# Paternal imprint essential for the inheritance of telomere identity in *Drosophila*

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**Chromatin remodeling during sperm maturation could erase epigenetic landmarks on the paternal genome, creating a challenge for its reestablishment on fertilization. Here, we show that selective retention of a chromosomal protein in mature sperm protects the identity of paternal telomeres in *Drosophila*. The *ms (3)k81* (*k81*) gene is a duplication of *hiphop* that encodes a telomeric protein. Although HipHop protects telomeres in somatic cells, K81 is produced exclusively in males and localizes to telomeres in postmitotic cells, including mature sperm. In embryos fathered by *k81* mutants, the maternal supplies fail to reestablish a protective cap on paternal telomeres, leading to their fusions. These fusions hinder the segregation of the paternal genome and result in haploid embryos with maternal chromosomes. The functional divergence between *hiphop* and *k81* manifests not only in their expression patterns but also in the protein functions that they encode. By swapping the two coding regions, we show that K81 can replace HipHop for somatic protection; however, HipHop cannot replace K81 in the germ line to specify telomere identity, because HipHop ectopically expressed in the testis is removed from chromatin during sperm maturation. HipHop lacks a short motif in K81 that is essential for K81 to survive the remodeling process. We show that the combined functions of HipHop and K81 are likely fulfilled by the single ancestral *hiphop* locus in other *Drosophila* species, supporting the hypothesis that the evolutionary process of subfunctionalization was responsible for the preservation of the *hiphop-k81* duplicate.**

evolution of new genes | telomere capping | epigenetic marker | chromatin condensation | spermatogenesis

Telomeres protect ends of linear chromosomes from being recognized as DNA breaks. Loss of telomere identity leads to end to end fusions and ensuing genome instability. Telomere identity is protected by multisubunit capping complexes that specifically localize to chromosome ends. For multicellular organisms, telomere identity is maintained throughout the cell cycle in proliferating tissues, and it may also be protected through various developmental programs. How that is accomplished remains unknown.

During sperm maturation in animals from flies to men, chromatin undergoes massive remodeling that involves replacement of the bulk of chromosomal proteins, including histones, with protamines and similar proteins (1). This creates a challenge for reestablishing chromatin structures on the paternal genome on fertilization. It is believed that selective retention of paternal proteins on chromatin in mature sperm, such as centromeric histones and some histone modifications (2, 3), provides epigenetic landmarks on the paternal genome. It has been difficult to formally test this hypothesis, because loss of these proteins also disrupts somatic development. Moreover, a mark has not been identified for the paternal telomeres.

We recently identified a capping complex in *Drosophila* that includes the HP1 and Orc-associated protein (HOAP) and its interacting partners, HP1 and HOAP-interacting protein (HipHop) (4). HipHop, along with HOAP, specifically binds telomeres to prevent end to end fusions. A gene duplication event of *hiphop*, which is limited to the melanogaster subgroup, created the *ms(3) k81* (*k81*) locus (5, 6). Males mutant for *k81* are sterile (7), consistent with the testis-specific expression of *k81* (6, 8).

Here, we show that K81 marks *Drosophila* telomeres during spermatogenesis, and its loss specifically destabilizes the paternal genome postfertilization. Our study identified a paternal imprint on sperm chromatin that is essential for the functional reestablishment of the paternal genome on fertilization. By identifying residues critical for the functional divergence between HipHop and K81 and by studying the localization of the ancestral HipHop protein in other *Drosophila* species, we suggest that subfunctionalization is the underlying evolutionary force that drove both genes to fixation, illustrating a mechanism by which telomere functions drive the evolution of new genes.

## Results and Discussion

**K81 Specifically Marks Telomeres in the Male Germ Line.** To localize K81 in the male germ line, we constructed a *k81* gene with a tag expressing GFP (*gfp-k81*) under the regulatory control of the *k81* locus. *gfp-k81* rescued male fertility in *k81* males. In fixed preparations of testes from these rescued flies that had been stained with anti-GFP, K81 decorates telomeres on condensed meiotic chromosomes (Fig. 1*A*). Using both live imaging of GFP fluorescence and anti-GFP staining, we observed discrete GFP-K81 foci in spermatids of all stages, including the fully mature ones in the seminal vesicle (Fig. 1 *B–D*) (review on spermatogenesis in *Drosophila* in ref. 9). Considering that GFP-K81 localizes to meiotic telomeres and that we did not observe a postmeiotic zone in which cells lack GFP-K81 foci, we suggest that the foci on sperm chromatin are also at telomeres.

K81 is absent in the mitotic compartments of the testis, where we observed HipHop foci using an anti-HipHop antibody. On the contrary, HipHop is absent in postmeiotic cells of the testis. This is consistent with previous results from RNA in situ hybridization experiments (8) and our hypothesis that K81 replaces HipHop in cells undergoing germ-line differentiation.

**Loss of K81 Leads to Fusion of Paternal Telomeres After Fertilization.** Despite being sterile, *k81* males make motile sperm that can fertilize normal eggs. Analyses under either light microscopy or EM did not uncover any defect during spermatogenesis in these males (5, 7). Because HipHop loss in somatic cells results in telomere fusions (4), we suspected that K81 loss in the germ line might have the same outcome. We then examined meiotic chromosomes from fixed *k81* mutant testes for signs of telomere fusions but detected none in over 50 meioses from over 10 testes. In addition, if loss of K81 had led to end to end fusions, it would have interfered with chromosome segregation in either mitosis or meiosis, which we showed previously (10). Unequal segregation of chromosomal materials would have led to variable nu-

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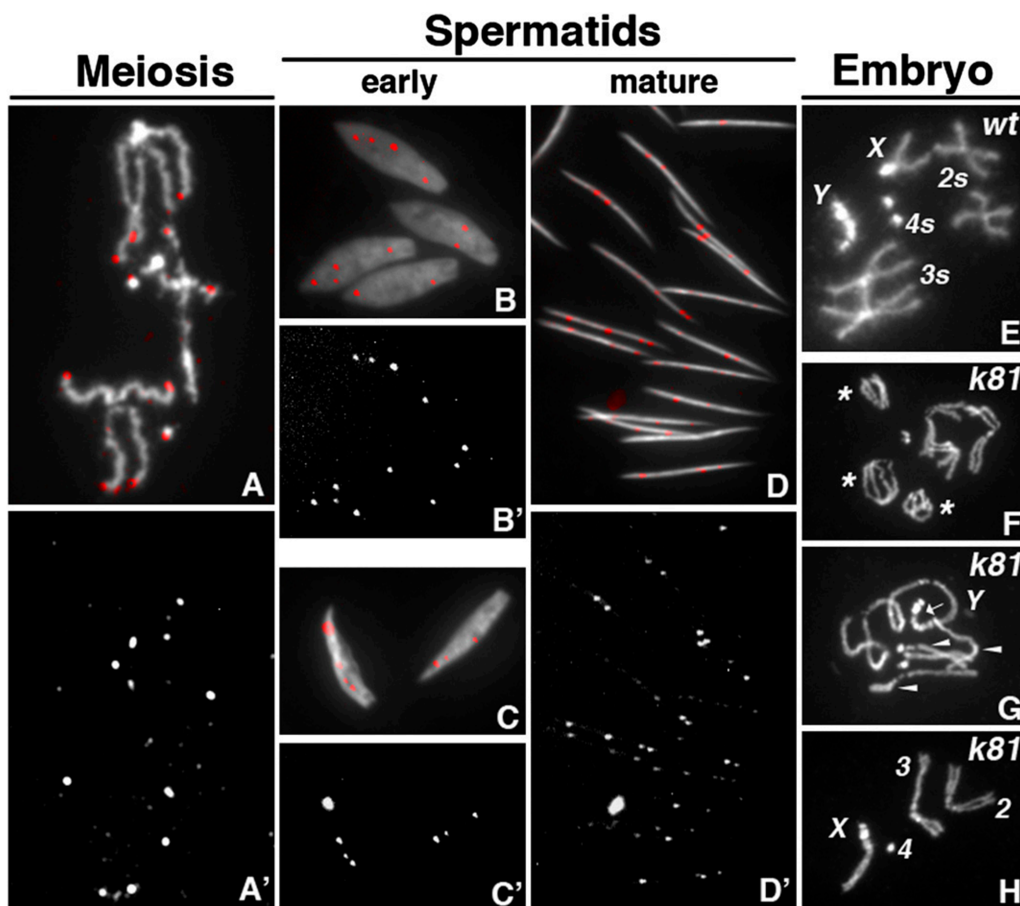



**Fig. 1.** K81 binds and protects paternal telomeres. For *A–D*, samples were prepared from testes mutant for *k81* but expressing GFP-K81. (*A*) A nucleus in meiosis II was stained with anti-GFP. Chromosomes are in white, and K81 signals in red. (*A′*) GFP signals only. (*B–D*) Merged images of GFP-K81 foci in spermatids, with DAPI signals in white and GFP signals in red. (*B′*, *C′*, and *D′*) GFP signals only. (*E–H*) Images of mitotic chromosomes from embryos. (*E*) From embryo with a WT father. (*F–H*) From embryos with *k81* fathers. In *F*, one-half of the genome engaged in intrachromosomal telomere fusions forming circular chromosomes (marked by asterisks), and the other one-half had normal, separated telomeres. In *G*, one-half of the genome (marked by arrowheads) has separated telomeres, and the other one-half engaged in interchromosomal fusions forming a chain starting from the paternal *Y* chromosome (marked with arrow). *H* shows a haploid nucleus with all of the chromosomes labeled.

clear sizes within individual sperm bundles, which we never detected in *k81* testes. Therefore, the cause for sterility is likely because of defects in postfertilization development.

*k81* is one of very few mutants that cause paternal effect lethality (11). WT eggs fertilized by *k81* mutant sperm never hatch. As previously reported, the first zygotic mitosis is invariably defective in these embryos, with a set of chromosomes failing to segregate to the daughter nuclei (5, 6). In addition, some of the embryos proceed with development, resulting in haploid embryos. Through painstaking genetic analyses, Fuyama (12) gathered convincing evidence suggesting that these haploid embryos consist of the maternal genome only, which implies that the segregation of the paternal genome is defective during the first division. We hypothesized that, in sperm lacking the protection of K81, the paternal telomeres are recognized as DNA breaks on chromatin decondensation in the embryo. Subsequently, end to end fusions involving only the paternal chromosomes prevent their segregation. The maternal telomeres, however, are protected by the HipHop proteins provided by the mother.

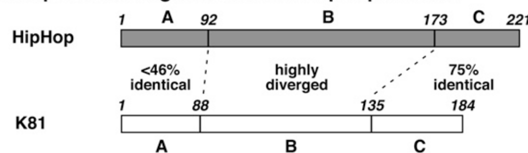
Using a recently developed cytology protocol (10), we performed mitotic squashes on embryos fertilized by *k81* sperm to provide direct evidence for the presence of telomere fusions. This has been difficult considering the fact that these embryos should have no more than one nucleus with potential telomere fusions. Nevertheless, we identified chromosome configurations that are clearly indicative of end to end fusions (Fig. 1 *F* and *G*). In some cases, fusions involved the *Y* chromosome (Fig. 1*G*), strongly suggesting that the paternal genome suffers telomere dysfunction.

We cannot rule out that the fusions that we observed in the embryos actually occurred during postmeiotic development in *k81* fathers. We consider this unlikely based on three lines of evidence. First, *Drosophila* sperm DNA carries extensive damage as assayed by Tunel staining (13). Second, DNA breaks on sperm DNA induced by radiation are not repaired until after fertilization (14). Third, postmeiotic telomeres in WT males lack the protection of the HOAP capping protein (*K81 Retains Telomere Protecting Function*), but they do not engage in telomere fusion as they would have in somatic cells that lack HOAP (4, 15). Taken together, these results suggest that the postmeiotic male germ line lacks DNA repair activities, and they are consistent with our proposition that telomere fusions did not occur until after fertilization in *k81*-fathered embryos.

In summary, we have identified the HipHop homologous K81 protein as a component of the paternal imprint that marks sperm telomeres for protection after fertilization.

**K81 Retains Telomere Protecting Function.** Although *k81* is a recent duplication of *hiphop* limited to the melanogaster subgroup, the two proteins have rapidly diverged at the sequence level (6) (Fig. 2, *I*). Because both HipHop and K81 carry essential functions, we are interested in how they have diverged functionally. We generated null mutations of *hiphop* by mobilizing a P element inserted at its 5′ UTR (Fig. S1). The mutant animals die early in development as first instars, and this lethality can be fully rescued by a *hiphop* transgene. We expressed a *gfp-k81* transgene under the regulatory control of *hiphop* in an attempt to complement HipHop functions in the soma of *hiphop* mutants. The GFP-K81 protein correctly localizes to telomeres (Fig. S2*B*). Remarkably, GFP-K81 produced in the soma was sufficient to rescue lethality, with many animals developing into adults (Fig. 2, *II*). Up to 5% of the expected class ($n > 2,000$) survives as adults, with the rest either dying as pharate adults or soon after eclosion, suggesting a near complete rescue. We examined mitotic chromosomes from larval neuroblasts of these rescued animals and observed 13 telomere fusions in 62 nuclei. This is a level fairly low compared with other telomere uncapping

**I. Sequence divergence between HipHop and K81**




**II. *hiphop* transgenes**

| Protein coding region | Total lines | Rescue *hiphop*⁻ lethality |
|---|---|---|
| mCherry HipHop | 3 | +++ |
| GFP K81 | 7 | ++[1] |

**III. *k81* transgenes**

| Protein coding region | Total lines | Nuclear foci in spermatids | | Rescue *k81*⁻ sterility |
|---|---|---|---|---|
| | | early | mature | |
| GFP K81 | 3 | yes | yes | +++ |
| mCherry HipHop | 5 | yes | no | -- |
| HipHop | 9 | yes | no | -- |
| GFP $A^HB^KC^K$ | 10 | yes | yes | +++ |
| GFP $A^KB^HC^K$ | 9 | yes | yes | +[2] |
| GFP $A^KB^KC^H$ | 8 | yes | no | -- |
| GFP $A^HB^HC^K$ | 9 | yes | yes | +[2] |
| GFP $K81^{PTV}$ * | 8 | yes | no | -- |

**Fig. 2.** Functional divergence between HipHop and K81. In I, protein-coding regions are represented as rectangular boxes, with the residues defining the *A*, *B*, and *C* segments numbered on top of the boxes. In II, transgenes with either an mCherry or GFP tag were expressed under the control of *hiphop* regulation, and all lines were able to rescue *hiphop* lethality. 1, partial rescue with some animals dying as pharate adults (in the text). In III, transgenes were under the control of *k81* regulation and were tested for their ability to rescue *k81* sterility. These transgenes were also tested for their ability to form nuclear foci in spermatids of different stages under both WT and *k81* mutant background. In each chimeric gene, a segment and its corresponding parental gene have the same shading. The asterisk in the last gene corresponds to the QFVH to PTV mutation in $k81^{PTV}$. 2, partial rescue (in the text).

mutations, but it is orders of magnitude higher than that of WT cells (16). Therefore, K81 can fulfill HipHop function in the soma, although with less efficacy. Our results contradicts those from a recent study on *k81* (17) in which it was concluded that K81 cannot substitute HipHop when expressed in the soma. The nature for this inconsistency requires further investigation.

**Chromatin Retention in Sperm Drives HipHop and K81 Divergence.** Although somatically expressed K81 can substitute for most HipHop functions, the converse is not true. We expressed HipHop, tagged with mCherry at its N terminus, in the soma and rescued *hiphop* lethality (Fig. 2, *II*). However, the same gene expressed in the testes under the control of *k81* regulatory elements failed to rescue *k81* sterility (Fig. 2, *III*). We detected mCherry foci during most stages of spermatogenesis, except in highly elongated spermatids or mature sperm. In addition, our anti-HipHop antibody, which did not detect any HipHop signal in WT postmitotic cells, was able to detect HipHop on meiotic telomeres in testes expressing mCherry-HipHop (Fig. S2*C*). We constructed a *k81*-driven *hiphop* transgene without the mCherry tag and obtained identical results (Fig. 2, *III*), ruling out any negative effect from the fluorescent tag. Therefore, HipHop expressed in the testis can correctly localize to telomeres but is excluded from chromatin in mature sperm, and this is likely responsible for HipHop's inability to substitute for K81.

To further dissect functional divergence between the two proteins, we constructed *gfp*-tagged chimeric *hiphop-k81* genes under the control of *k81* regulatory elements and tested their ability to form telomeric foci in spermatids from different stages and rescue *k81* sterility. For that purpose, we arbitrarily divided each protein into three segments (Fig. 2, *I A–C*). The N-terminal A segments between the two proteins share less than 45% residue identity. The $A^{HipHop}$ ($A^H$) fragment expressed from bacteria was able to interact with HOAP and HP1, two other components of the capping complex (4). We discovered that the N terminus of K81 possessed similar properties (Fig. S3). The B segments are the most divergent between the two proteins, with minimal similarity. There is an apparent deletion in $B^{K81}$ ($B^K$), making it about 40 residues shorter than $B^{HipHop}$ ($B^H$). The C segments are the most similar, sharing about 75% residue identity. This segment is also the most conserved region among HipHop proteins from other *Drosophila* species and among K81 proteins from the melanogaster subgroup. The chimeric $A^HB^KC^K$ protein, when expressed in the testis, formed foci in nuclei of both spermatids and mature sperm (Fig. 3 *A–D*) and fully rescued *k81* sterility. This suggests that the N-terminal fragment contributes minimally to the divergence in male functions between HipHop and K81. However, the $A^KB^KC^H$ protein formed foci in nuclei of spermatids but was excluded from chromatin in mature sperm, and it was not able to rescue fertility to *k81* males (Figs. 2, *III* and 3 *I* and *J*). This suggests that the C terminus of K81 is essential for its ability to be incorporated in the highly condensed chromatin in mature sperm and that this property was lost for the C terminus of HipHop. The behavior of the $A^KB^HC^K$ chimera was not as clear as that of the others. We observed lower levels of GFP fluorescence, suggesting a lower level of chimeric protein in the male germ line. Nevertheless, we detected nuclear GFP foci in both early and mature spermatids (Fig. 3 *E–H*). Consistent with its localization pattern, all transgenic insertions of this chimera rescued *k81* sterility, although rather poorly (Fig. 2, *III*), with the number of progeny from each mating ranging from 3 to 30. These results suggest that, although the B segment is not absolutely essential for K81 function, it nevertheless contributes significantly to the optimal performance in the male germ line of K81.

To test whether the $C^{K81}$ segment is both necessary and sufficient for K81-specific function, we expressed the $A^HB^HC^K$ chimera and discovered that it behaved essentially identical to $A^KB^HC^K$ in its ability to rescue *k81* sterility (Fig. 2, *III*). We, therefore, conclude that the C terminus of K81 is both necessary and sufficient for specifying K81 function, whereas the middle portion ensures optimal K81 functions in the testis.

The C segments share the highest degree of residue identity between HipHop and K81 and yet, account for most of the different properties between the two proteins in the male germ line. Through sequence analyses of these segments from HipHop and K81 proteins from sequenced *Drosophila* species, we identified that the conserved PTV peptide in HipHops from the melanogaster subgroup likely corresponds to QFVH in K81s from the same species (Fig. 4). Remarkably, the timing of this near bimodal division roughly correlates with that of *k81* duplication (with *D. ananassae* and possibly, *D. willistoni* as exceptions) so that the modern K81 proteins retain the QFVH motif that is present in the ancestral HipHop proteins (Fig. 4). Because one might expect that the ancestral HipHop fulfills both somatic and germ-line functions in telomere protection, we hypothesized that this bimodal peptide is likely a critical contributor to HipHop-K81 divergence. This is supported by our finding that a K81 protein with QFVH replaced with PTV ($K81^{PTV}$) formed nuclear foci in early but not mature spermatids and failed to rescue *k81* sterility (Fig. 2, *III*). Therefore, the HipHop protein from the melanogaster subgroup has lost the critical QFVH motif, which is necessary for its chromatin retention in mature sperm.

**Paternal Imprint Does Not Contain All Somatic Capping Proteins.** We showed previously that HipHop is a component of a capping complex that includes HOAP (4). It is possible that the entire complex needs to be inherited on the paternal telomeres for

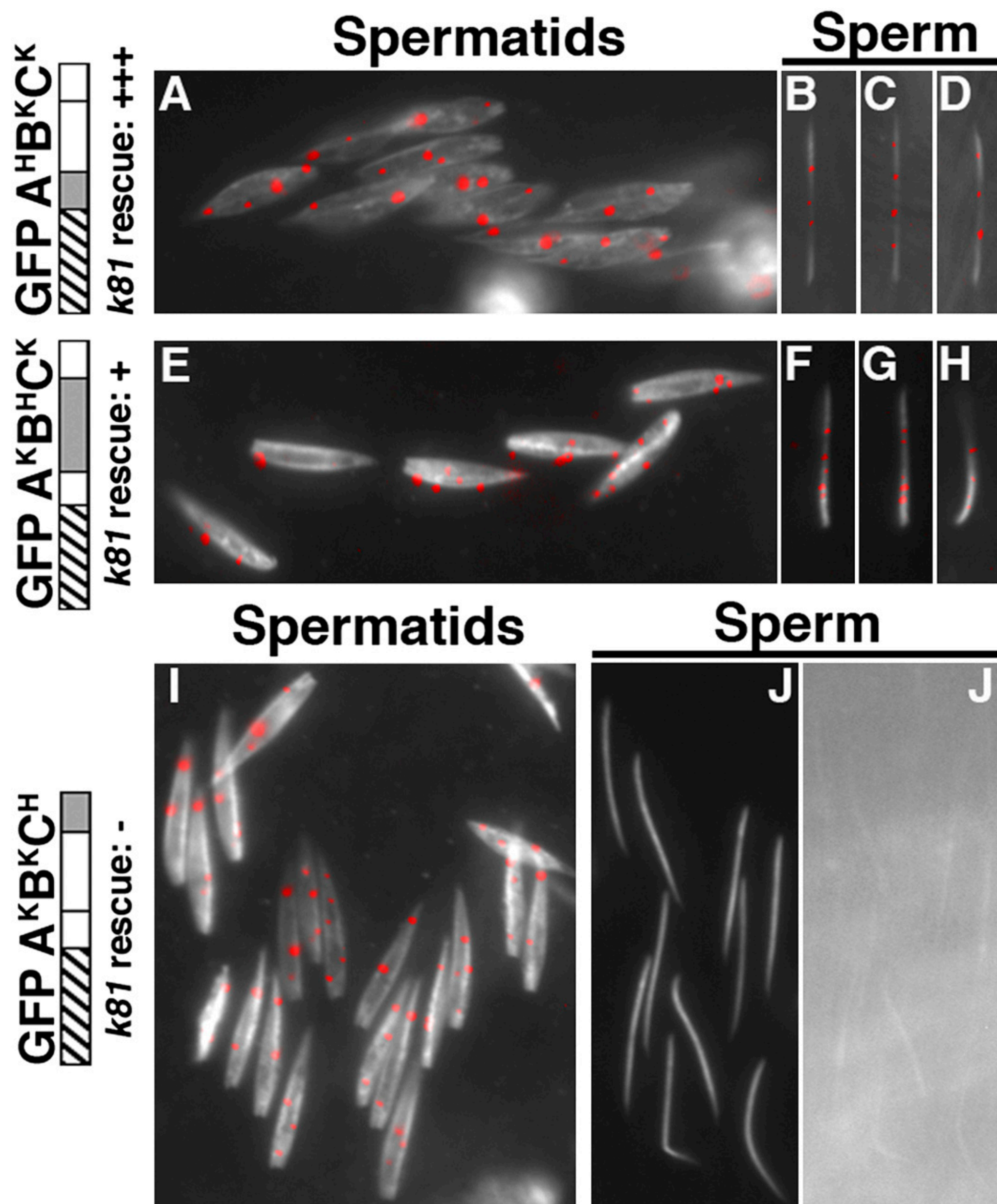



**Fig. 3.** Chromatin retention in mature sperm distinguishes K81 from HipHop. (*A*–*I*) Merged images of K81 foci in spermatids or sperm in WT males expressing various *hiphop-k81* chimeric genes tagged with GFP, with DAPI signals in white and GFP signals in red. The names and schematics of the chimeric proteins are shown to the left of picture panels. For name and shading definitions for the chimera, see Fig. 2 and the text. The extent of *k81* rescue for each construct is stated under the schematics for the chimeras. In *J*, DAPI signals from mature sperm are in white. *J*′ is an overexposed picture of the GFP channel showing no GFP foci on sperm chromatin shown in *J*.

their postfertilization protection. We investigated whether the telomere-specific HOAP protein is part of the paternal imprint.

By immunostaining with an anti-HOAP antibody, we detected HOAP on telomeres of meiotic chromosomes (Fig. 5*C*). However, we did not consistently detect HOAP foci in nuclei from spermatids in that HOAP signals were occasionally seen in elongated spermatids but never in mature sperm. To rule out antigen accessibility as a possible reason for the lack of HOAP signals in some of the postmeiotic compartments, we endogenously tagged the *hoap* locus with a *gfp* gene using the recently developed site-specific integrase mediated repeated targeting (SIRT) gene-targeting method (*SI Materials and Methods*) (18). We observed GFP foci in the premeiotic male germ line and very young spermatids (Fig. 5 *A* and *B*) but not in nuclei of older spermatids (Fig. 5*D*), which is consistent with our immunostaining results. Therefore, HOAP is displaced from chromatin soon after the completion of meiosis, and the composition of the paternal imprint is not identical to that of the conventional capping machinery.

We do not consider this result unexpected. On the contrary, it makes economical sense. Telomere protection in postmeiotic male germ line does not require activities that prevent telomere fusions but involves epigenetically marking the paternal telomeres for their protection in postfertilization embryos. A marker with fewer protein components would be more efficient, because fewer proteins would have to evolve the ability to survive the chromatin remodeling process during sperm maturation.

Our results refute the proposition by Dubruille et al. (17) that the essential function of K81 is to maintain capping complex on postmeiotic telomeres, because we showed with two different

**HipHop C-termini**

```
mel -DDYDPNSNLSLNAVNGTRLPHIQPIPKRRPT-VLDKQSMDIYLMGTTGG
sim -DDYDPNSNLSLNAVNGTRLPHIQPIPKRRPT-VLDKPSMDIYLMGTTGG
sec -DDYDPNSNLSLNAVNGTRLPHIQPIPKRRPT-VLDKPSMDIYLMGTTGG
yak -DDYDPNSNLSLNAVNGTRLPHIQPIPKRRPT-VLDQPFMDIYLQGTTGG
ere -DDYDPNSNLSLNAVNGTRLPHIQPIPKRRPT-VLDKQSMDIYLMGTTGG
ana -DD-DPNSNLSLNAVNGTRLPHIQPIPKRRPTIILNKAVMDIYLAGTTGG
pse -DD-DANSDLSLNAVNGTRLPHIQPIPKRRQVVHLNKTAMDIYLAGTTGG
per -DD-DANSDLSLNAVNGTRLPHIQPIPKRRQVVHLNKTAMDIYLAGTTGG
wil --D-DDEPLLSLNAVNGTRLPHIQPIPKRRQ-IQLTGPHLDIYLAGTTGG
moj -DD-DGESMLSLNAVNGTRLPHIQPIPKRRQFVHLNKDVMDIYLSGTTGG
vir IDD-DEEPLLSLNAVNGTRLPNIQPIPKRRQFVHLNKDVMDIYLSGTTGG
gri -SD-DDEQLLSLNAVNGTRLPNVQPIPKRRQFVSLNKDVMDIYLNGTTGG
     * * :   ************:;*******    *     :**** *****
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**K81 C-termini**

```
HipHop DDYDPNSNLSLNAVNGTRLPHIQPIPKRRPTV-LDKQSMDIYLMGTTGG
   mel DDYDPSSNLSLNAVNGTRLPNVQPIPKRRQFVHLNREAMAIYLAGTTGG
   sim DD-DPSSNLSLNAVNGTRLPNVQPIPKRRQFVHLNHQAMAIYLAGTTGG
   sec DD-DPSSNLSLNAVNGTRLPNVQPIPKRRQFVHLNHQAMAIYLAGTTGG
   yak DDDDPSSNLSLNAVNGTRLPNVQPIPKRRQFVHLNKEAMDIYLAGTTGG
   ere DDYDANSNLSLNAVNGTRLPNVQPIPKRRQFVHLNKEAMDIYLAGTTGG
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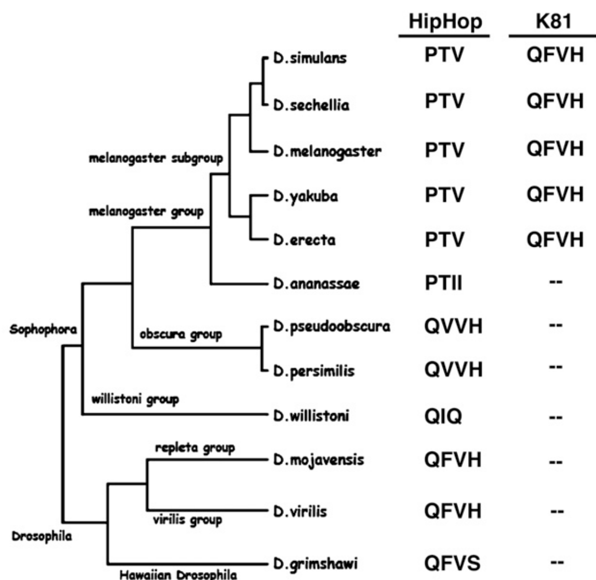


**Fig. 4.** The bimodal PTV-QFVH motif in HipHop and K81 proteins. An alignment of the Fig. 2*I* C segment (Fig. 2*I*) of HipHops from sequenced *Drosophila* species is shown at the top, with identical residues marked by asterisks underneath. The PTV and QFVH motifs are shaded gray. Species with K81 are labeled with a vertical line to the left. An alignment of the C segment of K81s from the sequenced melanogaster subgroup is shown in the middle, with melanogaster HipHop included at the top. At the bottom, the status of the PTV/QFVH motif is indicated for each species in reference to their evolutionary relationship and the status of the *k81* duplication. –, the absence of *k81*.

approaches that HOAP is undetectable from chromatin in mature sperm. Instead, we propose that the essential function of K81 is to prevent telomere fusion in the zygote by directly inhibiting end ligation and/or recruiting other members of the capping complex.

**Subfunctionalization Preserves the *hiphop–k81* Duplication.** Both HipHop and K81 carry out essential functions for the organism: HipHop for viability and K81 for male fertility. Neofunctionalization and subfunctionalization are two evolutionary models commonly put forth to explain the preservation of duplicated genes (reviewed in ref. 19). In neofunctionalization, the duplicated copy (i.e., *k81*) acquired beneficial mutations bestowing on it a new function that the ancestral copy (i.e., *hiphop*) lacks. Subfunctionalization, however, does not require the acquisition of beneficial mutations but involves degenerative mutations in both copies that lead to division of ancestral functions between the duplicates. The *hiphop-k81* case has been suggested as a case of neofunctionalization (6). As we have shown, the molecular function for both HipHop and K81 is to prevent telomere fusion, either in the soma or immediately after fertilization in embryos, although we cannot experimentally rule out that K81 had not acquired additional functions. We suggest that subfunctionalization would be the most parsimonious process that drove both genes to fixation. We propose the following scenario. Because *k81* likely duplicated through a retrotransposition-like process (6), it lost the ancestral expression regulation, making it unable to function in somatic cells. However, *hiphop* likely acquired a mutation that disrupted the QFVH motif, making it nonfunctional in testis because of the protein's inability to be retained on chromatin in mature sperm. In addition, the extra 40 residues in the B segment of HipHop reduce its efficacy in the testis. The coding changes in *hiphop* need not have happened right after the *k81* duplication. Because *k81* acquired strong expression in postmeiotic germ line, which is transcriptionally quiescent for most genes, including *hiphop* (8, 20), the K81 protein might have outcompeted the ancestral HipHop protein simply by being more abundant. This would have relieved positive selection on HipHop residues that are essential for chromatin retention in the sperm, allowing them to accumulate degenerative mutations over time. However, the fact that the PTV motif is invariant in HipHops from the melanogaster subgroup argues that the QFVH to PTV change might have occurred shortly after the *k81* duplication. However, because telomere capping is dispensable in the postmeiotic male germ line, positive selection might have been reduced on K81 residues that would have been essential for its somatic function. This is consistent with our results showing that K81 cannot fully replace HipHop in the soma.

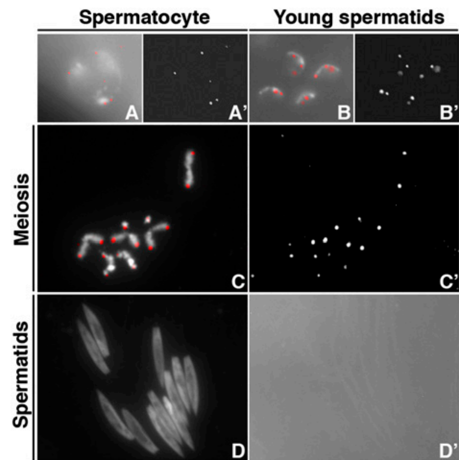


**Fig. 5.** The paternal imprint on telomeres does not contain HOAP. Samples are taken from testes with a *gfp*-tagged *hoap* locus under either live (*A*, *A*′, *B*, *B*′, *D*, and *D*′) or fixed conditions (*C* and *C*′). In the merged images *A*–*C* and image *D*, DAPI signals are in white. GFP signals are in red in *A* and *B*. In *C*, signals in red from anti-HOAP staining decorate telomeres on condensed chromosomes during meiosis II. *D*′ is an overexposed picture of the GFP channel showing no GFP foci in spermatids shown in *D*.

To provide support for our model that HipHop-K81 divergence involves subfunctionalization, we transformed *D. virilis* with a transgene in which the single *virilis hiphop* gene had been tagged with *gfp*. We observed GFP-HipHop foci, possibly telomeric, in somatic cells (Fig. 6*A*). Remarkably, we also observed nuclear foci in mature sperm from these animals (Fig. 6 *B* and *C*). Our results strongly suggest that the combined functions of HipHop and K81 are being fulfilled by the ancestral *hiphop* locus in *Drosophila* species without *k81*, such as *virilis*.

**Fig. 6.** *D. virilis* HipHop foci in both somatic and sperm nuclei. *A–C* are merged images from a *D. virilis* strain transformed with a *gfp*-tagged *hiphop* transgene. DAPI signals are in white, and GFP signals in red. (*A′–C′*) GFP signals only. (*A* and *A′*) Cells from the mitotic compartment at the tip of the testis showing GFP-HipHop nuclear foci. (*B* and *B′*) A bundle of spermatids with HipHop foci. (*C* and *C′*) Two mature sperm with HipHop foci.

In summary, the process of subfunctionalization is sufficient to account for the preservation of both *hiphop* and *k81* in the melanogaster subgroup. Interestingly, previous cases of subfunctionalization exclusively involve complementary patterns of gene expression for the duplicates. In our case, subfunctionalization also involves protein domains with complementary functions: the QFVH motif essential for sperm chromatin retention and the rest of the protein essential for the capping function.

## Materials and Methods

***Drosophila* Stocks and Genetics.** All melanogaster stocks were obtained from the Bloomington Stock Center unless otherwise noted. The *k81* alleles were gifts from Barbara Wakimoto (University of Washington, Seattle, WA). The $k81^1$ and $k81^2$ alleles were used in this study. *Df(3R)ro80b* was used as a chromosomal deficiency for *k81*. Null mutations of *hiphop* were generated as described in *SI Materials and Methods*. *Df(3L)Cat* was used as a deficiency for *hiphop*. A white-eyed stock of *virilis* was obtained from *Drosophila* Species Center at San Diego.

In both *hiphop* and *k81* rescue experiments, transheterozygotes of different mutant alleles and chromosomal deficiency were used, and they gave identical results. For testing male fertility, each mating involved two to three males with five or more virgin females for a period of 14 d. At least 10 males were tested for each line.

**Antibodies, Immunostaining, and Live Imaging.** Anti-HOAP and anti-HipHop have been previously described (4). The anti-GFP antibody was from Abcam. Immunostaining of squashed testis was performed as described (21). For live imaging of fluorescent proteins, animals were dissected in PBS, testes or salivary glands were stained with DAPI in PBS plus 0.1% Triton-X for 5 min, and they were mounted in PBS.

**Transgenes.** All transgenes are based on a modified pUAST vector with an FseI site added after the XhoI site. Upstream regulatory elements were cloned into BglII and XhoI sites. The coding region for EGFP or mCherry was cloned into XhoI and FseI sites. Downstream elements were cloned into the XbaI site. The coding regions of various *hiphop* or *k81* genes were inserted between FseI and XbaI sites. Coding regions for *hiphop* and *K81* were cloned by PCR using genomic DNA as a template, with primers carrying FseI site at the 5′ and XbaI site at the 3′. Coding regions for all of the *hiphop-k81* chimeric genes (Fig. 2, *III*) flanked by FseI and XbaI sites were synthesized by Celtek Bioscience.

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