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The *Drosophila* Ortholog of *MLL3* and *MLL4*, *trithorax related*, Functions as a Negative Regulator of Tissue Growth

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The human MLL genes (*MLL1* to *MLL4*) and their *Drosophila* orthologs, *trithorax* (*trx*) and *trithorax related* (*trr*), encode proteins capable of methylating histone H3 on lysine 4. MLL1 and MLL2 are most similar to *trx*, while MLL3 and MLL4 are more closely related to *trr*. Several MLL genes are mutated in human cancers, but how these proteins regulate cell proliferation is not known. Here we show that *trr* mutant cells have a growth advantage over their wild-type neighbors and display changes in the levels of multiple proteins that regulate growth and cell division, including Notch, Capicua, and cyclin B. *trr* mutant clones display markedly reduced levels of H3K4 monomethylation without obvious changes in the levels of H3K4 di- and trimethylation. The *trr* mutant phenotype resembles that of *Utx*, which encodes a H3K27 demethylase, consistent with the observation that Trr and Utx are found in the same protein complex. In contrast to the overgrowth displayed by *trr* mutant tissue, *trx* clones are underrepresented, express low levels of the antiapoptotic protein Diap1, and exhibit only modest changes in global levels of H3K4 methylation. Thus, in *Drosophila* eye imaginal discs, Trr, likely functioning together with Utx, restricts tissue growth. In contrast, Trx appears to promote cell survival.

In multicellular organisms, tissue growth and cell proliferation are regulated by diffusible growth factors and by cell-cell interactions. Extracellular signals eventually regulate gene expression by modulating the activity of proteins that bind to specific DNA sequences and influence the recruitment of the transcriptional machinery to specific promoters. A second layer of regulation is provided by the simultaneous recruitment of proteins that influence chromatin structure and thereby determine the accessibility of DNA for transcription (1, 2). While transcription factors have been studied intensively in the context of growth control, the role of chromatin-modifying proteins is less well understood.

Studies in *Drosophila* led to the discovery of two of the main classes of genes that regulate gene expression by influencing chromatin structure, namely, the trithorax group (TrG) and polycomb group (PcG) (3, 4). While specific mutations in TrG and PcG genes were initially found to alter the expression of Hox genes during development, it is now known that both PcG and TrG genes can have more widespread effects on gene expression. Both classes include members that encode proteins capable of regulating the methylation status of specific lysine residues on histone H3 (reviewed in references 4, 5, and 6). Activity of PcG proteins is associated with the methylation of lysine 27 on histone H3 (H3K27), typically a marker of genes that are repressed. In contrast, some members of the TrG are capable of methylating lysine 4 (H3K4), a modification often found in transcriptionally active genes.

The *Drosophila* genome encodes at least three genes encoding H3K4 methyl transferases, *Set1* (7), *trithorax* (*trx*) (8–10), and *trithorax related* (*trr*) (11, 12). The catalytic portion of these proteins is the Su(var)3-9/Enhancer of zeste/Trithorax (SET) domain. The Set1, Trx, and Trr proteins are each incorporated into a multiprotein complex that has similarities to the COMPASS (complex of proteins associated with Set1) complex found in yeast (13). Recent studies (7, 13, 14) suggested that these three COMPASS-like complexes differ in their effects on global levels of H3K4 methylation. The Set1-containing complex appears to account for the majority of trimethylation of H3K4. Trx appears to account for very little H3K4 methylation in cell culture or in imaginal discs. Conflicting results have been reported for Trr; using Western blotting, two studies have reported decreased levels of H3K4 trimethylation in mutant embryos and prepupae (12, 15) whereas a recent study of knockdown in S2 cells showed that the main effect was a decrease in H3K4 monomethylation (7).

Our knowledge of the biological function of these *Drosophila* genes and their mammalian orthologs is extremely limited. *Drosophila trx* and its mammalian orthologs *MLL1* and *MLL2* appear to regulate the expression of homeotic genes (16, 17). The Trr protein, whose mammalian orthologs are MLL3 and MLL4, has been shown to modulate the function of the Ecdysone receptor (EcR) (12, 15); ecdysone is the steroid hormone that promotes important developmental transitions in *Drosophila*. Similarly, the MLL4 protein in mammals is capable of interacting with the estrogen receptor (ER) (18). Since Trx and Trr have been shown to bind to many sites in the genome (12, 13, 19), it is likely that they also regulate the expression of genes other than those characterized in these studies.

Several MLL-family members have been implicated in mammalian cancer. MLL1, a mammalian ortholog of Trx, is translocated in several types of leukemia (20–22). In the course of these translocations, the N terminus of MLL1 is fused to a variety of proteins, generating a chimeric protein that lacks the SET domain. Interestingly, a property shared by these chimeric proteins is that

Received 24 November 2012 **Returned for modification** 28 December 2012
Accepted 22 February 2013
Published ahead of print 4 March 2013
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doi:10.1128/MCB.01585-12

they are incorporated into a complex that regulates transcriptional elongation (5). While this observation provides an important mechanistic advance in our understanding of the pathogenesis of MLL-induced leukemias, the links between possible perturbations in transcriptional elongation and changes in cell proliferation are still not known. Both orthologs of *trr*, *MLL3* and *MLL4*, have been implicated in mammalian cancer (5). MLL4 is mutated in 32% of diffuse B-cell lymphomas and 89% of follicular lymphomas (23). Currently, little is known about how these mutations contribute to lymphoma development. Thus, understanding how MLL-family proteins regulate cell proliferation is crucial to unraveling the pathogenesis of these types of leukemias and lymphomas.

Here we show that mutations in the *Drosophila trr* gene provide mutant cells with a growth advantage over their wild-type neighbors. Mutant tissue is characterized by a strong reduction in H3K4 monomethylation and alterations in multiple growth-promoting pathways. In contrast, *trx* mutations result in a decrease in tissue mass as a result of increased levels of apoptosis. Thus, inactivation of each of these two H3K4 methyltransferases, which are orthologs of MLL3 and -4 and MLL1 and -2, respectively, can have very different functions with respect to cell proliferation and survival *in vivo*.

MATERIALS AND METHODS

Fly stocks. All four *trr* alleles were generated by ethylmethanesulfonate (EMS) mutagenesis of w^{1118} sn^{3} *FRT19A* (24). *y w Ubi-GFP FRT19A*; *eyFLP (II)* (25), w^{1118} *sn FRT19A*; *eyFLP*, *y w l (1)cl 8.7 P*[*m-w*$^{+}$ *arm-lacZ*] *FRT19A/FM7a*; *eyFLP (II)* (26), *y w eyFLP*; *FRT82B P*[*mini-w*$^{+}$, *armLacZ*] (24, 27), and *y w eyFLP glass-lacZ*; *FRT82B* w^{+} *cl3R3/TM6B*, y^{+} (28) flies were used to generate mitotic clones. *y*; *FRT82B* trx^{E2}*/TM6C* (29) was obtained from the Bloomington Stock Center. UAS*trrRNAi* was obtained from the Bloomington Stock Center (stock number 29563) and expressed using *eyGal4*.

Quantification of imaginal disc clone size. Eye imaginal discs were imaged using a TCS confocal microscope (Leica). Images were edited in Adobe Photoshop, and areas were measured using ImageJ software.

Immunohistochemistry. Third-instar imaginal discs were dissected and fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS). Washes were performed with 0.1% Triton X-100–PBS. Primary antibodies used included rabbit anti-cleaved caspase 3 (Cell Signaling) (1:200), mouse anti-cyclin B (Developmental Studies Hybridoma Bank [DSHB]) (F2F4; 1:5), mouse anti-Diap1 (from Bruce Hay) (1:200), guinea pig anti-Capicua (anti-Cic) (1:300) (30), rabbit anti-phospho-Mad (Cell Signaling) (1:100), mouse anti-Notch intercellular domain (ICD) (DSHB) (9C6; 1:200), mouse anti-Dlg (DHSB) (4F3; 1:100), mouse anti-Crumbs (anti-Crb) (Cq4; 1:50) (31), guinea pig anti-cyclin E (from Terry Orr-Weaver), mouse anti-cyclin D (from Wei Du and Nick Dyson), mouse anti-Myc (from Robert Eisenman), rabbit anti-green fluorescent protein (anti-GFP) (Torrey Pines Biolabs Inc.) (1:500; TP401), mouse anti-GFP (Roche) (1:500), mouse anti-β-galactosidase (Sigma) (1:500), and rabbit anti-β-galactosidase (Promega) (1:500). Secondary antibodies used were anti-rabbit antibody–Alexa Fluor 555, anti-mouse antibody–Alexa Fluor 555, anti-rabbit antibody–Alexa Fluor 488, anti-mouse antibody-Alexa Fluor 488 (all from Invitrogen), and anti-guinea pig antibody–Alexa Fluor 555 (Molecular Probes) at 1:500.

Reverse transcription-quantitative PCR (RT-qPCR). Fifty third-instar eye imaginal discs were dissected and placed in RNAlater (Qiagen). The discs used had *trr* or *trx* clones or clones from the parent chromosome generated by mitotic recombination with a chromosome bearing a recessive cell-lethal mutation such that the twin spots were eliminated, leaving the mutant clones and some heterozygous tissue. Total RNA was extracted using TRIzol (Invitrogen) and then an RNAeasy minikit (Qiagen). cDNA synthesis was performed with a Transcriptor First-Strand Synthesis kit (Roche) using the oligo(dT) priming method. Samples were diluted (1:20 to 1:40) and amplified by quantitative PCR (qPCR) using SYBR GreenER SuperMix (Invitrogen) and an Applied Biosystems StepOnePlus real-time PCR system. Reactions were run in triplicate using the standard curve method. Melting curve analysis and conventional PCR were used to confirm primer specificity. Eight potential endogenous reference genes were analyzed, and *actin5C* was selected as the normalization transcript. Three biological replicates of each genotype were tested. A two-tailed Student's *t* test was used for statistical analysis.

RESULTS

In order to identify genes that regulate cell proliferation in *Drosophila* imaginal discs, we have screened the five main chromosome arms for mutations that allow mutant cells to outgrow their wild-type neighbors. By generating mosaic eyes that contained clones of mutant cells (marked white) and sister clones (marked red), we were able to screen for mutations that resulted in an increase in the relative representation of mutant versus wild-type tissue (27). In our screen of the X chromosome, we recovered a lethal complementation group consisting of four members (Fig. 1A to E) where each had a nonsense mutation in the *trithorax related* (*trr*) gene.

The *trr* gene encodes two protein isoforms that differ by 21 amino acids near the N terminus, the larger being 2,431 amino acids long (11) (Fig. 1F). Both isoforms include a C-terminal SET domain that accounts for its histone lysine methyltransferase activity. The human orthologs of Trr are MLL3 and MLL4. However, in some insects, including *Drosophila*, the domains found in the N-terminal portion of human MLL3 or -4 are found in a separate protein, Lost PHDs of Trr (LPT) (also known as Cara Mitad [Cmi]), that remains physically associated with Trr (13, 15) (Fig. 1F). Of the original four alleles recovered in the screen, only trr^{K662X} and trr^{K2375X} were still being maintained (Fig. 1C and E). Data pertaining to the other two alleles (trr^{K414X} and trr^{C1738X}) are therefore marked with an asterisk.

For all four alleles, mosaic eyes containing *trr* mutant clones exhibit a relative overrepresentation of mutant over wild-type tissue, indicating that the mutant tissue had a growth advantage. This disparity is more evident in the anterior part of the eye, likely resulting from the more prolonged period of cell proliferation that occurs in the anterior part of the eye disc. However, for two of the alleles, trr^{K662X} or trr^{K414X}, each of which is predicted to generate an extremely truncated protein, two additional phenotypic abnormalities are observed. First, the mutant portion of the eye appears rough, which is indicative of irregularities in the size or organization of the individual facets (ommatidia) of the compound eye. Indeed, sections of adult retinas reveal occasional ommatidia with missing photoreceptor cells as well as abnormalities in the orientation of individual ommatidia that often reflect defects in planar cell polarity (Fig. 1G, G′, H, and H′). Second, despite the relative growth advantage displayed by mutant clones, there is a small decrease in the overall size of the mosaic eyes. Additionally, when the wild-type cells are eliminated by a cell-lethal mutation (Fig. 1I and J) or when *trr* gene function is uniformly reduced by expression of a hairpin RNA interference (RNAi) transgene (Fig. 1K and L), the resulting eye is slightly reduced in size and roughness is observed similar to that observed with the strongest alleles of *trr*. All of the phenotypic abnormalities found in mutant clones are rescued by a transgene containing a wild-type copy of the *trr* gene (data not shown). These findings suggest that disruption of the

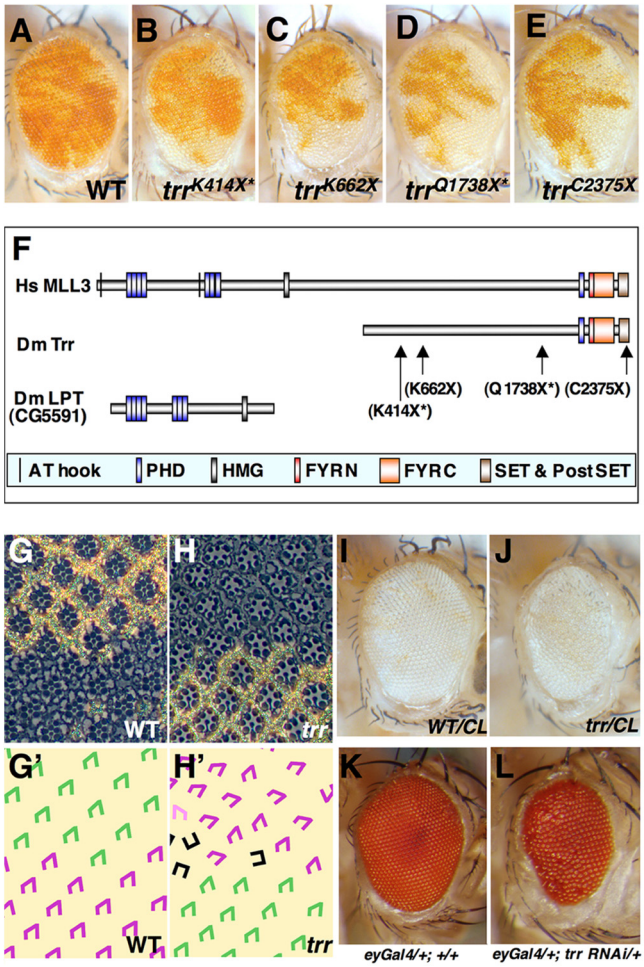

FIG 1 Mutations in *trr* result in a relative overrepresentation of mutant tissue. (A to E) Mosaic adult eyes generated by mitotic recombination, where the tissue homozygous for the parent chromosome (A) or four different *trr* alleles (B to E) appears white. The *trr* mutant tissue is overrepresented (B to E) compared to the relative amounts of nonmutant tissue (red) and mutant tissue (white) found in mosaic eyes generated using the isogenic parent chromosome (A). The alleles that are no longer being maintained are indicated with an asterisk. Genotypes are w^{1118} sn^3 *FRT19A/yw P*[w^+ *Ubi-GFP*] *FRT19A*; *eyFLP/+* for panel A and *trr* w^{1118} sn^3 *FRT19A/yw P*[w^+ *Ubi-GFP*] *FRT19A*; *eyFLP/+* for panels B to E. WT, wild type. (F) Schematic representation of the Trr protein of *Drosophila melanogaster* (Dm Trr) as well as its human ortholog MLL3 (Hs MLL3). In *Drosophila melanogaster* and some other insects, a portion of the protein orthologous to MLL3 is found in a separate gene Lost PHD domains of Trr (LPT). The major domains found in the proteins are shown, as are the changes caused by two of the *trr* mutations characterized. (G and H) Sections of adult eyes showing the arrangement of tissue homozygous for the FRT19A parent chromosome (G) or the chromosome bearing the trr^{K662X} mutation (H). (G′ and H′) The trapezoidal arrangement of photoreceptor cells in the ommatidia in panels G and H is shown in panels G′ and H′. Ommatidia from the mutant portion of the retina (shown in purple) show defects in rotation and sometimes have a rectangular rather than trapezoidal arrangement (black) suggestive of defects in the specification of the R3 and R4 photoreceptor cells. (I and J) Generation of eyes composed mostly of tissue homozygous for the FRT19A parent chromosome (I) or the trr^{K662X} chromosome (J) using *eyFLP*-induced mitotic recombination with a chromosome bearing a recessive cell-lethal (CL) mutation that eliminates the wild-type twin spots. The genotypes were w^{1118} sn^3 *FRT19A/y w l* (*1*)*cl 8.7 P*[*m-*w^+ *arm-lacZ*] *FRT19A*; *eyFLP/+* (I) and w^{1118} sn^3 trr^{K662} *FRT19A/y w l* (*1*)*cl 8.7 P*[*m-*w^+ *arm-lacZ*] *FRT19A*; *eyFLP/+* (J). (K and L) Expression of an RNAi transgene directed at *trr* in the eye primordium using the *eyGAL4* driver reduces eye size and causes an irregularity in the arrangement of ommatidia. The genotypes were *y w/+*; *eyGAL4/+* for panel K and *y w/+*; *eyGAL4/+*; UAS-$trr^{TRiP\_JF03242}$/+ for panel L.

C-terminal portion of the protein, which has the histone methyltransferase activity, is sufficient to give mutant cells a slight growth advantage over their neighbors. Elimination of functions involving regions nearer the N terminus would be predicted to disrupt the interaction of Trr with the Ecdysone receptor (12) and thus impair progression of the morphogenetic furrow, as has previously been shown by others (12, 32, 33), which could explain the reduction in eye size. Importantly, loss of *trr* function does not appear to prevent photoreceptor differentiation *per se*, as assessed by the presence of rhabdomeres in *trr* mutant photoreceptor cells in sections from adult eyes.

***trr* mutant clones have a growth advantage as well as alterations in cell survival.** To determine whether the increased representation of mutant tissues in adult eyes reflects a growth advantage, we examined imaginal discs from third-instar larvae. The Trr protein is expressed throughout the eye disc and cannot be detected in mutant clones of the trr^{K662X} allele (data not shown). Clones of the *trr* mutant occupied a significantly larger portion of the disc than clones homozygous for the isogenic parent chromosome (Fig. 2A and B; quantified in Fig. 2C). Despite their growth advantage, mutant cells appear to exit the cell cycle at the appropriate time since we did not observe ectopic bromodeoxyuridine (BrdU) incorporation posterior to the second mitotic wave as occurs in cells mutant for several negative regulators of cell proliferation such as components of the Hippo pathway (Fig. 2F and F′). Thus, *trr* mutant cells outgrow their wild-type neighbors but still exit the cell cycle at the appropriate time.

We also observed changes in the pattern of cell death in discs containing mutant clones. In pupal retinas, supernumerary cells are normally eliminated by a wave of apoptosis, leaving a regular array of cells with specified fates (Fig. 2D and D′). In contrast, in *trr* clones, additional interommatidial cells were observed, indicating that this wave of cell death may not have proceeded to completion (34) (Fig. 2E and E′). Additionally, in the larval disc, we observed clumps of cells expressing activated caspase basal to the disc but seldom observed these cells in the disc epithelium itself (Fig. 2G to J), suggesting that cells undergoing apoptosis are rapidly extruded from the epithelium. The mass of activated caspase 3-positive cells included material that was GFP positive, suggesting that at least some wild-type cells were present. These wild-type cells could have derived from the disc epithelium or, alternatively, could have been hemocytes that were engulfing the corpses of dead cells. Thus, while decreased *trr* function seems to protect cells from the normal developmental apoptosis that occurs at the pupal stage, it appears to promote some apoptosis of either mutant cells or their wild-type neighbors at an earlier stage.

***trr* mutations have markedly reduced levels of H3K4 monomethylation.** The Trr protein belongs to a family of proteins that contain SET domains that are capable of methylating the N-terminal tail of histone H3 on the lysine residue at position 4 (H3K4). In *Drosophila* cells, Trr proteins, along with two other SET domain-containing proteins, Set1 and Trithorax (Trx), assemble into multiprotein complexes that share several of their components (13). Of these, Trr alone forms a complex that includes Utx, a protein that is capable of removing methyl groups attached to the lysine residue at position 27 on histone H3 (H3K27). Interestingly, *Utx* mutants were previously isolated from our screen based on the phenotype of a relative overrepresentation of mutant tissue (35).

Mutations in *Drosophila trr* have been described previously

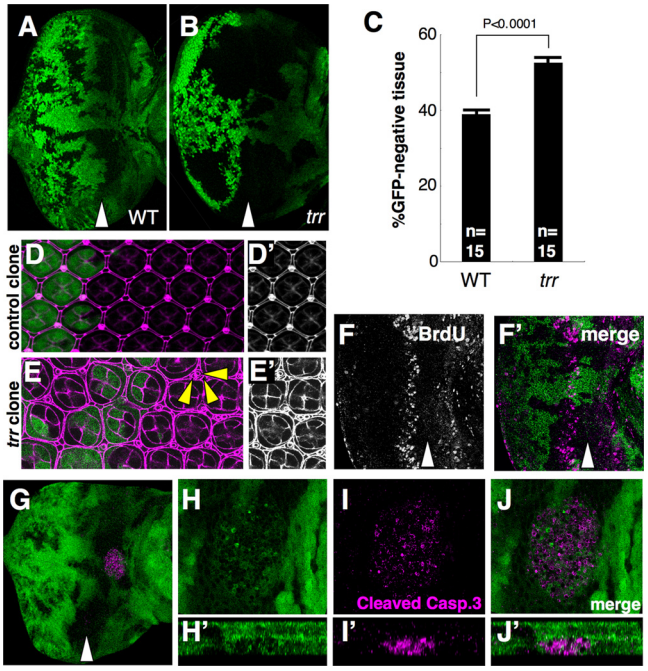

FIG 2 Mutations in *trr* result in increased growth and alterations in the pattern of apoptosis in the eye imaginal disc. (A to C) The ratio of mutant (black) to nonmutant (green) tissue in the eye is increased in trr^{K662X} mutants (B) compared to the *FRT19A* parent chromosome (A) as quantified in panel C. The data were compared using a 2-tailed *t* test ($n = 15$ eye discs each). $P = 1.4e\text{-}5$. Error bars indicate the standard errors of the means (SEM). (D and E) Mosaic pupal retinas showing a single layer of interommatidial cells in tissue homozygous for the FRT19A chromosome. In contrast, there are additional interommatidial cells in the trr^{K662X} tissue. Anti-Dlg staining is shown in magenta. GFP (green) indicates the presence of the wild-type chromosome. Yellow arrowheads indicate some examples of extra interommatidial cells. (F) Incorporation of BrdU in trr^{K662X} mosaic larval discs shows no differences between the wild-type and mutant portions of the disc. (G to J) A clump of tissue expressing activated caspase is observed basal to the epithelium near the junction of the eye and antennal portions of the disc in discs bearing *trr* clones. Mutant tissue does not express GFP. Z projections shown in panels H′ to J′ show that the tissue expressing activated caspase (Casp.) is basal to the epithelium of the disc proper. The genotypes were w^{1118} sn^{3} *FRT19A/yw Ubi-GFP FRT19A*; *eyFLP/+* for panels A, D, and D′ and trr^{K662X} w^{1118} sn^{3} *FRT19A/yw Ubi-GFP FRT19A*; *eyFLP/+* for panels B and E to J.

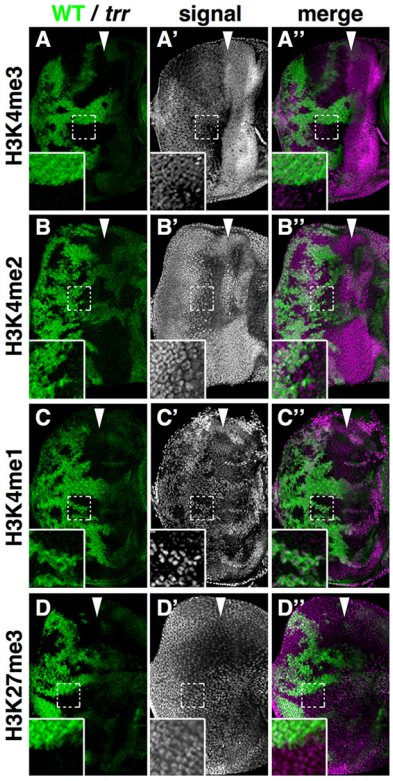

FIG 3 *trr* mutant cells have decreased levels of H3K4 monomethylation. (A to D) Mosaic eye imaginal discs stained with antibodies that detect methylation of H3K4 (A to C) and H3K27 (D). (A to C) Mutant tissue does not express GFP (green). *trr* mutant cells express greatly diminished levels of H3K4 monomethylation (H3K4me1) (C) but not di- or trimethylation (A and B). (D) Trimethylation of H3K27 was not detectably changed. The genotypes were *trrK662X w1118 sn3 FRT19A/y w P*[*wUbi-GFP*] *FRT19A*; *eyFLP/+* for panels A to D.

and were shown to modulate the function of the receptor for the steroid hormone ecdysone (12, 15), a function similar to that described for its human ortholog, *MLL4*, which has been shown to interact with the estrogen receptor (18). The authors (12) also reported that the levels of dimethylation and trimethylation of H3K4 were much reduced in *trr* mutant embryos. Another study, also using Western blotting, documented decreased levels of H3K4 trimethylation in both embryos and prepupae (15). However, two recent studies using RNAi-based approaches have reached a different conclusion. In one study, knockdown of Trr function in wing imaginal discs resulted in only a marginal decrease in di- and trimethylation of H3K4 (13). In another, knockdown of Trr in cell culture primarily caused a decrease in H3K4 monomethylation and the Trr protein showed robust H3K4 monomethylation activity *in vitro* (7).

We therefore examined H3K4 mono-, di-, and trimethylation levels in mosaic eye imaginal discs, where the adjacent wild-type cells provide an ideal reference for comparison. Consistent with the recent findings in *Drosophila* cell culture (7), we found no detectable change in the levels of di- or trimethylation of H3K4 (Fig. 3A to A″ and B to B″). However, monomethylation of H3K4 was dramatically reduced (Fig. 3C to C″). These findings are also similar to those observed in *Utx* mutants (35) and are consistent with the finding that Utx and Trr are found in the same protein complex. Thus, reducing *trr* or *Utx* function seems to primarily impair H3K4 monomethylation, a posttranslational modification that appears to be associated with enhancers (36–38). We did observe one difference between *Utx* and *trr* mutants. In *Utx* clones, a slight elevation of H3K27 trimethylation that is consistent with the ability of the Utx protein to demethylate H3K27 has been observed (35). In *trr* clones, H3K27 trimethylation levels appeared unchanged (Fig. 3D to D″). Thus, even though Trr and Utx are in the same complex, loss of either protein results in reduced H3K4 monomethylation. However, loss of Utx function but not Trr function results in slightly increased H3K27 trimethylation. These results suggest that the decrease in H3K4 monomethylation in either *trr* or *Utx* mutants is not a consequence of altered H3K27 trimethylation but is more likely the result of a direct loss of H3K4 monomethylation activity of the complex. Additionally, loss of Trr function may not compromise the H3K27 demethylase activity of the complex. Alternatively, the Utx protein could make only a minor contribution to the overall levels of H3K27 demethylation

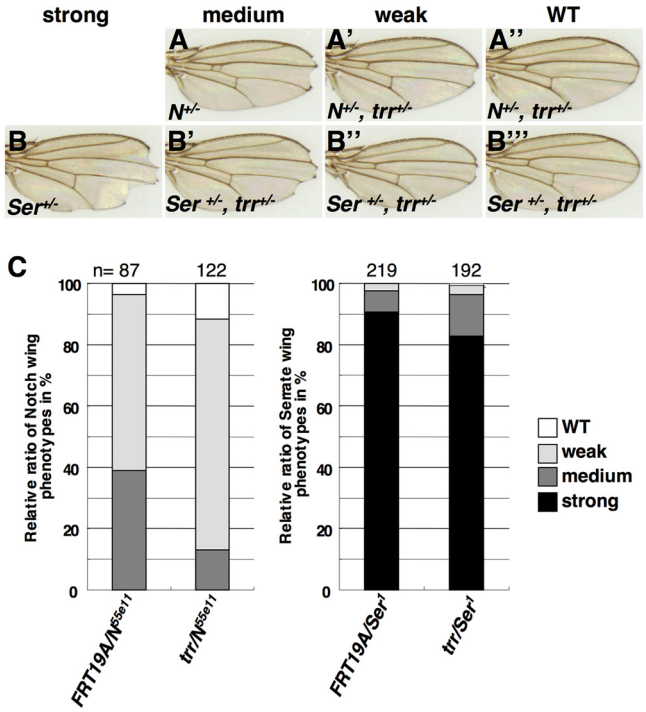

FIG 4 *trr* mutations suppress the dominant wing-notching phenotypes of *Notch* (*N*) and *Serrate* (*Ser*) mutants. The frequencies of different classes of notched wings were compared between flies heterozygous for the parent chromosome (*FRT19A*) and a chromosome bearing the trr^{K662X} mutation. The genotypes were N^{55e11} *FRT19A*/w^{1118} sn^{3} *FRT19A* for panel A, N^{55e11} *FRT19A*/trr^{K662X} w^{1118} sn^{3} *FRT19A* for panels A′ and A″), w^{1118} sn^{3} *FRT19A*/+; *TM3*, *Ser*/+ for panel B, and trr^{K662X} w^{1118} sn^{3} *FRT19A*/+; *TM3*, *Ser*/+ for panels B′ to B‴.

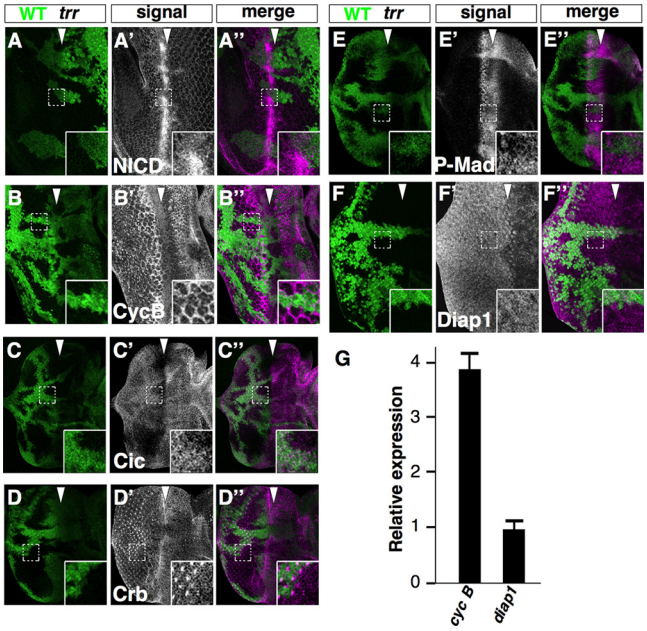

FIG 5 *trr* cells have altered levels of multiple regulators of tissue growth and cell survival. (A to F) Eye imaginal discs mosaic for clones homozygous for trr^{K662X} stained with antibodies to the intracellular domain of Notch (NICD; A to A″), cyclin B (CycB; B to B″), Capicua (Cic; C to C″), Crumbs (Crb; D to D″), phospho-Mad (P-Mad; E to E″), and Diap1 (F to F″). Mutant tissue did not express GFP (green). (G) Relative levels of *cyclin B* and *diap1* RNA as assessed by RT-qPCR. Error bars indicate the SEM of the results determined with three biological replicates. The genotypes were trr^{K662X} w^{1118} sn^{3} *FRT19A*/*yw Ubi-GFP FRT19A*; *eyFLP*/+ for panels A to F″.

and a slight increase might not be obvious using immunofluorescence.

Indeed, while this work was being prepared for submission, another study used both immunohistochemistry and genome-wide chromatin immunoprecipitation (ChIP) studies to demonstrate that decreasing *trr* function reduces levels of both H3K4 monomethylation and H3K27 acetylation (which is normally a mark of enhancer elements) (39). Moreover, the absence of Trr results in diminished levels of Utx, likely by destabilizing the Utx protein.

***trr* mutations impact Notch signaling as well as levels of other growth regulators.** Mutations in *Drosophila Utx* augment Notch signaling, as shown by both an increase in the expression level of genes downstream of Notch and the ability of *Utx* mutants to dominantly suppress phenotypes resulting from decreased Notch signaling (35). To determine whether this aspect of *Utx* function is shared by *trr*, we examined the ability of *trr* mutations to modify the wing-notching phenotype of *Notch* (*N*) and *Serrate* (*Ser*) mutants (Fig. 4). As with *Utx* mutants, *trr* mutants demonstrated some suppression of both phenotypes. We also examined the level of Notch protein in mutant clones and observed markedly increased staining with an antibody directed against the intracellular domain of Notch (Fig. 5A to A″).

Since mutations in epigenetic regulators such as *trr* are likely to affect multiple genes and signaling pathways, we surveyed a number of regulators of growth and cell cycle progression. We found a decrease in the levels of the corepressor Capicua (Cic) (Fig. 5C to C″), which is indicative of increased receptor tyrosine kinase (RTK) signaling (40), and an increase in phospho-Mad levels, which is indicative of increased Dpp signaling (Fig. 5E to E″). Additionally, levels of the cell-surface protein Crumbs were decreased (Fig. 5D to D″). Complete inactivation of *crumbs* (*crb*) results in a modest upregulation of Yorkie-regulated genes such as *diap1* (41–44). However, there does not appear to have been an increase in *diap1* expression (Fig. 5F), suggesting that alterations in Hippo pathway signaling were perturbed even less than they are in *crb* mutant clones. The level of the mitotic cyclin, cyclin B, was also elevated (Fig. 5B to B″). However, the levels of the G1 cyclins, cyclin D and cyclin E, as well as of the Myc protein which promotes growth and cell cycle progression were unchanged (data not shown). We tested whether these changes in the levels of growth and cell cycle regulators were the result of changes in RNA levels by RT-PCR. For the genes examined, only *cyclin B* showed a 4-fold increase in RNA levels (Fig. 5G); others showed little change. Thus, the overgrowth phenotype cannot easily be explained by a simple effect on a single pathway but likely results from a complex interplay of transcriptional and posttranscriptional changes involving multiple genes that eventually result in altered activity of multiple growth-promoting pathways.

Mutations in *trithorax* (*trx*) affect tissue growth and gene expression in ways that differ from those seen with *trithorax related* (*trr*). *Drosophila* has three SET domain-containing proteins that assemble into COMPASS-like complexes (13). The Set1-containing complex accounts for most of the H3K4 trimethylation observed. The complexes containing the MLL-family

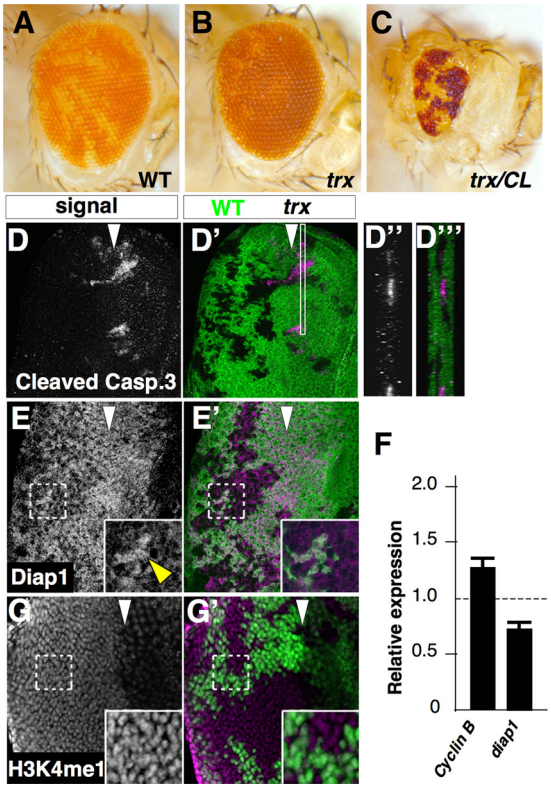

FIG 6 Mutations in *trithorax* (*trx*) elicit phenotypes that are qualitatively distinct from those observed in *trr* mutants. (A and B) Eyes mosaic for the *FRT82B* chromosome (A) or the trx^{E2} mutation (B) show a relative underrepresentation of *trx* mutant tissue. (C) Eyes composed mostly of *trx* tissue were generated by mitotic recombination with a chromosome bearing a recessive cell-lethal mutation and were much smaller. The genotypes were *y w eyFLP*; *FRT82B P*[*mini-w*$^+$, *armLacZ*]/*FRT82B* for panel A, *y w eyFLP*; *FRT82B P*[*mini-w*$^+$, *armLacZ*]/*FRT82B* trx^{E2} for panel B, and *y w eyFLP glass-lacZ*; *FRT82B w*$^+$ *cl3R3*/*FRT82B* trx^{E2} for panel C. (D) Increased staining with an antibody that recognizes cleaved caspase is observed in *trx* clones. Z projections of the region boxed in panel D′ are shown in panels D″ and D‴. (E) Staining of mosaic discs with an antibody to Diap1 shows reduced staining in mutant clones compared to wild-type tissue (green). The yellow arrowhead indicates the expression of Diap1 in wild-type tissue. (F) Relative levels of *cyclin B* and *diap1* RNA in discs containing *trx* clones as measured using RT-qPCR. Error bars indicate the SEM of the results determined with three biological replicates. (G) Staining with an antibody to the monomethylated form of H3K4 (H3K4me1) shows no change in the level of signal in mutant clones. The genotypes were *y w eyFLP*; *FRT82B P*[*mini-w*$^+$, *armLacZ*]/*FRT82B* trx^{E2} for panels D to G.

proteins Trr and Trx appear to contribute very little to the trimethylation (7, 13). Inhibiting *trr* function reduces H3K4 monomethylation in cell culture (7, 13) and also in imaginal disc cells (Fig. 3) (39). In contrast, reducing *trx* function did not lead to marked changes in the global levels of H3K4 monomethylation in imaginal discs (Fig. 6G and G′). Moreover, levels of H3K4 di- or trimethylation were also not different (data not shown). When we generated mosaic eyes containing *trx* clones, the mutant tissue in adult eyes was greatly underrepresented (Fig. 6A and B). Indeed, a *trx* allele was previously identified in a mosaic screen for mutant clones that reduced eye size or caused scarring (45). This was in marked contrast to the mild overgrowth phenotype displayed by *trr* mutant clones (Fig. 1A to E). When mitotic recombination was induced with a chromosome bearing a recessive cell-lethal mutation such that most of the eye was mutant for *trx*, the eye was markedly reduced in size (Fig. 6C). These results indicate that *trx* cells either had a severe growth defect or had been eliminated during development.

An examination of imaginal discs containing *trx* clones demonstrated high levels of cell death, as assessed by elevated levels of activated caspase in the disc epithelium. Moreover, the activated caspase 3-positive material was found to have been extruded basally from the epithelium (Fig. 6D to D‴). Levels of the antiapoptotic protein Diap1 (46) were reduced in *trx* clones (Fig. 6E and E′). This is likely to have resulted, at least in part, from decreased transcription, since *diap1* RNA levels were also reduced (Fig. 6F). Moreover, this measurement of *diap1* RNA levels is likely to underestimate its reduction in *trx* mutant cells since the samples were prepared from discs that also contained heterozygous (*trx*/+) cells. In contrast, the levels of Notch, cyclin B, phospho-Mad, Capicua, and Crumbs, as assessed by antibody staining, were all unchanged in *trx* mutant clones compared to adjacent wild-type tissue (data not shown). Of these, there was a small increase in *cyclin B* RNA (Fig. 6F). A previous study demonstrated a reduction in the expression of *eyeless* and *teashirt* and increased expression of *homothorax* in *trx* clones that together resulted in a fate change from eye to head cuticle; sometimes these transformed clones appeared larger than those confined to the eye primordium (45).

Thus, *trr* and *trx* mutant cells differ considerably in their phenotypic characteristics. Consistent with the growth advantage exhibited by *trr* clones, several growth-promoting signaling pathways appear to function at higher levels whereas Diap1 RNA or protein levels were unchanged. In contrast, these same growth-promoting pathways were unchanged in *trx* clones whereas Diap1 RNA and protein levels were reduced. The reduction in Diap1 levels likely contributes to the apoptosis observed in *trx* clones. Additionally, *trx* promotes the specification of retinal fates in the eye disc; mutant cells can often fail to express eye-specific markers (45). In contrast, photoreceptor cell specification and differentiation appear to occur relatively normally in *trr* clones.

DISCUSSION

Several members of the MLL family of genes have been implicated in mammalian cancer. However, we still know very little about how these genes regulate cell proliferation. Study of the function of MLL-family genes is simplified in *Drosophila* because *trr* appears to correspond to both MLL3 and MLL4 whereas *trx* corresponds to MLL1 and MLL2. Here we have characterized the properties of each of these major subclasses of MLL genes. First, we show that mutations in *trr* promote tissue overgrowth without obviously interfering with differentiation. In contrast, inactivating mutations in *trx* cause increased levels of cell death. Second, we demonstrate that *trr* mutant clones, but not *trx* clones, are characterized by a marked decrease in H3K4 monomethylation. Third, we find that multiple pathways that promote growth and cell cycle progression are altered in *trr* cells, indicating that the phenotypic abnormalities are unlikely to result from the deregulation of a single pathway. Finally, we observe considerable similarities between the phenotypic abnormalities displayed by *trr* and *Utx* clones, consistent with the finding that the two proteins are present in the same complex.

In the past, studies of H3K4 methyltransferase complexes have emphasized their role in trimethylation. However, the main

change observed *in vivo* with mutations in *Utx* or in *trr* is a decrease in H3K4 monomethylation. Recent work indicates that H3K4 monomethylation is characteristic of enhancer sequences and genome-wide surveys of H3K4 monomethylation can be used to predict the location of enhancers (36–38). Thus, it is likely that the function of a number of enhancers in the genome is altered in some way in *trr* mutants.

trr mutants and *Utx* mutants display remarkable similarities in their mutant phenotypes. Alleles of *trr* (this work) and alleles of *Utx* (35) were both identified in unbiased genetic screens for mutations that increased the relative overrepresentation of mutant tissue compared to wild-type tissue. Additionally, *trr* and *Utx* mutations display similar genetic interactions, with mutations in *Notch* (*N*) and *Serrate* (*Ser*). These findings indicate that these phenotypic consequences are likely to arise from a disruption of the complex that includes both proteins and that likely regulates the levels of H3K4 monomethylation at multiple enhancers.

In a survey of the main pathways that are known to regulate imaginal disc growth in *Drosophila*, we found multiple changes in *trr* mutants. Consistent with the genetic interactions with *N* and *Ser*, we found increased levels of the Notch protein in mutant clones. Thus, the increased activity of the Notch pathway in *trr* mutants could result from increased levels of the Notch protein itself. This would explain why this phenotype could suppress the wing-notching phenotype of *N* heterozygotes, where Notch protein levels are reduced. We also observed reduced levels of Capicua, which is indicative of increased RTK/Ras signaling (40), and increased levels of phospho-Mad, which is suggestive of increased signaling via Dpp receptors (47). Despite at least three growth-promoting pathways being activated at increased levels, the overgrowth phenotype of *trr* mutants is relatively subtle, suggesting that the extent of change in these pathways has a small impact on tissue growth or that there are also unknown changes in other pathways that counteract these effects.

Although *trr* and *trx* encode proteins of the same superfamily, their effects on tissue growth are completely different. Each of the growth-regulatory pathways that displayed alterations in *trr* mutant clones was unchanged in *trx* clones. In contrast, levels of the antiapoptotic protein Diap1 are reduced in *trx* clones and likely contribute to the increased cell death in *trx* mutants. Additionally, in screens of the right arm of the third chromosome conducted in our laboratory for adult eyes with an increased representation of mutant tissue, despite obtaining multiple alleles of several negative regulators of growth, including *Tsc1*, *salvador*, *warts*, and *capicua*, we never obtained any alleles of *trx* with an overgrowth phenotype. Thus, in contrast to *trr*, inactivating mutations in *trx* do not promote tissue growth while preserving the ability of cells to differentiate.

Mutations that are predicted to decrease the function of MLL3 and MLL4, the mammalian proteins most similar to Trr, are being found in an increasing number of solid tumors (see, for example, references 48, 49, and 50) in addition to the mutations described in lymphomas (23). This suggests that, as seen with *trr*, inactivation of MLL3 or MLL4 also promotes cell proliferation. In leukemias, however, MLL1 is disrupted by a variety of chromosomal translocations, each of which fuses the N terminus of MLL1, which lacks the SET domain, to a protein that functions in transcriptional elongation, resulting in the incorporation of this chimeric protein into a multiprotein complex that regulates transcriptional elongation (reviewed in reference 5). Thus, the effect of MLL1 on leukemic cell proliferation may not reflect a normal role for MLL1 as a regulator of cell proliferation. At least in *Drosophila*, inactivation of its ortholog *trx* does not seem to result in excess proliferation. Rather, *trx* mutant cells display increased levels of apoptosis as well as cell fate changes. Thus, these two major subclasses of MLL genes appear to have different types of effect on cell proliferation and survival.

While genome-wide changes in histone methylation caused by manipulation of genes encoding histone methyltransferases and demethylases are being increasingly documented, we still lack a basic understanding of how these changes relate to biological consequences. In yeast, inactivation of Set1 abolishes most if not all H3K4 methylation. Yet mutant strains are viable, albeit with alterations in growth and in morphology, and display abnormalities in gene silencing at specific loci (51–53). Thus far, it has been difficult to ascribe the phenotypic abnormalities to specific changes in transcription. The demonstration that mutations in the *Drosophila* genes *trr* and *trx* elicit specific and distinct phenotypic abnormalities pertaining to growth and cell survival will provide a useful starting point for dissecting those aspects of the biological functions of MLL-class genes that are most relevant to human cancers.

ACKNOWLEDGMENTS

We thank Wei Du, Nick Dyson, Robert Eisenman, Bruce Hay, Alexander Mazo, and Terry Orr-Weaver for antibodies and fly stocks as well as the following stock centers: VDRC, Bloomington Drosophila Stock Center, NIG-FLY, the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947), and the Drosophila Genome Resource Center at Kyoto. We are especially grateful to Brett Pellock for initiating the FLP/FRT screen of the X chromosome and for providing stocks and encouragement during this project and to current and former members of the Hariharan, Bilder, and Okano laboratories for discussions and advice. We thank Rieko Shimamura for technical support.

I.K.H. is funded by the NIH (R01 GM61672) and a Research Professor Award from the American Cancer Society (120366-RP-11-078-01-DDC). H.K. and H.O. received support from the Japanese Ministry of Education, Science, Sports, Culture and technology (H.K. and H.O.), the Japan Society for the Promotion of Science (H.K.), Keio Gijuku Academic Development Funds (H.K.), Keio University Grant-in-Aid for Encouragement of Young Medical Scientists (H.K.), the Strategic Research Foundation Grant-aided Project for Private Universities from MEXT (H.K.), and the Grant-in-Aid for the G-COE program from MEXT to Keio University (H.K. and H.O.).

H.K. designed and conducted the genetic screen with the assistance of L.C. and identified the mutants. A.N. identified *trr* as the gene responsible for the mutant phenotype and conducted its molecular characterization. H.K. and A.N. characterized the mutant phenotypes and prepared the figures. Data were interpreted by H.K., A.N., and I.K.H. The manuscript was written by I.K.H., H.K., and A.N. I.K.H. oversaw the work conducted at Berkeley. H.O. oversaw the experiments conducted by H.K. after he moved to Keio University.

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