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Origin and Neofunctionalization of a *Drosophila* Paternal Effect Gene Essential for Zygote Viability

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Summary

Background: Although evolutionary novelty by gene duplication is well established, the origin and maintenance of essential genes that provide entirely new functions (neofunctionalization) is still largely unknown. *Drosophila* is a good model for the search of genes that are young enough to allow deciphering the molecular details of their evolutionary history. Recent years have seen increased interest in genes specifically required for male fertility because they often evolve rapidly. A special class of genes affecting male fertility, the paternal effect genes, have also become a focus of study to geneticists and reproductive biologists interested in fertilization and sperm-egg interactions.
Results: Using molecular genetics and the annotated *Drosophila melanogaster* genome, we identified *CG14251* as the *Drosophila* paternal effect gene, *ms(3)K81* (*K81*). This assignment was subsequently confirmed by P-element rescue of *K81*. A search for orthologous *K81* sequences revealed that the distribution of *K81* is surprisingly restricted to the 9 species comprising the *melanogaster* subgroup. Phylogenetic analyses indicate that *K81* arose through duplication, most likely retroposition, of a ubiquitously expressed gene before the radiation of the *melanogaster* subgroup, followed by a period of rapid divergence and acquisition of a critical male germline-specific function. Interestingly, *K81* has adopted the expression profile of a flanking gene suggesting that transcriptional coregulation may have been important in the neofunctionalization of *K81*.
Conclusion: We present a detailed case history of the origin and evolution of a new essential gene and, in so doing, provide the first molecular identification of a *Drosophila* paternal effect gene, *ms(3)K81* (*K81*).

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Introduction

Understanding the origin of genes, particularly genes that encode new functions, is an important aspect of evolutionary biology. The study of recently evolved genes is particularly informative as it helps uncover the molecular mechanisms underlying evolutionary novelty and the creation of new genetic units. *Drosophila* is well suited for the search of such genes because of the relatively large number of closely related species and the availability of the *D. melanogaster* genome and other genetic and molecular tools. The reported molecular evolution studies of recently evolved *Drosophila* genes show that gene duplication, by means of DNA transposition or RNA retroposition, is involved in all cases [1–6] (reviewed in [7]). Interestingly, three of these genes are male-specific genes, supporting the general idea that male reproductive functions are subject to rapid molecular evolution [8, 9]. Likewise, older genes such as the male-specific *Drosophila* gene *Odysseus*, required for sperm development, have undergone positive selection [10, 11].

It is also interesting to note that, to our knowledge, such recently created genes have not yet been studied by classical genetic approaches and, therefore, their functional significance has not been directly assessed. Genetic analysis has identified a particularly interesting class of male reproductive genes that act during fertilization, the paternal effect genes. Paternal effects identify male contributions to fertilization and zygote formation (reviewed in [12–14]). Paternal effects are extremely rare—of the approximately 1500 genes known to affect male fertility [15], only four strict paternal effect mutants have been reported to date. Males homozygous for the paternal effect mutation *ms(3)K81 (K81)* produce motile sperm capable of entering the egg, but the resulting embryos fail to hatch [16–18]. In wild-type eggs fertilized by sperm from *K81* homozygous males, paternal chromosomes systematically fail to properly separate sister chromatids during the first zygotic division (Figure 1, [17–19]). This unique phenotype could result from a defect of sperm chromatin remodeling or paternal DNA replication during male pronuclear formation and therefore suggests that *K81* function could be required for the proper organization of sperm chromatin that takes place during spermiogenesis.

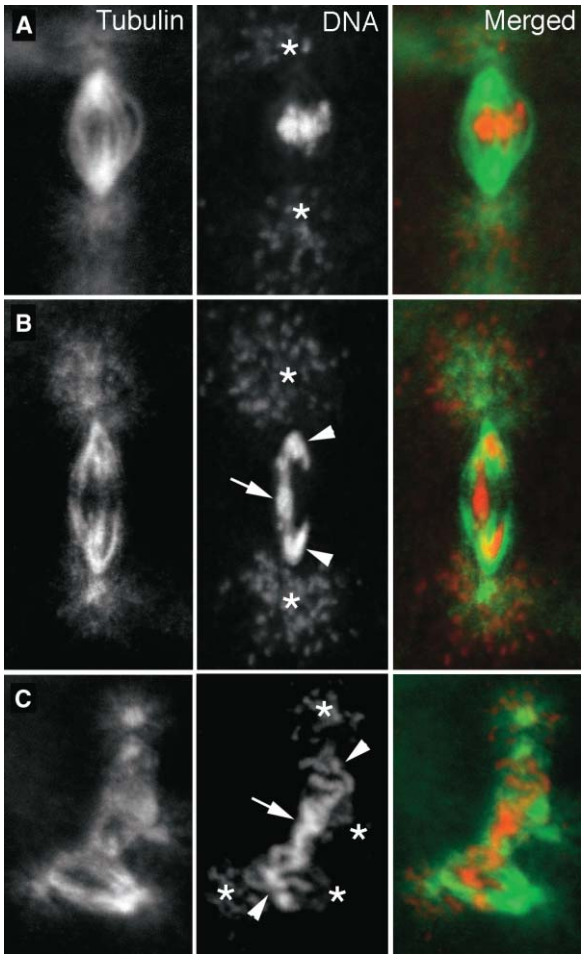

Figure 1. The *K81* Paternal Effect Phenotype at the First Zygotic Division

Confocal images of eggs from *y w*^{67} control females crossed with *K81*^{1}/ *K81*^{1} males and stained for tubulin (green, left panels) and DNA (red, middle panels).

(A) Metaphase of the first zygotic division with both sets of parental chromosomes visible on the metaphase plate.

(B) Anaphase of the first zygotic division: paternal chromosomes (arrow) are unable to separate sister chromatids and form a chromatin bridge that stretches between the spindle poles. The aberrant chromosomes were inferred as paternal based on the original work of Fuyama [16] demonstrating only genetically marked female chromosomes were transmitted to impaternate female progeny. The maternally derived chromatids separate normally (arrowheads).

(C) Metaphase of the second zygotic division. Paternal chromatin (arrow) typically bridges between the two haploid zygotic nuclei that contain maternally derived chromosomes (arrowheads). Asterisks indicate the presence of endosymbiotic bacteria *Wolbachia* that accumulate around spindle poles. *Wolbachia* naturally infects the *y w*^{67} strain but does not have an effect on the *K81* phenotype (B.L. and T.L.K, unpublished data).

In this study we provide the molecular identification of *K81* and further demonstrate that the phylogenetic distribution of *K81* is restricted to the *melanogaster* subgroup. We propose a model for the recent origin and evolution of *K81* and discuss a possible evolutionary scenario for its subsequent neofunctionalization.

Results

Molecular Identification of K81

The original *K81*^{1} mutant allele was mapped near the *rough* gene on chromosome 3R by its noncomplementation with the *Df(3R)ro*^{XB3} deficiency (97D2-9) [18]. The generation of small noncomplementing deficiency alleles of *K81* (*K81*$^{2\text{-}6}$) mapped this gene to an ~1 kb interval between *Rb97D* and *rough* [18]. The *Drosophila* genome project [20] predicts a single gene, *CG14251*, within this region with an ORF of 184 amino acids (Figure 2A). We amplified and sequenced *CG14251* from wild-type and *K81*^{1} homozygous flies. As expected, the 555 bp coding sequence was identical to the predicted *CG14251* gene. Sequences from *K81*^{1} were identical to *CG14251* with the exception of a 38 bp insertion near its 5′ end. This insertion contains a 30 bp match to the inverted terminal repeats of the *Drosophila* P-element transposon and an 8 bp duplication of the insertion site (Figure 2B) strongly suggesting that the mutation arose from the imperfect excision [21] of a natural P element from *CG14251*. This insertion results in a premature stop codon after the first 15 residues of the predicted protein (Figure 2B). Thus, the molecular nature of this lesion is consistent with genetic evidence that *K81*^{1} is an amorphic mutation. To demonstrate that this mutation in *CG14251* is responsible for the *K81* phenotype, we performed rescue experiments of the mutant phenotype with a transgene containing a wild-type copy of *CG14251* (Figure 2A). This transgene fully rescued the fertility of *K81*^{1}/*K81*^{1}, *K81*^{2}/*K81*^{2}, and *K81*^{1}/*K81*^{2} mutant males (data not shown), thus confirming that *CG14251* is the *ms(3)K81* gene.

K81 Is Expressed in the Male Germline

Expression of *K81* was analyzed by RT-PCR using primers designed from the coding sequence. As expected for a paternal effect gene, *K81* expression was not detected in agametic males derived from *tudor* mutant mothers [22]. However, very weak expression was also detected in wild-type females (Figure 3A). In males, *K81* expression is first detected during larval stages onward to adult (data not shown). We constructed a *K81::GFP* fusion reporter transgene under the control of the *K81* upstream regulatory sequences. In transgenic adult males, K81::GFP accumulated in primary spermatocyte nuclei, confirming that *K81* is expressed and translated in the male germline (Figure 3B). K81::GFP is not detected in later stages, suggesting that the postfertilization phenotype of *K81* could be a late consequence of a subtle and so-far-undetected premeiotic defect.

The K81 Gene Is Restricted to the *melanogaster* Subgroup

Extensive database searches for orthologs of *K81* failed to find significant homology outside *D. melanogaster*. Our unsuccessful BLAST search for *K81* orthologs included the recently available *D. pseudoobscura* genome sequence (Baylor Human Genome Sequencing Center, http://www.hgsc.bcm.tmc.edu/projects/Drosophila/). Although it is known that a significant fraction (18.6%) of

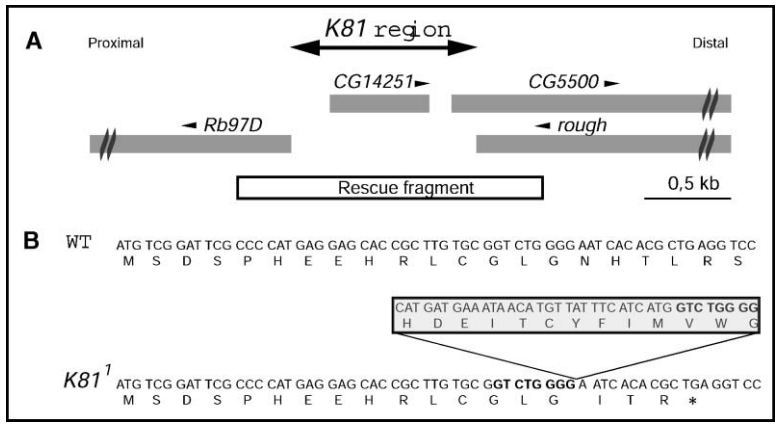

Figure 2. Identification of the *K81* Gene

(A) Representation of the *K81* genomic region (97D, chromosome 3R) with predicted genes (light gray bars) and the genomic fragment used in the *K81* rescue experiment.

(B) Comparison of nucleotide and deduced amino acid sequences at the 5′ region of wild-type (top) and *K81*^{1} (bottom) regions of the *CG14251* gene showing the 38 bp DNA insertion (box) and the 8 bp duplication (bold) indicative of a P-element insertion/excision event.

D. melanogaster genes have no ortholog in the distantly related mosquito *Anopheles gambiae* [23], *D. melanogaster* genes that are not conserved in *D. pseudoobscura* are relatively rare [24], which led us to search for the gene in species more closely related to *D. melanogaster*. We successfully amplified and sequenced *K81* orthologs in all members of the *melanogaster* subgroup. In contrast, PCR failed to amplify *K81*-related sequences from species outside the *melanogaster* subgroup. To further confirm this result, the distribution of *K81* throughout the genus *Drosophila* was surveyed by genomic DNA dot blot assays in representative *Drosophila* species for which we had been unsuccessful in obtaining PCR products (Figure 4). As expected, this probe gave positive signals for all tested species from the *melanogaster* subgroup. In contrast, dot blot analyses failed to detect *K81* in either distantly related species (*D. hydei*, *D. pseudoobscura*, and *D. willistonii*) or other more closely related members of the *melanogaster* group (*D. kikkawai, D. takahashii, D. ananassae, and D. elegans*). Sequence obtained from the *Rb97D-rough* region from *D. elegans* that gave the strongest background signal in the dot blot experiment confirmed the absence of *K81* at this locus (data not shown). Thus, we conclude that *K81* is restricted to the *melanogaster* subgroup.

Significant homology to *K81* was found to *CG6874,* a gene predicted to encode a 221 amino acid protein (Figure 5A). Homology is particularly strong with K81 at each end with a divergent central region (Figure 5A). *CG6874* maps to 75E1 on 3L and its protein, like K81,

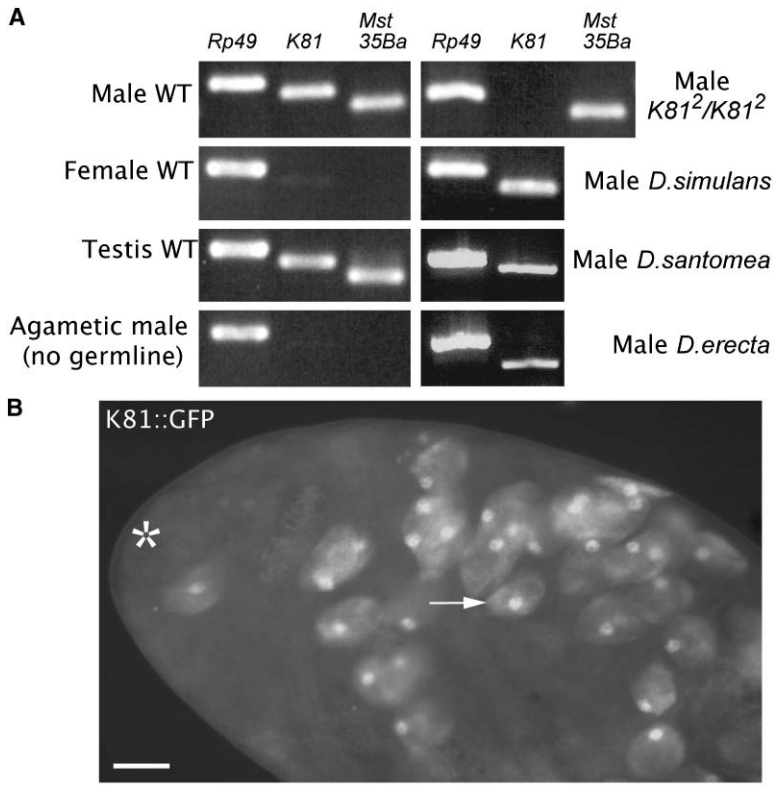

Figure 3. *K81* Is Expressed in the Male Germline

(A) RT-PCR analysis showing *K81* expression in adult males, testis, and very low levels in females. *K81* expression is not detected in agametic males from *tud1/tud1* mothers or in *K81*^{2}/*K81*^{2} control males. As a general PCR control, Ribosomal Protein gene *Rp49*, a ubiquitously expressed gene, was used. Also, an additional control for male germline-specific expression in *D. melanogaster* was performed with the *Male-specific-transcript-35Ba* [44].

(B) Testis of a transgenic male carrying a *K81::GFP* fusion construct. The K81::GFP protein is detected in primary spermatocyte nuclei (arrow) with an accumulation in the presumptive nucleoli (brighter spot in spermatocyte nuclei). Asterisk indicates the position of the apical tip of the testis. The scale bar represents 10 μm.

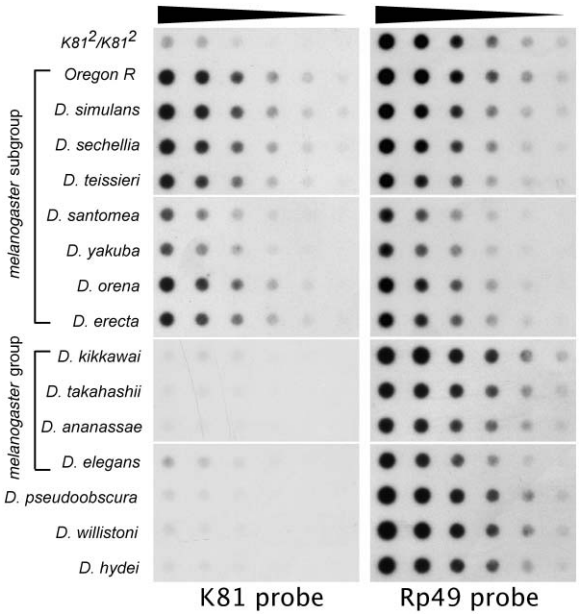

Figure 4. Genomic Dot Blot Analysis of *K81* Showing Its Phylogenetic Restriction to the *melanogaster* Subgroup

We used a *K81* probe to the most highly conserved 5′ region to avoid any cross hybridization with the conserved 3′ end of *CG6874*. Each horizontal lane contains six decreasing dilutions of genomic DNA from each indicated species. Hybridization with a *D. melanogaster K81* probe (left panel) gives positive signals only for the species of the *melanogaster* subgroup. The $K81^2/K81^2$ lane is used as a control for evaluating the level of nonspecific hybridization with the *K81* probe. Lanes that give signals weaker or equal to the $K81^2/K81^2$ signals are considered negative for the *K81* gene. The right panel shows the control hybridization with a *melanogaster Rp49* probe. This autoradiography was obtained after washing the membranes at an intermediary stringency (see Experimental Procedures).

is encoded by a single exon. *CG6874* has a clear ortholog in *D. pseudoobscura,* and microsynteny of the locus is conserved between the two species (data not shown). In contrast, no *K81* sequence homology was observed in the ~1 kb region that separates the *D. pseudoobscura Rb97D* and *rough* orthologs (Figure 5B).

Discussion

A Scenario for the Origin and Evolution of *K81*

The alignments of the *K81* sequences obtained from the *melanogaster* subgroup species clearly revealed a high degree of sequence identity and conservation. All evolutionary rate comparisons between sister species and across the *melanogaster* subgroup reveal conservative and homogeneous rates consistent with purifying selection throughout the subgroup (Table 1). We also verified that these *K81* orthologs are expressed in adult males (Figure 3A), suggesting a conserved function for *K81* within the subgroup.

The restricted distribution of *K81* with the *melanogaster* subgroup suggests that this gene was created and fixed in a common ancestor to this subgroup after the *obscura-melanogaster* group divergence (~30 Mya) [25, 26]. Previous studies have shown that genes restricted to the *melanogaster* subgroup were most probably created by either DNA duplication of a preexisting gene [4, 27] or through the retroposition of a processed mRNA from a precursor gene [2, 3, 6]. We thus considered the possibility that *K81* was created by a duplication of *CG6874* in the common ancestor of the *melanogaster* subgroup. Although the absence of introns in *CG6874* prevents us distinguishing either possible duplication mechanism, the fact that these two genes are in different genome regions supports the hypothesis that *K81* arose by random retroposition of an ancestral *CG6874* mRNA. Also supporting this idea is the fact that two more ancient genes tightly flank *K81* (Figure 5B). We could not find any clear evidence of known retroposition landmarks around the *K81* gene, such as the presence of a poly-A tract or flanking short direct repeats. These landmarks are present in retroposed genes that have a very recent origin [2, 6] but are presumably no longer detectable for the more ancient *K81* gene.

Theory predicts that a period of relaxed functional constraint follows gene duplication due to functional redundancy, and that fixation of duplicated genes involves subsequent functional diversification [28, 29]. Interestingly, *Rb97D*, the gene immediately upstream of *K81* (Figure 2A), is specifically expressed in primary spermatocytes [30], suggesting that *K81* could share *Rb97D*'s regulatory sequences. If this is the case, the ancestral *CG6874* duplicate could have rapidly acquired male germline-specific expression. Such spatiotemporal expression partitioning has been observed [31–33] and is a simple means to produce functional diversification between a progenitor gene and its duplicate. Interestingly, RT-PCR analysis showed that *CG6874* is ubiquitously expressed (including the testis) in adult flies of both sexes (data not shown). It therefore seems likely that acquisition of K81 function reflects the fortuitous male germline-specific expression of the new *CG6874* duplicate. Consistent with this evolutionary scenario, accelerated amino acid substitutions occurred on the lineage following the creation of *K81* prior to *melanogaster* subgroup divergence (Figure 5C), and may be attributed to relaxed purifying selection on the duplicated genes and/or the impact of Darwinian selection during the neofunctionalization of *K81* [1, 7, 10, 31, 34].

Although the function of *CG6874* has yet to be determined, P-element disruption of this gene is lethal, thus demonstrating that *CG6874* encodes an essential gene (B.L., unpublished data). Some indication of function is evidenced by two independent microarray analyses showing that *CG6874* expression is positively regulated by both the E2F1 transcription factor [35] and the oncogene Ras [36]. E2F1 is known to activate target genes required for G1/S progression, DNA replication, and mitosis, whereas Ras is a well-known inducer of cell proliferation. Thus, these data implicate *CG6874* in cell cycle progression. It will be of interest to determine the functional relationship between the presumed general role of *CG6874* and the function of *K81* in primary spermatocyte cells.

Conclusions

The combined use of molecular genetics and comparative evolutionary genomics has provided insights into

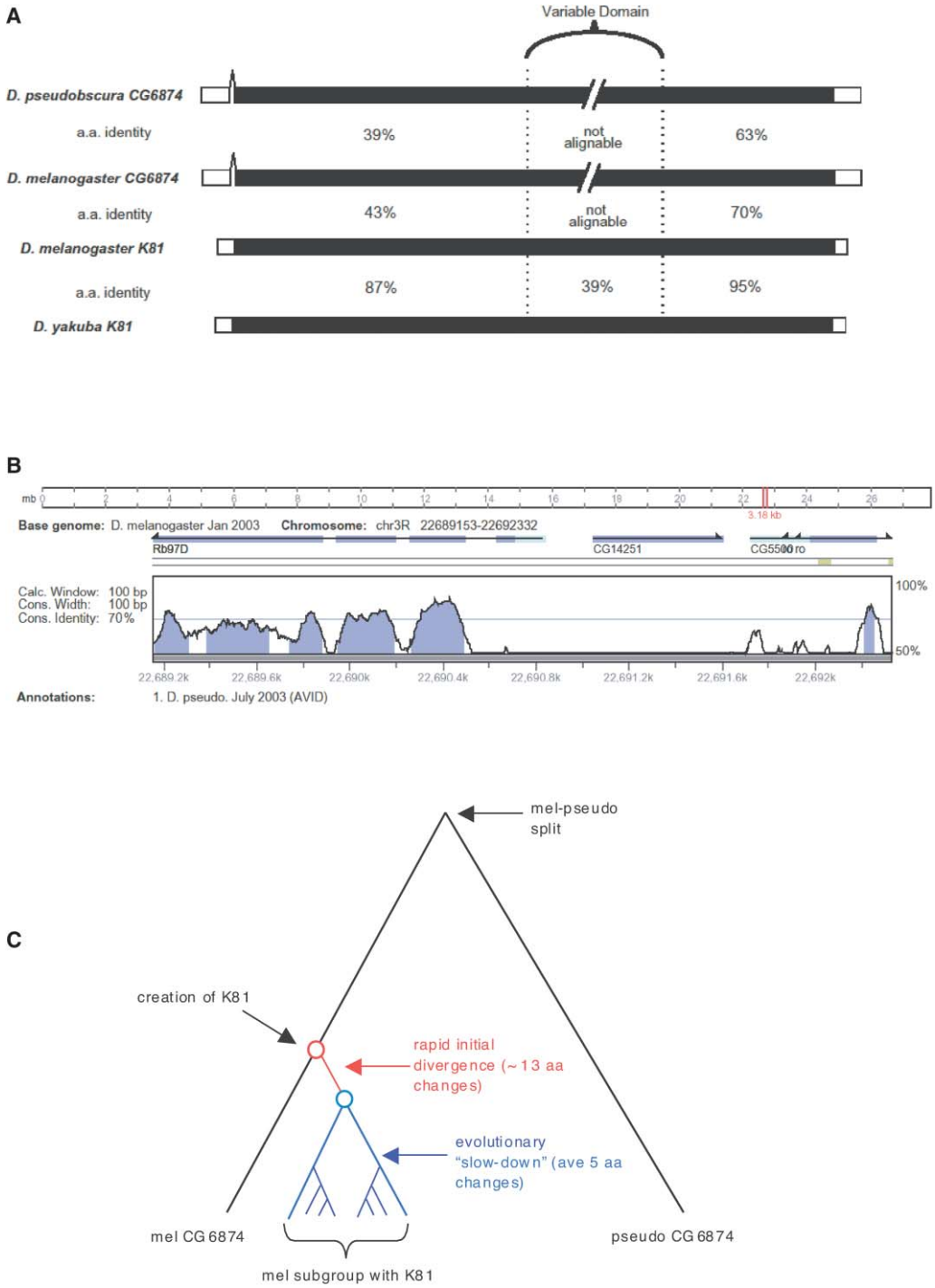

Figure 5. Evolutionary Genomics of *K81* and *CG6874* Genes

(A) Homology among representative *K81* and *CG6874* genes. Closed bars depict protein-coding regions; open bars depict untranslated portions of the cDNAs. Amino acid identities between proteins are given for individual domains.

(B) Vista plot comparison of annotated genes in the 97D region of *D. melanogaster* and *D. pseudoobscura* demonstrating lack of K81 sequence homology.

(C) Protein phylogeny of *K81* and *CG6874*. The phylogeny was created from alignable regions using *D. pseudoobscura CG6874* as an outgroup.

the origin of *K81* and the evolution of its essential germline function. The molecular evolutionary and phylogenetic reconstruction of *K81* suggests that gene duplication, transcriptional coregulation, and adaptative evolution were key steps in the neofunctionalization of *K81*. As such, *K81* is an example of the recent creation of an essential cellular function. This finding advances the concept that the evolution of novel and important

Table 1. Evolutionary Analysis of *K81* within the *melanogaster* Subgroup

	K_a	K_s	K_a/K_s
Within *mel* Clades			
D. mel versus *D. mau*	0.030	0.129	0.232
D. sim versus *D. sec*	0.010	0.051	0.188
D. yak versus *D. san*	0.018	0.057	0.327
D. ore versus *D. ere*	0.033	0.104	0.318
Across *mel* Subgroup			
D. ere versus *D. sim*	0.084	0.400	0.210
D. ere versus *D. mel*	0.073	0.409	0.179
D. yak versus *D. sim*	0.085	0.404	0.211
D. yak versus *D. mel*	0.083	0.453	0.183

All K_a/K_s ratios are significantly lower than unity, $p < 0.0001$ [41]. Note that comparisons across the *mel* subgroup are nonindependent.

functionality is an ongoing process and is contrary to the commonly held belief that critical biological functions represent ancient evolutionary origins. Finally, further study of *K81* and other paternal effect genes may provide additional insight into the roles gene duplication and neofunctionalization have played in the evolution of sperm function in *Drosophila*. The inferred role of *K81* also suggests male-contributed proteins may have a more prominent role in fertilization and early embryogenesis than currently recognized [37, 38].

Experimental Procedures

Immunofluorescence and Confocal Microscopy

Eggs were collected every 15-20 min, fixed and stained as described [19]. A monoclonal anti-α-tubulin (clone DM1A, Sigma) and a Alexafluor 488 Goat-anti-mouse secondary antibody (Molecular Probes) were used at a 1:200 and 1:500 dilution, respectively. Stained eggs were observed under a LSM 510 Confocal microscope (Zeiss) and images were processed using Photoshop software (Adobe).

Plasmid Constructs

K81 rescue plasmid : a 1781 bp DNA fragment covering the *CG14251* gene was amplified by PCR from genomic Oregon-R DNA using the primers 5′-AACATCGACCACCTTGCCCC-3′ and 5′-GGGCCTAA ACTACTATGCCG-3′, cloned into the pGEM-T vector (Promega). The insert fragment was excised by *SphI* and *NotI* and subcloned into the PW8 transformation vector [39].

K81::GFP plasmid : a 1228 bp genomic DNA fragment containing the complete coding sequence of *CG14251* plus 666 bp of upstream sequence was amplified by PCR from Oregon-R genomic DNA using primer 5′-AACATCGACCACCTTGCCCC-3′ and primer 5′-TTGGAT CCTTTCCCCCAGTAGTTCCCGCCAG-3′. This 3′ primer was designed to remove the STOP codon of K81 and to introduce a BamHI site that was used to fuse in frame a BamHI-PstI GFP-SV40 fragment from the pEGFP-N1 vector (Clontech, Palo Alto, CA). The resulting construct (K81-GFP-SV40) was cloned in the PW8 vector. Transgenic lines were established by P-mediated germline transformation of w^{1118} flies as described [40].

K81 Cloning, Sequencing, and Evolutionary Analysis

DNA fragments covering the *Rb97D-rough* intergenic region were amplified by PCR from genomic DNA of different *Drosophila melanogaster* subgroup species using the following primers (5′-AACATC GACCACCTTGCCCC-3′ and 5′-GGGCCTAAACTACTATGCCG-3′) cloned in the pGEM-T vector (Promega). At least two independent clones were sequenced for each species. In-frame alignments of coding regions were done with the Megalign program (DNASTAR, Madison, WI). The K_a and K_s values were calculated by using the Li method [41] as implemented by the Wisconsin Package v10.2 (Accelrys, San Diego, CA). Tests of purifying selection on *K81* were conducted using MEGA2 software package [42]. Reconstruction of the evolutionary history of *CG6874* and *K81* were conducted by placing total character differences on the phylogeny using the exhaustive search algorithm in PAUP* 4.0 [43].

Reverse Transcription Analysis

Total RNA was extracted by the Trizol method (Invitrogen) and first-strand cDNAs were synthesized with the Superscript II reverse transcriptase (Invitrogen) and oligo-dT primers. The following primers were used for PCR amplification of the cDNAs: *K81 simulans, K81 mauritiana,* and *K81 sechellia* (accession numbers: AJ627280, AJ627281, and AJ627282, respectively), (5′-ATGTCGGATTCGCCCC ATG-3′ and 5′-TAGTGGTTGATTCTTGCTCCTC-3′).

K81 yakuba and *K81 erecta* (accession numbers: AJ627283 and AJ627284, respectively), (5′- ATGTCGGATTCGCCCCATG-3′ and 5′-TTGTTCTTCAGCCTGTAGAGCC-3′).

K81 santomea (accession number: AJ627285), (5′-AGTTCGGTC GGTTGATAAAC-3′ and 5′- GTCGTGGCCAATGGTTTTATGAGC-3′). *K81 orena* (accession number: AJ745103), (5′-ACATCGACCACCTT GCCCCACTG-3′ and 5′- GTCGTGGCCAATGGTTTTATGAGC-3′).

Rp49 (5′-AAGATCGTGAAGAAGCGCAC-3′ and 5′-ACTCGTTCT CTTGAGAACGC-3′),

Mst35Ba (5′-CCAATAAGGAGAGCACCTCA-3′ and 5′-CTTTCT ATTCTCCGAGAGCCT-3′), and *CG6874* (5′-AGCGGAGATTCTGCG CAGTC-3′ and 5′- CTAACCACCTGTGGTTCCCA-3′).

Dot Blot Analysis

Genomic DNA were prepared from young adult males and were agarose gel quantified with the Bio-Profil image analysis software (Vilber Lourmat). Dilutions of genomic DNA were blotted on a nylon membrane with a 96-wells manifold. Membranes were prehybridized and hybridized overnight in low stringency conditions at 42°C in a Denhart's 5×, formamide 20%, 5× SSC, 0.5% SDS, and 0.1 mg/ml salmon sperm solution. Probes were purified PCR products labeled with dCTP-α^{32}P with a random priming labeling kit (Ready-to-go, Amersham). The *K81* and *Rp49* probes were amplified from *D. melanogaster* Oregon-R genomic DNA using the primers 5′-ATGTCG GATTCGCCCCATG-3′ and 5′-TAGTGGTTGATTCTTGCTCCTC-3′ (*K81*) and 5′-AAGATCGTGAAGAAGCGCAC-3′ and 5′-ACTCGTTCT CTTGAGAACGC-3′ (*Rp49*). Membranes were washed in 1× SSC, 0.1% SDS at 50°C (low stringency), 57°C (intermediary stringency), and 65°C (strong stringency). After each washing procedure, autoradiography was performed using X-Omat LS films (Kodak). Autoradiograms were scanned and images were processed with Photoshop and Illustrator software (Adobe).

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References

1. Long, M., and Langley, C.H. (1993). Natural selection and the origin of *jingwei,* a chimeric processed functional gene in *Drosophila*. Science *260*, 91–95.
2. Wang, W., Brunet, F.G., Nevo, E., and Long, M. (2002). Origin of *sphinx*, a young chimeric RNA gene in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *99*, 4448–4453.
3. Wang, W., Yu, H., and Long, M. (2004). Duplication-degeneration as a mechanism of gene fission and the origin of new genes in *Drosophila* species. Nat. Genet. *36*, 523–527.
4. Parsch, J., Meiklejohn, C.D., Hauschteck-Jungen, E., Hunziker, P., and Hartl, D.L. (2001). Molecular evolution of the *ocnus* and

janus genes in the *Drosophila melanogaster* species subgroup. Mol. Biol. Evol. *18*, 801–811.
5. Ranz, J.M., Ponce, A.R., Hartl, D.L., and Nurminsky, D. (2003). Origin and evolution of a new gene expressed in the *Drosophila* sperm axoneme. Genetica *118*, 233–244.
6. Wang, W., Zhang, J., Alvarez, C., Llopart, A., and Long, M. (2000). The origin of the *jingwei* gene and the complex modular structure of its parental gene, *yellow emperor*, in *Drosophila melanogaster*. Mol. Biol. Evol. *17*, 1294–1301.
7. Long, M., Betran, E., Thornton, K., and Wang, W. (2003). The origin of new genes: Glimpses from the young and old. Nat. Rev. Genet. *4*, 865–875.
8. Swanson, W.J., and Vacquier, V.D. (2002). The rapid evolution of reproductive proteins. Nat. Rev. Genet. *3*, 137–144.
9. Swanson, W.J., Nielsen, R., and Yang, Q. (2003). Pervasive adaptive evolution in mammalian fertilization proteins. Mol. Biol. Evol. *20*, 18–20.
10. Ting, C.T., Tsaur, S.C., Wu, M.L., and Wu, C.I. (1998). A rapidly evolving homeobox at the site of a hybrid sterility gene. Science *282*, 1501–1504.
11. Perez, D.E., Wu, C.I., Johnson, N.A., and Wu, M.L. (1993). Genetics of reproductive isolation in the *Drosophila simulans* clade: DNA marker-assisted mapping and characterization of a hybrid-male sterility gene, *Odysseus* (*Ods*). Genetics *134*, 261–275.
12. Fitch, K.R., Yasuda, G.K., Owens, K.N., and Wakimoto, B.T. (1998). Paternal effects in *Drosophila*: Implications for mechanisms of early development. Curr. Top. Dev. Biol. *38*, 1–34.
13. Karr, T.L. (1996). Paternal investment and intracellular sperm-egg interactions during and following fertilization in *Drosophila*. Curr. Top. Dev. Biol. *34*, 89–115.
14. Loppin, B., and Karr, T.L. (2004). Molecular Genetics of Insect Fertilization. In Comprehensive Insect Molecular Science, L.B. Gilbert and K. Iatrou, eds. (Oxford: Elsevier), pp. 213–236.
15. Fuller, M.T. (1993). Spermatogenesis. In The Development of *Drosophila melanogaster*, M.a.A. Bates, A.R., ed. (Cold Spring Harbor, NY: Cold Spring Harbor Press), pp. 71–147.
16. Fuyama, Y. (1984). Gynogenesis in *Drosophila melanogaster*. Jpn. J. Genet. *59*, 91–96.
17. Fuyama, Y. (1986). Genetics of Parthenogenesis in *Drosophila melanogaster*. I. The mode of diploidization in the gynogenesis induce by a male-sterile mutant, *ms(3)K81*. Genetics *112*, 237–248.
18. Yasuda, G.K., Schubiger, G., and Wakimoto, B.T. (1995). Genetic characterization of *ms (3) K81*, a paternal effect gene of *Drosophila melanogaster*. Genetics *140*, 219–229.
19. Loppin, B., Docquier, M., Bonneton, F., and Couble, P. (2000). The maternal effect mutation *sésame* affects the formation of the male pronucleus in *Drosophila melanogaster*. Dev. Biol. *222*, 392–404.
20. Consortium, T.F.B. (1996). FlyBase: The Drosophila database. The Flybase Consortium. Nucleic Acids Res. *24*, 53–56.
21. Beall, E.L., and Rio, D.C. (1998). Transposase makes critical contacts with, and is stimulated by, single-stranded DNA at the P element termini in vitro. EMBO J. *17*, 2122–2136.
22. Boswell, R.E., and Mahowald, A.P. (1985). *tudor,* a gene required for assembly of the germ plasm in *Drosophila melanogaster*. Cell *43*, 97–104.
23. Zdobnov, E.M., von Mering, C., Letunic, I., Torrents, D., Suyama, M., Copley, R.R., Christophides, G.K., Thomasova, D., Holt, R.A., Subramanian, G.M., et al. (2002). Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. Science *298*, 149–159.
24. Bergman, C.M., Pfeiffer, B.D., Rincon-Limas, D.E., Hoskins, R.A., Gnirke, A., Mungall, C.J., Wang, A.M., Kronmiller, B., Pacleb, J., Park, S., et al. (2002). Assessing the impact of comparative genomic sequence data on the functional annotation of the *Drosophila* genome. Genome Biol. *3*, RESEARCH0086.
25. Russo, C.A., Takezaki, N., and Nei, M. (1995). Molecular phylogeny and divergence times of drosophilid species. Mol. Biol. Evol. *12*, 391–404.
26. Tamura, K., Subramaniam, S., and Kumar, S. (2004). Temporal patterns of fruit fly (*Drosophila*) evolution revealed by mutation clocks. Mol. Biol. Evol. *21*, 36–44.
27. Nurminsky, D.I., Nurminskaya, M.V., De Aguiar, D., and Hartl, D.L. (1998). Selective sweep of a newly evolved sperm-specific gene in *Drosophila*. Nature *396*, 572–575.
28. Ohno, S. (1970). Evolution by gene duplication (Springer, Berlin, 1970).
29. Lynch, M., and Conery, J.S. (2003). The origins of genome complexity. Science *302*, 1401–1404.
30. Heatwole, V.M., and Haynes, S.R. (1996). Association of RB97D, an RRM protein required for male fertility, with a Y chromosome lampbrush loop in *Drosophila* spermatocytes. Chromosoma *105*, 285–292.
31. Dorus, S., Gilbert, S.L., Forster, M.L., Barndt, R.J., and Lahn, B.T. (2003). The CDY-related gene family: Coordinated evolution in copy number, expression profile and protein sequence. Hum. Mol. Genet. *12*, 1643–1650.
32. Lahn, B.T., and Page, D. (1999). Retroposition of autosomal mRNA yielded testis-specific gene family on human Y chromosome. Nat. Genet. *21*, 429–433.
33. Serluca, F.C., Sidow, A., Mably, J.D., and Fishman, M.C. (2001). Partitioning of tissue expression accompanies multiple duplications of the Na+/K+ ATPase subunit gene. Genome Res. *11*, 1625–1631.
34. Ting, C.T., Tsaur, S.C., and Wu, C.I. (2000). The phylogeny of closely related species as revealed by the genealogy of a speciation gene, *Odysseus*. Proc. Natl. Acad. Sci. USA *97*, 5313–5316.
35. Dimova, D.K., Stevaux, O., Frolov, M.V., and Dyson, N.J. (2003). Cell cycle-dependent and cell cycle-independent control of transcription by the *Drosophila* E2F/RB pathway. Genes Dev. *17*, 2308–2320.
36. Asha, H., Nagy, I., Kovacs, G., Stetson, D., Ando, I., and Dearolf, C.R. (2003). Analysis of Ras-induced overproliferation in *Drosophila* hemocytes. Genetics *163*, 203–215.
37. Karr, T. (1991). Intracellular sperm/egg interactions in *Drosophila*: A three-dimensional structural analysis of a paternal product in the developing egg. Mech. Dev. *34*, 101–112.
38. Karr, T.L., and Pitnick, S. (1996). The ins and outs of fertilization. Nature *379*, 405–406.
39. Klemenz, R., Weber, U., and Gehring, W.J. (1987). The *white* gene as a marker in a new P-element vector for gene transfer in *Drosophila*. Nucleic Acids Res. *15*, 3947–3959.
40. Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. Science *218*, 348–353.
41. Li, W.H. (1993). Unbiased estimation of the rates of synonymous and nonsynonymous substitution. J. Mol. Evol. *36*, 96–99.
42. Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M. (2001). MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics *17*, 1244–1245.
43. Swofford, D.L. (2004). PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods) 4.0 Beta (Sinauer).
44. Russell, S.H., and Kaiser, K. (1993). *Drosophila melanogaster* male germ-line specific transcripts with autosomal and Y-linked genes. Genetics *134*, 293–308.