Birth, Death, and Replacement of Karyopherins in Drosophila

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Abstract

Nucleocytoplasmic transport is a broadly conserved process across eukaryotes. Despite its essential function and conserved mechanism, components of the nuclear transport apparatus have been implicated in genetic conflicts in Drosophila, especially in the male germ line. The best understood case is represented by a truncated RanGAP gene duplication that is part of the segregation distorter system in *Drosophila melanogaster*. Consistent with the hypothesis that the nuclear transport pathway is at the heart of mediating genetic conflicts, both nucleoporins and directionality imposing components of nuclear transport have previously been shown to evolve under positive selection. Here, we present a comprehensive phylogenomic analysis of importins (karyopherins) in Drosophila evolution. Importins are adaptor molecules that physically mediate the transport of cargo molecules and comprise the third component of the nuclear transport apparatus. We find that importins have been repeatedly gained and lost throughout various stages of Drosophila evolution, including two intriguing examples of an apparently coincident loss and gain of nonorthologous and noncanonical importin-α. Although there are a few signatures of episodic positive selection, genetic innovation in importin evolution is more evident in patterns of recurrent gene birth and loss specifically for function in Drosophila testes, which is consistent with their role in supporting host genomes defense against segregation distortion.

Key words: gene duplication, importins, genetic conflict, Drosophila.

Introduction

All eukaryotes must transport proteins and RNA molecules across their nuclear membrane. This essential function is performed with a nuclear transport apparatus, which consists primarily of three components (Wente and Rout 2010): 1) factors that establish the directionality of nuclear transport, 2) the nuclear pore complex (NPC), and 3) the adaptor molecules (importins or karyopherins and exportins) that mediate the transport of cargo. Despite the conservation of the transport mechanism across eukaryotes, there is significant diversity in the components of the nuclear transport apparatus among eukaryotes (Wente and Rout 2010). Over the last decade, the nuclear transport pathway in Drosophila has emerged as a convergence point for evolutionary studies of genetic conflict, speciation, and gene duplication (Kusano et al. 2001; Presgraves 2007; Tracy et al. 2010).

The first connection between genetic conflict and the nuclear transport pathway emerged from the Segregation Distorter (SD) system in *Drosophila melanogaster* (Sandler et al. 1959). The SD system in *D. melanogaster* gains an evolutionary advantage by causing non-Mendelian segregation in its favor. *Drosophila melanogaster* males heterozygous for the SD chromosome preferentially transmit it at a frequency >95% compared with the non-SD chromosome (Sandler et al. 1959). This segregation distortion was mapped genetically to a partial duplication of the *RanGAP* gene (*Ran GTPase Activator Protein*) (Kusano et al. 2001), a component that is integral to the directionality of nucleocytoplasmic transport across eukaryotes (Stewart 2007). RanGAP is normally tethered to the cytoplasmic side of the nuclear membrane and stimulates the hydrolysis of RanGTP to RanGDP, thus maintaining a high RanGDP concentration in the cytoplasm. Ran guanine nucleotide exchange factor (RanGEF) is present inside the nucleus and catalyzes the conversion of RanGDP to RanGTP, thus maintaining a high RanGTP concentration inside the nucleus. This RanGDP/RanGTP concentration gradient determines the directionality of nucleocytoplasmic transport across eukaryotes (Wente and Rout 2010). In the SD system, the partial duplicate of RanGAP has lost its nuclear export signal (NES) and mislocalizes to the nucleus (Kusano et al. 2001). This causes a defect in the RanGTP gradient across the nuclear envelope and, in a mechanism that is yet unknown, preferentially kills sperm that contain chromosomes bearing Responder DNA satellite repeats via a chromatin condensation defect (Kettaneh and Hartl 1976; Tokuyasu et al. 1977; Hauschteck-Jungen and Hartl 1978, 1982). Even overexpressing wild type RanGAP can lead to segregation distortion (Kusano et al. 2002), confirming that a breakdown of the RanGDP/RanGTP gradient rather than a neofunction of SD-RanGAP that causes distortion. Wild type RanGAP itself evolves under positive selection between *D. melanogaster* and *D. simulans*, which is thought to be a consequence of genetic conflict tied between segregation distortion and its suppression (Presgraves 2007).

In addition, both *Ran* and *NTF-2* (*Nuclear Transport Factor 2*), which are involved in maintaining the gradient of RanGTP have formed retrogenes at least three independent times in Drosophila species, acquired testes-specific expression and show signatures of adaptive evolution (Tracy et al. 2010). These patterns of *Ran* and *NTF2* evolution and expression have been attributed to the recurrent pressure imposed on this system by segregation distortion during spermatogenesis and constant selection to suppress it (Tracy et al. 2010).

Studies of speciation in Drosophila have independently implicated a different component of the nuclear transport apparatus. Genetic mapping studies of hybrid incompatibilities have shown that the inviability of hybrid males between *D. melanogaster* and *D. simulans* is caused by two components of the NPC: nucleoporins *Nup96* and *Nup160* (Presgraves et al. 2003; Tang and Presgraves 2009). Furthermore, replacing a *D. melanogaster Nup160* with a *D. simulans* allele in an otherwise *D. melanogaster* genetic background causes hybrid female sterility (Sawamura et al. 2010). Molecular evolution studies have shown several nucleoporins to evolve rapidly under positive selection in Drosophila (Presgraves and Stephan 2007). The pattern of recurrent adaptive substitutions in nucleoporins has been proposed to result from its roles in multiple genetic conflicts (Presgraves 2007), although the precise biological processes that may have imposed positive selection on nucleoporins remain undefined.

A third component of the nuclear transport apparatus involves the adaptor molecules that mediate the transport of cargo across the nuclear membrane. While the NPC provides the channel between the nucleus and the cytoplasm, and the small GTPase Ran provides the directionality, the actual transport of cargo is carried out by specialized proteins called karyopherins (Goldfarb et al. 2004). Karyopherins that mediate nuclear import are called importins, whereas those that mediate nuclear export are called exportins. Import is carried out by importin-α and importin-β. A canonical importin-α consists of an N-terminal importin-β binding (IBB) autoinhibitory domain and a tandem array of Armadillo (ARM) repeats (Stewart 2007; Wente and Rout 2010). The IBB domain normally binds to the ARM repeats within the importin-α molecule. Cargo molecules that bear nuclear localization signals displace the IBB domain and bind to the ARM repeats of importin-α. Upon displacement from the ARM repeats by the cargo, the IBB domain is now free to bind importin-β (Stewart 2007). Importin-βs consist mostly of HEAT repeats that share ancestry with ARM repeats (Malik et al. 1997) required for binding to the IBB domain, RanGDP, and the NPC (Conti et al. 1998). This cargo-importin-α-importin-β ternary complex associates with the NPC and translocates across the nuclear membrane (Stewart 2007; Wente and Rout 2010). High RanGTP levels in the nucleus cause this complex to dissociate, thus releasing the cargo into the nucleus (Stewart 2007; Wente and Rout 2010). Conversely, cargo that need to be exported out of the nucleus bear a NES that is bound by exportins (Weis 1998).

Despite evidence of positive selection driving evolution of components of NPC and the gradient-establishing apparatus, few studies have directly investigated the evolution and genetic novelty in importins. Phylogenetic studies have previously shown that the number of these components can differ quite dramatically between eukaryotes (Mason et al. 2009). While the yeast *Saccharomyces cerevisiae* encodes a single importin-α, most metazoan importins fall into three phylogenetic groups, importin-α1, -α2, and -α3 (Mason et al. 2009). Previous studies have concluded that Drosophila genomes encode a single representative of each importin (Ratan et al. 2008; Mason and Goldfarb 2009). The Drosophila *importin-α2* (*aKap2*) is highly expressed in the ovaries, testes, and other somatic tissues. *Importin-α2* mutant males and females in Drosophila develop to adulthood but are sterile. This defect can be partially rescued by ectopic expression of *importin-α1* or *-α3* (Mason et al. 2002). Drosophila *importin-α3* (*aKap3*) mutants are embryonic lethals, but this lethality can be partially rescued by *importin-α1* and *-α2* transgenes (Mason et al. 2003). These results indicate a functional redundancy between the importins and support the idea that the three importin-α paralogs can be used interchangeably as transport factors (Goldfarb et al. 2004).

Here, we present a detailed analysis of importin evolution during Drosophila evolution, identifying several novel importins. On average, Drosophila genomes contain between four and five importins. We find that importins have been repeatedly gained and lost throughout various stages of Drosophila evolution, including two intriguing examples of an apparently coincident loss and gain of importin-α, which likely represent nonorthologous replacements. Although we find some episodic evidence of positive selection, the bulk of the genetic innovation appears to be manifest in terms of recurrent gene birth and loss, specifically for function in Drosophila testes. We hypothesize that this recurrent pattern of importin birth is consistent with their role in increasing the rate of nuclear import in the male germ line to defend host genomes against segregation distortion.

Materials and Methods

Drosophila species (table 1) were obtained from the *Drosophila* Species Center (presently at UC San Diego, CA). Genomic DNA was extracted from flies with Gentra Puregene Tissue Kit (Qiagen, Valencia, CA). To test whether a gene was present or absent in particular species, we first designed primers in genomic regions flanking our gene of interest. These primers were designed to syntenic regions using a *D. melanogaster–D. ananassae* genomic alignment, thus maximizing the chances of successful amplification in species that branched at intermediate divergence relative to *D. melanogaster* and *D. ananassae*. Polymerase chain reaction (PCR) was performed using Invitrogen Superscript HiFi supermix using a touchdown protocol. A large

Table 1. List of Species Used in This Study.

Species	Species Group	Strain
Drosophila melanogaster	melanogaster	W^{118}
Drosophila simulans	melanogaster	W^{501}
Drosophila mauritiana	melanogaster	14021-0241.1
Drosophila sechellia	melanogaster	Robertson1
Drosophila yakuba	melanogaster	Tai6
Drosophila santomea	melanogaster	14021-0271.00
Drosophila tiessieri	melanogaster	Brz7
Drosophila erecta	melanogaster	14021-0224.00
Drosophila orena	melanogaster	14021-0245.01
Drosophila eugracilis	melanogaster	14026-0451.03
Drosophila lutescens	takahashii	14022-0271.00
Drosophila mimetica	suzukii	14023-0381.00
Drosophila ananassae	ananassae	14024-0371.00
Drosophila pallidosa-like	ananassae	From A. Kopp
Drosophila papuensis	ananassae	From A. Kopp
Drosophila bipectinata	ananassae	14024-0381.10
Drosophila parabipectinata	ananassae	14024-0401.00
Drosophila malerkotliana	ananassae	14024-0391.01
Drosophila pseudoobbscura	pseudoobbscura	Flag14
Drosophila virilis	virilis	15010-1501.00
Drosophila americana americana	virilis	15010-0951.14
Drosophila americana texana	virilis	Unfused X CD97.5
Drosophila novamexicana	virilis	15010-1031.0

amplicon is generated when a particular gene is present, whereas a smaller amplicon is generated when the gene is absent. Gel electrophoresis was performed for all PCR products on a 1% agarose gel. To obtain the sequences of all successfully amplified PCR fragments, we first performed gel extractions with the Qiagen gel extraction kit. Amplicons were cloned using the TOPO 2.1 vector (Invitrogen) and sequenced using universal primers. When gel extractions were not performed, PCR products were cleaned up using ExoSAP-IT (US Biological, Inc.) and directly sequenced using ABI Bigdye terminator chemistry. Gene sequences obtained in this study are in the process of being deposited in NCBI GenBank. All primer sequences are included in supplementary table S1 (Supplementary Material online).

Total RNA extractions were performed on tissues dissected from *D. melanogaster*, *D. ananassae*, *D. pseudoobscura*, and *D. virilis* with TRIzol reagent (Invitrogen) according to the manufacturer's recommendations, followed by DNase treatment and cleanup with RNeasy kit (Qiagen, Valencia, CA). The panel consisted of RNA from whole males, male heads, testes, gonadectomized males, whole females, female heads, ovaries, and gonadectomized females. cDNAs for all tissues were synthesized using Superscript III reverse transcriptase (Invitrogen). Primers were designed flanking an intron for each gene when possible to distinguish between amplification of genomic DNA and cDNA.

Evolutionary and Bioinformatic Analyses

We identified new copies of karyopherins using tBLASTn searches of the 12 published Drosophila genomes using each of the *importin-α1*, *-α2*, *-α3* as a query sequence. Based on synteny, additional sequences were obtained by PCR from species that were not part of the original 12 Drosophila genomes project (Drosophila 12 Genomes Consortium 2007) as described above. All sequences were aligned at the amino acid level using ClustalX (Larkin et al. 2007), and the nucleotide sequence alignments were created using the amino acid alignment as a template by the Geneious software package (Drummond et al. 2011). Phylogenetic trees were created using both neighbor joining (Saitou and Nei 1987) and PhyML (Guindon et al. 2010). Signatures of selection along specific branches were evaluated using the free-ratio model implemented in PAML. Statistical significance for positive selection along a particular branch was assessed by fixing $dN/dS = 1$ and comparing likelihoods. To test whether a specific branch had undergone positive selection, dN/dS was set to 1 (Model 2) and maximum likelihood compared with when the branch was allowed to exceed 1 (Model 1). Positive selection at specific sites was evaluated using a comparison of the NSsites models M7 (that disallows positive selection) and M8 (that permits it) to assess whether positive selection had shaped the evolution of some importins (Yang 2007). Pairwise dN/dS analyses were also performed using *K* estimator (Comeron 1999). In a few instances, multiple strains of a related species were sequenced for population genetic analyses of their karyopherin sequences. The McDonald–Kreitman and Tajima's *D* tests were performed using DnaSP version 4.0 (Rozas et al. 2003); we used the two-tailed Fisher's exact test to evaluate significance.

All sequences obtained in this study have been deposited in GenBank under the accession numbers JQ173065–JQ173097.

Results

Birth of an Importin-α (*aKap4*) in the *melanogaster* Species Subgroup

In order to detect new karyopherin gene duplications, we performed tBLASTn searches of the *D. melanogaster* genome sequence using the three canonical importin-α protein sequences as queries. Using the *importin-α3* (*aKap3*) as a query, we identified a new importin-α gene *CG10478*, which we named as *aKap4* (*α Karyopherin-4*). *aKap4* bears armadillo/HEAT repeats characteristic of importin-αs. Although ARM repeats are also found in other proteins (Peifer et al. 1994), homology scores clearly indicated evolutionary relatedness of *aKap4* with other aKaps, especially *aKap3*. *aKap4* lacks the canonical IBB at its N-terminus (fig. 1A). Moreover, *aKap4* appears to be a partial retrogene, bearing one of the introns also present in *aKap3* at the same precise location (fig. 1A). *aKap3* has four introns, of which only one is retained in *aKap4*. This suggested that *aKap4* was derived from a partial retroposition of a parental *aKap3* gene rather than a DNA-based complete gene duplication followed by loss of introns. We did not detect an intact polyA tail immediately after *aKap4* sequence. We were interested in whether *aKap4* also had a broad expression pattern in adult Drosophila tissues, just like the parental *aKap3* gene

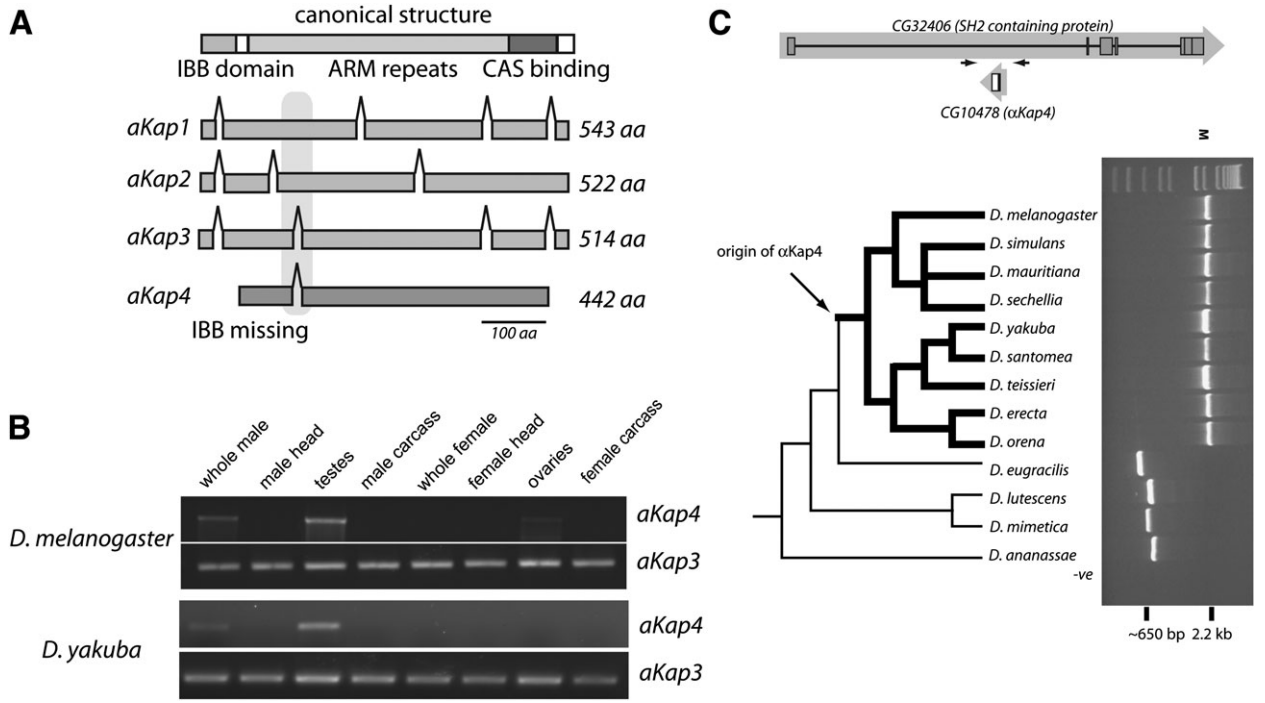


FIG. 1. (*A*) Gene structure and intron–exon boundary of importin-αs from the *Drosophila melanogaster* genome. *aKap4* is a partial retrogene, which has one intron in the same precise location as *aKap3*, suggesting its birth from *aKap3*. Note that introns are not shown to scale. (*B*) Expression patterns of *aKap4* and *aKap3* in *D. melanogaster* as revealed by reverse transcription polymerase chain reaction (RT-PCR). Whereas *aKap3* is ubiquitously expressed in adult *D. melanogaster* and *Drosophila yakuba* tissues, *aKap4* is only expressed in testes in both species. (*C*) Using PCR and primers flanking the syntenic location of *aKap4* (which is found in an intron of the *CG32406* gene in *D. melanogaster*), we amplified the genomic region containing *aKap4* gene from a variety of species. Subsequent sequence analysis of the approximately 2.2 kb products revealed that all members of the melanogaster species subgroup possess *aKap4*, whereas all other Drosophila species assayed had a much smaller (650 bp) band that corresponded to the syntenic region in the intron of the *CG32406* but lacked *aKap4*. We therefore presume that *aKap4* was born in the ancestor of the melanogaster species subgroup (bold lines in schematic phylogeny).

(Mathe et al. 2000; Mason et al. 2003). We profiled expression patterns using PCR from cDNA collected from various *D. melanogaster* and *D. yakuba* male and female tissues. These analyses revealed that in stark contrast to the ubiquitously expressed *aKap3*, *aKap4* had acquired a highly testis-specific expression (fig. 1*B*). Our findings are in strong agreement with published profiles of gene expression (Chintapalli et al. 2007) that find that *aKap4* is only expressed in testes among all adult tissues assayed.

Using *aKap4* as a query, we next queried the other 12 Drosophila genomes whose sequence was available (Drosophila 12 Genomes Consortium 2007). We found *aKap4* to be present in multiple species closely related to *D. melanogaster* but absent from *D. ananassae* and more distantly branching species. To more precisely date the evolutionary birth of *aKap4*, we amplified the *aKap4* syntenic region from a panel of Drosophila species in the melanogaster group via PCR and performed gel electrophoresis. Presence of *aKap4* manifests as a band of higher molecular weight as compared with when the gene is absent at its syntenic position (fig. 1*C*). Using this analysis, we found *aKap4* to be present in all species in the melanogaster species subgroup but absent in all other species assuming that the same syntenic location has been maintained. *aKap4* sequences were confirmed with direct sequencing of PCR products. The birth of *aKap4* can thus be traced to just before the diversification of the melanogaster species subgroup, estimated to be approximately 13 Ma (Russo et al. 1995; Hedges et al. 2006).

What selective forces drove the evolutionary origin of *aKap4*? To address this, we performed a sliding window analysis comparing rates of nonsynonymous to synonymous changes (d*N* vs. d*S*) in *aKap4* genes from *D. melanogaster* to *D. simulans* (supplementary fig. S1, Supplementary Material online). We found several domains of *aKap4* had $dN/dS > 1$, with a pattern suggestive of multiple ARM repeats being subject to positive selection. To follow up on this, we sequenced *aKap4* from nine strains of *D. melanogaster* and ten strains of *D. simulans*. Using the McDonald–Kreitman test (McDonald and Kreitman 1991), we compared the total number of fixed replacement (amino acid altering, Rf) and silent substitutions (Sf) with those that were still polymorphic within species (Rp and Sp, respectively, fig. 2*A*). We find a strong excess of fixed replacement substitutions indicative of positive selection ($P = 0.005$, fig. 2*A*) having acted on *aKap4* since the divergence of *D. melanogaster* and *D. simulans*. On polarizing changes to either lineage using *D. yakuba* as outgroup, we find strong evidence of positive selection acting on *D. simulans* but not *D. melanogaster* (fig. 2*A*). In the *D. simulans* lineage, the ratio of fixed replacement to synonymous changes is 42:12, whereas the ratio of polymorphic replacement to synonymous changes is 10:13, suggesting an excess

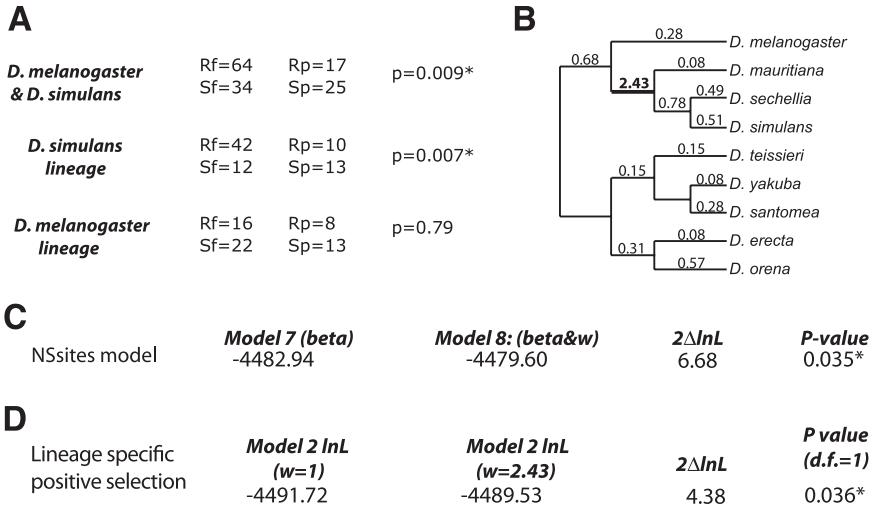


FIG. 2. (*A*) McDonald–Kreitman test for *aKap4* from *Drosophila melanogaster* and *Drosophila simulans*. Using a Fisher's exact test to evaluate significance, we find that *aKap4* has evolved under positive selection specifically in the lineage leading to *D. simulans*. (*B*) Free-ratio analysis for *aKap4* in the melanogaster group of species reveals a single lineage leading to the sister species *D. simulans*, *Drosophila mauritiana*, and *Drosophila sechellia* with a dN/dS ratio exceeding 1. This elevated dN/dS value of 2.43 was statistically significant when compared with the neutral expectation of 1 for this branch (see fig. 2*D*). (*C*) NSsites analysis for *aKap4* from the melanogaster group of species suggests that some residues in *aKap4* have evolved recurrently under positive selection. (*D*) Test for statistical significance of lineage-specific positive selection for *aKap4* in the branch highlighted in (*B*) using the branch-specific positive selection analysis.

of 12 replacement changes leading up to *D. simulans* (using the $P < 0.05$ threshold). The Tajima's *D* (Tajima 1989) value for *D. simulans* is 0.209, ($P > 0.05$), whereas for *D. melanogaster*, $D = -0.251$ ($P > 0.05$), suggesting that no recent selective sweep occurred in *aKap4* from either species. To address whether *aKap4* was subject to recurrent positive selection, we obtained sequences from all nine known members of the melanogaster species subgroup either via PCR or from genome databases. Using maximum likelihood based approaches to detect positive selection (Yang 2007), we find that the NSsites model M8, which permits positively selected codon residues, fits the nine *aKap4* sequences better than those models (M7, M8a) that disallow positive selection (fig. 2*C*). In a free-ratio analysis (fig. 2*B*), it appeared that the only branch-specific signature of positive selection (dN/dS > 1) emerged from branch leading to the triad of sister species *D. simulans*, *D. sechellia*, and *D. mauritiana*, which had a dN/dS of 2.43. We tested the statistical significance of this high dN/dS using a likelihood comparison either fixing the dN/dS of this branch to be 1 or 2.43. Indeed, we find that a dN/dS of 2.43 is much more likely than that of 1, indicating that this branch has evolved under with positive selection ($P < 0.05$) (fig. 2*D*). Barring this single episode of positive selection between 0.5 and 2.5 Ma, *aKap4* appears to have been preserved largely under purifying selection, with no apparent loss events in the melanogaster species subgroup (fig. 1*C*).

An Ancient Importin-α (*aKap5*) Was Lost in the melanogaster Species Subgroup

During our survey of the 12 Drosophila genomes via tBLASTn searches, we detected the presence of another importin-α gene, which we call *aKap5* in species such as *D. ananassae*, *D. pseudoobscura*, *D. virilis*, and *D. mojavensis* (fig. 3). *aKap5* appears only distantly related to any of the canonical importin-αs or to *aKap4*. The *aKap5* gene is intronless and encodes a protein that appears to lack an IBB domain at its N-terminus, similar to *aKap4* (fig. 3*A*). Intriguingly, we could not find any evidence for *aKap5* in the *D. melanogaster* genome or those of closely related species and in *D. grimshawi*. To more closely date the apparent loss of *aKap5* from the melanogaster group lineage, we PCR amplified the syntenic region of *aKap5* using primers in neighboring genes in a panel of Drosophila species and performed gel electrophoresis and sequencing analysis (fig. 3*C*). Sequencing results revealed that we had successfully amplified the syntenic regions of the genome. As we expected, the smaller PCR products accurately reflected the absence of *aKap5* in some species including *D. melanogaster*, as judged by the fact that a blastx search using these sequences revealed no homology to *aKap5* or any of the other importins. Conversely, the larger PCR products generally reflected the presence of *aKap5* in the genome. The only exceptions were the *D. yakuba*, *D. santomea*, and *D. teissieri* species that yielded a larger PCR band (fig. 3*C*), which was devoid of any *aKap5* sequence homology, instead reflecting an independent insertion. Furthermore, in species where whole-genome sequences are available such as those from the melanogaster group, we could confirm that no extra copy of aKap is present anywhere in the genome. *aKap5*, therefore, appears to have been lost through a deletion event ancestral to all species of the *D. melanogaster* species subgroup but present in most other Drosophila species.

Intriguingly, the loss of *aKap5* in the melanogaster species subgroup coincides exactly with the birth of *aKap4*—no species tested had both *aKap4* and *aKap5* simultaneously in their genomes. We were interested in whether *aKap5* also had an expression pattern in adult

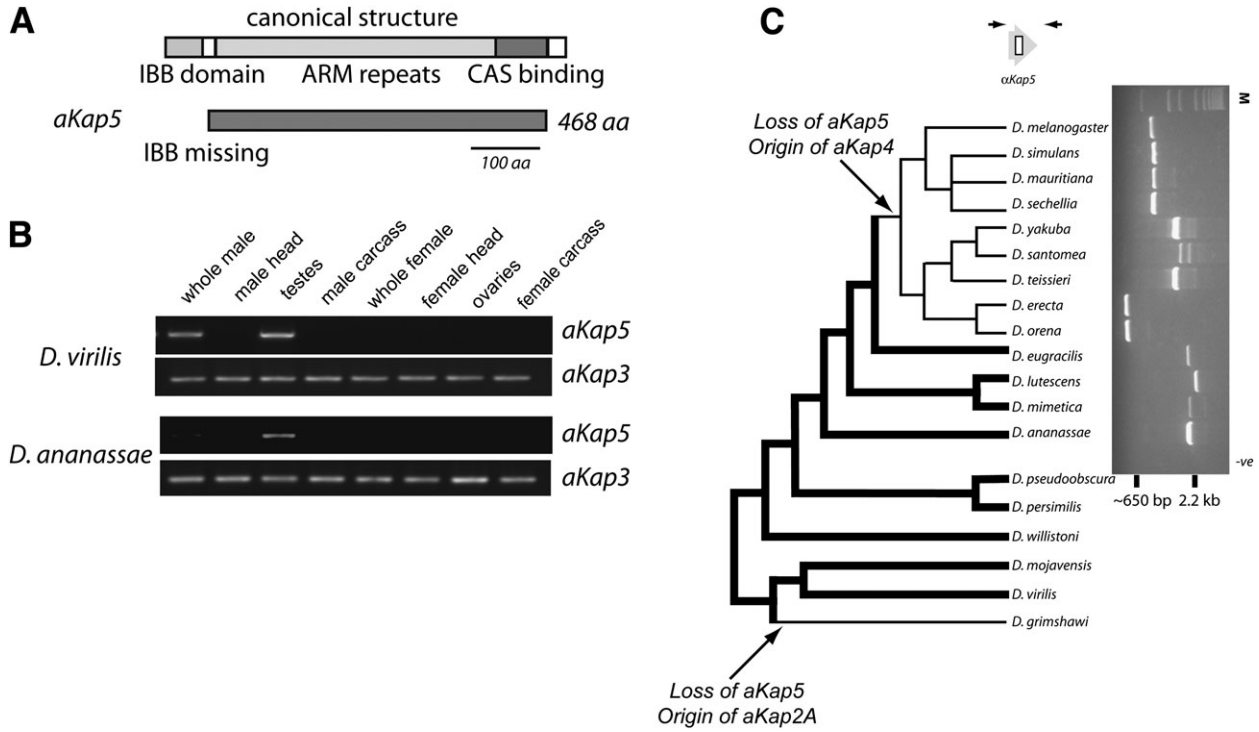


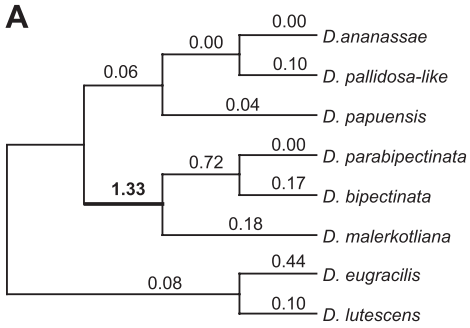
FIG. 3. (*A*) Gene structure of *aKap5* showing the absence of the canonical IBB domain. (*B*) Expression pattern of *aKap5* and *aKap3* in *Drosophila virilis* and *Drosophila ananassae* as revealed by RT-PCR. Like in *Drosophila melanogaster*, *aKap3* is expressed ubiquitously, whereas *aKap5* is expressed only in testes in both *D. virilis* and *D. ananassae*. (*C*) PCR surveys using *aKap5*-syntenic primers in the melanogaster group of species reveal that *aKap5* is ancestrally present in Drosophila species (bold lines on schematic phylogeny, larger band of 2.2 kb) but was lost in the melanogaster species subgroup (leading to the shorter band of ~800 bp) coincident with the gain of *aKap4* (fig. 1). Although the *Drosophila yakuba*, *Drosophila santomea*, and *Drosophila teissieri* species also yield a larger PCR product, sequencing of this product revealed no akap5 rather an independent genomic expansion. Further bioinformatic analyses of genome sequences in 12 Drosophila species further revealed not only the loss of *akap5* in *Drosophila grimshawi* but also the birth of *aKap2A* in this lineage.

Drosophila tissues, similar to the *aKap4* gene. We profiled expression patterns using PCR from cDNA collected from various male and female tissues from *D. virilis* and *D. ananassae*, two species that have *aKap5*. These analyses revealed that *aKap5*, similar to *aKap4*, shows a highly testis-specific expression (fig. 3*B*).

Is *aKap4* a transposed copy of *aKap5*? While gene movement may simultaneously explain both the loss of "older" *aKap5* and gain of "younger" *aKap4*, both the genomic organization of these genes as well as their phylogenetic relationships to each other (see below) argues against this scenario. *aKap5* is a retrogene lacking introns, whereas *aKap4* is a partial retrogene that harbors a single intron, which is present at the exact location as that in *aKap3*, indicating that *aKap4* was born from *aKap3*. We therefore consider the "gene movement" scenario unlikely and instead favor the more parsimonious "gene replacement" scenario in which *aKap4* is born from *aKap3* followed by the near simultaneous loss of *aKap5*.

To address the selective forces that drove the evolution of *aKap5*, we compared rates of nonsynonymous to synonymous changes (d*N* vs. d*S*) in *aKap5* sequences we obtained from *D. ananassae* and *D. bipectinata*. Sliding window and whole gene d*N*/d*S* analyses suggested that *aKap5* has evolved under purifying selection in these species (supplementary fig. S2, Supplementary Material online). We also sequenced *aKap5* from a panel of species in the ananassae species group to employ maximum likelihood approaches to detect positive selection (Yang 2007). These approaches also found no evidence for positive selection on *aKap5* sequences at least within the ananassae species subgroup (fig. 4*A* and *B*). In a free-ratio analysis, only a single branch leading to the bipectinata group of species shows a d*N*/d*S* ratio that is higher than 1. However, this branch does not meet a threshold of statistical significance ($P = 0.4$, fig. 4*C*), suggesting that *aKap5* has largely evolved under purifying selection throughout the evolution of the ananassae subgroup.

In addition to the melanogaster species subgroup, we found that *aKap5* has also been lost in *D. grimshawi*, as confirmed by tBLASTn searches of the region syntenic to *aKap5*. Intriguingly, we found yet another new nonorthologous copy of importin-α in *D. grimshawi*, which may serve as a "replacement" to *aKap5*. This importin-α is a retrogene that appears to be most closely related to *aKap2* based on amino acid similarity; we refer to this paralog as *aKap2A*. Strikingly, *aKap2A* is also missing its IBB domain similar to *aKap5* and *aKap4*. Because we did not have livestocks and cDNAs derived from specific tissues from *D. grimshawi*, we were unable to ascertain whether *aKap2A*, like *aKap4* and *aKap5*, is also restricted to the male germ line. Nevertheless, we can conclude that in



B NsSites model

Model 7 (beta)	Model 8: (beta&w)	2ΔlnL	P-value
-5274.76	-5274.76	0.001	0.99

C Lineage specific positive selection

Model 2 lnL (w=1)	Model 2 lnL (w=2.43)	2ΔlnL	P value (d.f.=1)
-5362.81	-5363.16	0.70	0.40

Fig. 4. (*A*) Free-Ratio analysis for *aKap5* in the ananassae species group reveals only one lineage with a dN/dS > 1; however, this branch did not meet the statistical threshold for significance using a branch-specific test (fig. 4*C*). (*B*) NSsites analysis for *aKap5* in the ananassae species group reveals no evidence for recurrently positively selected codons in *aKap5*. (*C*) Statistical tests for significance of the finding of lineage-specific positive selection for *aKap5* in (*A*) using the branch-specific test.

two independent lineages where *aKap5* has been lost, we observe a gain of new importin-α that also lacks an IBB domain (fig. 3*C*). Barring the loss events in the melanogaster species subgroup and in *D. grimshawi*, *aKap5* is otherwise preserved in all Drosophila species sampled in either the Drosophila or the Sophophora subgenus. Thus, our analyses have revealed that all Drosophila species without exception have a novel testes-specific importin-α that lacks an IBB domain.

Independent Duplications of Importin-α in *D. ananassae*, *D. pseudoobscura*, *D. virilis*

During our systematic survey of the 12 Drosophila genomes via tBLASTn searches, we detected the presence of additional importin-α gene paralogs (fig. 5). First, using Drosophila importin-α2 (*aKap2*) as a query, we identified a new importin-α gene *GF21735* in *D. ananassae* (fig. 5*A*). *GF21735* is a retrogene that appears to be born from *aKap2* in the *D. ananassae* subgroup (fig. 6) and is absent in other Drosophila lineages. We therefore refer to *GF21735* as *aKap2B*, to reflect its origin from *aKap2*. *aKap2B* bears armadillo/HEAT repeats typical of importin-αs. Characteristic of all previously identified canonical importin-α proteins, *aKap2B* also bears an IBB domain at its N-terminus. PCR analyses from cDNA collected from adult *D. ananassae* male and female tissues revealed that *aKap2B* has acquired a highly testis-specific expression after its birth from *aKap2*, which is enriched in the testes and ovaries (fig. 5*A*). Comparison of *aKap2B* orthologs between closely related species of the *D. ananassae* subgroup suggest that this paralog is also evolving under purifying selection (*D. ananassae* vs. *D. pallidosa-like*, whole gene dN/dS = 0.03; *D. ananassae* vs. *D. papuensis*, whole gene dN/dS = 0.41). If we make the simplifying assumption that retrogene formation was not accompanied by higher mutation rates, comparison between *aKap2B* and *aKap2* from *D. ananassae* shows dS = 1.5, suggesting that *aKap2B* is not a young duplication.

Second, in *D. virilis*, we identified another importin-α gene *GJ14921*, which appears to be a partial retrogene born from *aKap2* within the virilis subgroup (fig. 5*B*). We refer to this paralog as *aKap2C*. *aKap2C* bears armadillo/HEAT repeats characteristic of importin-αs and also bears an IBB domain at its N-terminus. *aKap2C* in *D. virilis* appears to be a young gene because it shows little sequence divergence from its parent gene *aKap2*. Because *aKap2C* is such a young gene, we were interested in further tracking its birth in the virilis subgroup, and whether this duplicate was fixed or polymorphic in *D. virilis* populations. We performed PCR with genomic DNA from *D. americana americana*, *D. americana texana*, *D. novamexicana*, and ten *D. virilis* strains. These analyses revealed that *aKap2C* is even younger than we anticipated, being born in the lineage leading to *D. virilis* and "after" the split between *D. virilis* and its most closely related species *D. am. americana* and *D. am. texana* (fig. 5*B*). The low dS value of 0.03 (*D. virilis aKap2* vs. *aKap2C*) is consistent with its recent origin. Based on sampling of multiple *D. virilis* strains, *aKap2C* appears to have gone to fixation in this species in a very short period of time. PCR analyses from cDNA collected from adult *D. virilis* male and female tissues shows that *aKap2C* has acquired a highly testis-specific expression after its birth from *aKap2*, which is enriched in the testes and ovaries (fig. 5*B*).

Third, an extra copy of *aKap5* is present in *D. pseudoobscura* as a part of tandem duplication of a 15 kb region that includes *aKap5* (*GA25249* and *GA25250*, which we call *aKap5A* and *aKap5B*, respectively) (fig. 5*C*). Based on the sequence of the two *D. pseudoobscura aKap5* copies and their common divergence from the single *D. persimilis aKap5*, we estimate that this duplication occurred less than 2 Ma (Hedges et al. 2006). The genomic duplication includes the coding region of *aKap5* along with >10 kb of upstream region. Therefore, we expect both copies of *aKap5* to show similar expression, which is likely testis specific like all assayed *aKap5* genes. Indeed, with primers common to both *aKap5* paralogs, we find testis-specific expression (fig. 5*C*). Because our primers target both *aKap5* paralogs, the expression profile observed is a composite of expression patterns of both *aKap5A* and *aKap5B* and does not allow us to discern whether one or both copies are actively transcribed in the testes.

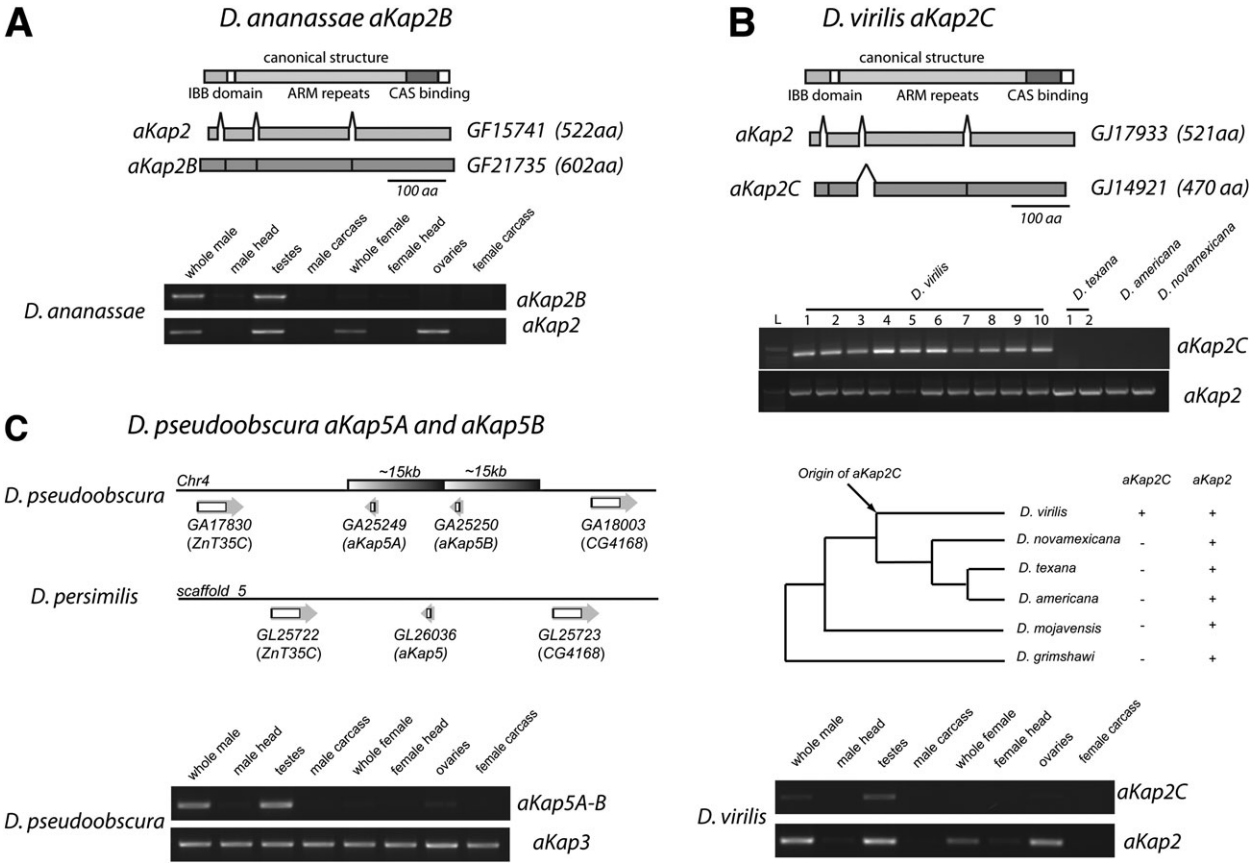


Fig. 5. (*A*) Gene structure and tissue-specific expression of *aKap2B* compared with the parental gene *aKap2* in *Drosophila ananassae*. Whereas parental *aKap2* is expressed in testes and ovaries, we find *aKap2B* to be exclusively expressed in testes of *D. ananassae* adults. (*B*) Gene structure of *aKap2C* compared with the parental *aKap2* gene in *D. virilis*. Genomic PCR analyses reveal that *akap2C* is a young *Drosophila virilis*–specific partial retrogene, whereas parental *aKap2* is present in other members of the virilis group. RT-PCR analyses on adult tissues reveal that *aKap2C* is testes specific in *D. virilis*, whereas *akap2* is expressed in both testes and ovaries. (*C*) Genomic region encompassing the *aKap5* duplication in *Drosophila pseudoobscura* compared with the syntenic region in *Drosophila persimilis*. Tissue-specific RT-PCR analyses of *aKap5* paralogs in *D. pseudoobscura* reveal testes-specific expression in contrast to the ubiquitously expressed akap3.

Phylogenetic Analyses of Importin-αs in Drosophila

To characterize the age and evolutionary relationships of importin-α genes, we performed phylogenetic analysis of aKap homologs from the 12 published Drosophila genomes based on an alignment of their amino acid sequences (fig. 6A). We also included mosquito homologs from *Anopheles gambiae* and *Aedes aegypti* genomes to represent ancient divergence within Diptera. We found that the importin-αs were easily distinguished into five monophyletic clades. The previously known, *aKap1*, *aKap2*, and *aKap3* lineages were found in all Drosophila as well as mosquito genomes. These lineages were associated with short branch lengths, indicative of a high degree of evolutionary constraint. In contrast, the testis-expressed *aKap5* clade had long branch lengths, which suggests a more relaxed constraint. We were unable to find *aKap5* orthologs in the two fully sequenced mosquito genomes—*Anopheles gambiae* and *Aedes aegypti*, As we expected, we do not find *aKap4* orthologs outside the melanogaster species subgroup. Although the identical intronic position between *aKap4* and *aKap3* led us to propose that *aKap4* derived from within *aKap3*, we found that *aKap4* represented a monophyletic clade distinct from both *aKap5* and *aKap3*. It is possible that the dramatically different evolutionary constraints acting on *aKap3* and *aKap4* clades have obscured their phylogenetic proximity to each other. Despite its presence in a more limited set of Drosophila species, the longer branch lengths of *aKap4* are more similar to *aKap5* than *aKap3*, suggesting a lower degree of functional constraint (fig. 6) and/or positive selection. The phylogenetic analysis also revealed that "new" importins had originated at several points in Drosophila evolution, representing both old and young events. These importins are spread throughout the Drosophila phylogeny and reveal that most Drosophila species carry between four and five importins (fig. 6B). In light of recent results that even young duplications can rapidly acquire essential function in Drosophila (Chen et al. 2010), these new genes warrant further study for their roles in nuclear import or other processes.

Discussion

Dynamic Repertoires of Importins in Drosophila

The nuclear import machinery in all eukaryotes performs the essential function of transporting molecules across the

B

	aKap1	aKap2	aKap3	aKap4	aKap5	aKap5B	aKap2A	aKap2B	aKap2C
Muller element	D	B	E	D	B	B	C	A	B
Size (aa)	543	522	514	442	468	468	464	602	470
D. melanogaster	+	+	+	+	-	-	-	-	-
D. simulans	+	+	+	+	-	-	-	-	-
D. sechellia	+	+	+	+	-	-	-	-	-
D. yakuba	+	+	+	+	-	-	-	-	-
D. erecta	+	+	+	+	-	-	-	-	-
D. ananassae	+	+	+	-	+	-	-	+	-
D. pseudoobscura	+	+	+	-	+	+	-	-	-
D. persimilis	+	+	+	-	+	-	-	-	-
D. wilistoni	+	+	+	-	+	-	-	-	-
D. mojavensis	+	+	+	-	+	-	-	-	-
D. virilis	+	+	+	-	+	-	-	-	+
D. grimshawi	+	+	+	-	-	-	+	-	-
IBB domain	Yes	Yes	Yes	No	No	No	No	Yes	Yes

Fig. 6. (*A*) Maximum-likelihood tree constructed using PhyML showing the phylogenetic relationship between all importins based on amino acid sequences. The five clades of aKap genes are highlighted *aKap1* through *aKap5*. Lineage-specific new aKap genes are highlighted in bold. Bootstrap values refer to 100 trials on PhyML performed using the webserver at www.phylogeny.fr. (*B*) A summary of the 12 sequenced Drosophila genomes indicating the presence and absence of importins across the Drosophila phylogeny. All importin-αs are indicated, along with their location in associated Muller elements. Also indicated is the size of the encoded proteins as well as all noncanonical IBB-domain lacking importins (in gray).

nuclear membrane through a broadly conserved mechanism. It is therefore surprising to observe several novelties in the evolution of Drosophila importins. We have uncovered an ancient importin-α gene—*aKap5*, which was missed in previous compendia of Drosophila importins. We have also found two intriguing instances where the loss of *aKap5* occurred near simultaneously with the gain of *aKap4* in the melanogaster species subgroup and *aKap2A* in *D. grimshawi*. Although *aKap4* was likely born out of a duplication of *aKap3* and *aKap2A* from *aKap2*, both appear to have acquired characteristics more reminiscent of *aKap5* (see below). In addition, among the 12 Drosophila genomes, we have discovered at least four separate gene duplications events, which are either subgroup

specific (*aKap2B* in *D. ananassae*), species specific (*aKap2C* in *D. virilis*), or may even be polymorphic within the species (*aKap5B* in *D. pseudoobscura*). We expect this pattern to recur in additional species of Drosophila that have not been assayed yet. Thus, there appears to have been significant diversity in the nuclear import adaptor molecules even within closely related Drosophila species. A comparable rate of gene loss may account for our finding that most Drosophila genomes only encode between four and five aKap genes (fig. 6*B*) despite a high rate of gene duplication; however, we have only documented two instances of loss in the same *aKap5* lineage (fig. 3, fig. 6*B*). Genome-wide estimates of gene duplication rates suggest that changes in gene copy number are very common (Lynch and Conery 2000; Hahn et al. 2007; Demuth and Hahn 2009; Rogers et al. 2009). The importin-α gene dynamics have clear and testable biological consequences that might allow organisms to compete in arms races against segregation distorters or transposable elements (see below).

Testes-Expressed Noncanonical Importins

The apparent genomic replacement of the ancestral importin *aKap5* by a new importin *aKap4* and *aKap2A* is particularly intriguing. *aKap5* is a testes-specific importin-α that lacks a canonical IBB domain. *aKap5* is present in most Drosophila species except for its loss in the lineages leading to the melanogaster species subgroup and to *D. grimshawi*. In the same lineages where *aKap5* is lost, we observe the birth of *aKap4* and *aKap2A*. *aKap4* and *aKap2A*, like *aKap5*, lack the canonical IBB domain and are also testes specific raising the exciting possibility that *aKap4* and *aKap2A* may be nonorthologous functional replacements of *aKap5*. The lack of IBB domain in these three importins is curious because the IBB domain is a universally conserved feature of importin-αs (Goldfarb et al. 2004). Furthermore, the IBB domain-mediated binding to importin-β is believed to be central to the translocation mechanism and is, therefore, often considered to be essential for nuclear transport. Can importin-αs that lack their IBB domain be functional in nuclear transport? Importin-αs lacking an IBB domain have been shown to be functional. GFP-tagged copies of importin-α that lack an IBB domain readily translocate into the nucleus in an importin-β and Ran independent manner in *S. cerevisiae* (Miyamoto et al. 2002). Furthermore, truncations of *aKap2* that lack the IBB domain have also been shown to localize inside the nucleus in *Drosophila* (Gorjanacz et al. 2006), suggesting that *aKap4*, *aKap2A*, and *aKap5* are viable candidates for functional nuclear importins. The wide conservation of *aKap5* and homology to other importin-αs also supports an important function in nuclear import. If so, the lack of an IBB domain might suggest importin β-independent nuclear import by *aKap4*, *aKap2A*, and *aKap5*. At this juncture, however, we cannot rule out the possibility of convergent evolution of these noncanonical importin-derived genes for some function that is independent of nuclear import.

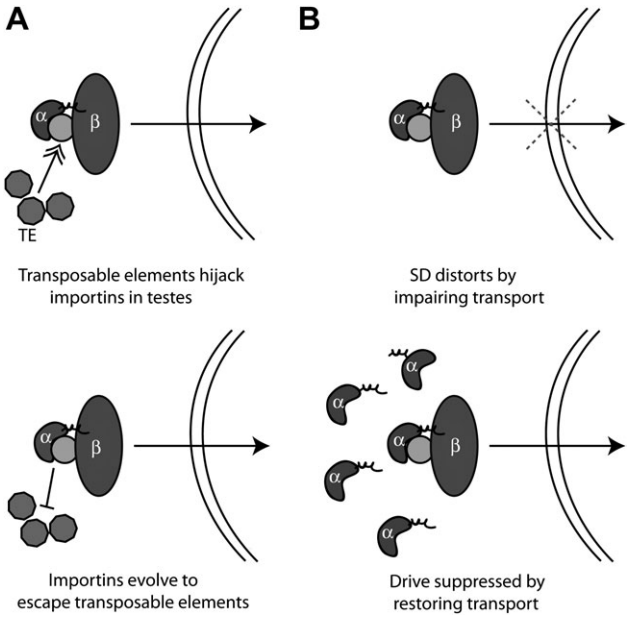


Fig. 7. (*A*) Model for importin evolution where pressure to escape from antagonism from transposable elements (or unknown viruses) that wish to access the germ line genome leads to amino acid sequence evolution in importins. (*B*) An alternate (favored) model for importin evolution where pressure for restoring transport to suppress segregation in male gametogenesis drives innovation in importins.

Evolutionary Forces Driving Gene Innovation in Importins

Molecular population genetic tests of selection show that most novel aKaps evolve under purifying selection, except for a single episode of positive selection acting on *aKap4* (fig. 2). Therefore, the bulk of the evolutionary innovation is manifest as the acquisition of extra importins in the testes. This raises the question as to why testes function requires so many extra importins. At least two evolutionary arms race scenarios can explain the dynamic evolution of nuclear import components specific to Drosophila testes.

The first possibility for an evolutionary arms race scenario that engages the nuclear transport machinery in the male germ line is that of transposable elements that require entry to the nucleus to be passed on to the gametes (fig. 7*A*). Studies in *S. cerevisae* have clearly demonstrated the impact the nuclear import has on the transposition rates of Ty elements (Irwin et al. 2005). Such transposable elements rely on the host nuclear import machinery to gain access to the nucleus and thus exert evolutionary pressure on nuclear transport components to avoid binding by these elements. Under such a model, importins could be targets of transposable element integration machineries to facilitate nuclear import especially in the male germ line. However, this scenario does not predict an increased dosage of importins in the male germ line; rather, it would predict more recurrent signatures of positive selection (Sawyer and Malik 2006) as seen in other instances of host–virus interactions (Elde et al. 2009). Our dual findings of recurrent birth but rare positive selection are inconsistent with

a simplistic model of an arms race with transposable elements. However, in the absence of experimental data especially on IBB-lacking importin-α proteins, we cannot rule out that such an arms race may be the driving force behind the gene dynamics we have observed.

We favor an alternate model of genetic conflict between nuclear import components and segregation distorters as a likely explanation for the dynamic evolution of the nuclear transport repertoire involved in male gametogenesis (fig. 7*B*). Segregation distortion in Drosophila male gametogenesis is known to operate through impairment of the nuclear transport. The SD system in *D. melanogaster* operates by impairing nuclear transport in the testes, via disruption of the RanGTP gradient across the nuclear membrane. A series of experiments showed that segregation distortion could be suppressed by restoring the RanGTP gradient and nuclear transport in the testes (Kusano et al. 2002). Although SD is caused by a partial duplication of RanGAP, the original copy of RanGAP itself evolves rapidly under recurrent positive selection, likely due to a constant and milder version of segregation distortion (Presgraves 2007). It is possible that male gametogenesis is particularly sensitive to slight perturbations of nuclear import efficiency. For instance, even the SD-mediated disruption of the RanGTP gradient appears to biologically manifest only or primarily in male gametogenesis (Kusano et al. 2001). Indeed, it seems likely that such constant pressure to suppress SDs that rely on impairing nuclear transport could lead to the birth of importins specific to the testes to improve nuclear transport as a mechanism of suppression of distortion. Such an evolutionary arms race between SDs that act during male gametogenesis and suppressors of distortion can manifest as the repeated innovation of nuclear transport components specific to the testes.

Given multiple pressures for constantly changing the rate of nuclear import in the male germ line, recurrent gene duplication of testes-specific importin-αs might also be expected under a sexual antagonism model (Gallach and Betran 2011). Rather than adapting the existing broadly expressed importins to accommodate the extra needs in male gametogenesis and risk maladaptation for their normal somatic or female germ line function, antagonistic functional constraints may be resolved by the propagation of gene duplications. Under this model, the novel importin-α can specialize for male gametogenic function without affecting the function of the other importin-αs. Similar arguments have been invoked to explain the presence of male gametogenesis-specific gene duplications for mitochondrial function (Gallach and Betran 2011).

Our expression analyses strongly implicate testes expression as the main driver behind importin evolution. However, since we have not assayed other developmental stages, we cannot exclude the possibility that these importins are acting at other important developmental stages. For instance, *aKap4* does appear to be expressed in pupal and adult male stages (Chintapalli et al. 2007). Moreover, our hypotheses for the evolutionary forces driving importin innovation specifically assume a germ line function, we do not yet know whether the testes-specific expression we have seen is actually limited to the germ line compartment. It is formally possible that the innovation is actually driven for somatic function in Drosophila testes. Nevertheless, given the analogous situation seen with both NPC and Ran gradient establishing components, we favor this common hypothesis for gene innovation in importins. Together with previous results on the evolutionary innovation among components of NPC and the Ran gradient transport factors, our studies highlight the important role played by genetic conflicts in the male germ line in driving novelty in the nuclear import pathway.

Supplementary Material

Supplementary table S1 and figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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