input signal for each primer pair. Error bars represent standard deviations. Total H3 levels (control) are shown in Supplementary Material, Figure S3.
RNAi knockdown of dhtt. Since dhtt is expressed ubiquitously (12,32) and affects PEV in both wing and bristles (Fig. 2), we predicted that dhtt functions in a wide variety of tissues. The majority of RNAi phenotypes examined were completely unaffected by loss of one copy of dhtt or by RNAi targeting of dhtt, arguing against the loss of dhtt having a general affect on RNA interference (Supplementary Material, Table S3). RNAi knockdown of dhtt identified several additional potential interactions that were not observed with a single dhtt int allele, presumably because RNAi resulted in almost complete loss of dhtt protein (Fig. 7A). We prioritized hits that interacted with both the dhtt int allele and dhtt RNAi for further analysis (Table 1).

Genetic interactions between dhtt and genes encoding heterochromatin proteins

We found interactions between dhtt and two members of the heterochromatin protein 1 (HP1) family, which encode non-histone chromosomal proteins that are important regulators of heterochromatin-mediated gene silencing and chromosome structure (33). These proteins each consist of an N-terminal chromodomain (CD), a chromo shadow domain (CSD) and a variable length C-terminal extension. Three of the five members of the Drosophila HP1 family, HP1a (HP1/Su(var)205), HP1b and HP1c, function in somatic nuclei (34). While HP1a is located primarily in heterochromatic domains of the chromocenter, telomeres and the fourth chromosome (35), HP1c is primarily associated with euchromatic domains, and HP1b overlaps with both (36). HP1a acts as a ‘hub protein,’ interacting with a variety of chromosomal proteins through its CSD, in addition to recognizing key histone modification sites (primarily histone H3K9me2/3) through the CD (37). HP1a thereby plays an important role in higher-order chromatin structures, affecting both gene expression and silencing. HP1b has been reported to counteract HP1a function in heterochromatin formation and in the transcriptional regulation of euchromatic genes (38). We found that loss of dhtt suppressed the notched wing phenotypes associated with knockdown of both HP1a and HP1b in the posterior of the wing (Fig. 4F, H, J, N and P) but, in contrast, strongly enhanced the appearance of ectopic vein phenotypes only in wings with HP1b knockdown (Fig. 4L and P). RNAi targeting HP1c in the developing wing resulted in unfurled and held-out wing phenotypes using nub-Gal4 and en-Gal4, respectively. However, no modification with the dhtt int allele was observed.

Table 1. dhtt modification of RNAi-induced phenotypes of genes previously shown to affect PEV

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>RNAi/allele</th>
<th>Modification with dhtt int allele</th>
<th>Modification with dhtt RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine specific demethylase 1/Suppressor of variegation 3-3 dLsd1/Su(var)3-3</td>
<td>Histone demethylase (H3K4)</td>
<td>v25128</td>
<td>ENH (ovary and wing)</td>
<td>ENH (ovary and wing)</td>
</tr>
<tr>
<td>little imaginal discs (lid)</td>
<td>Histone demethylase (H3K4)</td>
<td>dLsd1 int</td>
<td>ENH (wing)</td>
<td>ENH (wing)</td>
</tr>
<tr>
<td>JmjC domain-containing histone demethylase 2 (CG8165/JHDM2)</td>
<td>Histone demethylase (H3K9)</td>
<td>v10380</td>
<td>–</td>
<td>SUP (wing)</td>
</tr>
<tr>
<td>Jumonji, AT rich interactive domain 2 (Jrd2)</td>
<td>Histone demethylase</td>
<td>HM05155</td>
<td>–</td>
<td>SUP (wing)</td>
</tr>
<tr>
<td>grappa (gpp)</td>
<td>Histone demethylase (H3K79)</td>
<td>HMS00775</td>
<td>–</td>
<td>de novo (wing)</td>
</tr>
<tr>
<td>extra sex combs (esc)</td>
<td>PRC2 complex; histone H3K27 methyltransferase</td>
<td>JF02081</td>
<td>–</td>
<td>ENH (wing)</td>
</tr>
<tr>
<td>Su(var)205 Heterochromatin protein 1 (HP1)</td>
<td>Satellite DNA binding</td>
<td>v107477</td>
<td>SUP (wing)</td>
<td>SUP/ENH/de novo (wing)</td>
</tr>
<tr>
<td>Heterochromatin protein 1b (HP1b)</td>
<td>Chromo domain</td>
<td>v109735</td>
<td>ENH (wing)</td>
<td>SUP/ENH (wing)</td>
</tr>
<tr>
<td>brahma (brm)</td>
<td>ATPase/SWI/SNF complex</td>
<td>V37720</td>
<td>ENH (wing)</td>
<td>ENH (wing)</td>
</tr>
<tr>
<td>Enhancer of polycomb (E(Pc))</td>
<td>Unknown</td>
<td>JF03101</td>
<td>SUP (eye)</td>
<td>SUP (eye)</td>
</tr>
<tr>
<td>E(var)-95E (dE2F1)</td>
<td>Transcription factor</td>
<td>GL00169</td>
<td>n/d</td>
<td>SUP (eye)</td>
</tr>
<tr>
<td>Methyltransferase 2 (MT2)</td>
<td>DNA methyltransferase</td>
<td>UAS-E2F1 RNAi</td>
<td>ENH (eye)</td>
<td>ENH (eye)</td>
</tr>
<tr>
<td>Sin3A</td>
<td>Protein heterodimerization activity</td>
<td>v37815</td>
<td>ENH (eye)</td>
<td>ENH (eye)</td>
</tr>
<tr>
<td>Additional sex combs (Asx)</td>
<td>Non-catalytic component of the polycomb repressive deubiquitinase (PR-DUB) complex</td>
<td>HMS00359</td>
<td>SUP (eye and wing)</td>
<td>SUP/ENH (eye and wing)</td>
</tr>
<tr>
<td>Regena (Rga)</td>
<td>Protein-binding</td>
<td>v107274</td>
<td>–</td>
<td>de novo (wing)</td>
</tr>
<tr>
<td>absent, small, or homeotic discs 1 (ash1)</td>
<td>TRX-G—histone H3-K4 methyltransferase</td>
<td>GL00386</td>
<td>ENH (wing)</td>
<td>ENH (wing)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JF01498</td>
<td>ENH (wing)</td>
<td>ENH (wing)</td>
</tr>
</tbody>
</table>

Results from the genetic modifier screen are shown for both heterozygous loss of dhtt (using the dhtt int allele) and dhtt RNAi knockdown (using v29532). Modifications were scored as either enhancement (ENH) or suppression (SUP). In some cases, a de novo phenotype was observed when UAS-shRNA dhtt (v29532) was combined with a UAS-shRNA, which showed no discernible phenotype by itself.
Genetic interactions of \textit{dhtt} with genes encoding chromatin-remodeling complex components

Transcriptional regulation in eukaryotic cells occurs on DNA compacted in nucleosomes and involves ATP-dependent chromatin remodeling by a number of different protein complexes. The \textit{Drosophila} Brahma (Brm) complex is a SWITCH/Sucrose NonFermentable (SWI/SNF)-related chromatin-remodeling complex named for the gene encoding its ATPase subunit (\textit{brahma}; \textit{brm}) (39). We found that loss of \textit{dhtt} enhances \textit{brm} RNAi phenotypes in the wing (Fig. 5A–D). RNAi-induced \textit{brm} phenotypes in the eye were too strong to reliably assess modification. However, loss of \textit{dhtt} due to RNAi enhanced the eye phenotype caused by transgenic expression of the dominant-negative \textit{brm} protein (\textit{UAS-brm\textsubscript{K804R}}, Fig. 5E–H). Despite the strong interaction between \textit{dhtt} and \textit{brm}, we only detected very weak interactions with RNAi lines targeting genes encoding other SWI/SNF complex components, \textit{osa} and \textit{Snr1}, which were included in the screen (Supplementary Material, Table S3).

As part of a central chromatin-remodeling complex, \textit{brm} has been shown to genetically interact with many other epigenetic regulators and chromatin-modifying genes. Several of these were found in our screen, including \textit{Enhancer of Polycomb [E(Pc)]}, the transcription factor, \textit{\textit{dE2F1 [E(var)3-95E]}}, the atypical polycomb protein \textit{Additional sex combs (Asx)} and the HDAC, \textit{Rpd3} (Table 1, Fig. 5 and Supplementary Material, Table S3). Loss of \textit{dhtt} modified phenotypes resulting from RNAi against \textit{E(Pc)} and \textit{dE2F1} in the eye (Fig. 5L–Q).

The \textit{Drosophila} genome contains two \textit{E2F} genes, encoding the activator \textit{dE2F1} and the repressor \textit{dE2F2} proteins (43). Various chromatin regulatory complexes have been linked to the \textit{E2Fs} and the Retinoblastoma-family protein (Rbf1), while histone modifications have been shown to alter \textit{E2F}-dependent transcription (43). Loss of \textit{dhtt} enhanced the GMR\textgreater\textit{E2F1} RNAi rough eye phenotype, using either RNAi or the \textit{dhtt\textsubscript{int}} allele (Fig. 5P and Q).

The acetylation of lysine residues within the N-terminal tail of histones results in a relaxed chromatin structure associated with gene expression.
with transcriptionally active DNA. By altering the accessibility to basal transcription machinery and transcription factors, the dynamic enzymatic regulation of histone acetylation/deacetylation plays an important role in chromatin remodeling, affecting gene expression, cell cycle progression and developmental events (44). Drosophila Rpd3 encodes a class I HDAC homologous to mammalian HDAC1 and HDAC2, which catalyzes the deacetylation of lysine residues on core histones (H2A, H2B, H3 and H4) (45). We found that RNAi knockdown of Rpd3 in the eye using the ey-Gal4 driver resulted in small, rough eyes, and that this phenotype was weakly enhanced by introduction of the dhtt allele or by dhtt RNAi (data not shown; Supplementary Material, Table S3). Rpd3 has been reported to be associated with a number of important epigenetic regulatory complexes, including the PRC2 complex (46), NURD complex (47), DREAM complexes (48) and SIN3 complexes (49). It was therefore interesting to note that we found dhtt to be a potent suppressor of Sin3A RNAi phenotypes in eye and wing (Supplementary Material, Fig. S7). Different isoforms of Sin3A have been shown to partner with numerous histone-modifying enzymes and in this way, Sin3 complexes are thought to serve distinct roles in the regulation of chromatin function (49,50).

Interactions between dhtt and HDMs and HMTs

The methylation of lysine and arginine residues within histone side chains and core domains modulates the epigenetic landscape regulating transcriptional control during embryonic development, genomic imprinting and chromosome X inactivation (51,52). Histone lysine methylation is implicated in both gene activation and repression, depending on the site and state of methylation (mono-, di- or trimethylation). Methylation at histone H3-K4, -K36 and -K79 is typically associated with active transcription, whereas methylation on histone H3-K9, -K27 and histone H4K20 is linked to gene silencing. Taken together, HDMs and HMTs regulate the dynamics of histone methylation. We observed strong genetic interactions between dhtt and several HDMs and HMTs in our genetic screen (Table 1), suggesting that dhtt may play a general role in facilitating histone methylation.

The Jumonji C (JmjC) domain is found in Jumonji HDMs (JHDMs) that employ α-ketoglutarate (α-KG), Fe(II) and molecular oxygen as cofactors to demethylate specific histone mono-, di- and trimethyl lysine residues (53). The mammalian JHDM2 family is comprised of four proteins that function as histone H3K9 or histone H3K36 demethylases (53,54). The Drosophila genome encodes a single homolog of the JHDM2 family, CG8165/JHDM2 (55). We found that combined RNAi knockdown of both CG8165/JHDM2 and dhtt in the wing gave rise to a de novo ectopic vein phenotype (Supplementary Material, Fig. S8A and B).

Jarid2, another JmjC domain-containing protein, is a component of the PRC2, which is involved in implementing histone H3K27 methylation and transcriptional repression during development (56,57). In contrast to other JmjC domain-containing proteins, Jarid2 does not share the conserved residues that are essential for HDM activity and is predicted to be catalytically inactive (53,58). Loss of dhtt enhanced the appearance of ectopic veins at the PCV upon Jarid2 RNAi knockdown in the wing (Supplementary Material, Fig. S8C–F). However, the strongest

Figure 5. dhtt interacts with genes encoding chromatin-remodeling complex components. (A–D) The dhtt allele dominantly enhances the UAS-shRNA brm phenotype in the wing. Male (A and B) and female (C and D) wings are shown. Although dhtt RNAi knockdown has no effect by itself when driven with ey-Gal4 (F), it is able to enhance the rough eye caused by expression of dominant-negative brm from the UAS-brm transgene (H). Similarly, GMR>dhtt RNAi and the dhtt allele have no discernible phenotypes, but are able to suppress and enhance the RNAi phenotypes caused by knockdown of E(Pc) (L–N) and dE2F1 (O–Q), respectively. Full genotypes are described in Supplementary Material.
interaction of dhtt and an HDM uncovered in our screen was with dLsd1, which is discussed in further detail below.

In addition to interactions with HDMs, we also uncovered enhancement of ectopic wing veins caused by RNAi of an HMT, grappa (gpp), which modifies histone H3K79 residues. gpp mutants exhibit phenotypes characteristic of both Pc-G and TRX-G mutants and have been shown to result in disruption of telomeric silencing, but do not affect centric heterochromatin (59). RNAi of dhtt enhanced the disruption of veins in the posterior of wing upon gpp RNAi (Supplementary Material, Fig. S8G–J). Additionally, we found that dhtt interacts with extra sexcombs (esc; Supplementary Material, Fig. S8K–N). This WD repeat-containing protein physically associates with E(z), the histone H3K27 specific HMT in the PRC2 complex. Although esc is critically required for the establishment of PcG-mediated silencing during early embryogenesis, it is dispensable for its subsequent maintenance throughout development (60). We also noted a weak interaction between dhtt and
Loss of dhtt enhances dLsd1 mutant phenotypes

The Drosophila ortholog of lysine-specific demethylase 1, KDM1, also known as LSD1 [dLsd1/Su(var)3-3], is a flavin adenine dinucleotide-containing enzyme that functions as a histone H3K4 demethylase, acting specifically on mono- and dimethyl residues (28). Homozygous loss of dLsd1 has been reported to result in reduced male viability, a held-out wing phenotype, ectopic vein material emanating from the posterior cross vein and defects in oogenesis (26,28). In the dLsd1 loss-of-function mutant, oogenesis is arrested at a very early stage and ovaries consist of germaria that fails to form egg chambers (26). This very strong phenotype makes the identification of putative genetic enhancers very difficult. However, we found that ubiquitous RNAi knockdown of dLsd1 driven by Act5C-Gal4 partially recapitulates the ovary defects of the dLsd1 loss-of-function mutant, giving rise to a milder phenotype (Fig. 6D). The dhtt<sup>mt</sup> allele dominantly enhanced this phenotype (Fig. 6E), an interaction confirmed by RNAi targeting of dhtt driven with Act5C-Gal4. Although Act5C>dhti RNAi produced no ovarian phenotype by itself (Fig. 6C), it resulted in a more severe dLsd1 RNAi oogenesis phenotype (Fig. 6F).

We also found that dLsd1 and dhtt genetically interact in the wing. dLsd1 null flies (dLsd1<sup>ΔN</sup>) or those with dLsd1 RNAi driven in the posterior half of the wing imaginal disc (en-Gal4>UAS-dLsd1 RNAi) have ectopic vein material at the posterior cross vein (PCV; (26); Fig. 6L and K). The severity and penetrance of this phenotype was dominantly enhanced by introduction of either the dhtt<sup>mt</sup> allele or dhti RNAi (Fig. 6J, L, N and P).

Another histone H3K4 demethylase, lid (little imaginal discs), has been shown to antagonize dLsd1 function (26,29). In contrast to the enhancement of dLsd1 phenotypes by loss of huntingtin, dhti RNAi partially suppressed the incomplete PCV phenotype caused by lid knockdown with the nud-Gal4 driver (Fig. 6Q and R).

Drosophila huntingtin facilitates histone H3K4 demethylation activity of dLsd1

Since dhtt genetically interacts with a number of genes encoding chromatin-associated proteins, including HDMs and HMTs, we tested whether loss of dhtt affects global levels of histone modifications. Extracts from adult female flies were probed on date, Mt2 has only been shown to be capable of methylating tRNAs (62–64).

Since we found that dhtt plays a role in heterochromatin silencing by influencing histone H3K9me2 levels at specific loci (Fig. 3), we were surprised that our screen did not uncover interactions with any of the three known H3K9-specific HMTs in Drosophila: Su(var)3-9, Setdb1 or G9a. Su(var)3-9 has been identified as the key HMT in Drosophila acting on histone H3K9 sites to establish heterochromatin gene silencing (65–67). However, our inability to observe an interaction between dhti and Su(var)3-9 may have been confounded for several reasons. Not only do the available Su(var)3-9 RNAi lines induce very strong phenotypes (Supplementary Material, Table S1 and Fig. S5), these lines also are predicted to target both Su(var)3-9 and elf2<γ as these genes share several common exons.
western blots using antibodies against a range of histone methylation and acetylation marks. However, we found no effect of either complete loss of d htt (d htt\(^{null}\) homozygous mutants) or overexpression of transgenic d htt (Supplementary Material, Fig. S9). Since d htt dominantly enhances phenotypes caused by loss of dLsd1 in two different tissues, wing and ovary, we decided to explore the hypothesis that d htt might modify the function of dLsd1 as an HDN. To test this, we examined the global levels of histone H3K4 mono- and di-methylation in adult female flies in which dLsd1 or d htt or both dLsd1 and d htt were knocked down using shRNA with the ubiquitous Act5C-Gal4 driver. As expected, levels of histone H3K4me1 and histone H3K4me2 were elevated in animals depleted of dLsd1 (Fig. 7A). Although a reduction in d htt alone had no obvious effect on global levels of histone H3K4me1 or me2, simultaneous dLsd1 and d htt RNAi knockdown resulted in a significant increase in the mono- and di-methylation status of histone H3K4 over that of dLsd1 knockdown alone (Fig. 7B and C). In contrast, when the same experiment was performed using loss-of-function dLsd1\(^{1 \text{AV}}\) homozygotes, we were unable to detect any further increase in methylation of histone H3K4 by reducing d htt levels (data not shown), suggesting d htt acts as a facilitator of dLsd1 demethylase activity.

**DISCUSSION**

The studies of the inverse relationship between the age at onset of clinical symptoms and the size of the HTT CAG repeat mutation have revealed that the repeat confers on the mutant allele a fully dominant gain-of-function property. However, it is unknown whether this is the acquisition of enhanced normal huntingtin function or the acquisition of a novel opportunistic function, although targeted null and CAG expansion mutations at the mouse homolog provide support for both possibilities (5,68). Despite recent studies, details of the normal function of huntingtin remain relatively elusive, hindering further investigation into the molecular mechanisms underlying the disease (5,13,69–74).

In this study, we used a novel d htt allele to examine the normal function of Drosophila huntingtin, focusing on its potential role in chromatin function during development. Although d htt flies are viable and appear grossly normal, our genetic findings indicate that d htt influences chromatin regulation: (i) d htt is a suppressor of PEV, suggesting that it is involved in heterochromatin formation; (ii) d htt affects heterochromatin spreading in a PEV model; (iii) d htt genetically interacts with a number of genes encoding proteins known to affect chromatin organization and function and (iv) d htt genetically interacts with the HDM dLsd1 and facilitates its ability in demethylating histone H3K4.

PEV is a powerful genetic assay that has been used previously to identify genes that can regulate chromatin structure (24). In PEV models, a chromosomal rearrangement or transposition abnormally juxtaposes a reporter gene with heterochromatin. A variegated phenotype is produced since the gene is stochastically silenced in some of the cells in which it is normally active. The silencing that occurs in PEV is attributed to the ‘spreading’ of heterochromatin along the chromosome into a region that would normally be in a euchromatic form. Thus, since the reporter gene is on the boundary between these two states, PEV provides a sensitive system in which to test genetic modifiers of heterochromatin formation. In this report, we used two independent PEV assays [T(2;3)Sb\(^{P}\) and In(1)\(^{P}\)y3P] to demonstrate that d htt facilitates heterochromatin formation, thereby suppressing variegated phenotypes (Fig. 2). To date, approximately 500 dominant Su(var) and E(var) mutations have been isolated from PEV screens and it is estimated that these affect about 150 unique genes (24). Those that have been molecularly characterized so far have been revealed to generally encode chromosomal proteins or modifiers of chromosomal proteins.

Histone PTMs play essential roles in the transition between active (euchromatin) and inactive (heterochromatin) chromatin states (75). In particular, histone methylation has been widely studied in nearly all model systems and is generally recognized as an epigenetic marker for transcriptionally silent heterochromatin. High levels of methylated histone H3K9me2 are associated with heterochromatin loci (75). Using the established PEV model, \(w^{med}\), we demonstrated that the d htt allele dominantly reduces the level of histone H3K9me2 at the white locus and the adjacent CG12498 gene at the heterochromatin– euchromatin boundary (Fig. 3). This level of histone H3K9me2 reduction was comparable to that caused by a dLsd1/Su(var)3-3 null allele, an established suppressor of variegation (27,28). The loss of d htt therefore significantly influences chromatin structure, thereby shifting the euchromatin–heterochromatin boundary.

Where in the cell might huntingtin function to affect chromatin structure and act as a suppressor of variegation? Although the majority of huntingtin in human and mouse cells have been shown to reside within the cytoplasm, about 5% is estimated to be nuclear (5,76,77). A previous report suggested that Drosophila huntingtin is solely cytoplasmic, but this was based solely on ectopic d htt overexpression (12). Since both fly and mouse loss-of-function huntingtin models show defects in mitotic spindle orientation in neuroblast precursors, it is clear that huntingtin does have a nuclear function (13). However, it is possible that d htt could also influence chromatin structure by acting in the cytoplasm.

Based on our finding that d htt dominantly suppresses PEV and affects chromatin function, we hypothesized that it may genetically interact with previously identified PEV modifiers. Our approach to screening for possible d htt interactors utilized a collection of RNAi lines targeting known suppressors and enhancers of PEV. Such a screen has a number of caveats: first, it relies on RNAi producing a modifiable phenotype in a relevant tissue. Secondly, due to the nature of our screen, we were largely limited to looking for interactions in the adult eye and wing. Interestingly, in some cases, we found interactions between d htt and genes in the wing, but not in eye and vice versa. This may reflect tissue-specific requirements for different genes, or that d htt functions only within certain complexes in certain tissues. Nevertheless, we identified a number of strong genetic interactors of d htt, which included central regulators of chromatin architecture and function, such as the heterochromatin proteins, HP1 and HP1b, brm—the ATPase subunit of the SWI/SNF (Brm) complex, the transcription factor dE2F1 and various HDMs and HMTs (Table 1). We focused our immediate attention on the interaction between d htt and dLsd1 for the following reasons: dLsd1 interacted with both the d htt allele and d htt RNAi and loss of d htt caused enhancement of dLsd1 phenotypes in both wing and ovary. Additionally, we found that d htt...
and \( \text{dLsd1} \) both affected PEV and heterochromatin formation to comparable extents (Figs 2 and 3).

Although \( \text{d htt} \)-deficient flies are fertile and display no obvious ovarian phenotype, loss of \( \text{d htt} \) strongly enhanced the \( \text{dLsd1} \) ovary defects. We hypothesize that \( \text{dLsd1} \) and \( \text{d htt} \) collaborate in the regulation of histone H3K4 methylation at specific loci to control gene expression critical for oogenesis. Similarly, in contrast to \( \text{dLsd1} \) mutant flies which show elevated levels of histone H3K4me1 and H3K4me2 (28), we could not detect any changes in the global levels of these modifications in \( \text{d htt} \)-deficient flies. However, simultaneous knockdown of \( \text{dLsd1} \) and \( \text{d htt} \) resulted in a significant increase in histone H3K4me1 and H3K4me2 levels over that of the \( \text{dLsd1} \) knockdown alone (Fig. 7). Human LSD1 is a component of the CoREST/REST (repressor element silencing transcription factor) complex, which represses the transcription of neuronal genes in non-neuronal cell lineages (78, 79). Within this complex, LSD1 acts to demethylate histone H3K4 residues in nucleosomes at REST target genes, thereby contributing to their transcriptional repression (80–82). Mammalian full-length huntingtin has been shown to physically interact with this complex and contribute to its regulation (70), and it will therefore be interesting to determine whether \( \text{dLsd1} \) and \( \text{d htt} \) similarly associate with each other. Unfortunately due to the lack of phenotype upon knocking down the \( \text{Drosophila} \) ortholog of CoREST, \( \text{dCoREST} \), with the available RNAi lines, we were unable to test for a potential interaction with \( \text{d htt} \) in our screen. The genetic interaction between \( \text{d htt} \) and \( \text{dLsd1} \) could potentially account for the strong effect of \( \text{d htt} \) seen on H3K9 methylation at the heterochromatin/euchromatin boundary in the \( \text{w}^\text{med} \) PEV model. \( \text{dLsd1} \) has been shown to physically associate with Su(var)3-9 and to control Su(var)3-9-dependent spreading of histone H3K9 methylation along euchromatin (27).

There is considerable evidence suggesting a link between aberrant acetylation and methylation marks and HD (14, 15, 83–86). Mouse \( \text{Htt} \) has been implicated in facilitating the trimethylation of histone H3K27 in developing murine embryoid bodies (5). Recently, the levels of histone H3K4me3 have been shown to change at dysregulated promoters in a mouse HD model (R6/2) and human HD postmortem brain tissue (20). Our screen uncovered interactions between \( \text{d htt} \) and \( \text{Drosophila} \) HDM and HMTs with a variety of different histone H3 specificities (H3K4, H3K27, H3K9 and H3K79). It is therefore possible that \( \text{d htt} \) has a general role, possibly as a scaffold protein, in facilitating a number of complexes containing histone-modifying enzymes with different specificities. Since mammalian full-length huntingtin has been implicated in the trimethylation of histone H3K27 by facilitating PRC2 function (5), we were surprised that the histone H3K27 methyltransferase, \( \text{esc} \), was the only component of PRC2 found to interact with \( \text{d htt} \) in our screen. Although we detected no effect of the \( \text{d htt} \) null mutation on the global levels of histone H3K27me levels, it is possible that \( \text{d htt} \) may play a similar role to mouse \( \text{Htt} \) in modulating histone H3K27me during development, with histone H3K27me differences only observed at specific loci.

A number of the \( \text{d htt} \) interacting genes found in our screen encode important chromatin regulating proteins that have previously been found to genetically interact with each other. For example, we detected a strong interaction between \( \text{d htt} \) and \( \text{brm} \), the central subunit of the Brm SWI/SNF complex. \( \text{brm} \) is known to interact with \( \text{E(Pc)} \), \( \text{dE2F1} \), \( \text{Asx} \) and \( \text{Rpd3} \), which were also found in our screen. The SIN3 corepressor complex is a class I HDAC complex conserved from \( \text{Drosophila} \) to humans and regulates gene transcription through deacetylation of nucleosomes. Loss of \( \text{d htt} \) suppresses both eye and wing phenotypes caused by \( \text{Sin3A} \) RNAi (Supplementary Material, Fig. S7). \( \text{Drosophila Sin3A} \) has been shown to interact with the HDAC \( \text{Rpd3} \) and the HDM, \( \text{lid} \)—both of which were also scored as hits in our screen. Furthermore, mammalian \( \text{Sin3A} \) was previously reported as a huntingtin N-terminal yeast two-hybrid interactor (87).

\( \text{Drosophila} \) has proved to be a useful model to investigate polyglutamine-fragment toxicity (88, 89). Expression of an N-terminal fragment with an expanded polyglutamine tract in the fly has been shown to accumulate in the nucleus (90). It would therefore be interesting to evaluate whether the normal chromatin regulatory functions of \( \text{d htt} \) are perturbed in the fly polyglutamine-fragment models. Although our \( \text{Drosophila} \) screen was designed to look initially for phenotypes in visible external structures of the adult fly (wing and eye), many of the genes that we found to interact with \( \text{d htt} \) are known to also be expressed and function in the developing nervous system. It is therefore possible that \( \text{d htt} \) may also exert a role in regulating chromatin function during neurogenesis and neural function in the fly, leading to subtle behavioral mutant phenotypes that have been described previously (12). The current study establishes \( \text{Drosophila} \) as a system in which to investigate the normal role of \( \text{d htt} \) in chromatin regulation. It will be particularly useful in examining \( \text{d htt} \) functions that are evolutionarily conserved as these provide assays with which to determine the impact of the expanded polyglutamine region on full-length huntingtin function, thereby deepening our understanding of the mechanism that initiates the HD disease process.

**MATERIALS AND METHODS**

**Fly strains and culturing**

The following fly stocks were obtained from Bloomington Stock Center: \( T(2;3) \text{Sh}/\text{TM3} \#(878), \text{In}(1)\text{y}^{10P}; \text{Su(var)3-9} /\text{Me1} \#(4246) \), \( \text{P}[w^{+}; mC] = \text{Act5C-GAL4}\{25FO1\}\text{CyO} \#(4414) \), \( \text{P}[w^{+}; mC] = \text{GAL4-cy.HI}3-8 \#(5534) \), \( \text{P}[w^{+}; mC] = \text{GAL4-ninaE.GMR}12 \#(1104) \), \( \text{P}[\text{nub.PK}] = \text{nub-GAL4.K6} \#(42699) \). The \( \text{d htt}^{60A} \) allele was kindly provided by Dr Sheng Zhang. The \( \text{P}[\text{XP}d01701] \) and \( \text{P}[\text{PBac}\{\text{WH}\}]05417 \) lines were obtained from the Exelixis Collection at Harvard Medical School. Transgenic RNAi lines were obtained from the Vienna \( \text{Drosophila} \) Research Center (VDR) and the TRiP Collection at Harvard Medical School. The \( \text{UAS-brm}^{K0047} \) transgene was kindly provided by Dr A. Dingwall. \( \text{w}^{1118} \) was used as a wild-type control in all experiments. Flies were cultured on standard cornmeal, molasses \( \text{Drosophila} \) medium and maintained at 25°C unless otherwise stated.

**Generation and analysis of the \( \text{d htt}^{60A} \) and \( \text{d htt}^{A} \) lines**

Ends-in homologous recombination was carried out according to Rong and Golic (23). The following primers were used to generate a construct for homologous recombination consisting of a
9.1 kb genomic fragment from exon 2 to exon 8 of dhtt, flanked with KpnI and NotI sites for subcloning:

FOR: CGTATAGGTACGCATTCTTATGGATCTGTATGG
REV: CGTATAGCCGCGCAGCTAAGAAAGGATTAGCTAA

The underlined sequences represent nucleotides 3873–3995 and 12 929–12 904 corresponding to the dhtt reference sequence AF147779. The dhtt locus at the integration site of the resulting dhtt<sup>tm1</sup> line was sequenced. qRT-PCR analysis was performed using total RNA extracted from w<sup>1118</sup> and dhtt<sup>tm1</sup> homozygous adult flies, with random priming to produce first-strand cDNA which was subsequently DNAse-treated to remove any traces of genomic DNA. Primer pairs used were as follows:

Exons 1–2 FOR: TTAAGGACGCGAAAAATGGAC
Exons 1–2 REV: AAATCCACATCCTCGCAGAAC;
Exons 3–4 FOR: TCAAGTGGTATGCCGTGAGA,
Exons 3–4 REV: ATTGCGTGCATTCTCAATCA;
Exons 12–13 FOR: TTAAGGACGCGAAAATGGAC
Exons 12–13 REV: CGGACCGGTTTGAGAGAG

The deletion line dhtt<sup>x</sup> was generated using the Exelixis transposons d01701 and f05417 using the protocol of Parks et al. (2004) (91). This resulted in deletion of CG9990 and most of the dhtt gene.

Transgenic flies

RT-PCR was performed using cDNA generated by random priming of total RNA from Drosophila embryos. Several overlapping cDNA clones were generated across the entire length of the dhtt coding region and unique restriction sites were used to construct a full-length clone. We found the cDNA encoding the C-terminus of dhtt to be highly unstable, with frequent deletions occurring in this region using standard culturing conditions. To facilitate subcloning we included a genomic fragment, which includes intron 10 to produce a dhtt ‘mini-gene.’ Additionally, the use of XL-10 Gold Escherichia coli and incubating liquid LB plates and plates at 25°C with lower concentrations of ampicillin (25 mg/ml) reduced the frequency of deletions during subcloning steps. The dhtt mini-gene was finally subcloned into the pUAST vector with a single in-frame myc-tag added at the C-terminus. Transgenic flies were balanced and expression of dhtt was assessed using western blots.

Longevity

Adult flies were maintained on normal fly media in batches of 25 per vial at 25°C. For each genotype, 10 vials were used which were changed every 2–3 days and the number of dead flies counted.

PEV assays

To test for PEV of the Stubble (Sh<sup>v</sup>) translocation, T(2;3)Sh<sup>v</sup>/TM3 virgin females were crossed to male flies of the genotype being examined. The ratio of stubble and non-stubble bristles of the relevant progeny was determined by scoring the six bristles on the thorax, as shown in Figure 2A and B. Males and females were scored separately, but produced the same effect.

In each case, at least 50 flies of each genotype were counted. For the yellow variegation, In(1)Sh<sup>BP</sup>/FM7c females were crossed to males of different genotypes being tested. The relevant progeny were scored for the number of y<sup>+</sup> and y<sup>−</sup> hairs on the anterior edge of the wing. At least 12 flies of each genotype were assessed.

Screen for dhtt genetic interactors

RNAI lines targeting candidate genes were obtained from the TRiP or VDRC collections and initially screened for visible phenotypes by crossing to wing (en- and nubbin-) and eye (ey- and GMR-) Gal4 drivers at 25°C. The severity and/or penetrance of the phenotypes varied considerably depending on the gender of the parent from which the Gal4 line was derived. Therefore, in all subsequent tests, virgin females of the UAS-shRNA lines were crossed to male Gal4 flies. Stocks were generated for each Gal4 driver in both dhtt<sup>int</sup> (testers: Act5C-Gal4/CyO; dhtt<sup>int</sup>, en-Gal4; dhtt<sup>int</sup>, nub-Gal4; dhtt<sup>int</sup>, ey-Gal4; dhtt<sup>int</sup> and GMR-Gal4; dhtt<sup>int</sup>) and wild-type backgrounds (controls: Act5C-Gal4/CyO; +, en-Gal4; +, nub-Gal4; +, ey-Gal4; +, GMR-Gal4; +). Stocks for testing the effect of dhtt RNAI were generated using a VDRC RNAI line (v29532): en-Gal4; v29532/TM3, nub-Gal4; v29532/TM3, ey-Gal4; v29532/TM3 and GMR-Gal4; v29532/TM3. Controls consisted of Gal4 drivers alone. Crossing schemes are described in Supplementary Material, Figure S2 and in each case progeny was scored for modification of RNAI phenotypes in the respective tissue, compared with the relevant control. For RNAI lines that gave very strong phenotypes, the incubation temperature was reduced to 18°C to produce a more moderate effect suitable for screening.

Antibodies and western blot analysis

Rabbit polyclonal anti-dhtt sera (#3526) were raised against a bacterially expressed maltose-binding protein (MBP) fusion of amino acids 2458–2570 of the dhtt protein (encoded within exons 18–20) and immunizations were carried out by Invitrogen. Affinity purification of sera was performed using MBP-dhtt coupled with CNBr-activated Sepharose 4B beads (GE Healthcare). Lysates of embryos (0–12 h) and whole adult flies for western blot analysis were prepared using RIPA buffer supplemented with protease inhibitors (Sigma). For huntingtin blots, embryo lysates were resolved on NuPAGE Novex 4–12% gradient Bis-Tris gels with MOPS-SDS running buffer (Invitrogen); whole adult lysates were resolved on NuPAGE 3–8% gradient Tris-Acetate gels with Tris-Acetate running buffer. To examine histone modifications using western blot analysis, histones were prepared by acid extraction (as below) and separated using NuPAGE 12% Bis-Tris gels and MOPS-SDS running buffer. In each case, after transfer to polyvinylidene difluoride (PVDF) membranes, 5% non-fat powdered milk or 5% BSA in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) was used for blocking. Antibodies used in this study were: affinity purified dhtt (3526; 1 : 4000), H3 (Santa Cruz Biotechnology, sc-10809 or Abcam, ab1791, both at 1 : 20 000), H3K4me1 (ab8895; 1 : 5000), H3K4me2 (ab103938; 1 : 10 000), H3K27me3 (Millipore, ABE44; 1 : 2000), H3K9me2 (ab1220; 1 : 1000), H3K9me3 (ab8898; 1 : 2000); H3K9ac,
Acid extraction of histones

Adult flies were homogenized in TEB buffer (Trition Extraction Buffer: PBS, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02% NaN3) at a protein concentration of 200 µg/ml and centrifuged (10,000 rpm) in an Eppendorf microfuge for 1 min at 4°C. Pellets were resuspended in three volumes of acid extraction buffer (0.5 M HCl, 10% glycerol), incubated on ice for 30 min and centrifuged at 12,000 rpm for 5 min at 4°C. Eight volumes of acetic acid were added to the supernatant and incubated overnight at −20°C. Following centrifugation at 12,000 rpm for 5 min, pellets were air-dried prior to suspension in distilled water. Histone concentrations were determined by Bradford assay and confirmed by western blot analysis with an antibody specific for histone H3.

Chromatin immunoprecipitation

Flies were resuspended in Buffer A [60 mM KCl, 15 mM NaCl, 4 mM MgCl2, 15 mM HEPES (pH 7.6), 0.5% Triton X-100, 0.5 mM DTT, protease inhibitors] and fixed in 1% formaldehyde for 15 min at room temperature. Samples were washed three times in Buffer A, resuspended in lysis buffer (140 mM NaCl, 15 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 0.1% SDS and 0.5% N-lauroylsarcosine) and lysed by sonication. The lysates were cleared by centrifugation, preabsorbed by incubation with protein G and A sepharose beads (GE Healthcare) and incubated overnight at 4°C with 1 µg of anti-H3 (AB1791) or anti-H3K9me2 (AB1220). Antibody complexes were recovered with a mixture of protein A- and G-sepharose beads. After extensive washes, immunocomplexes were eluted from the beads and cross-link-reversed, and the DNA was recovered by phenol/ chloroform extraction and ethanol precipitation. DNA was resuspended in 150 µl of water and 7.5 µl was used for real-time qPCR.

Immunostaining of ovaries

Ovaries were dissected and fixed in 4% paraformaldehyde in PBS and stained overnight with anti-Fasciclin III (7G10, Developmental Studies Hybridoma Bank). DNA was stained using DAPI (Molecular Probes), and ovaries were mounted for confocal microscopic imaging.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Genetic Services, Inc., Cambridge for transgenic fly injections; Sheng Zhang, Nick Dyson and Andy Dingwall for fly stocks. We thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) and the Vienna Drosophila RNAi Center for providing transgenic RNAi fly stocks used in this study.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Huntington’s Disease Society of America (grant NS16367) and CHDI Foundation, Inc. LDS was funded by the Fondation pour la Recherche Médicale (FRM).

REFERENCES


