The *Drosophila melanogaster importin α3* Locus Encodes an Essential Gene Required for the Development of Both Larval and Adult Tissues

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ABSTRACT

The nuclear transport of classical nuclear localization signal (cNLS)-containing proteins is mediated by the cNLS receptor importin α. The conventional importin α gene family in metazoan animals is composed of three clades that are conserved between flies and mammals and are referred to here as α1, α2, and α3. In contrast, plants and fungi contain only α1 genes. In this study we report that Drosophila *importin α3* is required for the development of both larval and adult tissues. *Importin α3* mutant flies die around the transition from first to second instar larvae, and homozygous *importin α3* mutant eyes are defective. The transition to second instar larvae was rescued with *importin α1*, *α2*, or *α3* transgenes, indicating that Importin α3 is normally required at this stage for an activity shared by all three importin α's. In contrast, an α3-specific biochemical activity(s) of Importin α3 is probably required for development to adults and photoreceptor cell development, since only an *importin α3* transgene rescued these processes. These results are consistent with the view that the importin α's have both overlapping and distinct functions and that their role in animal development involves the spatial and temporal control of their expression.

MOST proteins targeted to the nucleus contain nuclear localization signals (NLSs) that are recognized by soluble receptors called karyopherins (Macara 2001; Bednenko *et al.* 2003; Weis 2003). Proteins containing classical NLSs (cNLSs) are imported bound to the importin α/β1 heterodimer. Importin α serves as an adapter that links cNLS cargo to the karyopherin importin β1, which ferries the complex through the nuclear pore complex (Macara 2001; Bednenko *et al.* 2003; Weis 2003).

The genomes of metazoan organisms encode multiple importin α genes. For example, the human genome encodes six importin α's (Köhler *et al.* 2002; Cabot and Prather 2003). Phylogenetic analyses of the importin α gene family revealed that most importin α's belong to one of three evolutionarily conserved clades, designated by our nomenclature as conventional α1's, α2's, and α3's (Köhler *et al.* 1997, 1999; Malik *et al.* 1997; Máthé *et al.* 2000; Mason *et al.* 2002). Conventional importin α's from plants and fungi are all α1's. In contrast, metazoan animals, with the exception of *Caenorhabditis elegans* (Geles and Adam 2001), contain representatives from each of the three groups. Parsimony arguments suggest that metazoan α2 and α3 genes arose from α1 progenitors in ancestral single-cell eukaryotic lineages.

Vertebrate importin α's show distinct tissue- and cell-type-specific expression patterns (Prieve *et al.* 1996; Köhler *et al.* 1997, 2002; Tsuji *et al.* 1997; Nachury *et al.* 1998; Kamei *et al.* 1999), and human importin α paralogs are differentially regulated in quiescent and proliferating cultured cells and tissue differentiation models (Köhler *et al.* 2002). *In vitro* binding assays and permeabilized cell transport assays indicate that α1's, α2's, and α3's have both overlapping and distinct sets of transport cargoes (Miyamoto *et al.* 1997; Nadler *et al.* 1997; Sekimoto *et al.* 1997; Prieve *et al.* 1998; Köhler *et al.* 1999, 2001; Welch *et al.* 1999; Kumar *et al.* 2000; Nemergut and Macara 2000; Talcott and Moore 2000; Jiang *et al.* 2001; Guillemain *et al.* 2002; Melén *et al.* 2003). For example, a vertebrate α3 has unique specificity for RCC1 (Köhler *et al.* 1999; Nemergut and Macara 2000; Talcott and Moore 2000), Ran BP3 (Welch *et al.* 1999), interferon regulatory factor 3 (Kumar *et al.* 2000), and adenoviral E1A (Köhler *et al.* 2001). An α2 selectively bound the glucose transporter GLUT2 (Guillemain *et al.* 2002) and an α1 specifically transported STAT1 and STAT2 transcription factors (Sekimoto *et al.* 1997; Melén *et al.* 2003). Importantly, the preference of importin α's for NLS cargo can be altered when two different substrates are presented together in permeabilized cell transport assays (Kohler *et al.* 1999). This latter finding underscores the complexity of the functional interactions between importin α's and different NLS cargo and indicates that *in vivo* studies are needed to unravel the physiological roles of individual importin α's.

In vivo studies are consistent with the notion that the different importin α's play distinct roles in animal development. The RNAi-mediated disruption of an α3 paralog, but not an α2, had a severe effect on the devel-

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opment of porcine embryos (Cabot and Prather 2003). Likewise, RNAi-mediated reductions in the expression of different importin α's caused distinct developmental defects in *C. elegans* (Geles and Adam 2001; Askjaer *et al.* 2002; Geles *et al.* 2002). Specifically, the *C. elegans* α3 paralog ima-3 is required for meiosis in the developing female germline (Geles and Adam 2001), while the nonconventional ima-2 is required for mitosis (Geles *et al.* 2002). Proper spindle formation requires ima-2 but not ima-3 (Askjaer *et al.* 2002).

The Drosophila genome encodes four importin α's (Küssel and Frasch 1995; Török *et al.* 1995; Dockendorff *et al.* 1999; Adams *et al.* 2000; Máthé *et al.* 2000; Giarrè *et al.* 2002; Mason *et al.* 2002), three of which contain conserved Importin β1 (Görlich *et al.* 1996; Weis *et al.* 1996) and cNLS-binding domains (Conti *et al.* 1998; Dockendorff *et al.* 1999). The fourth predicted Drosophila importin α, *cg14708* (Adams *et al.* 2000), is extremely divergent, has a weakly conserved IBB domain, and is missing the conserved tryptophan-asparagine array that is crucial for binding to cNLS cargo (Conti *et al.* 1998).

The three conventional Drosophila importin α paralogs have different developmental stage- and cell-type-specific expression patterns (Küssel and Frasch 1995; Török *et al.* 1995; Dockendorff *et al.* 1999; Máthé *et al.* 2000; Fang *et al.* 2001; Giarrè *et al.* 2002). In addition, α1 and α2, but not α3, accumulate in the nucleus at the onset of mitosis (Küssel and Frasch 1995; Török *et al.* 1995; Máthé *et al.* 2000; Giarrè *et al.* 2002). Null α*2* mutations result in defects in gametogenesis that cause incompletely penetrant male sterility and complete female sterility (Giarrè *et al.* 2002; Gorjánácz *et al.* 2002; Mason *et al.* 2002). The α2 activity essential for female fertility appears to be unique to α2 since it cannot be replaced by the ectopic expression of α*1* or α*3* transgenes. In contrast, male sterility was rescued to a similar extent by the expression of α*1*, α*2*, and α*3* (Mason *et al.* 2002).

Drosophila Importin α3 has been identified as a binding partner of germ cell-less (Dockendorff *et al.* 1999), DNA polymerase α (Máthé et *al.* 2000), and heat-shock factor (HSF; Fang *et al.* 2001). *Importin* α*3* mRNA and protein were not detected in early embryos, coincident with the restriction of HSF to the cytoplasm (Fang *et al.* 2001). Finally, defects in α3 nuclear export correlate with specific cell fate transformations in mechano-sensory organs observed in hypomorphic mutations in the importin α recycling factor *Dcas* (Tekotte *et al.* 2002). In this study we describe the developmental defects associated with severe mutations in α*3* and conclude that α3 is required for the development of both larval and adult tissues. Transgene rescue studies demonstrate that the requirement for α3 in larval development can be partially replaced by ectopic expression of α*1* or α*2*. In contrast, only ectopic α*3* expression can support development to adults. In the eye, α1, but not α2, can partially replace α3 in at least some cell types, but α3 appears to be uniquely required for the proper differentiation of photoreceptor cells.

MATERIALS AND METHODS

Genetic stocks and markers: Flies were kept on standard cornmeal-dextrose media and grown at 25° unless indicated otherwise. The *importin* $\alpha 3^{1}$*/TM6C* stock is described in Máthé et *al.* (2000). The FRT82B, $\alpha 3^{17\text{-}7}$*/TM3* {Kr-GFP}, Sb^{1} and FRT82B, $\alpha 3^{w73}$*/TM3* {Kr-GFP}, Sb^{1} stocks were created and provided by Tory Herman and Larry Zipursky [University of California (UCLA), Los Angeles]. The $\alpha 2^{D14}/y^{+}$ *CyO* stock was created by Bernard Mechler (Department of Developmental Genetics, DKFZ, Heidelberg, Germany) and provided by István Kiss (Hungarian Academy of Sciences, Szeged, Hungary) (Török *et al.* 1995; Giarrè *et al.* 2002; Gorjánácz *et al.* 2002). The $Gal4^{tpmo\text{-}VP16}$ stock (Rørth 1998) was provided by Pernille Rørth (EMBL, Heidelberg, Germany). The (1) $Gal4^{tubP}$ (P{w + mC = tubP-GAL4}LL7)*/TM3,*Sb^{1}; (2) $Gal4^{Act5C}$ (P{w[+mC] = Act5CGAL4} 17bFO1)*/TM6B,* Tb^{1}; (3) $Gal4^{arm}$ (P{w[+m W.hs] = GAL4-armS}4a P{w[+mW.hs] + GAL4-arm.S}4b)*/TM3,* Sb^{1}; (4) Sb^{1}*/TM3* P{w + mC = ActGFP}JMR2, Ser^{1}; (5) *Df(3R)GB104/TM3,* Sb^{1}; (6) *Df(3R)by416/TM3,* Sb^{1}; (7) *Df(3R)by62/TM1*; (8) *TM3, Sb1,* P{ry[+t7.2] = Δ2-3}99B*/Df(3R)C7, ry[506]*; and (9) $Gal4^{eve}$, UASt FLP*/CyO* ; FRT82B, GMR-*hid, l(3)CL-*R^{1}*/TM2* (Stowers and Schwarz 1999) stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University.

PCR of *importin* $\alpha 3^{1}$: Genomic DNA was prepped from single flies of the indicated genotypes and used for PCR. PCR conditions were: 2 μM primers; 1.5 mM $MgCl_2$; 2.5 units Taq DNA polymerase; and 2 mM dATP, dCTP, dGTP, and dTTP (annealing temperature is 62°, 30 cycles). The 5′ *P*-element primer, PF-2 or primer 1 in Figure 1, had the sequence CGAC GGGACCACCTTATGTTAT (Eggert *et al.* 1998), and the 3′ primer, α*3* 3′N*sad*I or primer 2 in Figure 1, had the sequence CGCACGCCGCGGCCTTTGCCAGCTTCTTCAGG. The resulting band that appears only when *importin* $\alpha 3^{1}$ is present was sequenced and shown to correspond to a *P*-element insertion ~780 bp from the ATG of α*3* (see also Máthé et *al.* 2000).

Expression constructs and germline transformations: UASp Importin α transgenes were created by cloning α*1* (Mason *et al.* 2002), α*2*, or α*3* PCR fragments containing a 5′ Cavener consensus sequence (AAAATG; Cavener 1987) and the ~1.5-kb coding region into *Kpn*I and *Not*I sites in the UASp *P*-element transformation vector (Rörth 1998). In contrast to transgenes used previously (Mason *et al.* 2002), the α*2* and α*3* transgenes do not contain any α*2*- or α*3*-specific 5′ or 3′ untranslated region (UTR) sequences. The UASp α*1* transgene contains 1 nucleotide of α*1* 3′UTR. Transgenic UASp α*1*, α*2*, and α*3* lines were created using standard germline transformation procedures (Spradling 1986). The UASp α*1*, α*2*, and α*3* inserts used in this study were all located on the second chromosome.

Larval cuticle preps: *Importin* α*3* alleles were balanced with a green fluorescent protein (GFP)-tagged *TM3* chromosome and the appropriate crosses were set up in egg-laying cups. Eggs were laid on apple juice plates overnight. Resulting first instar larvae were examined with a UV dissecting microscope and nonfluorescent larvae were removed to a plate containing standard cornmeal-dextrose media. After ~24–48 hr dead larvae were isolated and larval cuticles were prepared as previously described (Stern and Sucena 2000), except that 4% paraformaldehyde was used as the fixative. Cuticles were observed by differential interference contrast (DIC) imaging with a Leica TCS NT microscope equipped with UV, Ar, Kr/Ar, and

He/Ne lasers. Digital images were processed using Adobe PhotoShop (Adobe Systems, San Jose, CA).

Northern and Western blots: Total RNA and protein were isolated from Drosophila tissues with Tri-Reagent LS (Molecular Research Center, Cincinnati; Chomczynski 1993) following the recommended protocols. Protein isolated from larvae of the indicated stage and genotype was analyzed by Western blot with rabbit anti-Importin α2 (Török *et al.* 1995) provided by Istvan Török (DKFZ, Heidelberg, Germany), rabbit anti-α3 (Máthé *et al.* 2000), or a mouse anti-α-tubulin antibody (Amersham Biosciences, Piscataway, NJ). Blots were developed using alkaline phosophatase-tagged goat anti-rabbit secondary antibodies. The Fermentas Prestained Protein Ladder, an ~10- to 180-kD size marker (Fermentas, Hanover, MD; Figure 3, A and C), or the GIBCO BRL (Gaithersburg, MD) benchmark size marker (Life Technologies, Grand Island, NY; Figure 3B) were used as size markers. RNA isolated from heterozygous and homozygous $\alpha 3^{D93}$ and $\alpha 3^{D165}$ mutant first instar larvae was analyzed by Northern blot with an *α3* full-length random prime ^{32}P-labeled probe (not shown).

Scanning electron microscopy of adult eyes: Flies of the indicated genotypes were dehydrated in a graded ethanol series and stored in 100% ethanol. Flies were critical point dried, coated with gold, and examined by scanning electron microscopy with a LEO 982 FESEM microscope. Digital images were processed using Adobe PhotoShop (Adobe Systems).

Eye sectioning: Fly eyes of the indicated genotypes were embedded in Durcapan resin according to standard procedures (Wolff 2000), sectioned to 1 μm thickness, stained with toluidine blue, and observed by DIC imaging. Digital images were processed using Adobe PhotoShop (Adobe Systems).

Immunofluorescence: Ovaries were dissected from females of the indicated genotypes, fixed in 1× PBS, 4% paraformaldehyde, and blocked in PBS-saponin (1× PBS, 0.1% saponin, and 1% normal goat serum). Ovaries were incubated with a mouse anti-Kelch antibody (Xue and Cooley 1993) diluted 1:1 (Gorjánácz *et al.* 2002) in PBS-saponin, followed by a goat anti-rabbit FITC-labeled secondary antibody diluted 1:300 in PBS-saponin. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Samples were examined by confocal microscopy and digital images were processed using Adobe PhotoShop (Adobe Systems). The anti-Kelch antibody was developed by L. Cooley and provided by the Developmental Studies Hybridoma Bank (Iowa City, IA).

Crosses: *Recombination of importin* $\alpha 3^1$*:* $Gal4^{arm}$*/TM3, Sb*^{1} or $Gal4^{Act5C}$*/TM6B, Tb*^{1} males were crossed to *importin* $\alpha 3^1$*/TM6C, Sb*^{1}*, Tb*^{1} virgin females. $Gal4^{arm}/\alpha 3^1$ or $Gal4^{Act5C}/\alpha 3^1$ virgin female offspring were collected and mated to *TM3, Sb*^{1}*/TM6B, Tb*^{1} males. Resulting male offspring were selected for a dark red eye. These males were utilized to make stocks and the presence of the *P* element in $\alpha 3^1$ was verified by PCR (see above).

To allow *importin* $\alpha 3^1$ to recombine with a "wild-type" third chromosome, w^{1118} males were crossed to $\alpha 3^1$*/TM6C, Sb*^{1}*, Tb*^{1} females. *Importin* $\alpha 3^1$/+ virgin female offspring were collected and mated to *TM3, Sb*^{1}*/TM6B, Tb*^{1} males. Recombinant $\alpha 3^1$*/TM6B, Tb*^{1} males were mated individually to virgin females from the original $\alpha 3^1$*/TM6C, Sb*^{1}*, Tb*^{1} stock. Crosses were incubated at room temperature and each vial was examined for the presence of non-*Tb*^{1} pupal offspring. If non-*Tb*^{1} pupae were observed the adult offspring from this cross were analyzed. Four recombinant $\alpha 3^1$ chromosomes were viable over the original $\alpha 3^1$ chromosome [designated $\alpha 3^{1(R1)}$, $\alpha 3^{1(R2)}$, $\alpha 3^{1(R3)}$, and $\alpha 3^{1(R4)}$]. Stocks were made for the $\alpha 3^{1(R1)}$ and $\alpha 3^{1(R2)}$ chromosomes, and the presence of the *P* element was verified by PCR (Figure 1).

Analysis of importin $\alpha 3^1$ *viability: Importin* $\alpha 3^1$*/TM6C, Sb*^{1}*, Tb*^{1} females were crossed to: (a) $\alpha 3^1$*/TM6C, Sb*^{1}*, Tb*^{1}; (b) $\alpha 3^{1(R1)}$*/TM3, Sb*^{1}; (c) $\alpha 3^{1(R2)}$*/TM6C, Sb*^{1}*, Tb*^{1}; (d) *Df(3R)by416/TM3, Sb*^{1}; (e) *Df(3R)GB104/TM3, Sb*^{1}; (f) *Df(3R)by62/TM1*; or (g) $Gal4^{arm,\ \alpha 3^1}$*/TM6B, Tb*^{1} males. Likewise, $\alpha 3^{1(R1)}$*/TM3, Sb*^{1} flies were crossed to (a) $\alpha 3^{1(R1)}$*/TM3, Sb*^{1}; (b) *Df(3R)by416/TM3, Sb*^{1}; (c) *Df(3R)GB104/TM3, Sb*^{1}; or (d) *Df(3R)by62/TM1* flies. Finally, $\alpha 3^{1(R2)}$*/TM6C, Sb*^{1}*, Tb*^{1} flies were crossed to (a) $\alpha 3^{1(R2)}$*/TM6C, Sb*^{1}*, Tb*^{1} or (b) *Df(3R)GB104/TM3, Sb*^{1}. For all crosses offspring were analyzed for the presence of the appropriate markers on balancers and viability indices were calculated by dividing the number of observed offspring by the number of expected offspring if all nonhomozygous balancer genotypes were equally viable (Table 1). Offspring inheriting two copies of the same balancer (*e.g., TM3, Sb*^{1}*/TM3, Sb*^{1}) were assumed to be completely lethal. Offspring inheriting two different balancers (*e.g., TM3, Sb*^{1}*/TM6C, Sb*^{1}*, Tb*^{1}) were often lethal. However, in crosses in which these offspring were observed the viability was calculated assuming them to be fully viable.

Creating deletions in importin $\alpha 3$*: Importin* $\alpha 3^{1(R1)}$*/TM3, Sb1* virgin females were crossed to *TM3, Sb1,* P{ry[+t7.2] = Δ2-3}99B*/Df(3R)C7, ry[506].* "Jump start" male offspring of the genotype $\alpha 3^{1(R1)}$*/TM3, Sb*^{1}, P{ry[+t7.2] = Δ2-3}99B were collected and mated to *TM3, Sb*^{1}*/TM6B, Tb*^{1} virgin females. Resulting white-eyed, non-ebony, *TM3, Sb*^{1} or *TM6B, Tb*^{1} male offspring were selected and mated individually to *TM3, Sb*^{1}*/TM6B, Tb*^{1} virgin females. After ~5 days of mating 10–20 males were pooled together for a genomic DNA extraction. Approximately 3 μl of this genomic DNA was then used in a PCR reaction (60° annealing temperature, 1.5 mm $MgCl_2$) with the *α3* 3′N*Sac*II primer, primer 2 in Figure 1 (sequence above), and the *α3* 5′ prom 2 primer, primer 3 in Figure 1 (α3 5′ prom 2 sequence, CCAGTTCATTGCTGTTGCTCC). Small deletions in *α3* were detected by the presence of a smaller PCR product. If a pool of DNA was shown to contain an *α3* deletion, DNA was extracted separately from offspring of each of the 10–20 males. This DNA was utilized in the PCR reaction as described above, enabling the identification of the specific line that contained the deletion. The shortened PCR products were gel purified with QiaQuick columns (QIAGEN, Valencia, CA) and sequenced.

Analysis of importin $\alpha 3$ *mutant alleles: Importin* $\alpha 3^{D93}$*/TM3, Sb*^{1} flies were crossed to: (a) $\alpha 3^{D93}$*/TM3, Sb*^{1}; (b) FRT82B, $\alpha 3^{17\text{-}7}$*/TM3* {GFP}, *Sb*^{1}; (c) FRT82B, $\alpha 3^{w73}$*/TM3* {GFP}, *Sb*^{1}; (d) *Df(3R)by416/TM3, Sb*^{1}; (e) *Df(3R)GB104/TM3, Sb*^{1}; (f) *Df(3R) by62/TM1*; or (g) $\alpha 3^1$*/TM6C, Sb*^{1}*, Tb*^{1} flies. Likewise, $\alpha 3^{D165}$*/TM3, Sb*^{1} or *TM6B, Tb*^{1} or *TM3* {GFP}, *Ser*^{1} flies were crossed to: (a) $\alpha 3^{D165}$*/TM3, Sb*^{1}; (b) FRT82B, $\alpha 3^{17\text{-}7}$*/TM3* {GFP}, *Sb*^{1}; (c) FRT82B, $\alpha 3^{w73}$*/TM3* {GFP}, *Sb*^{1}; (d) *Df(3R)by416/TM3, Sb*^{1}; (e) *Df(3R)GB104/TM3, Sb*^{1}; (f) *Df(3R)by62/TM1*; or (g) $\alpha 3^{D93}$*/TM6B, Tb*^{1}. Finally, *Df(3R)GB104/TM3, Sb*^{1} flies were crossed to (a) FRT82B, $\alpha 3^{17\text{-}7}$*/TM3* {GFP}, *Sb*^{1} or (b) FRT82B, $\alpha 3^{w73}$*/TM3* {GFP}, *Sb*^{1} flies. For all crosses offspring were analyzed for the presence of the appropriate markers on balancers and viability indices were calculated as described above (Table 2).

To determine the approximate stage of lethality the *importin* *α3* mutant alleles and deficiency chromosomes were balanced with *TM3* {GFP} chromosomes and the appropriate crosses were repeated in egg-laying cups. Embryos were allowed to hatch and early first instar larvae were sorted by fluorescence. Nonfluorescent first instar larvae were collected on a cornmeal agar plate and their development was observed at 25°.

Rescue of importin $\alpha 3^{D93}/\alpha 3^{D93}$ *lethality:* Male flies of the genotype UASp *importin* *α1*, *α2* or *α3*/UASp *α1*, *α2*, or *α3*; FRT82B, $\alpha 3^{D93}$*/TM3* {GFP}, *Ser*^{1} (males carrying UASp *α1* had the original $\alpha 3^{D93}$ chromosome instead of FRT82B, $\alpha 3^{D93}$) were crossed to virgin females of the genotype $Gal4^{tubP}$, $\alpha 3^{D93}$*/TM3* {GFP}, *Ser*^{1}. Progeny were scored at the onset of pupariation for fluorescence when observed through the side of the vial with a

UV dissecting microscope. The resulting adult progeny were scored for the presence of the *Ser*^{1} marker on the *TM3*{GFP}, *Ser*^{1} chromosome. Viability indices were calculated by dividing the number of observed offspring by the number of expected offspring if all nonhomozygous balancer genotypes were viable to the indicated stage (Table 3). Due to the partial penetrance of *Ser*^{1}, all non-*Ser*^{1} flies were assayed for fluorescence with a UV dissecting microscope to conclusively determine their genotype. As a negative control, $\alpha 3^{D93}$/*TM3*{GFP}, *Ser*^{1} males were mated to Gal4^{tubP}, $\alpha 3^{D93}$/*TM3*{GFP}, *Ser*^{1} females.

The approximate stage of lethality for UASp *importin* $\alpha 1$, $\alpha 2$, or $\alpha 3$;$\alpha 3^{D93}$/Gal4^{tubP}, $\alpha 3^{D93}$ offspring was determined as previously described. FRT82B, $\alpha 3^{D93}$/Gal4^{tubP}, $\alpha 3^{D93}$ and $\alpha 3^{D93}$/Gal4^{tubP}, $\alpha 3^{D93}$ offspring served as negative controls.

Rescue of importin $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ *lethality:* Male flies of the genotype UASp *importin* $\alpha 1$, $\alpha 2$, or $\alpha 3$/*CyO*; Gal4^{tubP}, $\alpha 3^{D93}$/*TM3* {GFP}, *Ser*^{1} were crossed to females of the genotype FRT82B, $\alpha 3^{17\text{-}7}$/*TM3* {GFP}, *Sb*^{1}. Progeny were scored at the onset of pupariation as previously described. It was assumed that all nonfluorescent pupae had inherited the UASp importin α transgene and not *CyO*. Adult offspring were scored for the presence of the *CyO*, *TM3* {GFP}, *Ser*^{1}, and *TM3* {GFP}, *Sb*^{1} balancers using the appropriate markers. Viability indices were calculated as previously described, except we assumed that all *CyO*; Gal4^{tubP}, $\alpha 3^{D93}$/ FRT82B, $\alpha 3^{17\text{-}7}$ offspring died as first/second instar larvae and, therefore, zero offspring of this genotype were expected at later stages. The viability of adult progeny was calculated for (a) UASp $\alpha 1$, $\alpha 2$ or $\alpha 3$; Gal4^{tubP}, $\alpha 3^{D93}$/ FRT82B, $\alpha 3^{17\text{-}7}$ experimental flies and (b) UASp $\alpha 1$, $\alpha 2$, or $\alpha 3$; Gal4^{tubP}, $\alpha 3^{D93}$/*TM3* {GFP}, *Sb*^{1} positive control flies (Table 4). As a negative control Gal4^{tubP}, $\alpha 3^{D93}$/*TM3* {GFP}, *Ser*^{1} males were crossed to FRT82B, $\alpha 3^{17\text{-}7}$/*TM3*{GFP}, *Sb*^{1} females and the viability of Gal4^{tubP}, $\alpha 3^{D93}$/FRT82B, $\alpha 3^{17\text{-}7}$ offspring was calculated at puparium, pharate adult, and adult stages (Table 4). Due to the partial penetrance of *Ser*^{1}, all non-*Ser*^{1} flies were assayed for fluorescence with a UV dissecting microscope to conclusively determine their genotype. The approximate stage of lethality for UASp $\alpha 1$, $\alpha 2$, or $\alpha 3$;FRT82B, $\alpha 3^{17\text{-}7}$/Gal4^{tubP}, $\alpha 3^{D93}$ off-spring was determined as previously described. FRT82B, $\alpha 3^{17\text{-}7}$/Gal4^{tubP}, $\alpha 3^{D93}$ and FRT82B, $\alpha 3^{17\text{-}7}/\alpha 3^{D93}$ offspring served as negative controls.

Generating homozygous importin $\alpha 3^{D93}$ *eyes using EGUF/hid:* UASt FLP, Gal4^{eye}/*CyO*; FRT82B, GMR-*hid, l(3)CL-R*^{1}/*TM6B* females were crossed to: (a) FRT82B, $\alpha 3^{+}$, 87E P-lacW [w^{+}]/ FRT82B, *importin* $\alpha 3^{+}$, 87E P-lacW [w^{+}] or (b) FRT82B, $\alpha 3^{D93}$/*TM6B* flies. Adult offspring of the genotype (a) UASt FLP, Gal4^{eye}/+; FRT82B, GMR-*hid, l(3)CL-R*^{1}/FRT82B, $\alpha 3^{+}$, 87E P-lacW [w^{+}] or (b)UASt FLP, Gal4^{eye}/+; FRT82B, GMR-*hid, l(3)CL-R*^{1}/FRT82B, $\alpha 3^{D93}$ were selected for and their eyes were analyzed by scanning electron microscopy (SEM) and tangential sectioning.

Rescue of homozygous importin $\alpha 3^{D93}$ *eyes:* UASt FLP, Gal4^{eye}/*CyO*; FRT82B, GMR-*hid, l(3)CL-R*^{1}/*TM6B* females were crossed to UASp *importin* $\alpha 1$, $\alpha 2$, or $\alpha 3$/*CyO*; FRT82B, $\alpha 3^{D93}$/*TM6B* males. Adult offspring of the genotype UASt FLP, Gal4^{eye}/UASp $\alpha 1$, $\alpha 2$, or $\alpha 3$; FRT82B, GMR-*hid, l(3)CL-R*^{1}/FRT82B, $\alpha 3^{D93}$ were selected for and their eyes were analyzed by SEM and tangential sectioning.

RESULTS

Phenotypes associated with *importin* $\alpha 3^{1}$ can be removed by recombination: A Drosophila *importin* $\alpha 3$ hypomorphic mutation, $\alpha 3^{1}$, was reported to greatly reduce viability, and all surviving females were sterile (Máthé *et al.* 2000). The $\alpha 3^{1}$ allele is associated with a *P*-element insertion (P-lacW [w^{+}]) located ~780 bp upstream of the start codon (Máthé *et al.* 2000). To determine if UASp $\alpha 1$, $\alpha 2$, or $\alpha 3$ transgenes could rescue the homozygous $\alpha 3^{1}$ phenotypes a Gal4^{arm} driver was recombined onto the same chromosome as $\alpha 3^{1}$. Unexpectedly, the Gal4^{arm}, $\alpha 3^{1}$ chromosome was homozygous viable (Table 1) and homozygous females were fertile (not shown). The presence and correct position of the $\alpha 3^{1}$ *P* element was confirmed by PCR (Figure 1). Therefore, the low viability and female sterility of $\alpha 3^{1}$ flies is likely not due to the hypomorphic α3 mutation. Alternatively, it is possible that the chromosome containing the Gal4^{arm} driver carries a suppressor of the $\alpha 3^{1}$ mutation.

TABLE 1

Viability of *importin* $\alpha 3^{1}$ chromosomes

Genotype[a]	Viability[b]
importin $\alpha 3^{1}$/*importin* $\alpha 3^{1}$	0 (0/119)
importin $\alpha 3^{1}$/*Df (3R)by416*	1.2 (131/109)
importin $\alpha 3^{1}$/*Df (3R)GB104*	0.7 (40/56)
importin $\alpha 3^{1}$/*Df (3R)by62*	1.4 (82/59)
importin $\alpha 3^{1}$/Gal4^{arm}, *importin* $\alpha 3^{1}$	0.6 (66/103)
importin $\alpha 3^{1}$/*importin* $\alpha 3^{1(R1)}$	0.9 (73/85)
importin $\alpha 3^{1}$/*importin* $\alpha 3^{1(R2)}$	1.1 (62/57)
importin $\alpha 3^{1(R1)}$/*importin* $\alpha 3^{1(R1)}$	0.8 (30/40)
importin $\alpha 3^{1(R1)}$/*Df (3R)GB104*	0.7 (55/74)
importin $\alpha 3^{1(R1)}$/*Df (3R) by62*	1.4 (201/145)
importin $\alpha 3^{1(R2)}$/*importin* $\alpha 3^{1(R2)}$	0 (0/39)
importin $\alpha 3^{1(R2)}$/*Df (3R)GB104*	0.8 (69/85)

[a] *importin* $\alpha 3$ alleles and deficiency chromosomes are described in the text.

[b] Viability was determined as described in MATERIALS AND METHODS. The viability index is shown on the left side of the column and the (observed/expected) is shown on the right.

To distinguish between these possibilities we repeated the recombination experiment using an independent third chromosome from a "normal" *w*^{1118} stock. This analysis showed that 4 out of 74 recombinant P-lacW [w^{+}]-containing chromosomes [*importin* $\alpha 3^{1(R1)}$, $\alpha 3^{1(R2)}$, $\alpha 3^{1(R3)}$, and $\alpha 3^{1(R4)}$] supported good viability over the original $\alpha 3^{1}$ chromosome (Table 1; not shown). The presence and position of the *P*-element insert was confirmed by PCR for two of the recombinant chromosomes [$\alpha 3^{1(R1)}$ and $\alpha 3^{1(R2)}$; Figure 1]. Flies homozygous for $\alpha 3^{1(R1)}$ were viable (Table 1) and the females were fertile (not shown). These results confirm that the original P-lacW[w^{+}] insertion could not have been solely responsible for the reported phenotypes. For unknown reasons flies homozygous for $\alpha 3^{1(R2)}$ were homozygous lethal despite the fact that they were viable over the original $\alpha 3^{1}$ allele (Table 1).

We also examined the viability of the original and recombinant *importin* $\alpha 3^{1}$ alleles over various deficiencies. Flies carrying the original $\alpha 3^{1}$ allele or the recombinant $\alpha 3^{1(R1)}$ were viable and female progeny were fertile over *Df(3R)by416*, breakpoints 085D10–12;085E01–03;

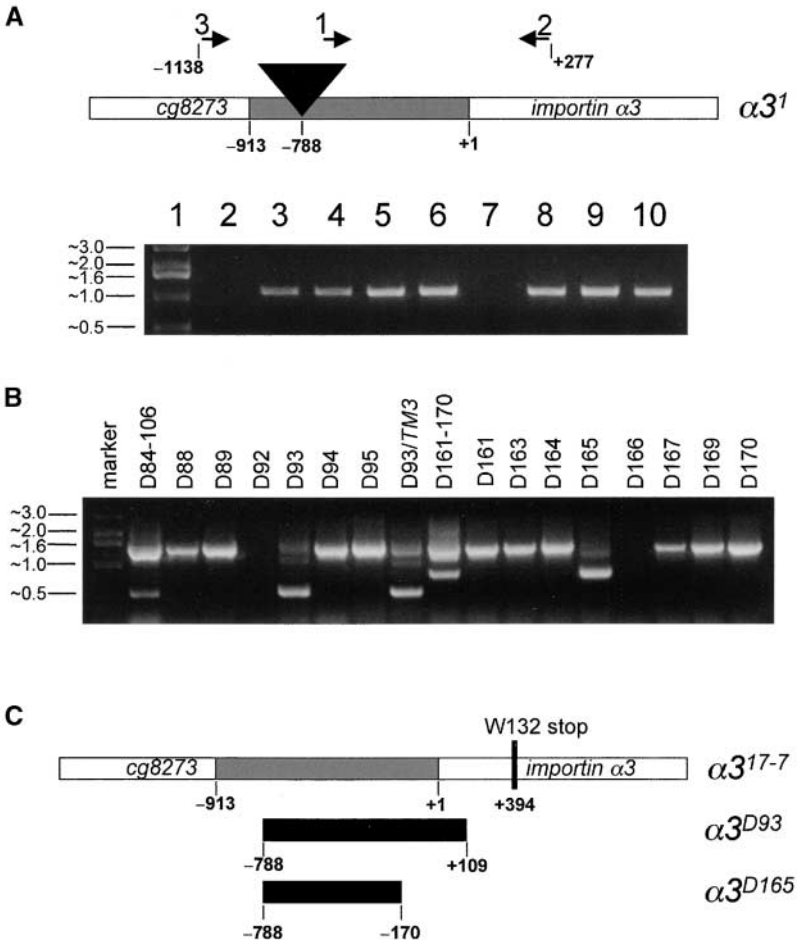

FIGURE 1.—PCR of *importin* $\alpha 3^{1}$ chromosomes and creation of deletion mutants in *importin* α*3*. (A) PCR to detect the *P* element in *importin* $\alpha 3^{1}$. *Importin* α*3* and *cg8273* coding regions are shown in white, the noncoding region is shown in gray, the *P*-element insertion in $\alpha 3^{1}$ is indicated by the black triangle, and primers used for PCR are indicated with arrows. Nucleotide numbers are indicated relative to the start of the α*3* coding region (ATG = +1). To verify the presence of the *P* element in $\alpha 3^{1}$, DNA was isolated from single flies of the indicated genotypes and analyzed by PCR using a *P*-element primer (primer 1) and a primer in α*3* (primer 2). Lane 1, 1-kb DNA ladder; lane 2, w^{1118}; lane 3, $\alpha 3^{1}/TM6C$; lane 4, $Gal4^{Act5C}$, $\alpha 3^{1}/TM6B$; lane 5, $Gal4^{arm}$, $\alpha 3^{1}/TM6B$; lane 6, $Gal4^{arm}$, $\alpha 3^{1}/Gal4^{arm}$, $\alpha 3^{1}$; lane 7, $Gal4^{arm}/TM6B$; lane 8, $\alpha 3^{1(R1)}/TM6B$; lane 9, $\alpha 3^{1(R1)}/\alpha 3^{1(R1)}$; lane 10, $\alpha 3^{1(R2)}/TM6C$. DNA size markers are shown in kilobases. (B) PCR to detect *P*-element excision-induced deletions in *importin* α*3*. DNA isolated from *P*-element-excised flies was analyzed by PCR using primers that flank the *P*-element insertion site (primers 2 and 3 in A). Two lines, *importin* $\alpha 3^{D93}$ and $\alpha 3^{D165}$, carrying small deletions in the α*3* locus were identified (lanes containing bands <1.4 kb). DNA size markers are shown in kilobases. (C) Diagram of *importin* α*3* alleles. *Importin* α*3* and *cg8273* coding regions are shown in white, the noncoding region is shown in gray, and deleted regions in $\alpha 3^{D93}$ and $\alpha 3^{D165}$ are indicated by black boxes. The $\alpha 3^{17\text{-}7}$ allele is also used in this study and contains a stop codon in place of amino acid W132 (T. HERMAN and L. ZIPURSKY, personal communication). Nucleotide numbers are indicated relative to the start of the α*3* coding region (ATG = +1).

Df(3R)GB104, breakpoints 085D12;085E10; and *Df(3R) by62,* breakpoints 085D11–14; 085F06 (Table 1; MÁTHÉ *et al.* 2000; not shown). Further experiments demonstrated that all three of these deficiencies uncover α*3* (Table 2; Figure 3; not shown). Taken together, these results indicate that a second site mutation(s) on the original $\alpha 3^{1}$ chromosome either caused or contributed strongly to the published phenotypes (MÁTHÉ *et al.* 2000). The discovery that the $\alpha 3^{1}$ allele is viable and female fertile over deficiencies and loss-of-function α*3* alleles (see below) suggests that the second-site mutation is the major contributor to these phenotypes. Although $\alpha 3^{1}$ flies were demonstrably hypomorphic for α3 protein expression (MÁTHÉ *et al.* 2000), the reduced α3 levels are apparently not deleterious to the organism.

***P*-element excision-induced alleles of *importin* α*3*:** In search of more severe *importin* α*3* mutations, a *P*-element excision mutagenesis was used to create small deletions in the α*3* coding sequence. The *P* element in the clean $\alpha 3^{1(R1)}$ stock was mobilized and offspring were selected for the loss of the *P* element (loss of $white^{+}$). A PCR assay using primers flanking the *P*-element insertion site was used to screen for imprecise *P*-element excision events (Figure 1). Two small deletions in the α*3* gene ($\alpha 3^{D93}$ and $\alpha 3^{D165}$) were identified (Figure 1). Sequencing of the shortened PCR products revealed that the $\alpha 3^{D93}$ deletion removes 897 bp from the 5′ region of α*3*,

TABLE 2

Viability of *importin* α*3* mutants

Genotype[a]	Viability[b]
importin $\alpha 3^{1}$/*importin* $\alpha 3^{D93}$	1.3 (138/109)
importin $\alpha 3^{D93}$/*importin* $\alpha 3^{D93}$	0 (0/166)
importin $\alpha 3^{D165}$/*importin* $\alpha 3^{D165}$	0 (0/180)
importin $\alpha 3^{D93}$/*importin* $\alpha 3^{D165}$	0 (0/67)
importin $\alpha 3^{D93}$/*Df (3R)by416*	0 (0/131)
importin $\alpha 3^{D93}$/*Df (3R)GB104*	0 (0/58)
importin $\alpha 3^{D93}$/*Df (3R)by62*	0 (0/152)
importin $\alpha 3^{D165}$/*Df (3R)GB104*	0 (0/75)
importin $\alpha 3^{D93}$/*importin* $\alpha 3^{17\text{-}7}$	0 (0/183)
importin $\alpha 3^{D93}$/*importin* $\alpha 3^{w73}$	0 (0/121)
importin $\alpha 3^{17\text{-}7}$/*Df (3R)GB104*	0 (0/62)

[a] *importin* α*3* alleles and deficiency chromosomes are described in the text.

[b] Viability was determined as described in MATERIALS AND METHODS. The viability index is shown on the left side of the column and the (observed/expected) is shown on the right.

including the coding sequence for the first 20 amino acids. The $\alpha 3^{D165}$ deletion removes 619 bp but no coding sequence (Figure 1). Because the original $\alpha 3^{1}$ *P* element was inserted in the 5′ region between $\alpha 3$ and the convergently transcribed predicted open reading frame *cg8273* (Adams *et al.* 2000), only ~125 nucleotides remain upstream of one of the predicted start sites of *cg8273* in $\alpha 3^{D93}$ and $\alpha 3^{D165}$ (Figure 1). Thus, it is possible that the expression of *cg8273* will be affected by $\alpha 3^{D93}$ and $\alpha 3^{D165}$ deletions.

The *importin* $\alpha 3^{D93}$ and $\alpha 3^{D165}$ mutations were both homozygous lethal and lethal over each other (Table 2). In addition, $\alpha 3^{D93}$ was lethal over *Df(3R)by416, Df(3R)GB104*, and *Df(3R)by62* (Table 2). *Importin* $\alpha 3^{D93}/\alpha 3^{1}$ flies were completely viable (Table 2) and the females were fertile (not shown), confirming our conclusion that the $\alpha 3^{1}$ allele does not yield severe phenotypes. In conclusion, this strategy produced recessive lethal $\alpha 3$ mutants, thereby demonstrating that $\alpha 3$ is an essential gene in flies.

***Importin* α*3* deletion mutants do not develop past larval stages:** To determine when *importin* $\alpha 3$ mutant flies die, $\alpha 3^{D93}$ and $\alpha 3^{D165}$ chromosomes were balanced with a *TM3* chromosome marked by GFP (TM3{GFP}, Ser^{1}). In this fashion homozygous mutant offspring could be distinguished from heterozygotes by GFP fluorescence. Homozygous $\alpha 3^{D93}$ and $\alpha 3^{D165}$ offspring completed embryogenesis and formed normal-appearing first instar larvae. Most homozygous mutant $\alpha 3^{D93}$ and $\alpha 3^{D165}$ first instar larvae were able to duplicate their mouth hooks in preparation for molting (Figure 2, B and C), but most died before completing ecdysis. Of the offspring that did begin ecdysis most died while still attached or immediately adjacent to their first instar cuticles. Rarely first instar cuticles were found that were not associated with dead larvae. These larvae presumably died as very early second instar larvae, since we never observed any crawling homozygous mutant second instar larvae. Offspring carrying $\alpha 3^{D93}$ or $\alpha 3^{D165}$ deletions over *Df(3R)by416* or *Df(3R)GB104* also died around the first instar molt (not shown). We conclude that $\alpha 3$ serves an essential role in larval development and that the majority of $\alpha 3$ mutant offspring die before or during ecdysis of the first instar larval molt.

***importin* α*3*^{D93} and α*3*^{D165} larvae express little if any full-length Importin α3 protein:** Since the phenotypes of *importin* $\alpha 3^{D93}$ and $\alpha 3^{D165}$ were no more severe over deficiencies than when homozygous, it is likely that both mutations are either null or strongly hypomorphic. This conclusion was supported by Northern blot analysis showing that $\alpha 3^{D93}/\alpha 3^{D93}$ and $\alpha 3^{D165}/\alpha 3^{D165}$ first instar larvae contained very little or no $\alpha 3$ mRNA (not shown, see materials and methods). This was expected since the two deletions removed significant portions of the $\alpha 3$ 5′UTR (Máthé *et al.* 2000; Figure 1C). Immunoblot analysis was used to investigate whether the first-second instar arrest phenotypes of homozygous $\alpha 3^{D93}$ and $\alpha 3^{D165}$ mutants are due to the complete or partial absence of α3 protein. Total protein was isolated from first instar larvae and examined by immunoblotting with an antiserum against the C-terminal domain of α3 (Máthé *et al.* 2000). As shown in Figure 3, homozygous $\alpha 3^{D93}$, $\alpha 3^{D165}$, and $\alpha 3^{D93}/Df(3R)GB104$ first instar larvae contained very little or no full-length α3 protein. Curiously, a faster-migrating anti-α3 cross-reactive band appeared in both wild-type and mutant larvae (Figure 3A; see below). The identity of this band is currently unknown.

Rescue of *importin* α*3*^{D93} larval lethality: If the developmental defects of *importin* $\alpha 3^{D93}$ and $\alpha 3^{D165}$ mutants are due to the lack of α3 protein, the defects should be rescued by an $\alpha 3$ transgene. A $Gal4^{tubP}$ driver was used to express a UASp $\alpha 3$ transgene in a homozygous $\alpha 3^{D93}$ background. As shown in Table 3, the $\alpha 3$ transgene rescued many $\alpha 3^{D93}/\alpha 3^{D93}$ and $\alpha 3^{D93}/\alpha 3^{D165}$ offspring to the pigmented pharate adult stage and $\alpha 3^{D93}/Df(3R)GB104$ offspring to pupal stages, but none to full adulthood. Some rescued larvae completed the second and third instar molts to form morphologically normal pupae before dying, and a few became well-developed pigmented pharate adults that never eclosed (Table 3). When expressed with a $Gal4^{Act5c}$ driver the $\alpha 3$ transgene rescued $\alpha 3^{D93}/\alpha 3^{D93}$ offspring to wandering third instar larvae. These partially rescued larvae sclerotized their cuticles but did not extend their spiracles and died before becoming puparia. Full rescue may require the expression of the $\alpha 3$ transgene in the correct tissues at the correct time and at appropriate levels. The fact that $Gal4^{tubP}$ and $Gal4^{Act5c}$ drivers rescued to different degrees supports this possibility. Another concern is the likelihood that the $\alpha 3^{D93}$ and $\alpha 3^{D165}$ deletions affected not only the expression of both $\alpha 3$'s but also the divergently transcribed *cg8273* (Figure 1). The partial rescue by the $\alpha 3$ transgene does, however, demonstrate that the death of $\alpha 3^{D93}/\alpha 3^{D93}$ larvae around the first molt is due to defects in $\alpha 3$ and not in *cg8273*.

An objective of this study is to determine if importin α1's, α2's, and α3's have distinct and/or overlapping functions. Previously, using the Gal4/UAS expression system (Brand and Perrimon 1993), we showed that $\alpha 1$, $\alpha 2$, and $\alpha 3$ transgenes all rescued the partial male sterility of $\alpha 2$ null flies, but only $\alpha 2$ transgenes rescued the sterility of $\alpha 2$ null females (Mason *et al.* 2002). Thus the role of α2 in gametogenesis appears not to be redundant with α1 and α3 in females but is redundant in males. A similar approach was taken to determine if $\alpha 1$ and $\alpha 2$ transgenes could rescue the death of $\alpha 3$ mutant offspring.

For these rescue experiments to be meaningful it is important that the transgenes be expressed at reasonable levels in mutant first instar larvae. Extracts from homozygous *importin* $\alpha 3^{D93}$ first instar larvae expressing UASp $\alpha 1$, $\alpha 2$, or $\alpha 3$ transgenes were examined by Western blot using antibodies directed against α2 (Török *et al.* 1995), α3 (Máthé *et al.* 2000), or α-tubulin (Figure

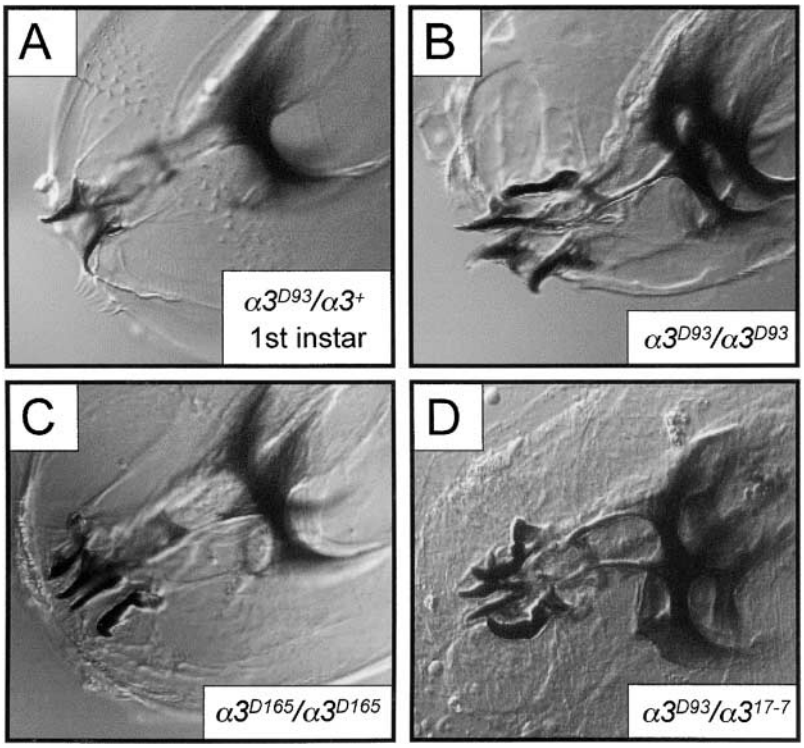

FIGURE 2.—Stage of lethality for *importin α3* mutant larvae. Larval cuticles were prepared from (A) first instar larvae of the genotype *importin* $\alpha 3^{D93}$*/TM3* {GFP} or dead larvae of the genotypes (B) $\alpha 3^{D93}/\alpha 3^{D93}$, (C) $\alpha 3^{D165}/\alpha 3^{D165}$, and (D) $\alpha 3^{17\text{-}7}/\alpha 3^{D93}$. Note the duplicated mouth hooks in B–D.

3B). As shown in Figure 3B, α2 and α3 were both expressed at high levels in first instar larvae carrying the UASp α*2* or α*3* transgenes, respectively. A slower-migrating anti-α3 cross-reactive band in mutant first instar larvae expressing UASp α*1* (* in Figure 3B) is consistent with results observed when UASp α*1* was expressed in α*2* mutant ovaries with the $Gal4^{pnos\text{-}VP16}$ driver (MASON *et al.* 2002). Since α1 is predicted to be ~60 kD and α3 is predicted to be ~56.6 kD, it is likely that this band represents a cross-reaction of α1 with the anti-α3 antiserum. We conclude that all three transgenes are expressed at high levels in first instar larvae.

The expression of either the *importin* α*1* or α*2* transgene delayed the death of homozygous $\alpha 3^{D93}$ offspring. Many offspring expressing UASp α*1* with the $Gal4^{tubP}$ or $Gal4^{Act5c}$ drivers completed the first instar molt before dying as late-stage second instar larvae, although some larvae appeared to survive to early third instar stages (not shown). Similarly, many $\alpha 3^{D93}/\alpha 3^{D93}$ offspring expressing *UAS*p α*2* also survived the first instar larval molt before dying as late second or early third instar larvae. In some trials a few of these animals developed to puparia (not shown). We note that $\alpha 3^{D93}/\alpha 3^{D93}$ offspring expressing α*1* or α*2* were not able to reach pupal stages as efficiently as mutant offspring expressing α*3* (Table 3). We conclude that α1 and α2 can, at least partially, replace the function(s) of α3 during larval development.

Nonsense mutation alleles of *importin α3*: The results described above suggest that α3 is important for developmental events during or after the first larval molt. There are two caveats to this conclusion. First, the $\alpha 3^{D93}$ and $\alpha 3^{D165}$ deletions may affect the expression of *cg8273*, a divergently transcribed gene whose possible role in development is not known. Second, it is not certain whether the $\alpha 3^{D93}$ and $\alpha 3^{D165}$ alleles are null or hypomorphic. If hypomorphic, it is possible that α3 is required for even earlier stages of development. To address these issues we used two nonsense alleles, $\alpha 3^{17\text{-}7}$ containing a stop codon after amino acid (aa) 131 in the second ARM repeat and $\alpha 3^{w73}$ containing a stop codon after aa 158 within the third ARM repeat (kindly provided by T. Herman and L. Zipursky). Since the nonsense mutations were isolated in a screen using heavy mutagenesis they may carry multiple mutations (T. HERMAN and L. ZIPURSKY, personal communications). However, by working with independently derived nonsense mutations over the $\alpha 3^{D93}$ deletion chromosome we can alleviate the possible contribution of recessive mutations in *cg8273* or other loci to the phenotype. Both $\alpha 3^{17\text{-}7}$ and $\alpha 3^{w73}$ were completely lethal over $\alpha 3^{D93}$, $\alpha 3^{D165}$, and *Df(3R)GB104* (Table 2; not shown). *Importin* $\alpha 3^{w73}/\alpha 3^{D93}$ offspring died as mid to late second instar larvae, indicating that the $\alpha 3^{w73}$ allele may not be null. In contrast, many $\alpha 3^{17\text{-}7}/\alpha 3^{D93}$ and $\alpha 3^{17\text{-}7}$*/Df(3R)GB104* larvae died at the first/second instar molt with duplicated mouth hooks (Figure 2D; not shown), although some died as early second instar larvae. More $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$, like $\alpha 3^{D93}/\alpha 3^{D165}$, offspring appeared to complete ecdysis and died as early second instar larvae compared to $\alpha 3^{D93}/\alpha 3^{D93}$ and $\alpha 3^{D165}/\alpha 3^{D165}$ mutants. This is possibly due to subtle genetic background differences. Generally, then, $\alpha 3^{17\text{-}7}$, $\alpha 3^{D93}$, and $\alpha 3^{D165}$ alleles all cause death at or soon after the first larval molt. Therefore, the larval deaths of $\alpha 3^{D93}$

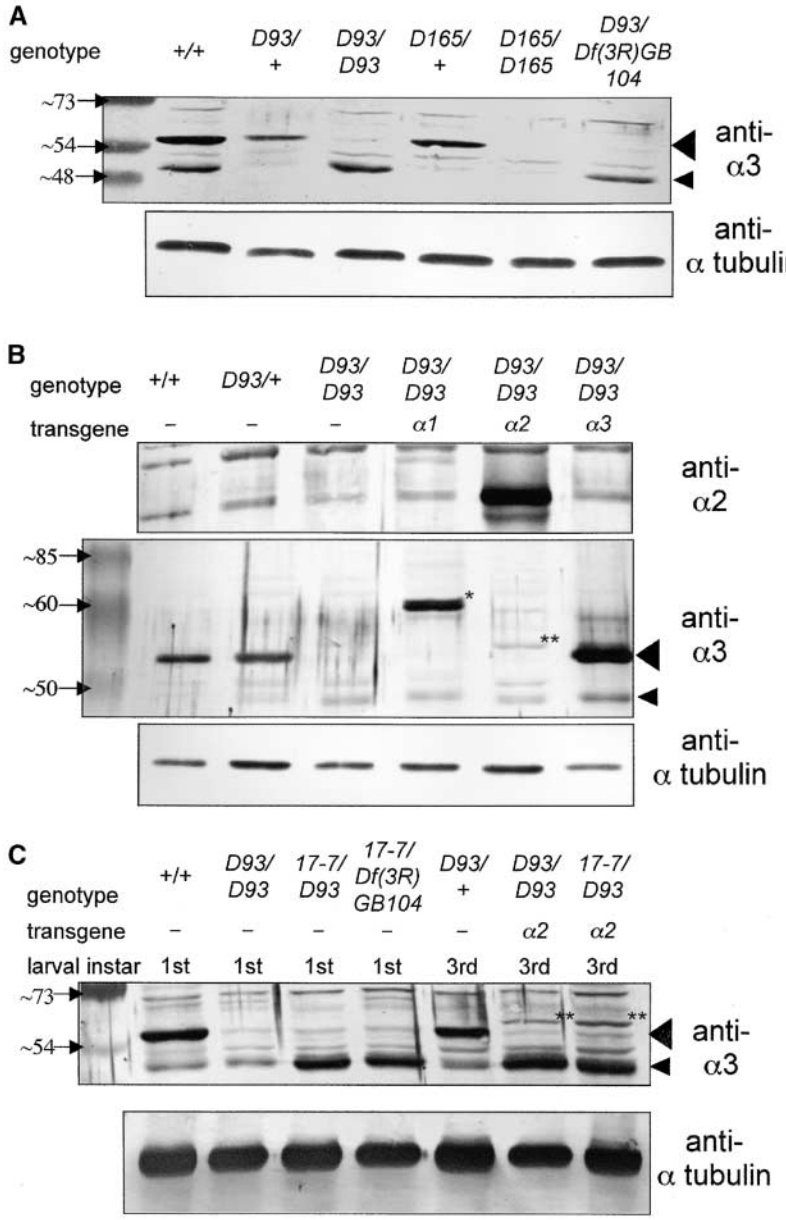

FIGURE 3.—Importin α3 protein levels in *importin α3* mutants and Importin α transgene expression. (A) Protein isolated from first instar larvae of the indicated genotype was examined by Western blot with anti-Importin α3 or anti-α-tubulin antibodies. (B) Protein isolated from first instar larvae of the indicated genotype was examined by Western blot with anti-α2, anti-α3, or anti-α-tubulin antibodies. (C) Protein isolated from first or third instar larvae of the indicated genotype was examined by Western blot with anti-α3 or anti-α-tubulin antibodies. The estimated sizes of the markers are indicated next to the arrows; the thick arrowhead indicates the size of the full-length α3 protein, and the thin arrowhead represents a smaller anti-α3-cross-reactive band (A–C).*, the slower migrating anti-α3-cross-reactive band that appears only when α*1* transgenes are expressed (B); **, a second slower-migrating anti-α3-cross-reactive band that appears only when an α*2* transgene is expressed (B and C).

and $\alpha 3^{D165}$ mutant flies reflect defects in α*3* expression that are independent of *cg8273*.

As expected, levels of Importin α3 protein in first instar larvae carrying $\alpha 3^{17\text{-}7}$ over $\alpha 3^{D93}$ or *Df(3R)GB104* were extremely low or undetectable (Figure 3C). The faster-migrating cross-reactive band described above is also apparent in extracts from $\alpha 3^{17\text{-}7}/Df(3R)GB104$ flies (Figure 3C). This finding rules out the possibility that this band is an N-terminal truncation expressed from the $\alpha 3^{D93}$ chromosome. The band is unlikely to be a degradation product of maternal α3 since the faster-migrating band is also present in extracts from $\alpha 3^{D93}/\alpha 3^{D93}$ and $\alpha 3^{17\text{-}7}/\alpha 3^{D93}$ larvae that were rescued to third instar by an α*2* transgene (see below). The identity of this faster-migrating band is a mystery, since it appears in extracts from flies (potentially) expressing native, N-terminally truncated ($\alpha 3^{D93}$ and $\alpha 3^{D165}$), and C-terminally truncated ($\alpha 3^{17\text{-}7}$) α3 proteins, each of which has a different predicted mass. The simplest explanation is that the band is a spurious cross-reactive species that is unrelated to α3.

Importantly, expression of UASp *importin α3* with Gal-4^{tubP} was able to rescue $\alpha 3^{17\text{-}7}/\alpha 3^{D93}$ and $\alpha 3^{w73}/\alpha 3^{D93}$ offspring to fully viable fertile adults (Table 4; not shown). Thus, it is likely that the inability to rescue $\alpha 3^{D93}/\alpha 3^{D93}$ flies to adulthood with an α*3* transgene is due to disruption of *cg8273* expression. In contrast, UASp α*1* and α*2* transgenes were both unable to rescue $\alpha 3^{17\text{-}7}/\alpha 3^{D93}$ flies to adulthood (Table 4). However, the α*2* transgene was able to rescue some offspring to abnormal pharate adults, although most died at earlier stages (Table 4). Dissecting these partially rescued pharate adults from

TABLE 3

Rescue of importin $\alpha 3^{D93}/\alpha 3^{D93}$ lethality

Genotype[a]	Transgene[b]	Viability[c]		
		Puparia[d]	Pharate adults[d]	Adults
$\alpha 3^{D93}/\alpha 3^{D93}$	α1	0 (0/76)	0 (0/76)	0 (0/82)
	α2	0[e] (0/121)	0 (0/121)	0 (0/105)
	α3	0.5 (44/82)	0.1 (9/82)	0 (0/75)
	—	0 (0/116)	0 (0/116)	0 (0/165)
$\alpha 3^{D93}/\alpha 3^{D165}$	α3	0.5 (30/66)	0.1 (9/66)	0 (0/39)
$\alpha 3^{D93}/Df(3R)GB104$	α3	0.3 (12/38)	0 (0/38)	0 (0/40)

[a] *importin α3* alleles and deficiency chromosomes are described in the text. The *importin α2* and $\alpha 3\ \alpha 3^{D93}/\alpha 3^{D93}$ rescue crosses used the FRT82B, $\alpha 3^{D93}$ chromosome.

[b] UASp *importin α1*, *α2*, and *α3* transgenes were expressed with the $Gal4^{tubP}$ driver. The "−" indicates that this cross did not contain a UASp transgene.

[c] Viability for each stage was determined as described in MATERIALS AND METHODS. The viability index is shown on the left side of each column and the (observed/expected) is shown on the right.

[d] For technical reasons not all pupae could be scored for fluorescence. Therefore, the pupal and adult total numbers are not directly comparable.

[e] In other experiments we have observed a low number of *importin α2*-rescued $\alpha 3^{D93}/\alpha 3^{D93}$ progeny surviving to puparia (not shown).

the pupal cases revealed that some adult structures were at least partially formed, including wings, tergites, sternites, thorax, and bristles. Expression of the *α1* transgene rescued less well, as these offspring survived at best to late second or early third instar larvae. It is worth noting that heterozygous $\alpha 3^{D93}$ flies expressing UASp *α1* have a lower than expected viability (Table 4). Thus, it is possible that the overexpression of α1 causes a partial-dominant lethal phenotype. On the basis of these rescue experiments we conclude that α3 serves a mostly redundant function during larval development. However, since *α1* and *α2* transgenes do not rescue to adult stages it is likely that the role of α3 in the development of some adult tissues cannot be replaced by α1 or α2.

Analysis of *importin* $\alpha 3^{D93}$ mutant eyes: The observation that *importin α3* does not appear to play an exclusively paralog-specific role in larval development led us to examine the effects of the loss of *α3* on the development of adult tissues. To address this issue we created an FRT82B, $\alpha 3^{D93}$ chromosome that can be used to create clones of homozygous $\alpha 3^{D93}$ cells in an otherwise heterozygous fly using the FLP/FRT recombinase system (Xu and Harrison 1994). We subsequently generated eyes that were homozygous for $\alpha 3^{D93}$ using a stock that expresses the FLP recombinase in the eye and contains a FRT82B, GMR-*hid* chromosome (Stowers and Schwarz 1999). The GMR-*hid* element drives the expression of the apoptosis-inducing gene *hid* under the control of

TABLE 4

Rescue of *importin* $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ lethality

Genotype[a]	Transgene[b]	Viability[c]		
		Puparia[d]	Pharate adults[d]	Adults
$\alpha 3^{D93}/\alpha 3^{17\text{-}7}$	*α1*	0 (0/134)	0 (0/134)	0 (0/173)
	α2	0.4 (52/121)	0.1 (8/121)	0 (0/131)
	α3	1.3 (165/127)	ND	1.3 (193/153)
	—	0 (0/100)	0 (0/100)	0 (0/170)
$\alpha 3^{D93}/\alpha 3^{+}$	*α1*	ND	ND	0.3 (45/173)
	α2	ND	ND	0.9 (126/131)
	α3	ND	ND	1.0 (148/153)

[a] *importin α3* alleles are described in the text. The $\alpha 3^{+}$ allele is on the *TM3*{GFP}, Sb^{1} chromosome.

[b] UASp *importin α1*, *α2*, and *α3* transgenes were expressed with the $Gal4^{tubP}$ driver. The "−" indicates that a transgene was not included in the cross.

[c] Viability for each stage was determined as described in MATERIALS AND METHODS. The viability index is shown on the left side of the column and the (observed/expected) is shown on the right. ND, the viability index at these stages was not or could not be determined.

[d] For technical reasons not all pupae could be scored for fluorescence. Therefore, the pupal and adult total numbers are not directly comparable.

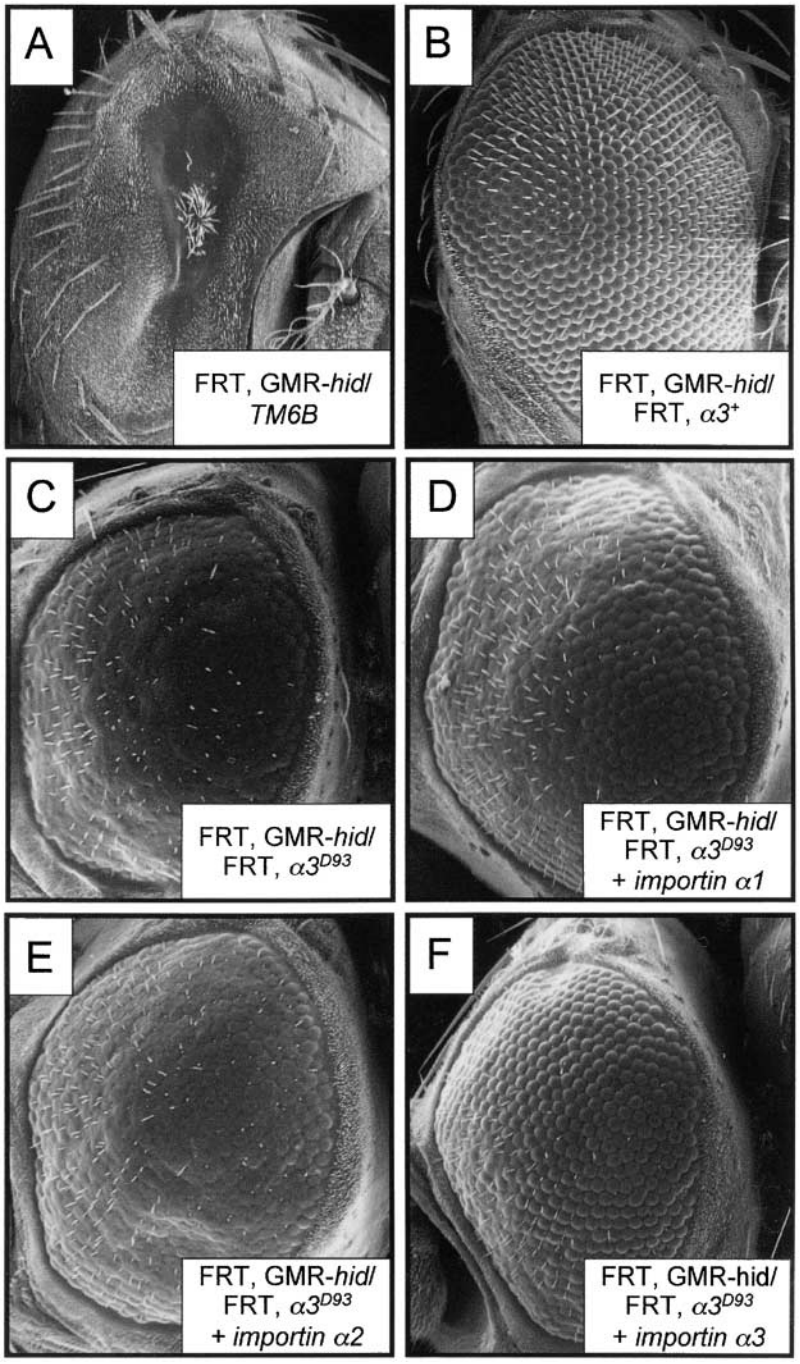

FIGURE 4.—SEM of *importin* $\alpha 3$ mutant and rescued eyes. Flies of the genotypes (A) Gal4^{eye}, UASt FLP; FRT82B, GMR-*hid/TM6B*; (B) Gal4^{eye}, UASt FLP; FRT82B, GMR-*hid*/FRT82B, *importin* $\alpha 3^{+}$; (C) Gal4^{eye}, UASt FLP; FRT82B, GMR-*hid*/FRT82B, $\alpha 3^{D93}$; (D) Gal4^{eye}, UASt FLP/UASp $\alpha 1$; FRT82B, GMR-*hid*/FRT82B, $\alpha 3^{D93}$; (E) Gal4^{eye}, UASt FLP/UASp $\alpha 2$; FRT82B, GMR-*hid*/FRT82B, $\alpha 3^{D93}$; and (F) Gal4^{eye}, UASt FLP/UASp $\alpha 3$; FRT82B, GMR-*hid*/FRT82B, $\alpha 3^{D93}$ were critical-point dried and examined by scanning electron microscopy.

the eye-specific GMR enhancer. Consequently, any eye cell that contains one or two copies of GMR-*hid* undergoes apoptosis during early pupal stages. Therefore, only cells that have lost this chromosome through mitotic recombination survive to form adult eyes (STOWERS and SCHWARZ 1999; Figure 4A). Consistent with previous results, we observed that FRT82B, $\alpha 3^{+}$/FRT82B, GMR-*hid* flies had well-formed eyes when FLP recombinase was expressed in the eye (STOWERS and SCHWARZ 1999; Figure 4B), although the photoreceptor patterning observed in eye sections appears to be slightly defective (Figure 5A).

Eyes homozygous for the *importin* $\alpha 3^{D93}$ allele were highly defective (Figure 4C). Under the light microscope these eyes appeared "glassy." The $\alpha 3^{D93}$ mutation did not cause a general cell-lethal phenotype in the eye, since ommatidial-like structures were visible (Figure 4C). However, the ommatidia were disorganized and did not fully develop, and interommatidial bristles were often missing (Figure 4C). Examination of tangential sections of homozygous $\alpha 3^{D93}$ eyes demonstrated that the ommatidia were defective, since the photoreceptor cell rhabdomeres were not visible and the ommatidia were severely misshapen (Figure 5B).

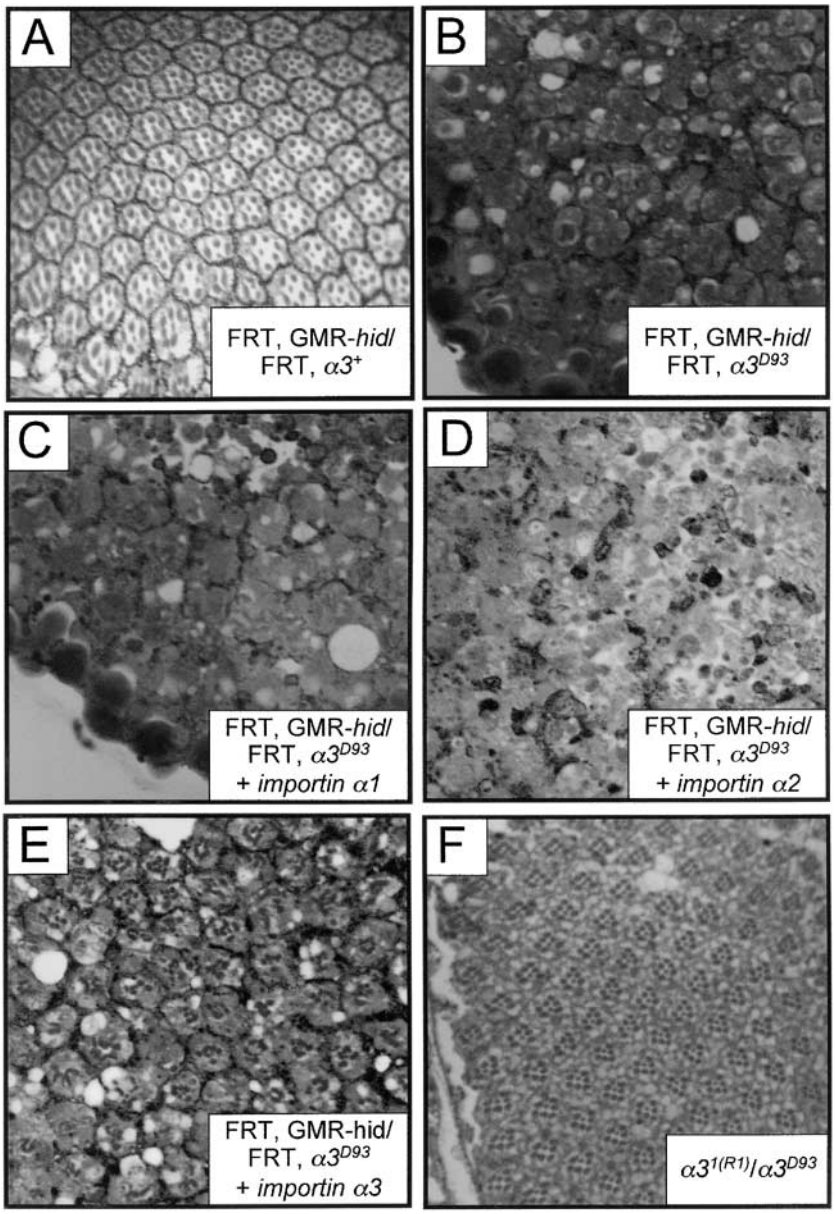

FIGURE 5.—Tangential sections through *importin* α*3* mutant and rescued eyes. Eyes dissected from flies of the genotypes (A) Gal4^{eye}, UASt FLP ; FRT82B, GMR-*hid*/FRT82B, α3^{+}; (B) Gal4^{eye}, UASt FLP; FRT82B, GMR-*hid*/FRT82B, *importin* α3^{D93}; (C) Gal4^{eye}, UASt FLP/UASp α*1* ; FRT82B, GMR-*hid*/FRT82B, α3^{D93}; (D) Gal4^{eye}, UASt FLP/UASp α*2*;FRT82B, GMR-*hid*/FRT82B, α3^{D93}; (E) Gal4^{eye}, UASt FLP/UASp α*3*;FRT82B, GMR-*hid*/FRT82B, α3^{D93}; and (F) α$3^{1(R1)}$/α3^{D93} were embedded in resin, sectioned, and toluidine blue stained. Note the absence of photoreceptor rhabdomeres in B–D.

To test whether the defective eye phenotype is truly the result of the lack of Importin α3 activity in the eye, UASp α*3* was expressed in α3^{D93} mutant eyes using the Gal4^{eye} driver. The α*3* transgene was able to partially rescue the defect in ommatidia formation (Figure 4F), demonstrating that the glassy-eye phenotype is indeed due to the lack of α3. However, expression of the α*3* transgene did not completely rescue the phenotype, as most interommatidial bristles were missing (Figure 4F). Tangential sectioning of these rescued eyes revealed that the photoreceptor cell rhabdomeres were visible, demonstrating that their loss was indeed due to a disruption in α*3*. However, the photoreceptors in the rescued eyes were not wild type in appearance. They appeared disorganized and misshapen and most ommatidia had an incorrect number of photoreceptors (Figure 5E). This partial rescue may be due to problems with the Gal4^{eye} expression pattern or may be the result of the disruption of the neighboring gene as previously discussed.

To examine the specificity of Importin α3 function in the eye, UASp α*1* and α*2* transgenes were expressed. Eyes mutant for α*3*, but ectopically expressing α*1*, appeared to be at least partially rescued (Figure 4D). Eyes rescued with α1 still appeared partially glassy by light microscopy, but when examined by SEM it was clear that they had more well-developed ommatidia than when the transgene was not expressed (Figure 4D). Tangential sections of these eyes revealed that α1-rescued ommatidia were still largely defective, since no photoreceptor cell rhabdomeres were observed (Figure 5C). Expression of UASp α*2* did not appear to affect the phenotype, since homozygous α3^{D93} eyes expressing α*2* looked identical to those not expressing the transgene (Figure 4E)

and tangential sections demonstrated that photoreceptor cell rhabdomeres were not present in these ommatidia (Figure 5D). These data suggest that α1, but not α2, is able to partially replace α3 in the eye.

We have also observed that a null mutation in *importin α2*, $\alpha 2^{D14}$ (Török *et al.* 1995; Giarrè *et al.* 2002; Gorjánácz *et al.* 2002), was able to enhance the eye defect observed in homozygous $\alpha 3^{D93}$ eyes. Eyes that were homozygous for the $\alpha 3^{D93}$ mutation and heterozygous for the $\alpha 2^{D14}$ allele lacked almost all ommatidial structures. These eyes appeared to be thin sheets of tissue with very little differentiation (not shown). However, for unknown reasons this enhancement was not rescued by the expression of an *α2* transgene. Specifically, flies expressing UASp *α2* in eyes that were homozygous for $\alpha 3^{D93}$ and heterozygous for $\alpha 2^{D14}$ appeared identical to those that were not expressing the transgene (not shown).

Finally, we looked at whether a hypomorphic *importin α3* condition caused slight defects in eye development. To examine this we looked at tangential sections of eyes from $\alpha 3^{1(R1)}/\alpha 3^{D93}$ flies. This analysis demonstrated that these ommatidia had the correct number, shape, and patterning of photoreceptor rhabdomeres (Figure 5F). We conclude that a hypomorphic *α3* condition does not cause defects in eye development.

Importin α3 does not function in ring canal formation: Gorjánácz *et al.* (2002) have recently demonstrated that Importin α2 is required in the female germline to correctly form ring canals. In homozygous $\alpha 2^{D14}$ ovaries, Kelch (Xue and Cooley 1993) is not targeted to the ring canal correctly, correlating with a defect in transfer of maternal material from the nurse cells to the developing oocyte (Gorjánácz *et al.* 2002). Expression of a UASp *α2* transgene is able to rescue the Kelch mislocalization phenotype observed in *α2* null females (Gorjánácz *et al.* 2002; Figure 6D). To examine the ability of α1 or α3 to function in ring canal formation we examined the localization of the Kelch protein in ovaries from homozygous $\alpha 2^{D14}$ females expressing UASp *α1*, *α2*, or *α3* transgenes. Consistent with previous observations that *α2* mutant females expressing *α1* or *α3* are sterile (Mason *et al.* 2002), we found that expression of *α1* or *α3* did not rescue the mislocalization of Kelch (Figure 6, C and E). In rare cases some accumulation of weak Kelch fluorescence was observed in mutant ovaries expressing α3 (Figure 6E, arrow). It is not known whether this signal represents poorly formed ring canals or, more likely, is a staining artifact. We conclude that α1 and α3 do not function to properly target Kelch to ring canals in the same manner as α2 does.

DISCUSSION

Nuclear transport is facilitated largely by members of the karyopherin gene family. These importins and exportins bind nuclear import or export signal-bearing proteins and ferry them across the nuclear pore complex. Importin α's are adaptors that link many cNLS-containing cargoes to the karyopherin importin β1 (Macara 2001; Bednenko *et al.* 2003; Weis 2003). The conventional importin α gene family is composed of three clades, α1's, α2's, and α3's, although fungi and plants contain only α1 genes. With the exception of *C. elegans*, invertebrate and vertebrate animal genomes encode at least one importin α from each clade (Köhler *et al.* 1997, 1999; Malik *et al.* 1997; Máthé *et al.* 2000; Mason *et al.* 2002). For example, humans contain three α1's, one α2, and two α3's. There is ample *in vitro* evidence that conventional importin α's mediate the import of cNLS-containing cargoes as well as paralog-specific NLS cargoes (Miyamoto *et al.* 1997; Nadler *et al.* 1997; Sekimoto *et al.* 1997; Prieve *et al.* 1998; Köhler *et al.* 1999, 2001; Welch *et al.* 1999; Kumar *et al.* 2000; Nemergut and Macara 2000; Talcott and Moore 2000; Jiang *et al.* 2001; Guillemain *et al.* 2002; Melén *et al.* 2003). The *in vivo* analysis of the importin α gene family in metazoan animals is complicated by the individual paralogs' poorly defined NLS-cargo-binding repertoires, their differing cell type- and tissue-specific expression patterns and levels, and the likelihood that some may perform non-transport-related activities (Matsusaka *et al.* 1998; Tabb *et al.* 2000; Gruss *et al.* 2001; Nachury *et al.* 2001; Wiese *et al.* 2001; Askjaer *et al.* 2002).

A previous study concluded that a hypomorphic mutation in *importin α3* was partially lethal with all surviving females being sterile (Máthé *et al.* 2000). However, these phenotypes did not cosegregate with the *P* element in the $\alpha 3^{1}$ allele, which itself caused no phenotypes. Therefore, even though the $\alpha 3^{1}$ allele is hypomorphic, the reported phenotypes were most likely due to a second-site mutation(s). To determine whether more severe mutations in *α3* cause phenotypes we generated new 5′ deletion alleles ($\alpha 3^{D93}$ and $\alpha 3^{D165}$) and studied the effects of nonsense mutation alleles ($\alpha 3^{17\text{-}7}$ and $\alpha 3^{w73}$) provided by T. Herman and L. Zipursky (UCLA). The fact that homozygous $\alpha 3^{D93}$ and $\alpha 3^{D165}$ flies and flies containing $\alpha 3^{D93}$, $\alpha 3^{D165}$, and $\alpha 3^{17\text{-}7}$ alleles over α3 deficiencies die at approximately the same stage is consistent with all three alleles being null. However, since we could not rule out by Western blot the possibility that these mutants retained low residual levels of α3 protein, they could be severe hypomorphs rather than nulls.

The analysis of these alleles demonstrates that Drosophila Importin α3 is required for the development of both larval and adult tissues. *Importin α3* mutant flies die around the first larval molt, and *α3* mutant eyes are severely defective and lack photoreceptor cells. The *α3* mutant phenotypes are dramatically different from those of *α2* mutant flies. Specifically, *α2* is required for gametogenesis and apparently not for somatic development (Giarrè *et al.* 2002; Gorjánácz *et al.* 2002; Mason *et al.* 2002). The loss of α2 causes sterility, total in females and partial in males. Interestingly, *α1*, *α2*, and *α3* transgenes all rescued the male sterility defect to the same

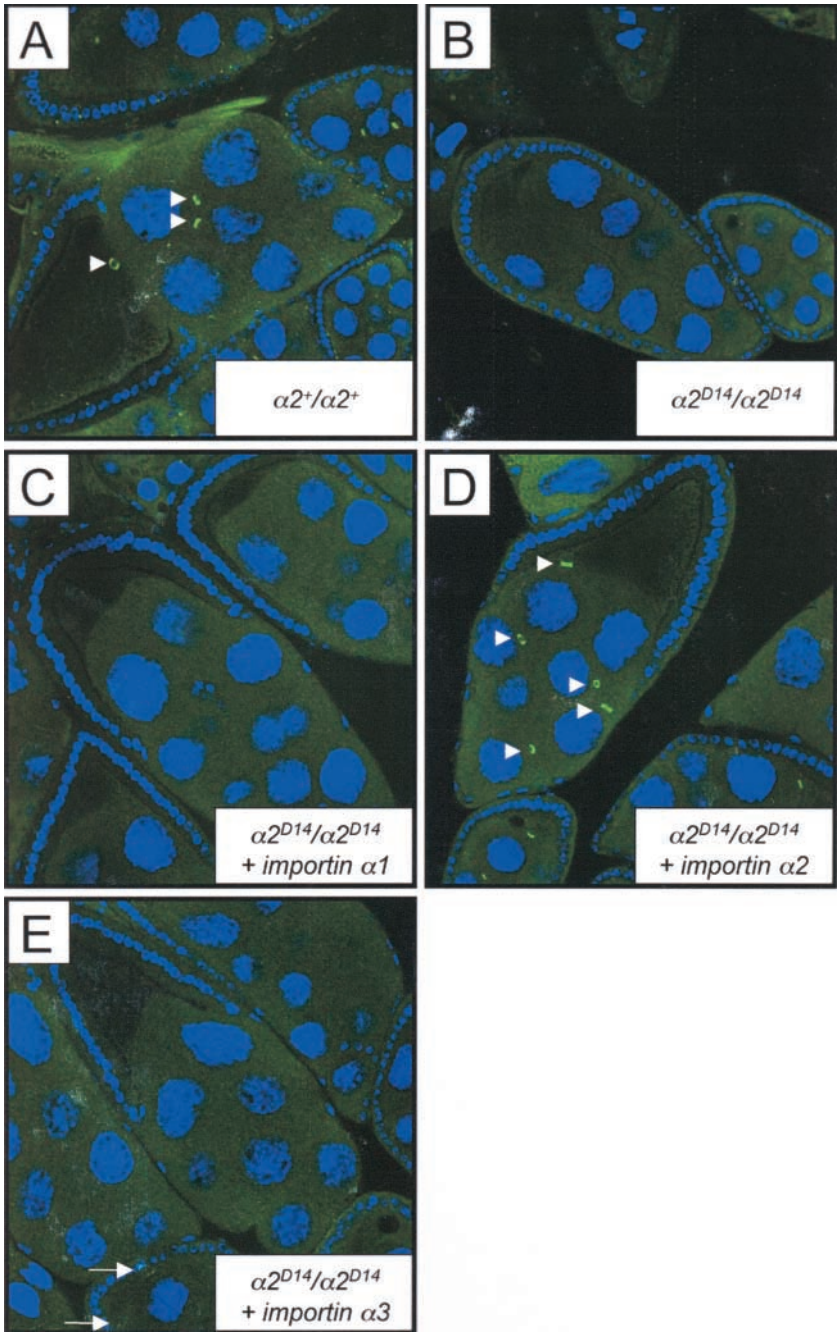

Figure 6.—Kelch staining of ovaries from *importin α2* null females rescued with importin α transgenes. Ovaries dissected from females of the genotypes (A) w^{1118}; (B) *importin* $\alpha 2^{D14}/\alpha 2^{D14}$; Gal4$^{pnos\text{-}VP16}$*/TM6B*; (C) $\alpha 2^{D14}$, UASp $\alpha 1/\alpha 2^{D14}$; Gal4$^{pnos\text{-}VP16}$; (D) $\alpha 2^{D14}$, UASp $\alpha 2/\alpha 2^{D14}$; Gal4$^{pnos\text{-}VP16}$; and (E) $\alpha 2^{D14}$, UASp $\alpha 3/\alpha 2^{D14}$; Gal4$^{pnos\text{-}VP16}$ were stained with an anti-Kelch antibody (green) and DAPI (blue). Note the Kelch-stained ring canals (arrowheads) in A and D and their absence in B, C, and E. Rarely, some very faint fluorescent anti-Kelch-stained structures are observed in $\alpha 2^{D14}$, UASp $\alpha 3/\alpha 2^{D14}$; Gal4$^{pnos\text{-}VP16}$ ovaries (arrows in E).

degree, but only α*2* transgenes rescued the female sterility defect. These results are consistent with α2 playing a paralog-specific role in oogenesis that cannot be performed by either α1 or α3 (Mason *et al.* 2002). Normally, α*2* mRNA is expressed in a number of larval tissues and imaginal discs (Török *et al.* 1995), but, since α*2* null flies develop normally to adulthood—the germline not withstanding—its role in somatic development must be either unimportant or redundant with at least one of the other paralogs.

Transgene rescue studies support the conclusion that Importin α3, like α2, serves both paralog-specific and redundant roles during development. On the basis of our criteria, paralog-specific roles for α3 are those that can be rescued by only α*3* transgenes. Redundant functions are those that could be rescued by an α*3* and α*1* and/or α*2* transgenes. The most likely redundant function is the housekeeping transport of cNLS cargoes. This class of phenotype probably arises when only the mutated importin α type, in this case α3, is normally expressed at sufficient levels in the relevant tissue or when a high level of general importin α activity is required.

Drosophila *importin α3* mutant offspring complete embryogenesis and hatch to first instar larvae without any apparent defects. The majority of α*3* mutant off-

spring die during the first instar molt. At the end of each larval stage ecdysone pulses signal significant changes in gene expression that are necessary for the generation of second instar larvae (Riddiford 1993; Bender 1995; Thummel 1996; Kozlova and Thummel 2000). It is possible that α3 plays a role in facilitating this transition, perhaps by mediating the nuclear transport of signaling proteins or transcription factors that specify the transition. In this regard, homozygous $\alpha 3^{D93}$ mutants containing a $Gal4^{Act5C}$-expressed α*3* transgene successfully completed first and second instar molts only to die near the transition from wandering third instar larvae to puparium. This is consistent with the notion that α3 is required for both the transition from first to second instar larva and the transition from larva to puparium. Thus, α3 may play a general role in developmental transitions.

Importin α3 is likely required during the first molt for a redundant function. First, since some α*3* mutant larvae reach the second instar, there may be enough endogenous α1 and α2 present to partially replace the loss of α3 during the first larval molt. Importin α1 in particular is well expressed in larval tissues (Giarrè *et al.* 2002). Alternatively, the requirement for α3 during the first molt may be important but nonessential, whether or not α1 or α2 is present. The most convincing argument that a redundant function of α3 is required during this developmental transition is the finding that the expression of α*1* and α*2* transgenes rescued α*3* mutant flies to later stages. In conclusion, it is likely that the cause of this phenotype is due to the preferential expression of redundant α3 activity(s) in one or more larval tissues, rather than of an α3-specific activity. Here, the preferential use of α3 to perform a redundant importin α function during larval development is analogous to the role of α2 in spermatogenesis.

The interpretation of the rescue experiments is complicated by differences in the capacity of the various transgenes to rescue the similar defects of homozygous *importin* $\alpha 3^{D93}$ *vs.* $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ flies. For example, only α*3* transgenes rescued $\alpha 3^{D93}/\alpha 3^{D93}$ flies to pupal stages, whereas both α*2* and α*3,* but not α*1*, transgenes rescued $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ flies to pupal stages. *Importin* α2-rescued $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ progeny do not properly complete pupation and, consequently, never eclose. Only α*3* transgenes are capable of rescuing the latter stages of development through eclosion. We trust the $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ results more because these flies would not suffer the effects of deleterious recessive alleles potentially present in homozygous $\alpha 3^{D93}/\alpha 3^{D93}$ offspring. Therefore, focusing on $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ results, we conclude that an activity of α3 that is essential for pupation is at least partially redundant with α2. These functional results are consistent with phylogenetic analyses showing that α3's are more closely related to α2's than to α1's (Köhler *et al.* 1999; Mason *et al.* 2002; not shown). However, the fact that only an α*3* transgene is able to rescue $\alpha 3^{D93}/\alpha 3^{17\text{-}7}$ progeny to adults suggests that α3 does serve an α3-specific function in the development of some adult tissues.

Importin α3 has both redundant and paralog-specific roles in eye development. Homozygous $\alpha 3^{D93}$ eyes appear glassy and lack photoreceptor cell rhabdomeres in adult ommatidia. These phenotypes can be mostly rescued by the expression of α*3* transgenes. Only an α*3* transgene was able to partially rescue the photoreceptor cell defect, indicating that α3 likely serves a paralog-specific function in the differentiation of these cells. A recent study demonstrated that a dominant-negative Importin β1 protein expressed in the eye caused defects in development of photoreceptor cells (Kumar *et al.* 2001), suggesting that α3 and Importin β1 may work together to perform a nuclear transport function essential for the development of the eye. We cannot rule out the possibility that α*3* is important for eye development only under the EGUF/*hid* experimental conditions (Stowers and Schwarz 1999).

Expression of *importin* α*1* improved the overall morphology of α*3* mutant eyes, but these eyes still lacked recognizable photoreceptor cell rhabdomeres. Importin α1 may rescue the differentiation of nonphotoreceptor accessory cells, like pigment and cone cells, or may improve photoreceptor development enough to allow more efficient specification of accessory cell lineages. *Importin* α*2* expression has little if any effect on the development of α*3* mutant eyes. Curiously, an α*2* null mutation enhanced the α*3* glassy eye phenotype, suggesting that endogenous α2 and α3 may function together during eye development. However, this enhancement could not be rescued by the expression of an α*2* transgene. Flies homozygous for the null α*2* allele have morphologically wild-type eyes, so α2 does not appear to be required for eye development when α3 is present (Giarrè *et al.* 2002; Gorjánácz *et al.* 2002; Mason *et al.* 2002). Interestingly, α1 was better than α2 at rescuing eye development, but the opposite was true for pupation, where α2 was better than α1. Rather than being contradictory, we believe these results underscore just how complex the physiology of the importin α gene family is likely to be.

We cannot rule out the possibility that the differing capacity of UASp *importin* α*1,* α*2,* or α*3* transgenes to rescue α*2* and α*3* mutant phenotypes is due to differences in transgenic protein expression levels, protein stability, or post-translational modifications. However, we do note that all three transgenes appear by Western blot analyses to be well expressed (Mason *et al.* 2002; Figure 3). In addition, a UASp α*2,* but not α*3*, transgene fully rescued phenotypes associated with the loss of α*2* (Figure 6 and not shown), while the same α*3* transgene, but not the α*2,* fully rescued phenotypes caused by the loss of α*3* (Table 4). These results strongly suggest that α2 and α3 differ in their ability to perform cellular functions *in vivo.*

Previously, *in vitro* studies showed that vertebrate im-

portin α3's specifically transported presumably essential cellular proteins, such as RCC1 and RanBP3 (Köhler *et al.* 1999; Welch *et al.* 1999; Nemergut and Macara 2000; Talcott and Moore 2000). Our finding that embryos and larvae do not appear to require an α3-specific activity was, therefore, surprising. It is possible that α*3* protein or mRNA may be stored maternally at a low level and maintained until larval stages. However, Fang *et al.* (2001) did not observe any α*3* mRNA or protein in 0- to 2-hr embryos, suggesting that α*3* is not stored maternally. Small amounts of α3 protein were observed in 0- to 2-hr embryos by Máthé *et al.* (2000) and the source of this discrepancy is currently unclear. Therefore, residual α3 activity may be present in mutant embryos and α*1*- and α*2*-rescued mutant larvae to perform all α3 functions necessary for cell survival. Alternatively, α3-specific nuclear transport of RCC1 and RanBP3 observed *in vitro* may not be specific for α3 *in vivo* or these α3-specific functions may not be conserved from vertebrates to flies. Finally, the nuclear import of α3-specific essential cellular proteins may also be imported by a redundant nuclear targeting pathway. This appears to be the case for the import of RCC1 in vertebrate cells. RCC1 import is mediated by two distinct pathways, only one of which requires α3 (Nemergut and Macara 2000).

The analyses of *importin* α*2* and α*3* mutant phenotypes demonstrate that α2 is essential only for gametogenesis, while α3 appears to serve a more widespread developmental role (Giarrè *et al.* 2002; Gorjánácz *et al.* 2002; Mason *et al.* 2002; this study). These observations are consistent with the defects in somatic tissues associated with RNAi-mediated disruption of α3 paralogs in *C. elegans* and porcine embryos (Geles *et al.* 2002; Cabot and Prather 2003) and suggest that the α2's are the most derived of the three importin α types. In addition, rescue experiments with UASp α*1*, α*2*, and α*3* transgenes suggest that these differential developmental roles are due, at least partly, to distinct α2 and α3 biochemical activities (Mason *et al.* 2002; this study). Much work remains to be done to determine the precise cellular processes and molecular mechanisms that yield the observed mutant phenotypes. In conclusion, these studies lay the groundwork for future *in vivo* and *in vitro* studies of the importin α gene family.

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