Evidence for Transgenerational Transmission of Epigenetic Tumor Susceptibility in *Drosophila*

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Transgenerational epigenetic inheritance results from incomplete erasure of parental epigenetic marks during epigenetic reprogramming at fertilization. The significance of this phenomenon, and the mechanism by which it occurs, remains obscure. Here, we show that genetic mutations in *Drosophila* may cause epigenetic alterations that, when inherited, influence tumor susceptibility of the offspring. We found that many of the mutations that affected tumorigenesis induced by a hyperactive JAK kinase, Hop^{Tum-l}, also modified the tumor phenotype epigenetically, such that the modification persisted even in the offspring that did not inherit the modifier mutation. We analyzed mutations of the transcription repressor *Krüppel (Kr)*, which is one of the *hop^{Tum-l}* enhancers known to affect *ftz* transcription. We demonstrate that the *Kr* mutation causes increased DNA methylation in the *ftz* promoter region, and that the aberrant *ftz* transcription and promoter methylation are both transgenerationally heritable if Hop^{Tum-l} is present in the oocyte. These results suggest that genetic mutations may alter epigenetic markings in the form of DNA methylation, which are normally erased early in the next generation, and that JAK overactivation disrupts epigenetic reprogramming and allows inheritance of epimutations that influence tumorigenesis in future generations.

Introduction

Epigenetic regulation of gene expression refers to repression or activation of gene expression via covalent modifications of DNA or histones, such as methylation or acetylation, without changing the DNA sequence of the gene [1–3]. Epigenetic modifications are usually stably heritable through subsequent cell divisions, resulting in permanent changes in gene expression profiles, such as those associated with terminal differentiation. However, at critical stages in normal development or disease situations, cells undergo genome-wide epigenetic reprogramming, erasing preexisting epigenetic marks and establishing a new set of marks. For instance, major epigenetic reprogramming occurs at fertilization prior to zygotic development, at dedifferentiation that leads to cancer development, and during somatic cell nuclear transfer, a procedure used for cloning or obtaining embryonic stem cells [4–7].

However, epigenetic marks are not always completely erased from one generation to the next. For instance, genomic imprinting, where clusters of genes or whole chromosomes are preferentially inactivated depending on their parental origin [8,9], can be considered an exception to epigenetic reprogramming, because in this case parental epigenetic markings are retained in the zygote. Loss of imprinting has been shown to increase the likelihood that cancer will develop [10–12]. Furthermore, human diseases, such as Prader-Willi and Angelman syndromes [13] and hereditary nonpolyposis colorectal cancer [14], are associated with germline inheritance of epimutations. Though transgenerational epigenetic inheritance has been documented for a variety of eukaryotic organisms ranging from plants to humans [15], the precise mechanisms that regulate epigenetic marking and erasure, as well as those that protect certain epigenetic marks from being reset, are not clear.

We have previously undertaken a genetic approach in order to identify genes that are important for *hop^{Tum-l}*-induced tumorigenesis in *Drosophila*, and in the process, have found that JAK signaling globally counteracts heterochromatin formation [16]. Further analyses of the identified mutations indicated that a number of those mutations that genetically modify *hop^{Tum-l}* tumorigenicity also do so epigenetically. In fact, *hop^{Tum-l}* itself plays an essential role in the maintenance of parental origin epigenetic alterations that subsequently affect tumorigenesis in a transgenerational manner. These results indicate a novel function for the *hop^{Tum-l}* oncogene: it interferes with the epigenetic reprogramming process.

Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; C, cytosine; G, guanine; HDAC, histone deacetylase; T, thymidine; TI, tumor index; TSA, tricostatin A

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Author Summary

It is well known that many genetic mutations in oncogenes or tumor suppressors can cause or greatly increase a person's susceptibility to cancer. It is generally assumed that persons should feel relieved if they have not inherited the particular "cancer-causing" mutation carried by their parents. However, we found that, under certain circumstances, fruit flies carrying tumor suppressor gene mutations can pass the increased tumor risk to all offspring, even those that have not inherited the particular mutation. A likely scenario is that many genetic mutations can lead to epigenetic alterations, that is, changes in the chemical modifications of DNA or the proteins that bind to DNA in the chromosomes, and these changes can have global effects on cell function. Normally, these epigenetic alterations are wiped out and reset in the early embryo, but under certain circumstances such alterations can be inherited. Interestingly, we found evidence that a particular oncoprotein, an overactivated form of a cell-signaling molecule called JAK kinase, can counteract the epigenetic resetting program that normally operates in the early embryo. Thus, the failure of epigenetic reprogramming allows the inheritance of parental epigenetic alterations that affect susceptibility to tumors.

Results

Paternal-Effect Modification of *Tum-l* Tumorigenicity

We previously conducted a genetic screen for modifiers of the hop^{Tum-l} hematopoietic tumorigenic phenotype and identified 37 modifier mutations [M(Tum-l)] that dominantly enhanced or suppressed hop^{Tum-l} tumorigenesis in $hop^{Tum-l}/+$; M(Tum-l)/+ transheterozygotes [16]. Hematopoietic tumors in hop^{Tum-l}-containing flies were quantified by tumor index (TI) (see Materials and Methods and also [16]). Interestingly, many of the M(Tum-l) mutations (24/37) exhibited paternal-effect modification of hop^{Tum-l} tumorigenicity, such that when $hop^{Tum-l}/+$ females were mated to males heterozygous for the modifier mutation (M[Tum-l]/+), tumorigenesis associated with hop^{Tum-l} was modified (enhanced or suppressed) in the F1 generation regardless of the inheritance of M(Tum-l) (Table 1). The transgenerational effects were confirmed with rebalanced stocks, indicating that they are unlikely to be due to different genetic backgrounds. Since little or no paternal cytoplasmic proteins are carried in the sperm, the observed paternal effects on the zygote suggest an epigenetic mechanism. Possibly, the M(Tum-l) mutations caused epigenetic alterations in the paternal chromosomes and these epigenetic changes were maintained through male meiosis and transmitted to the F1 generation, thereby influencing hop^{Tum-l} tumorigenicity.

Transgenerational Tumor Modification Depends on Maternal hop^{Tum-l} Mutation

To understand the nature of the transgenerational epigenetic modification of hop^{Tum-l} tumorigenicity by the M(Tum-l) mutations, we conducted a detailed analysis of Kr, which is one of the first zygotically transcribed "gap" genes whose activity is required for the correct segmentation of the embryo [17]. First, we tested two loss-of-function alleles of Kr (Kr^1 and Kr^2) and found that they both enhanced hop^{Tum-l} genetically and epigenetically (Figure 1A; unpublished data), confirming Kr as an E(Tum-l) with epigenetic effects.

To rule out any genetic background effects, we extensively outcrossed a Kr^1 allele, and isogenized and rebalanced it over a CyO balancer chromosome that in previous testing showed no enhancement of hop^{Tum-l} (see Materials and Methods). The new iso-Kr^1/CyO stock again enhanced hop^{Tum-l} tumorigenicity both genetically and epigenetically, such that when $hop^{Tum-l}/+$ females were crossed to iso-Kr^1/CyO males both $hop^{Tum-l}/+$; $Kr^1/+$ and $hop^{Tum-l}/+$; $+$/CyO progeny exhibited significantly higher TI (Figure 1B, columns 2 and 3). Interestingly, when F1 males of $+$/Y; $+$/CyO, which did not inherit Kr^1, were backcrossed to $hop^{Tum-l}/+$ females, we found that the enhancement persisted in the F2 generation in the absence of Kr^1, but diminished in the F3 (Figure 1B, columns 4 and 5). Since half and a quarter of the P0 paternal DNA contents (originally exposed Kr^1) are inherited in the F2 and F3 generation, respectively, the diluting effect of the enhancement in the absence of the original mutation (Kr^1) is consistent with the idea that the modification is epigenetic in nature and is distributed genome wide at multiple loci. To rule out the possibility that Kr^1 induced genome-wide genetic mutations, we conducted the reciprocal cross, mating iso-Kr^1/CyO females with rare escaper hop^{Tum-l}/Y males. We found that Kr^1 enhanced hop^{Tum-l} only genetically but not epigenetically, such that the TI increased in $hop^{Tum-l}/+$; $Kr^1/+$ but not in $hop^{Tum-l}/+$; $+$/CyO female progeny flies (Figure 1C). The result of the reciprocal cross confirms that the modification is epigenetic in nature, as genetic mutations (changes in DNA sequence) would not be reversible under normal circumstances. However, such a result could also suggest a parent-specific effect of Kr on the hop^{Tum-l} mutation.

To test whether the epigenetic effects of Kr^1 are specific for the male genome, we mated $hop^{Tum-l}/+$; Kr^1/CyO recombinant females to wild-type males. In this cross, the tumor phenotype associated with hop^{Tum-l} was enhanced in both $hop^{Tum-l}/+$; $Kr^1/+$ and $hop^{Tum-l}/+$; CyO/+ progeny flies (Figure 1D), indicating that the presence of Kr^1 in the female parent can also have epigenetic effects on hop^{Tum-l} tumorigenicity in the F1 generation. Thus, it appeared that Kr^1 was capable of epigenetically altering both male and female genomes, and these alterations could be transmitted through both male and female meioses to the F1. However, the inheritance and/or ability of these parental origin alterations to modify hop^{Tum-l} tumorigenicity epigenetically in the F1 progeny appeared to depend on the presence of hop^{Tum-l} as a maternal mutation.

Transgenerational Epigenetic Effects of Histone Deacetylase Inhibitors on *Tum-l* Tumorigenicity

To further test the ability of maternal hop^{Tum-l} to maintain parental origin epigenetic changes, we examined the effects of histone deacetylase (HDAC) inhibitors on hop^{Tum-l} tumorigenicity. Since Rpd3, encoding an HDAC, was identified as one of the genes which, when mutated, exhibited both genetic and epigenetic enhancement of hop^{Tum-l} tumorigenicity (Table 1), we reasoned that the epigenetic effect of an Rpd3 mutation on hop^{Tum-l} tumorigenicity might be mimicked by HDAC inhibitors such as tricostatin A (TSA) and sodium butyrate. Indeed, TSA treatment caused increased levels of acetylated histone H3 (Figure 1E), and increased the tumor index of $hop^{Tum-l}/+$ flies from 0.38 to 0.96 ± 0.06 ($p < 0.01$). Consistent with a transgenerational epigenetic effect, when wild-type flies that had been treated with TSA were mated with untreated $hop^{Tum-l}/+$ females and the progeny were raised in the absence of the drug, the TI of $hop^{Tum-l}/+$ F1 progeny was also significantly increased (Figure 1F). As with Kr^1, no

Table 1. Genetic and Epigenetic Modification of *hopTum-l* Tumorigenicity

Category	Modifier of Tum-l	Allele Tested	Tumor Index		Epigenetic Effect
			Tum-l/+; m/+	*Tum-l/+; Bal./+*	
E(tum-l)	Tp(3;Y)ry506-85C	—	1.89 ± 0.16	0.95 ± 0.20	+
	unchained	unch[k15501]	1.74 ± 0.19	1.20 ± 0.11	+
	TBP-associated factor 1	Taf1[1]	1.63 ± 0.06	0.74 ± 0.06	+/−
	Df(2R)H3C1	—	1.58 ± 0.09	0.60 ± 0.09	−
	Cdc27	Cdc27[L7123]	1.45 ± 0.05	1.02 ± 0.08	+
	abnormal wing discs	awd[j2A4]	1.42 ± 0.06	0.41 ± 0.10	−
	Ets at 97D	Ets97D[tne-4]	1.39 ± 0.12	0.90 ± 0.08	+
	spindle E	spn-E[1]	1.38 ± 0.08	0.71 ± 0.12	−
	HEM-protein	Hem[03335]	1.34 ± 0.09	0.92 ± 0.11	+
	Toll	Tl[r4]	1.33 ± 0.04	1.00 ± 0.19	+
	sallimus	sls[1]	1.31 ± 0.08	0.47 ± 0.09	−
	Krüppel	Kr[1]	1.29 ± 0.16	1.24 ± 0.14	+
	plume	plume[k00308]	1.28 ± 0.11	0.35 ± 0.05	−
	rhino	rhi[02086]	1.24 ± 0.12	1.01 ± 0.14	+
	Suppressor of variegation 2–5	Su(var)205[5]	1.18 ± 0.06	0.65 ± 0.26	+/−
	Rpd3	Rpd3[04556]	1.17 ± 0.08	0.68 ± 0.21	+/−
	bellwether	blw[1]	1.07 ± 0.06	0.92 ± 0.05	+
	knirps	kni[6]	0.98 ± 0.07	0.84 ± 0.06	+
	Suppressor of variegation 3–4	Su(var)3–4[1]	0.94 ± 0.04	0.81 ± 0.08	+
	moira	mor[1]	0.93 ± 0.06	1.08 ± 0.19	+
	hairy	h[08247]	0.91 ± 0.07	1.04 ± 0.22	+
	labial	lab[4]	0.91 ± 0.06	0.86 ± 0.03	+
	polo	polo[01673]	0.89 ± 0.05	0.49 ± 0.12	−
	Deformed	Dfd[6]	0.88 ± 0.05	0.50 ± 0.13	−
	Enhancer of bithorax	E(bx)[ry122]	0.87 ± 0.21	0.77 ± 0.26	+
	Vacuolar H+-ATPase 55kD B subunit	Vha55[j2E9]	0.85 ± 0.13	0.60 ± 0.19	+/−
	gooseberry	gsb[01155]	0.84 ± 0.06	0.96 ± 0.17	+
	Protein tyrosine phosphatase 69D	Ptp69D[1]	0.84 ± 0.04	0.39 ± 0.04	−
	Suppressor of variegation 3–9	Su(var)3–9[1]	0.83 ± 0.03	0.82 ± 0.27	+
	domino	dom[k08108]	0.82 ± 0.03	0.89 ± 0.01	+
	piwi	piwi[06843]	0.82 ± 0.04	0.36 ± 0.05	−
	Suppressor of variegation 2–10	Su(var)2–10[03697]	0.80 ± 0.04	0.34 ± 0.13	−
Su(Tum-l)	even-skipped	eve[3]	0.08 ± 0.02	0.05 ± 0.02	+
	baboon	babo[32]	0.02 ± 0.01	0.25 ± 0.14	−
	Serrate	Ser[VX82]	0.01 ± 0.00	0.01 ± 0.00	+
	Df(3L)Exel6111	–	0.00 ± 0.00	0.02 ± 0.01	+
	lethal with a checkpoint kinase	lack[KG07014]	0.00 ± 0.00	0.34 ± 0.02	−

Tum-l/FM7 females were mated to *mutant/Balancer* (*m/Bal.*) males. Mutant alleles used for testing are listed in column 2. Tumor indices were calculated for *Tum-l/+; m/+* F1 progeny flies and their *Tum-l/+; Bal./+* siblings. Epigenetic effect (column 5) was assessed by the tumor phenotypes of the *Tum-l/+; Bal./+* F1 progeny flies. Epigenetic effects are indicated by the following categories. (+): TIs of the *Tum-l/+; Bal./+* F1 flies that were significantly different from control cross (Tum-l/FM7 females crossed to wild-type males) but not from their Tum-l/+; m/+ siblings. (+/−): the TIs were significantly different from both the control cross and siblings. (−): the TIs were significantly different from the siblings but not from the control cross. Chi-squared test was used for analyzing statistical significance.
doi:10.1371/journal.pgen.0030151.t001

epigenetic effect was found in the reciprocal cross (Figure 1F), suggesting that the presence of *hop*[Tum-l] in the early embryo is important for TSA treatment to have a trans-generational epigenetic effect on *hop*[Tum-l] tumorigenicity. A similar transgenerational epigenetic effect on *hop*[Tum-l] tumorigenicity was also found with another HDAC inhibitor, sodium butyrate (unpublished data).

Maternal *hop*[Tum-l] Mutation Maintains *Krüppel* Mutant Phenotypes in Its Absence

To investigate the maternal *hop*[Tum-l]-dependent transgenerational inheritance of epigenetic changes at the level of gene expression, we examined the effects of *hop*[Tum-l] on *Kr*-dependent expression of the pair-rule gene *fushi-tarazu (ftz)*, which encodes a homeodomain protein required for embryonic patterning [18]. It has been shown that *Kr* heterozygous embryos exhibit defects in *ftz* expression [19]. In wild-type embryos, *ftz* is expressed in seven stripes at the onset of

gastrulation (Figure 2A). In *Kr*[1]/+ embryos, however, *ftz* stripe 3 is narrow or weak (Figure 2B; also see [19]). The same *ftz* stripe 3 phenotype was found in *Kr*[2]/+ embryos (unpublished data). We wondered whether the defects in *ftz* expression might involve epigenetic alterations, and whether these defects could be passed to the next generation in the presence of maternal *hop*[Tum-l] mutation. Indeed, we found that the *ftz* promoter region is differentially methylated in *Kr* heterozygotes (see below).

We reasoned that if *hop*[Tum-l] promotes transmission of parental origin epigenetic alterations to the next generation, then the *ftz* stripe 3 defect caused by *Kr*[1] could be retained in embryos from *hop*[Tum-l]/+ mothers and *Kr*[1]/+ fathers that did not inherit *Kr*[1]. To test this, we examined *ftz* expression from a *ftz-lacZ* transgene carried on the *CyO* balancer chromosome, which contains the *Kr*[+] allele and segregates from *Kr*[1] in the F1 when *Kr*[1]/CyO *ftz-lacZ* flies are used as a parent. In embryos from male and female *Kr*[1]/CyO *ftz-lacZ* flies, 70% (n = 61/87) of
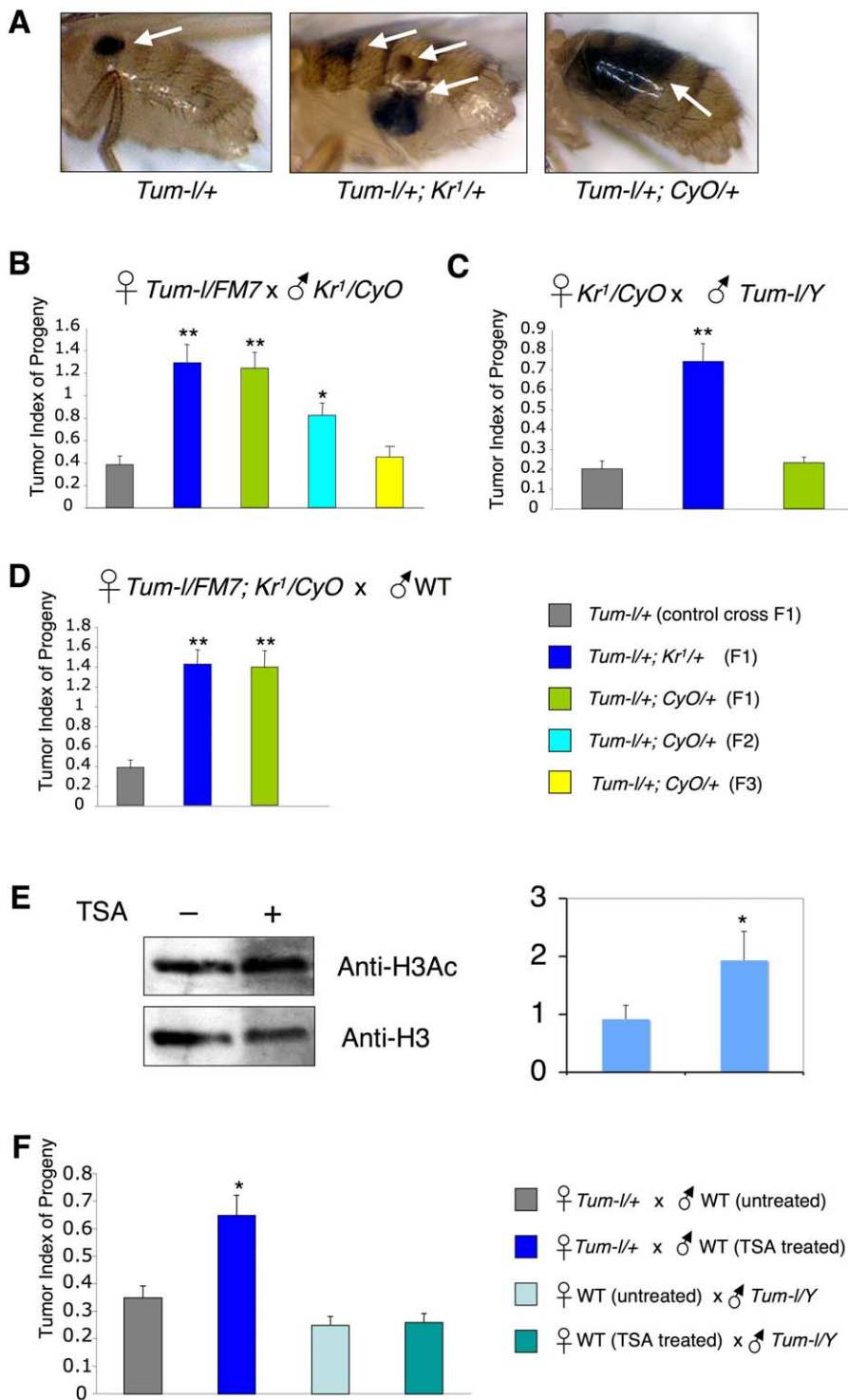
Figure 1. Epigenetic Enhancement of *hop^Tum-l* Tumorigenicity by *Kr^1* or TSA Treatment Requires Maternal *hop^Tum-l*

(A) Representative F1 progeny adult flies of indicated genotypes with blood tumors (black masses; arrows) in the abdomen are shown. The parents of these flies were *hop^Tum-l/+* females and wild type males (left), or *hop^Tum-l/+* females and *Kr^1/CyO* males (center and right).

(B–D) The tumor indices of progeny flies (genotypes are indicated in bottom right) are shown as mean and standard deviation of at least three independent crosses. "Control cross F1" were from *hop^Tum-l/+* crossed to wild type. Parental genotypes are indicated on the top. *FM7* and *CyO* are marked balancer chromosomes for the X and second chromosomes carrying a wild-type copy of the *hop* and *Kr* genes, respectively. Note that when *hop^Tum-l* was inherited from the mother (B, D), but not from the father (C), *Kr^1* epigenetically enhanced *hop^Tum-l* tumorigenicity.

(E) Total protein extracts from adult flies raised on food containing 4.5 μM TSA were subjected to SDS-PAGE and blotted with anti-acetyl-H3. The membrane was stripped and reblotted with anti-H3 (full-length gel image is shown in Figure S1). Quantification of three independent blots is shown to the right.

(F) Tumor indices of F1 progeny from wild-type flies treated or untreated (control) with TSA and *hop^Tum-l/+* females or males as shown. The F1 were raised in the absence of TSA. Tumors were counted in F1 that inherited *hop^Tum-l*. Note the parent-of-origin differential effects on the tumorigenesis of F1 flies. Three independent crosses with >200 progeny from each cross were counted. *, $p < 0.01$; **, $p < 0.001$, Student's *t*-test.

doi:10.1371/journal.pgen.0030151.g001
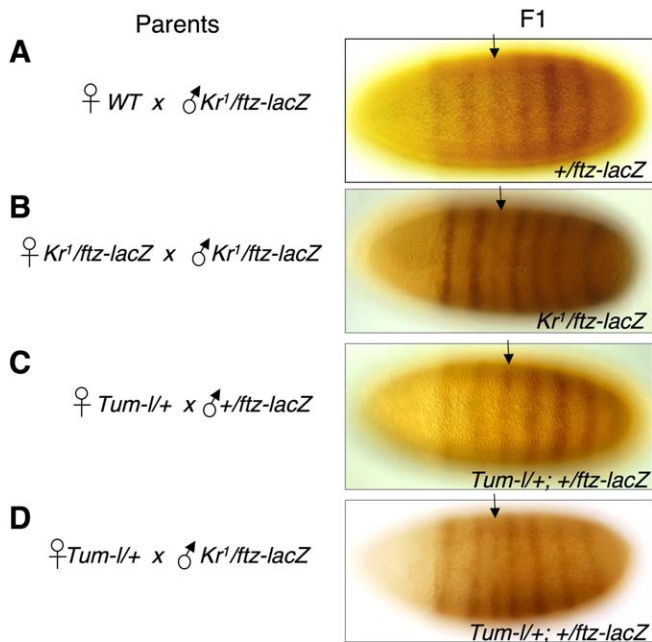
Figure 2. Maternal hop^{Tum-l} Maintains Kr^1 Mutant Phenotypes in Absence of Kr^1

ftz expression pattern in stage 4 embryos from a *ftz-lacZ* transgene carried on the *CyO* balancer chromosome was analyzed by anti-β-gal staining. Embryos were produced by crosses indicated to the left. Note the wild-type *ftz* expression pattern in (A) and (C). Also note that the *ftz* stripe 3 (arrow) is faint or missing Kr^1 heterozygotes (B) or in progeny from $hop^{Tum-l/+}$ females and $Kr^1/ftz-lacZ$ males (D), which did not inherit Kr^1.

doi:10.1371/journal.pgen.0030151.g002

the β-gal⁺ embryos exhibited the typical Kr^1 heterozygous defects, characterized by weakened or narrowed stripe 3 expression (Figure 2B), suggesting that all embryos that are genotypically Kr^1/CyO *ftz-lacZ* exhibit the stripe 3 defect. When Kr^1/CyO *ftz-lacZ* flies were crossed to wild-type flies, in the F1 embryos, *ftz-lacZ* was expressed in seven stripes identical to those in the wild-type background, such that these stripes were more or less evenly spaced and similar in intensity (Figure 2A; n = 54). When hop^{Tum-l} females were mated to +/CyO *ftz-lacZ* males, we found wild-type *ftz-lacZ* pattern and no stripe 3 defects similar to those in Kr^1 heterozygotes in the F1 embryos (Figure 2C; n = 78). Notably, although the JAK/STAT pathway is involved in regulating *even-skipped* stripe 3 expression [20,21], *ftz* expression seemed not affected in hop^{Tum-l} mutants. This is consistent with a lack of STAT-binding sites in the *ftz* promoter region (unpublished data). However, when Kr^1/CyO *ftz-lacZ* males were mated to hop^{Tum-l} females, 94% of the F1 β-gal⁺ embryos retained the stripe 3 defect characteristic of Kr^1 heterozygotes (Figure 2D; n = 48/51). Since in this mating scheme *ftz-lacZ* segregated from Kr^1, embryos that expressed the *ftz-lacZ* transgene would not inherit Kr^1 and were genotypically +/+ for the *Kr* locus. Thus, the presence of hop^{Tum-l} caused retention of the Kr^1-specific defective *ftz* expression pattern in embryos that did not inherit the Kr^1 mutation. These results demonstrate that hop^{Tum-l} can cause transgenerational inheritance of epigenetic changes at a transcriptional level.

## Transgenerational Inheritance of Kr-Induced ftz Promoter Methylation

To identify the epigenetic alterations caused by *Kr* mutations, we examined the DNA methylation status of the 620-bp minimal *ftz* enhancer in the *ftz-lacZ* transgene, as the expression of this *ftz-lacZ* is epigenetically modified by Kr^1. DNA methylation is the predominant epigenetic modification, and methylation of CpG islands is responsible for gene silencing and genomic imprinting in mammals [5–7]. There is evidence for the presence of DNA methylation in *Drosophila* [22,23]. *Drosophila* has a Dnmt2-like DNA methyltransferase that mediates methylation of cytosine residues in vivo [24], although the biochemical activity of *Drosophila* Dnmt2 as a DNA methyltransferase is still to be shown. Methylated cytosines in both CG and CT dinucleotides have been found in many transposons and repetitive sequences in *Drosophila* genomic DNA [25], and increased promoter DNA methylation is associated with gene silencing [26].

We first assessed the methylation status of the *ftz* minimal enhancer (Figure 3A) by digesting total genomic DNA with a methylation-sensitive restriction enzyme BstUI, which cuts unmethylated but not methylated CGCG sequences, followed by quantification of the undigested DNA by PCR. By comparing the time courses of BstUI digestion of genomic DNA samples isolated from $Kr^{+/-}$ versus wild-type control flies, we concluded that the former is more resistant to BstUI digestion (Figure 3B and 3C, top panels). Digestion of the same DNA samples with a methylation-insensitive restriction enzyme HaeIII produced no differences between the two samples (Figure 3B and 3C, bottom panels). These results suggest that the minimal enhancer of *ftz-lacZ* in $Kr^{+/-}$ flies is more methylated than in wild-type flies.

We next investigated whether the *Kr*-dependent differential methylation of the *ftz* minimal enhancer can be passed to the next generation. We crossed Kr^1/CyO *ftz-lacZ* flies to $hop^{Tum-l}/+$ and wild type females, respectively, and isolated genomic DNA from the F1 flies that inherited the *ftz-lacZ* transgene. We analyzed the methylation status of the 620-bp minimal *ftz* enhancer using methylation-sensitive and -insensitive restriction digests as described above. Indeed, we found the *ftz* enhancer in F1 flies of $hop^{Tum-l}/+$ females and Kr^1/CyO *ftz-lacZ* males was more resistant to a methylation-sensitive restriction enzyme than the *ftz* enhancer in F1 flies of +/+ females and Kr^1/CyO *ftz-lacZ* males (Figure 3D and 3E), consistent with the idea that hop^{Tum-l} promotes transgenerational inheritance of epigenetic changes.

We employed a second method to confirm that the promoter of the *ftz-lacZ* transgene has increased DNA methylation in *Kr* mutants and that this methylation status is transgenerationally inheritable in the presence of hop^{Tum-l} maternal mutation. We isolated total genomic DNA from embryos of different parental genotypes, digested with restriction enzymes, and incubated with antibodies against methylated cytosine. Quantification of immunoprecipitated DNA indicates that that the *ftz-lacZ* fragment was more methylated in embryos of Kr^1/CyO *ftz-lacZ* flies (Figure 3F) and the higher levels of methylation was maintained in embryos from Kr^1/CyO *ftz-lacZ* fathers and hop^{Tum-l} mothers (Figure 3G).

Finally, to further demonstrate the differential methylation of the *ftz* minimal enhancer in different genetic backgrounds or pedigrees, we treated the genomic DNA samples with
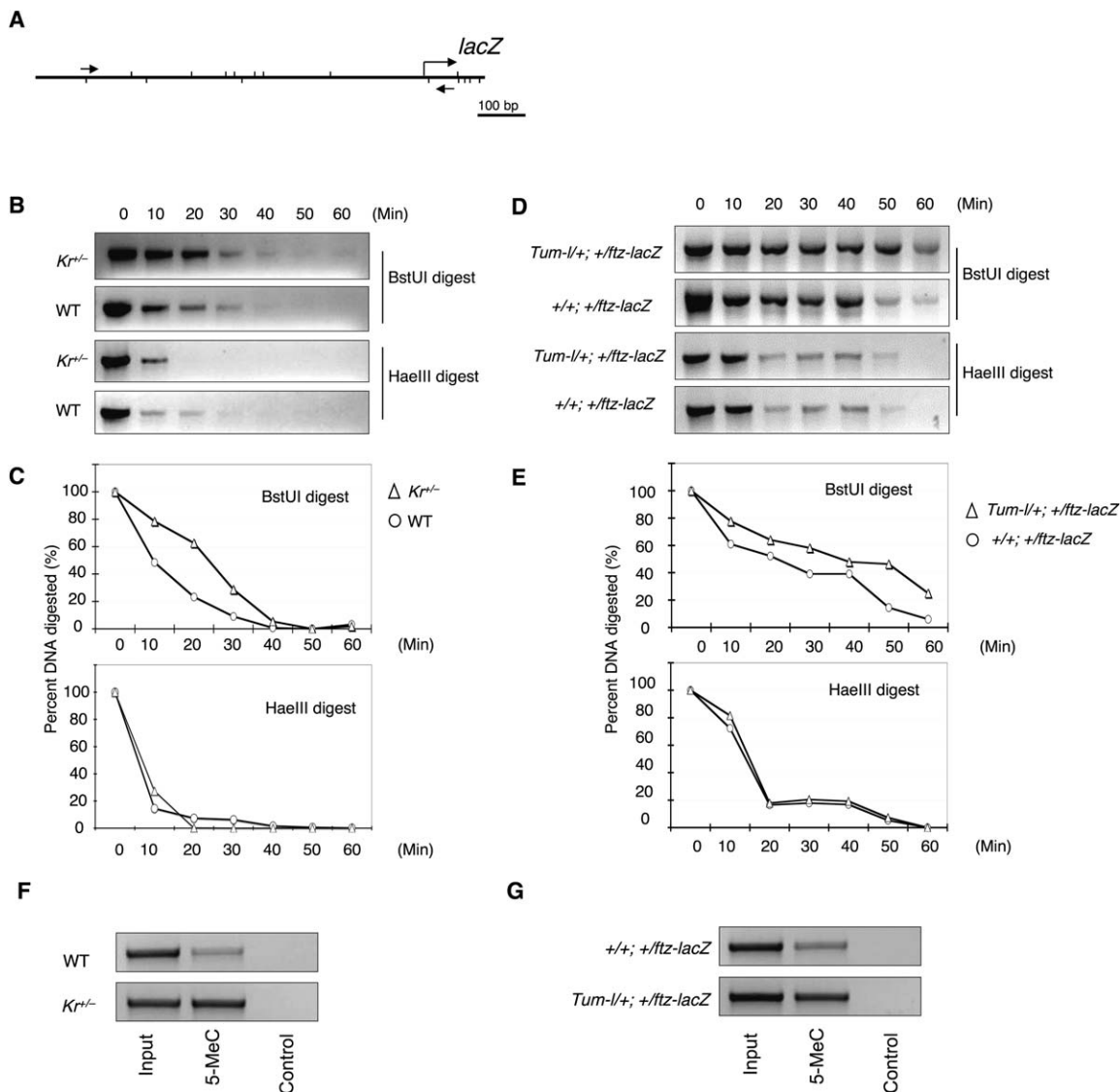
Figure 3. Increased Methylation of *ftz* Regulatory Region in *Kr*^{+/−} Animals and Its Inheritance

(A) Schematic representation of the *ftz-lacZ* reporter, showing the minimal *ftz* 5′ regulatory region previously shown to be sufficient to drive expression of a *ftz-lacZ* reporter transgene in *ftz* patterns [30]. Arrows above and below the horizontal line represent PCR primers used to amplify a 778-bp fragment, encompassing the 620-bp minimal *ftz* enhancer (see Figure S2 for sequence). Bent arrow indicates the start of the *lacZ* sequence. Vertical bars above and below the line represent positions of recognition sequences for restriction enzymes BstUI (CGCG) and HaeIII (GGCC), respectively.

(B–E) Time courses of restriction digests of genomic DNA with enzymes sensitive (BstUI) or insensitive (HaeIII) to methylated DNA are shown as agarose gel pictures and quantifications. Genomic DNA was isolated from *Kr*[1]*/CyO ftz-lacZ* and *+/CyO ftz-lacZ* (wild-type control) adult flies (B, C), or from the F1 progeny flies of *Kr*[1]*/CyO ftz-lacZ* males crossed to *hop*[Tum-l]/+ females or wild-type females (D, E), and digested with the indicated enzymes for the indicated times (minutes). Digested DNA was amplified with PCR primers shown in (A) and run on an agarose gel. Note that the genomic DNA from *Kr*^{+/−} flies or from the F1 progeny of *hop*[Tum-l]/+ females and *Kr*[1]*/CyO ftz-lacZ* males is more resistant to BstUI digestion than the controls.

(F, G) Digested genomic DNA purified from embryos was immunoprecipitated by antibodies to methylated cytosine and amplified by PCR primers shown in (A). Embryos derived from wild-type or *Kr*^{+/−} parents (F), or from *Kr*[1]*/ftz-lacZ* males crossed to wild-type (top) or *Tum-l/+* (bottom) females (G) were used for DNA isolation. Note the presence of higher levels of 5-meC in the *ftz-lacZ* promoter in *Kr*^{+/−} embryos or in those from *Tum-l/+* females crossed to *Kr*[1]*/ftz-lacZ* males.

doi:10.1371/journal.pgen.0030151.g003

sodium bisulfite, which converts cytosines (C) to thymidines (T), and then cloned and sequenced independent clones for each sample. Sequencing results indicated the presence of two CG (or CT)-rich "islands" in the *ftz* minimal enhancer that are preferentially methylated in *Kr*^{+/−} samples or in embryos of *Kr*[1]*/CyO ftz-lacZ* father and *hop*[Tum-l] mothers (Figure 4). Thus, *Kr* mutations indeed induce epigenetic alterations, as exemplified by increased DNA methylation in the *ftz* minimal enhancer, and such alterations are normally

erased, but are transmitted to the next generation if an overactivated JAK kinase is present in the early embryo.

5-Aza-dC Treatment Promotes Tumorigenesis but Inhibits the Epigenetic Effects of *Kr* on *hop*[Tum-l] Tumorigenicity

Since the epigenetic effects of *Kr* mutations involve DNA methylation, we investigated the effects of inhibiting DNA methylation on the ability of *Kr* mutations in promoting *hop*[Tum-l] tumorigenesis. We raised flies in food containing the
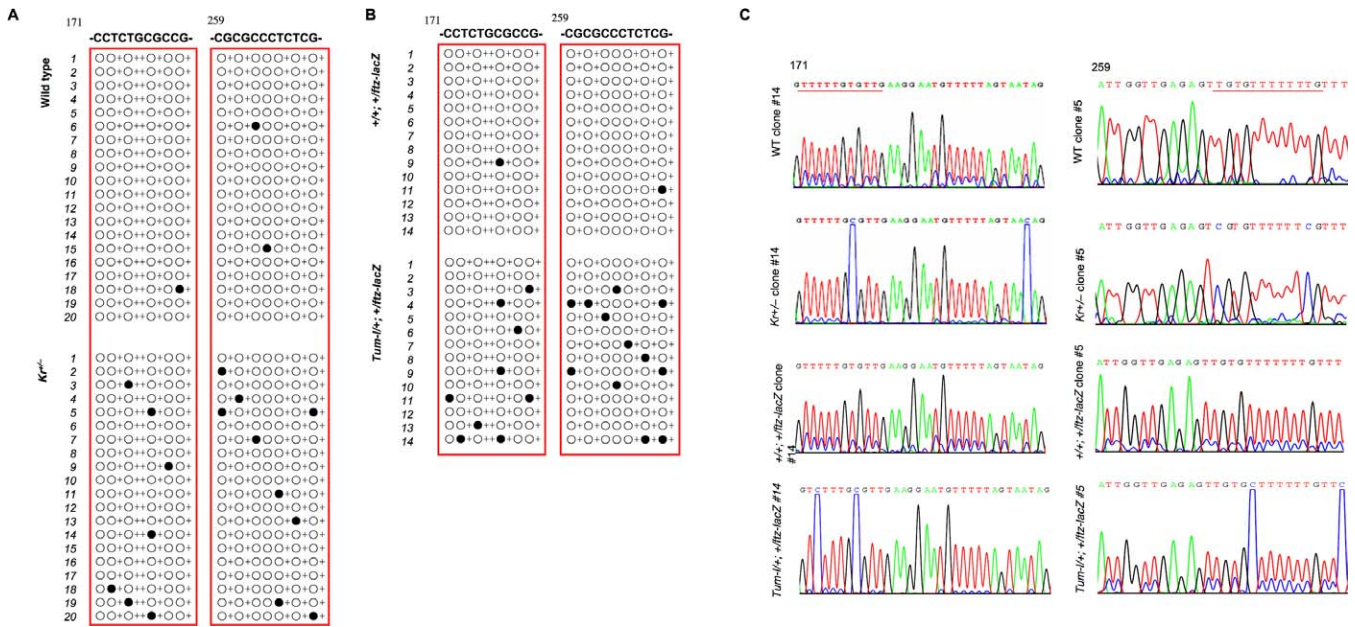
Figure 4. Methylation of CG/T "Islands" in the *ftz* Minimal Enhancer

Genomic DNA isolated from animals of different genotypes was treated with sodium bisulfite and independent clones of the *ftz-lacZ* were sequenced. Sequencing results of two CG- or CT-rich boxes in the *ftz* minimal enhancer from animals of indicated genotypes are shown in red boxes, with the original sequences on top. Numbers above the sequences indicate positions from the forward PCR primer (see Figure S2 for primer sequence). Numbers to the left indicate sample numbers of independent clones. Unmethylated C's, which are converted to T's following bisulfite treatment, are indicated by open circles. Methylated C's, which remain as C's following bisulfite treatment, are represented as filled circles. (A) Genomic DNA was isolated from Kr^1/CyO ftz-lacZ and +/CyO ftz-lacZ (wild-type control) adult flies (clones 1–15) and embryos (clones 16–20), and (B) from embryos of Kr^1/CyO ftz-lacZ males crossed to $hop^{Tum-l/+}$ females or wild-type females. Note that there are more unconverted C's in lower panels, indicating higher levels of methylation in these samples. Low background levels (<1%) of unconverted C's were found in other regions for both samples. (C) Sample chromatograms with clone number indicated.

doi:10.1371/journal.pgen.0030151.g004

DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) and determined the effects of drug treatment on hop^{Tum-l}–dependent blood tumor formation. When raised at 100 μM 5-aza-dC (a nonlethal dose), $hop^{Tum-l/+}$ flies exhibited dramatically increased tumors compared with untreated $hop^{Tum-l/+}$ flies, with TI increased from 0.41 ± 0.05 (untreated; $n = 116$) to 1.27 ± 0.15 (treated; $n = 68$; $p < 0.001$). Such results are in line with TSA treatment (see above). Similar to the effects of TSA treatment, when wild-type male flies raised in 5-aza-dC were crossed to $hop^{Tum-l/+}$ females and allowed to produce eggs in the absence of the drug, the F1 flies exhibited increased TIs (Figure 5), but no TI increase was detected in the reciprocal cross (unpublished data), suggesting a maternal hop^{Tum-l}-dependent transgenerational inheritance. Interestingly, we found that treatment with 5-aza-dC, although by itself promotes hop^{Tum-l} tumorigenesis, abolished the ability of *Kr* mutations to epigenetically enhance tumors, such that when Kr^1/CyO male flies raised on 5-aza-dC food were crossed to $hop^{Tum-l/+}$ females, the epigenetic effects (associated with CyO), but not the genetic effects of *Kr*, were abolished (Figure 5). Thus, the DNA methylation methyltransferase inhibitor 5-aza-dC both promotes hop^{Tum-l} tumorigenesis and inhibits *Kr* epigenetic effects. These results suggest that hop^{Tum-l}-induced blood tumors can be both enhanced by a general loss of genomic DNA methylation and suppressed by preventing *Kr* mutation–induced methylation in specific promoters.
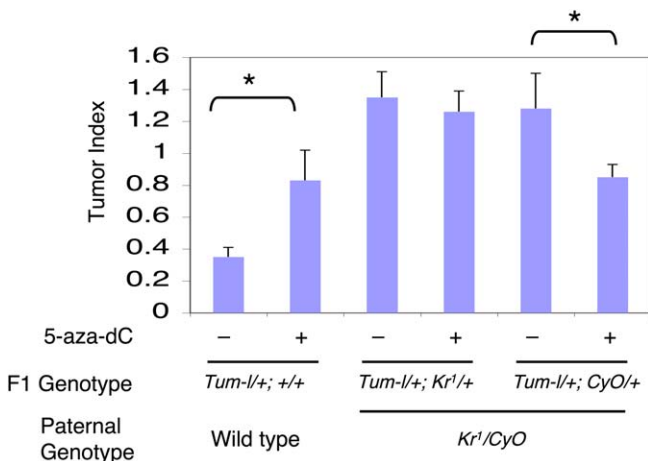
Figure 5. Effects of the Methyltransferase Inhibitor 5-Aza-dC on Tumorigenesis

Wild-type or Kr^1/CyO flies were raised in food supplemented with (+) or without (−) 5-aza-dC. Male flies of indicated genotypes were then crossed to $hop^{Tum-l/+}$ females and the F1 was raised in regular food in the absence of 5-aza-dC. F1 flies of the indicated genotypes were scored for the presence of melanotic tumors. TIs are shown as average ± standard deviation. * indicates significant difference by Chi-squared test.

doi:10.1371/journal.pgen.0030151.g005

Discussion

We have investigated the effects of genetic and epigenetic mutations on the tumorigenicity of the *Drosophila* hematopoietic oncogene hop^{Tum-l}, and found that hop^{Tum-l} and its modifier mutations mutually influence each other, both

genetically and epigenetically. In particular, we have shown that a *Kr* mutation that enhances hop^{Tum-l} tumorigenicity induces *ftz* promoter methylation, which is associated with repression of *ftz* stripe 3, and that *Tum-l* promotes trans-generational inheritance of *ftz* stripe 3 silencing in the F1 generation in the absence of the *Kr* mutation.

Taken together, these results suggest that the oncogenic JAK kinase encoded by hop^{Tum-l} is able to antagonize a cellular program that erases epigenetic markings of parental origin, allowing such epigenetic alterations to be maintained in the F1 even in the absence of the original genetic mutation. The epigenetic alterations in turn influence the risk of hop^{Tum-l}-induced tumorigenesis in the F1 generation.

Epigenetic Effects of hop^{Tum-l} Modifier Mutations

Many of the *M(Tum-l)* genes that exhibited paternal-effect modifications encode products with known chromatin remodeling functions. These include HP1, Rpd3, and several Suppressor of variegation (Su[var]) mutations. It is conceivable that flies heterozygous for these mutations have altered chromatin states that could directly influence the epigenetic state of the zygote, leading to paternal effects as shown recently in mice [27]. However, the *M(Tum-l)* genes that exhibited epigenetic effects on *Tum-l* tumorigenicity also include those whose functions in chromatin modification are not obvious. These include transcription factors such as Kr and signaling molecules such as the Notch ligand Serrate (Ser). This observation suggests that genetic mutations in genes other than those encoding chromatin remodeling proteins may also cause epigenetic alterations.

Although *Kr* is expressed only in 20% of the early embryo, lacking Kr causes profound patterning defects, resulting in deletion or defects in over 70% of embryonic segments [28]. As a first zygotically expressed "gap" gene, *Kr* is in the top tier of the regulatory hierarchy that controls pattern formation of the whole organism [28]. Thus, *Kr* mutations can affect expression of genes that are not directly regulated by Kr. A Kr neomorphic allele (Kr^{if}) has been shown to affect eye development by an epigenetic mechanism [29]. Our results indicate that the *Kr* mutation, which likely acts early on, results in the establishment of an epigenetic signature in the genome in the form of methylation of particular promoters, such as the *ftz* promoter. Repression of certain "tumor suppressor genes" may explain the enhancement of the hop^{Tum-l} tumorigenic phenotype by *Kr* mutations. As an epigenetic modification, DNA methylation is believed to be mitotically stable. In support of this notion, we detected similar methylation patterns in the *ftz-lacZ* promoter in embryos and adult flies of *Kr* heterozygotes (Figure 4). Although we have not directly examined germ cells, the transgenerational phenomenon suggests that the *Kr*-dependent epigenetic signature extends to germ cells, which give rise to sperm and eggs. We envision the possibility that the epigenetic signature of germ cells is established early together with somatic cells, and can be affected by mutations in *Kr*, which might have a global reach in the early embryo. Alternatively, there is constant communication between germ cells and somatic cells during animal development, such that their epigenetic states will stay in "sync." The precise mechanisms by which germ cells acquire the epigenetic states of somatic cells remain to be investigated.

Requirement of Maternal hop^{Tum-l} for Inheritance of Epigenetic Mutations

When hop^{Tum-l} is inherited from the mother, its product, a hyperactive JAK kinase, is present in the embryo from the very beginning as a maternal contribution. In contrast, when inherited from the father, the hop^{Tum-l} gene product is not present in the early embryo but is expressed as a zygotic gene. Zygotic genes are not transcribed until the midblastula transition or later. The parent-of-origin effect of hop^{Tum-l} on the ability of Kr^1 to modify its tumorigenicity suggests the following scenario. The *M(Tum-l)* mutations are capable of altering the state of the chromatin, resulting in epigenetic changes in the genome. These "epigenetic marks" can be maintained through mitosis and meiosis and transmitted to the F1 progeny, where they are normally erased in the zygote during early embryogenesis. However, the hop^{Tum-l} mutation, if present in the early embryo as a maternal-effect mutation, is able to preserve certain epigenetic alterations of parental origin. In other words, hop^{Tum-l} may play a role in counter-acting a mechanism that erases epigenetic marks of parental origin during early embryogenesis.

Materials and Methods

Fly stocks and genetics. All crosses were carried out at 25 °C on standard cornmeal/agar medium. All fly stocks, including hop^{Tum-l}, *Kr* alleles, *CyO [ftz-lacZ]*, and the Bloomington Deficiency Kit Stocks, are from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/). Accession numbers for mutations used in this study are list in Table S1.

Hematopoietic tumors induced by hop^{Tum-l} were scored in adult flies, which manifest as melanotic masses most frequently found in the abdomen (see Figure 1A), but were also found in other parts of the body. Tumors of all sizes and locations were scored. Typically more than 200 progeny flies were scored for each cross. More than two independent crosses were scored and the results averaged. Tumorigenicity was quantified by TI, which is defined as the sum of tumor size times occurrence, and divided by the total number of flies of a particular genotype (TI = $\sum$[tumor size × n]/N, where n is the number of occurrences for a particular tumor size and N is the total number of flies counted for a particular genotype). Tumor size 1 is defined as a tumor with a diameter equal to the width of an average abdominal segment (see Figure 1A). TI 1.0 is equivalent to all flies of a category each having a 1.0 size tumor.

To eliminate genetic background effects, hop^{Tum-l} and Kr^1 hetero-zygotes were outcrossed to a $y^1 w^1$ stock for ten generations. hop^{Tum-l} was monitored by the presence of melanotic tumors in the females in each generation. To recover Kr^1 from the outcrossed progeny, ten $y w$ virgin females were selected after five generations of outcrossing and individually crossed to a $y^1 w^1$; Sco/CyO $ftz-lacZ$ stock (in $y^1 w^1$ background). Three males from the F1 of each cross were individually backcrossed to $y^1 w^1$; Sco/CyO $ftz-lacZ$ flies (to maintain a stock) and the same male was testcrossed to Kr^2/CyO flies. The presence of Kr^1 was inferred by noncomplementation in the testcross, and a $y^1 w^1$; Kr^1/CyO $ftz-lacZ$ male was used to repeat the same outcrossing procedure one more round to establish an outcrossed $y^1 w^1$; Kr^1/CyO $ftz-lacZ$ stock.

Antibodies, drug treatment, and embryonic phenotypes. Anti-H3Ac and anti-H3 (both from Upstate, http://www.upstate.com/) were used as 1:1,000 dilutions in Western blots, sheep anti-5-meCytidine (Abcam, http://www.abcam.com/) was used for precipitating methylated DNA. For treatment with HDAC or methyltransferase inhibitors, flies were cultured in food containing TSA (4.5 µM; Sigma, http://www.sigmaaldrich.com/), sodium butyrate (10 mM, Sigma), or 5-aza-dC (100 µM; MP Biomedicals, http://www.mpbio.com) at 25 °C. To detect β-gal expression from the *ftz-lacZ* transgene, mouse anti-β-gal (1:1,000; Promega, http://www.promega.com/) and a biotinylated secondary antibody and the ABC Elite Kit (Vector Laboratories, http://www.vectorlabs.com/) were used for whole-mount immunos-taining of embryos. Signals were detected with DAB solution according to the manufacturer's recommendations. Stained embryos were dehydrated with ethanol, mounted with Euparal, and photo-graphed with an Axiophot microscope using DIC optics.

Analyses of genomic DNA methylation. Gemonic DNA was isolated

using the DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions with minor modifications. Thirty 1–2-d-old adult flies or 100 μl of 0–12-h embryos of desired genotypes were homogenized in 180 μl of PBS and 20 μl of proteinase K (1 mg/ml) per manufacturer's protocol. The samples were treated with DNase-free RNase A (Sigma) for 2 h at 37 °C prior to column purification. For restriction digests, 3 μg of genomic DNA was incubated with 10 units of BstUI (New England Biolabs, http://www.neb.com/) or 10 units of HaeIII (New England Biolabs) in 60 μl of total volume at 37 °C. At different time points, an aliquot of the digests was removed and heated at 80 °C to inactivate the restriction enzyme. One microliter of each sample was used for PCR amplification with primers specific to *ftz-lacZ* (forward: 5′-CCCAGGGATCGGACGTAATGTTAT-3′; reverse: 5′-GGATGTGC TGCAAGGCGATTAAGT-3′). Bisulfite treatment was carried out with the EpiTect Bisulfite Kit (Qiagen, http://www1.qiagen.com/) according to the manufacturer's instructions. Genomic DNA (2 μg) was treated in Bisulfite Mix. Treated genomic DNA was amplified with the following strand-specific primers (forward: 5′-TTTAGGGATTG GATGTAATGTTAT-3′; reverse: 5′-AAATATACTACAAAACAATTA AAT-3′). The PCR fragments were cloned into pGEM-T vectors (Promega) and independent plasmid DNA isolates were sequenced. Sequencing was carried out by Gene Gateway (http://www.genegateway.com/). For immunoprecipitation, genomic DNA was first digested to completion with EcoRI and BamHI (New England Biolabs). Digested genomic DNA (2 μg) in 200 μl was used for immunoprecipitation with 5 μl of anti-5-meC (Abcam) or control antibody at 4 °C overnight, together with protein-G beads that had been preabsorbed with sonicated single-stranded salmon sperm DNA. The antibody complex was centrifuged and washed and eluted. The presence of *ftz-lacZ* promoter sequence was quantified by PCR with the above primers.

Supporting Information

Figure S1. Full-Length Western Gel Images for Figure 1E

Found at doi:10.1371/journal.pgen.0030151.sg001 (1.5 MB JPG).

Figure S2. The *ftz* Minimal Enhancer

Partial sequence of the *ftz-lacZ* transgene is shown. PCR primers used in amplification of the genomic fragment are indicated in green and purple. Numbers correspond to nucleotide position of the PCR fragment. Two CG or CT-rich sequences are boxed in red.

Found at doi:10.1371/journal.pgen.0030151.sg002 (1.6 MB JPG).

Table S1. Accession Numbers for Mutations Used in This Study

Found at doi:10.1371/journal.pgen.0030151.st001 (21 KB XLS).

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