Genetic Analyses of Elys mutations in Drosophila Show Maternal-effect Lethality and Interactions with Nucleoporin Genes

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ELYS determines the subcellular localizations of Nucleoporins (Nups) during interphase and mitosis.

We made loss-of-function mutations of Els in Drosophila melanogaster and found that ELYS is dispensable for zygotic viability and male fertility but the maternal supply is necessary for embryonic development. Subsequent to fertilization, mitotic progression of the embryos produced by the mutant females is severely disrupted at the first cleavage division, accompanied by irregular behavior of mitotic centrosomes. The Nup160 introgression from D. simulans shows close resemblance to that of the Els mutations, suggesting a common role for those proteins in the first cleavage division. Our genetic experiments indicated critical interactions between ELYS and three Nup107–160 subcomplex components: hemizygotes of either Nup37, Nup96 or Nup160 were lethal in the genetic background of the Els mutation. Not only Nup96 and Nup160 but also Nup37 of D. simulans behave as recessive hybrid incompatibility genes with D. melanogaster. An evolutionary analysis indicated positive natural selection in the ELYS-like domain of
ELYS. Here we propose that genetic incompatibility between *Elys* and *Nups* may lead to reproductive isolation between *D. melanogaster* and *D. simulans*, although direct evidence is necessary.

**KEYWORDS** nuclear pore complex; maternal-effect lethal; fertilization; interspecific hybrids; centrosome

The nucleoporins (Nups) consist of ~30 distinct proteins that constitute the nuclear pore complex (NPC; for recent reviews, see Dickmanns *et al.*, 2015; Hurt and Beck, 2015; Kabachinski and Schwartz, 2015). NPCs are distributed throughout the nuclear envelope and provide the gate for nucleocytoplasmic transport of macromolecules like proteins and RNAs during interphase. They are disassembled and reassembled in open mitosis and have roles in mitosis, such as spindle assembly, kinetochore function, chromosome segregation and possibly centrosome formation (Resendes *et al.*, 2008; Güttinger *et al.*, 2009).
The Nup107–160 subcomplex, which consists of nine Nups, is the early key player for NPC assembly. ELYS (embryonic large molecule derived from yolk sac), which was originally discovered in mice as a transcription factor (Kimura et al., 2002), recruits the NPC to the nuclear envelope, kinetochore and mitotic spindle via the association between ELYS and the Nup107–160 subcomplex (Fernandez and Piano, 2006; Galy et al., 2006; Franz et al., 2007; Gillespie et al., 2007; Rasala et al., 2006, 2008; Chatel and Fahrenkrog, 2011; Clever et al., 2012; Bilokapic and Schwartz, 2013; Inoue and Zhang, 2014; Morchoisne-Bolhy et al., 2015; Schwartz et al., 2015; Gómez-Saldivar et al., 2016).

ELYS is essential for mice; a null mutant is lethal at the early embryonic stage (Okita et al., 2004). In contrast, the Caenorhabditis elegans homolog, MEL-28 (maternal-effect embryonic-lethal-28), which—as its name suggests—has a required maternal effect and is dispensable for zygotic development (Fernandez et al., 2014). Although a BLAST search against the Drosophila melanogaster genome suggested that gi:24643345 (= CG14215) encodes the ELYS homolog (Rasala et al., 2006), no analyses of the gene were undertaken in Drosophila (Chen et al., 2015). Ilyin et al. (2017)
recently conducted the immunological staining of ELYS in ovarian somatic cells of Drosophila.

Here we disrupted the X-linked CG14215 (hereafter, Elys) of D. melanogaster and analyzed the mutant phenotypes. Surprisingly, the D. melanogaster mutants exhibited an effect similar to the C. elegans mutants; homozygotes (or hemizygotes) were viable and male-fertile but female-sterile (maternal-effect lethal). Sperm penetrated the eggs produced by the mutant females, but the first mitotic division was never completed. This is one of the earliest developmental defects caused by D. melanogaster mutations (for the list of the genes, see Loppin et al., 2015) and will provide a rare opportunity to analyze Drosophila fertilization (Callaini and Riparbelli, 1996; Kawamura, 2001). In the present report we will describe in detail the developmental defects of the embryos in which maternally supplied ELYS is depleted.

The introgression of the Nup160 allele from D. simulans (Nup160sim) causes recessive female sterility in the D.
melanogaster genetic background (Sawamura et al., 2010). Females homozygous or hemizygous for Nup160sim produce eggs capable of sperm entry, but the embryos never develop (Sawamura et al., 2004). As this is similar to the maternal-effect phenotype of the Elys mutations, we wanted to compare these phenotypes in detail. We also show genetic interaction between Elys and the Nups, and discuss the possible involvement of ELYS in reproductive isolation between D. melanogaster and D. simulans.

Materials and Methods

Fly strains

For D. melanogaster strains used, see FlyBase (Gramates et al., 2017; http://flybase.org/). Int(2D)D+S carries D. simulans introgressions including Nup160sim (Sawamura et al., 2000), and Df(2L)Nup160M190 is a deficiency that only disrupts Nup160 (Maehara et al., 2012). The Nup98–96 gene is dicistronic and the Nup98–96339 mutation only disrupts Nup96 (Presgraves et al., 2003).
To eliminate endosymbiotic bacteria (presumably *Wolbachia*) from fly stocks used for embryo immunostaining, we fed flies with medium containing 0.03% tetracycline for one generation (Hoffmann *et al.*, 1986). This allowed us to analyze chromosomal DNA exclusively with DAPI staining, but not coexistent bacterial DNA, in the early Drosophila embryo (Lin and Wolfner, 1991; Kose and Karr, 1995).

**Establishment of Elys mutations**

No *Elys* mutations had been reported in *D. melanogaster*. Generation of *Elys* alleles was carried out with the CRISPR/Cas9 system described previously (Kondo and Ueda, 2013). The guide RNAs (gRNAs) were selected using CRISPR Optimal Target Finder (Gratz et al., 2014; http://tools.flycrispr.molbio.wisc.edu/targetFinder/). To generate a double gRNA construct to target the *Elys* locus, two pairs of oligonucleotides were annealed and cloned into the pBFv-U6.2B vector: one of the pairs of oligonucleotides is 5′-CTT CGC TGC ACT CGG TCT GCT ACA-3′ and 5′-AAA
CTG TAG CAG ACC GAG TGC AGC-3', and the other is 5'-CTT CGG CCA CTG ACT CGT TGC TCG-3' and 5'-AAA CCG AGC AAC GAG TCA GTG GCC-3'. The Elys gRNA vector was injected into embryos of y¹ v¹.

\[ P^{y^\text{att}7.7=\text{nos-phiC31}\int.NLS/X}; P^{y^\text{att}7.7=\text{CaryP/attP40}}. \]

The transgenic U6-Elys-gRNA flies were established, and mutations in the Elys locus were recovered in offspring from nos-Cas9 (y² cho² v¹; attP40/nos-Cas9/CyO) and the U6-Elys-gRNA flies. Cas9-mediated targeted mutagenesis of the Elys locus was introduced on the X chromosome of y² cho² v¹. Potential mutations of the Elys locus were identified by genomic PCR using the primers 5'AAG ACG GCC GAA TCC TGA TCT ACG-3' and 5'AGA CCA CTA GAC TGC GTT GCT TGC-3'; these primers sandwich the potential deletions (the former is on exon 3 and the latter is on exon 7). Sequencing of the obtained PCR products confirmed mutations of the corresponding genomic region (Figure 1).

Embryo collection and immunostaining

Well-fed virgin females were mated with wild-type (Oregon-R) males and allowed to lay eggs in short vials containing...
fly medium on which yeast was seeded. Embryos were collected at 20-min intervals, and the following fixation was completed within an additional 10 min. After dechorionation with 50% bleach for 1.5 min, embryos were washed with water and then fixed and devitellinized by shaking in a mixture of equal volumes of heptane and methanol. Fixed embryos were stored in methanol.

Embryos were rehydrated with PBT (PBS with 0.1% Triton X-100), blocked in PBT and 2% normal goat serum (Vector Laboratories) for 3 hr at room temperature and incubated with primary antibodies in PBT for 24 hr at 4°C. We used rat monoclonal anti-Tubulin (YL1/2, 1:300; Abcam) and rabbit anti-Centrosomin (Cnn) (1:3000; Lucas and Raff, 2007). Cnn, a component of pericentriolar material crucial for mitotic centrosome assembly (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Lucas and Raff, 2007), is a mitotic centrosome marker. Embryos were washed in PBT and incubated with secondary antibodies Alexa Fluor 488-conjugated goat anti-rat IgG (1:800; Thermo Fisher Scientific) and Cy3-conjugated AffiniPure goat anti-rabbit IgG (1:800; Jackson ImmunoResearch Laboratories) in PBT overnight.
at 4°C. After an addition of DAPI (final concentration, 2 μg per ml) to stain DNA, incubation was continued for an additional 3 hr at 4°C. After extensive washing in PBT, embryos were mounted in Fluoro-KEEPER antifade reagent (Nacalai Tesque). The preparations were imaged as z-series acquired at 0.5-μm intervals on a FLUOVIEW FV1000 with a 60×/1.30 Sil UPlanSApo objective (Olympus). Images were then processed as maximum-intensity projections using ImageJ (NIH) and Adobe Photoshop CS6 (Adobe Systems).

To visualize sperm in the eggs, females were crossed with w; dj-GFP/CyO males, which produce fluorescent sperm tails (dj, don juan; Santel et al., 1997). Egg collection, dechorionation and methanol fixation were performed as described above, followed by replacement of methanol with ethanol. Fixed eggs were stepped gradually into PBT by sequential transfers into PBT containing 75%, 50%, 25% and 0% ethanol and then were stored at 4°C. For observation, eggs were incubated in 25% glycerol in PBS, mounted on glass slides with SlowFade Gold antifade reagent (Thermo Fisher Scientific) and then coverslipped by using a small amount of silicone grease (HIVAC-G, Shinetsu Silicone) to avoid
Evolutionary analyses of Elys

By using *Elys* of *D. melanogaster* (CG14215) as a query, homologs of *D. simulans* (GD26978), *D. sechellia* (overlapping GM22978 and GM22979) and *D. yakuba* (GE15862) were obtained by a BLAST search (blastn in FlyBase). The sequences were aligned by using Clustal X ver. 2.1 (Larkin *et al.*, 2007) and corrected manually. The number of nonsynonymous substitutions per nonsynonymous site (*K*$_a$) and the number of synonymous substitutions per synonymous site (*K*$_s$) were calculated, and the *K*$_a$/*K*$_s$ ratio test (Li, 1993) was conducted by using the kaks function in the seqinR package for the R environment (Charif and Lobry, 2007; http://seqinr.r-forge.r-project.org). The *K*$_a$/*K*$_s$ ratio was also calculated within a 180-bp sliding window to increase the sensitivity. PAML (Phylogenetic Analysis by Maximum Likelihood) ver. 4.9d (http://abacus.gene.ucl.ac.uk/software/paml.html; Yang, 2007) was also applied for the test.
The sequences of the common ancestors, node 1 (sechellia/simulans) and node 2 (node 1/melanogaster), were estimated, and the substitution history of the ELYS-like domain was reconstructed on the consensus unrooted phylogenetic tree: 
((sechellia, simulans), melanogaster), yakuba (Lachaise and Silvain, 2004). The ancestral state of node 2 was not determined unambiguously for three sites. We assumed that each replacement substitution took place with an equal probability in three branches (node 2–yakuba, node 2–melanogaster and node 2–node 1). Thus, these were in total calculated as $1/3 \times 3 = 1$ replacement in each branch.

**Data availability**

All *Drosophila* stocks, DNA clones and reagents are available upon request. Viability test for the *Elys* mutations is shown in Table S1. Sperm penetration to the eggs is shown in Table S2. Interaction between *Elys* and *Nup37* is shown in Table S3. The lethal stage of *Elys/Y; Df-Nup160/+* males was determined (Table S4). The lethal stage of *Elys/Y:
$Df\text{-}Nup96^+/+$ males was determined (Table S5). The cross between $Elys/FM7c; Df(2L)Nup160M190/CyO$ females and

$Elys/Y$ males is shown in Table S6. The cross between $Df(3R)/TM6C$ females and $D. simulans$ $Lhr$ males is shown in Table S6. Sperm were visualized by $dj\text{-}GFP$ in the eggs from $Elys$ mutant females (Figure S1). Mating scheme to
determine the lethal stage of $Elys/Y; Df\text{-}Nup160^+/$ is shown in Figure S2. Supplemental material available at Figshare:

http://doi.org/xxx/xxx.

Results

Description of the Elys mutations

X-linked $CG14215$ (X:19,652,305–19,659,407 [+]) of $D. melanogaster$ (FlyBase ID FBgn0031052) encodes a protein of

2,111 amino acids (aa) that includes an ELYS-like domain at aa 714–922 (InterPro accession number Q9VWE6;

UniProtKB – X2JG50; Finn et al., 2017). We recovered two frameshift alleles ($Elys^2$ and $Elys^5$) that truncate the

majority of the coding potential; aa 372 and 367 are predicted to be stop codons, respectively (Figure 1). Surprisingly,
the mutants were viable and male-fertile (Supplemental Material, Table S1) but female-sterile in homozygotes (Table 1). Thus, the mutations can be maintained via heterozygous (Elys/FM) females and hemizygous (Elys/Y) males (or FM/Y males), where FM (first multiple) stands for a balancer X chromosome; rare FM homozygotes are also present in the stocks.

The homozygous (Elys/Elys) and hemizygous (Elys/Df) females produced eggs, but the eggs never hatched when crossed with wild-type males (Table 1). Furthermore, the Elys+ transgene on chromosome 3, Dp(1;3)DC365, rescued the effect of Elys (Table 1); the duplication segment (X:19,624,757–19,716,729; FlyBase ID FBab0046817) carries 22 X-linked protein-coding genes including Elys and two ncRNA genes (Venken et al., 2010). We can even maintain Elys: Dp(1;3)DC365 as a viable stock. Sperm were observed in the unhatched eggs when visualized by dj-GFP (Figure S1 and Table S2). Thus, the Elys mutations are recessive female-sterile or maternal-effect lethal.
Disruption of mitotic progression of the first cleavage division by maternal effects of Elys mutations and Nup160

introgression

The Drosophila embryo remains a syncytium for the first two hours of development, where 13 rounds of nuclear division take place rapidly (Foe and Alberts, 1983). To gain insights into the primary effect of the Elys mutations on embryonic development, we fixed embryos 10–30 min after deposition and carried out cytological analysis. Our comparative analysis of embryonic progeny produced by Elys mutant females (Elys\(^2\) or Elys\(^5\) homozygotes) and the control females (Elys\(^2\) or Elys\(^5\) heterozygotes) revealed significant differences in the progression of the earliest cycles. Embryos from females mutant for Elys did not display mitotic progression; there was instead the accumulation of characteristics representing the first mitotic cycle (Table 2). Further investigation uncovered the maternal-effect lethality resulting from a terminal arrest in a metaphase-like state of the first cleavage division (Table 3; see below). The phenotype was essentially identical in the two Elys mutant strains.
The normal mitosis of the first cleavage division in *Drosophila* is gonomeric (Huettner, 1924; Guyénot and Naville, 1929; Calliaini and Riparbelli, 1996; Williams *et al.*, 1997; Loppin *et al.*, 2015); after DNA replication in nuclei from the ovum and sperm, the haploid complements persist in separate groups on a bipolar spindle composed of two units of microtubule arrays, which we refer to as the dual spindle (Figure 2A). The two units of microtubule arrays share the spindle poles, where the entire set of chromosomes is gathered at telophase. The *Elys* mutations affected the arrangement of the chromosomes and microtubule configurations of the dual spindle, because only spindles that appeared to be composed of a single unit of microtubule arrays with indiscriminately conjugated chromosomes were observed among all 102 embryos obtained from *Elys*² and *Elys*⁵ females (Figure 2, D and E). In addition, centrosomes behaved in a peculiar manner in the embryos. An analysis of these centrosomes by Cnn immunolabeling showed that, in control embryos, the centrosome is present as a single focus at each of the spindle poles during metaphase of the first cleavage division but then splits into two adjacent foci as early as anaphase (Figure 2, A and B). In embryos of *Elys* mutant females in the first mitotic cycle, however, sister centrosomes were separate, giving rise to two discrete foci.
even when centrosomes were situated at the pole of the metaphase-like spindle (Figure 2D). Remarkably, individualized centrosomes often detached from the spindle poles and were randomly located in the cytoplasm. We detected free asters with Cnn labeling in >70% of the embryos from both Elys$^2$ and Elys$^5$ females, whereas these were never seen in control embryos (Table 3). We observed up to four free asters within an embryo, indicative of arrest at the first cleavage division. When a spindle pole was devoid of centrosomes, the spindle appeared to be shorter in length and roundish (Figure 2E). It is also noteworthy that, in some embryos from Elys mutant females, polar bodies anomalously formed bipolar spindles that lacked centrosomes (Figure 2F; for control see Figure 2C), although their location within the embryo was substantively unaffected, lying near the cortex.

We reported previously that Nup160$^{sim}$ induces maternal-effect lethality subsequent to sperm penetration in D. melanogaster (Sawamura et al., 2004), reminiscent of the above-mentioned embryonic phenotype that was due to the Elys mutations. Embryos from females hemizygous for Nup160$^{sim}$ generally arrested their development in a
metaphase-like state of the first mitotic cycle (Figure 2, G and H), as is the case with the embryos from Elys mutant females. Most (49/50) of the embryos had a total of two to four centrosome foci, whereas the one exception contained eight foci, which might have been attributable to another round of the centrosome cycle or the occurrence of dispermy (insemination by two sperm). Strikingly, Nup160\textsuperscript{sim} also caused abnormal centrosome behavior, which manifested as free asters in the cytoplasm in ~75% (38/50) of the embryos. A noticeable difference between the effect of the Elys mutations and that of Nup160\textsuperscript{sim} could be discerned in the deformed mitotic figures that they exhibited. In the embryos of the Nup160\textsuperscript{sim} females, the union within the dual spindle was partially (12/49, Figure 2G) or thoroughly (24/49, Figure 2H) dissolved, resulting in two distinct spindles, each of a small size. In addition, unlike the Elys mutations, Nup160\textsuperscript{sim} did not affect microtubule configurations of the polar bodies (Figure 2G). Taken together, both the Elys mutations and the Nup160\textsuperscript{sim} introgression commonly affected most, if not all, aspects of the first cleavage division, including mitotic centrosome behavior.
Synthetic lethality caused by Elys and Nups

Based on the phenotypic similarity between the Elys mutations and Nup160\textsuperscript{sim} introgression, we expected to find a genetic interaction between Elys and Nups. We thus made double mutants of D. melanogaster that carry an Elys mutation on the X chromosome and are hemizygous for either of nine autosomal Nup107–160 subcomplex genes. Elys/FM: +/+ females were crossed with +/Y; Df/Bal males, where Df and Bal stand for a Nup deficiency and a balancer, respectively (Table 4). Elys/Y: Bal/+ males were viable because the balancer contains the wild-type Nup\textsuperscript{+} (control), but Elys/Y: Df/+ males, which carried only one dose of the Nup, were lethal (Nup96: viability, 0), semi-lethal (Nup160 viability, 0.01–0.04) or had low viability (Nup37 viability, 0.13–0.14). It must be stressed here that the lethality caused by the Elys mutations or the Nup160\textsuperscript{sim} introgression is maternal but the synthetic lethality caused by Elys and Nups double mutants is zygotic. Even in the last case (Nup37), most of the Elys/Y: Df/+ males died during or just after emergence: 88.9% (24/27) in Elys\textsuperscript{2} and 82.9% (29/35) in Elys\textsuperscript{5}. The lethality of Elys/Y: Df/+ males was confirmed by using additional Nup37 deficiencies (Table S3; viability, 0.01–0.18). An exception is Df(3R)ED10946 (viability, 1.02), but
we suspect that this deficiency differs from the computational prediction and does not delete Nup37; in fact,

\textit{Df(3R)ED10946} was viable, although the other deficiencies were lethal, when they were made transheterozygous against \textit{Df(3R)ED10953}. Thus, three of the nine genes (\textit{Nup37}, \textit{Nup96} and \textit{Nup160}) exhibited haploinsufficiency (e.g., hemizygous lethal) in the genetic background of the \textit{Elys} mutations. The lethal stage of the \textit{Elys/Y; Df/+} males was late pupal in \textit{Nup160} and \textit{Nup96} (Figure S2, Table S4 and Table S5). We also determined that the lethality of the \textit{Elys} and \textit{Nup} double mutants is not sex-specific. Not only \textit{Elys/Y; Df/+} males but also \textit{Elys/Elys; Df/+} females were lethal when \textit{Nup160} was made hemizygous (Table S6).

\textbf{Not only Nup96 and Nup160 but also Nup37 may cause hybrid lethality}

In the cross between \textit{D. melanogaster} females and \textit{D. simulans} males, hybrid males are lethal but are rescued by the \textit{Lhr} (\textit{Lethal hybrid rescue}) mutation of \textit{D. simulans} (Sturtevant, 1920; Watanabe, 1979). When \textit{Nup96\textsubscript{sim}} or \textit{Nup160\textsubscript{sim}} is made hemizygous by a deficiency chromosome of \textit{D. melanogaster} or made homozygous by an introgression from \textit{D.}
simulans, the hybrid males cannot be rescued by *D. simulans* Lhr (Presgraves et al., 2003; Tang and Presgraves, 2009; Sawamura et al., 2010). This is because *Nup96^sim* and *Nup160^sim* behave as recessive hybrid incompatibility genes (Strategy 2 of Sawamura, 2016). In other words, a gene or genes from *D. melanogaster* (incompatibility partner) result in hybrid inviability in the genetic background of *Nup96^sim* or *Nup160^sim* homozygote (or hemizygote).

We reported above that not only *Nup96* and *Nup160* but also *Nup37* exhibited haploinsufficiency in the genetic background of the *Elys* mutations. This raises the possibility that *Nup37* is also a gene for hybrid incompatibility. We thus made crosses by using deficiency chromosomes that lack *Nup37*. The interspecific crosses were very difficult, presumably because the deficiencies affect mating behavior; the hemizygotes exhibited the Minute phenotype resulting from the haploinsufficiency of closely linked *RpS27* (*Ribosome protein S27*; Marygold et al., 2007). Crossing was successful only when *Df(3R)ED10953* was used, and the male hybrids hemizygous for *Nup37^sim* were not rescued by *Lhr* (Table S7), although we cannot rule out the possibility that the lethality is a secondary effect of *RpS27*. Thus, not
only Nup96 and Nup160 but also Nup37 may be hybrid incompatibility genes.

Adaptive evolution of Elys in Drosophila

Hybrid incompatibility genes generally evolve rapidly (Ting et al., 1998; Barbash et al., 2003; Presgraves et al., 2003; Brideau et al., 2006; Tang and Presgraves, 2009). We thus compared the Elys gene sequences of D. melanogaster and D. simulans. Although $K_a/K_s = 0.53$ when the entire coding sequence was used, the sliding window analysis indicated positive natural selection ($K_a/K_s > 1$) around the ELYS-like domain and the Glu-rich domain of the gene (Figure 3A). In fact, $K_a/K_s = 1.51$ and 1.10 for these two domains, respectively, even though the Glu-rich domain is 49 aa shorter in D. simulans. The sequences of D. yakuba and D. sechellia were added to the comparison of the ELYS-like domain, and amino acid replacements and synonymous substitutions were counted in each branch of the phylogenetic tree (Figure 3B). Positive natural selection seems to have occurred on the route from node 2 (the common ancestor of D. melanogaster and D. simulans) to D. simulans, as indicated by the 26 replacements vs. 3 synonymous substitutions.
This was confirmed by the branch model of PAML; not significant for the full-length *Elys* sequences but significant for the ELYS-like domain \((p = 0.008 \text{ for the } D. \text{ simulans branch after sprit from } D. \text{ melanogaster} \text{ and } p = 0.048 \text{ for } D. \text{ simulans branch after the sprit from } D. \text{ sechellia})\).

**Discussion**

**ELYS function in D. melanogaster**

ELYS plays an important role in the NPC assembly, as noted above. Therefore, it was a surprise that *Elys* is dispensable for viability and male fertility in *D. melanogaster* (Figure 1 and Table S1). *D. melanogaster* might have another gene or genes, the function of which is redundant with *Elys*, although we have not found genes with sequence similarity. Similar to mutations in the *C. elegans* homolog, *mel-28* (Fernandez *et al.*, 2014; Gómez-Saldivar *et al.*, 2016), *D. melanogaster Elys* exhibited a maternal effect (Table 1). Females mutant for the gene produced apparently normal eggs in which sperm can penetrate (Figure S1 and Table S2), but the development of the resulting embryos never
progressed beyond the first mitotic division (Figure 2 and Table 2).

In the present study, we carefully examined the maternal effect of the Elys mutations (Table 3) and Nup160\textsuperscript{sim} introgression in early Drosophila embryos and showed that they share the embryonic phenotype of developmental arrest in a metaphase-like state of the first cleavage division. Therefore, the Nup160\textsuperscript{sim} introgression in D. melanogaster appears to behave like a loss-of-function allele of Elys. The prior steps of fertilization, such as the establishment of the sperm aster and pronuclear apposition, were unaffected, and no figures showing anaphase of the first cleavage division or later were observed. In these embryos, abnormally individualized centrosomes and their dissociation from the spindle poles were obvious, implying ELYS and Nup160 in mitotic centrosome behavior. Consistently, a proteomic analysis of Drosophila embryonic centrosomes shows that ELYS is actually a centrosome component (see Table S1 of Müller et al., 2010), although its function has not yet been established. Centrosomal localization of Nup160 is unknown in Drosophila, but the protein has been detected in spindle poles and proximal
spindle fibers of HeLa cells (Orjalo et al., 2006).

The developmental arrest could be accounted for by failure in structural changes of the nuclear envelope during the semi-open mitosis of early Drosophila embryos and/or disrupted interactions between the kinetochore and microtubules (Güttinger et al., 2009). Both ELYS and the Nup107–160 subcomplex can be detected in an interdependent manner at spindle poles and kinetochores (Zierhut and Funabiki, 2015). Also, the halting of mitotic progression could reflect the abnormal persistence of spindle-associated Cyclin B owing primarily to the dissociation of centrosomes from spindle poles, as the polar localization of centrosomes is required to initiate local destruction of Cyclin B in mitotic spindles of the Drosophila syncytium (Huang and Raff, 1999; Wakefield et al., 2000). The fact that the Elys mutations and the Nup160<sup>sim</sup> introgression result in very different outcomes with respect to the deformed morphology of the first mitotic spindle suggests that the ELYS and Nup160 proteins may have both common and distinct roles in the spindle assembly characteristic of the first cleavage division.
The present cytological study clearly demonstrates that ELYS and Nup160 are commonly involved, at a minimum, in centrosome behavior during the first cleavage division. Studies on subcellular localization of the ELYS and Nup160 proteins and their protein-protein interactions are needed to further elucidate their functions.

Because ELYS determines the subcellular localization of the Nup107–160 subcomplex (Belgareh et al., 2001; Boehmer et al., 2003; Harel et al., 2003; Walther et al., 2003; Loïodice et al., 2004; Franz et al., 2007; Gillespie et al., 2007; Rasala et al., 2006, 2008; Doucet et al., 2010; Bilokapic and Schwartz, 2013; Inoue and Zhang, 2014), we expected genetic interaction between Elys and Nups. Among the nine Nup107–160 subcomplex components examined, Nup37, Nup96 and Nup160 indeed exhibited haploinsufficiency in the genetic background of the Elys mutations (Table 4, Table S3 and Table S6); Elys/Y; Df/+ males were lethal at the pupal stage (Figure S2, Table S4 and Table S5). Interestingly, those three Nups are located in close proximity in the NPC (see Figure 1 of Hurt and Beck, 2015). Furthermore,
Bilokapic and Schwartz (2012) have suggested that ELYS binds near an interface of the subcomplex consisting of Nup120 (the yeast homolog of Nup160) and Nup37 in *Schizosaccharomyces pombe*. This might cause the epistatic interaction detected in the present analysis. Notably, the effect of Elys mutations and *Nup160* introgression is different than that of double mutations of Elys and Nups: the former survived to adulthood on their own and the lethality was only revealed as maternal effect while the latter exhibited a strong zygotic phenotype. These results suggest that ELYS and Nups may act at the same component of the mitotic machinery, or at another unidentified biological process, resulting in more severe synthetic lethal interactions.

Although ELYS sequences are well conserved in metazoans (Rasala et al., 2006), our present analysis detected positive natural selection in the ELYS-like domain of the protein in the branch leading to *D. simulans* (Figure 3 and Table S8). This might be the consequence of coevolution between ELYS and Nups. Indeed, recurrent adaptive evolution has been detected in five Nup107–160 subcomplex components (Nup75, Nup96, Nup107, Nup133 and Nup160) and two mobile
Nups (Nup98 and Nup153) in *D. melanogaster* and *D. simulans* (Presgraves *et al.*, 2003; Presgraves and Stephan, 2007; Tang and Presgraves, 2009).

Possible involvement of ELYS in reproductive isolation

Several genes responsible for hybrid lethality between *D. melanogaster* and *D. simulans* have been identified (for recent reviews, see Sawamura, 2016; Castillo and Barbash, 2017). *Lhr* and *Hmr* (*Hybrid male rescue*), which encode chromatin binding proteins, are one such incompatibility pair (Watanabe, 1979; Hutter and Ashburner, 1987; Barbash *et al.*, 2003; Brideau *et al.*, 2006; Thomae *et al.*, 2013; Blum *et al.*, 2017), and *gfzf* (*GST*-containing FLYWCH zinc-finger protein) is an upstream gene in this incompatibility (Phadnis *et al.*, 2015).

*Nup96* and *Nup160* are also involved in reproductive isolation (Presgraves *et al.*, 2003; Tang and Presgraves, 2009; Sawamura *et al.*, 2010). *Nup96*\textsubscript{sim} and *Nup160*\textsubscript{sim} synergistically cause hybrid incompatibility (Sawamura *et al.*, 2014),
but the *D. melanogaster* alleles of *Nup160* and *Nup96* are not the dominant autosomal incompatibility partner of

*Nup96<sup>sim</sup>* and *Nup160<sup>sim</sup>* respectively (Tang and Presgraves, 2015). Then, what is (are) the incompatibility partner(s) of *Nup96<sup>sim</sup>* and *Nup160<sup>sim</sup>*? One can envision that at least one recessive gene must be located on the X chromosome of

*D. melanogaster* (*X<sub>mel</sub>*) because the hybrid inviability is revealed in *X<sub>mel</sub>Y<sub>sim</sub>* but not in *X<sub>mel</sub>X<sub>sim</sub>*, where *Y<sub>sim</sub>* and *X<sub>sim</sub>* stand for the Y and X chromosomes of *D. simulans*, respectively (Strategy 2 of Sawamura, 2016). We here propose that the X-linked *Elys* of *D. melanogaster* may be the incompatibility partner of *Nup96<sup>sim</sup>* and *Nup160<sup>sim</sup>*.

Our proposal is based on three observations. (1) *Elys* mutations mimic the maternal *Nup160<sup>sim</sup>* introgression phenotype in *D. melanogaster* (Figure 2), which suggests that *Elys* affects the same cascade as the *Nup160<sup>sim</sup>* incompatibility. (2) Epistatic interaction was detected between *Elys* and *Nup37, Nup96* or *Nup160* in *D. melanogaster* (Table 4). (3) Male hybrids between *D. melanogaster* and *D. simulans* cannot be rescued by the *Lhr* mutation if *Nup37, Nup96* or *Nup160* of *D. melanogaster* is deficient (Table S7; Presgraves *et al.*, 2003; Tang and Presgraves, 2009;
In this model we presume that *D. melanogaster* ELYS does not function properly—and thus NPC formation and mitotic centrosome behavior are compromised—if Nup37, Nup96 or Nup160 is from *D. simulans*. We must also note that the incompatible *D. simulans* allele of the Nup107–160 subcomplex genes is recessive; the presence of the *D. melanogaster* allele is enough to avoid incompatibility. Thus, hemizygous Nup160$^{sim}$ introgression causes female sterility (maternal-effect lethality) with a phenotype that is similar to the Elys mutations of *D. melanogaster* (Figure 2). But Nup96$^{sim}$ introgression does not cause female sterility (Sawamura et al., 2014) and Nup37$^{sim}$ has not been tested.

Recently, *rhi* (rhino) and *del* (deadlock), which encode piRNA pathway proteins, were shown to be another incompatibility pair (Parhad et al., 2017). This pathway might have been adapted to suppress the species-specific transposable element mobilization (Kelleher et al., 2012; Parhad et al., 2017). ELYS plays an important role in the
piRNA pathway; PIWI is released from messenger ribonucleoprotein particles by binding to NPCs via Xmas·2, ELYS and other NPC components (Ilyin et al., 2017). The piRNA pathway evolution might result in the incompatibility between *Elys* and *Nups*.

Thus, *Elys* is a candidate for a gene of reproductive isolation between *D. melanogaster* and *D. simulans*, but direct evidence is necessary. We are going to test the viability and female fertility of flies (*D. melanogaster* or the *D. melanogaster/D. simulans* hybrid) that carry various combinations of *Elys* and *Nup* alleles.

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**FIGURE LEGENDS**

**Figure 1** Structure of the *Elys* gene and its mutations. Box, exon; horizontal line, intron. 1–490 aa, seven-bladed beta propeller repeats; 714–922 aa, ELYS-like domain; 1,069–1,092 aa, coiled coil; 1,665–1,847 aa, Glu-rich. There was a
1-bp deletion (1,287T) in *Elys*² and a 3,475-bp deletion (1,293–3,512) in *Elys*⁵; 5′-CTC GGT CG-3′ was inserted at the latter site instead.

**Figure 2** Mitotic arrest phenotypes of embryos produced by *Elys*² mutant females and *Int(2L)D+S, Nup160<sup>sim</sup>/Df(2L)Nup160M190 females. Embryos fixed in 10–30 min after deposition were treated with antibodies against α-Tubulin (green in merged images) for microtubules and Centrosomin (Cnn, magenta) for centrosomes, as well as the DNA dye DAPI (light blue). (A–C) Embryos of *Elys*²/+ females were the control. (D–F) Embryos of *Elys*² homozygous females, showing developmental arrest at the first cleavage division. (G, H) Embryos of *Nup160<sup>sim</sup>/Df(2L)Nup160M190 females, showing developmental arrest at the first cleavage division. (A) Metaphase of the typical gonomeric mitosis of the first cleavage division. The dual spindle (see text) is organized around the two groups of the chromosomes in juxtaposition. The aster is present at each of the common poles with a single focus of Cnn labeling at each pole. (B) Anaphase of the first cleavage division. Chromosome groups of maternal and paternal origin
converge as they synchronously migrate toward the poles and appear as single chromosome masses. The growth of astral microtubules is prominent, and centrosomes are detected as two foci (shown in the upper left pole). (C) Polar bodies with the normal, diffuse or unfocused arrangement of microtubules in the same embryo as (B). (D, E) Note abnormal separation of sister centrosomes around the poles (D) and the individualized centrosomes detaching from the spindle as free asters (E). (F) Polar bodies of the same embryo as in (E). Acentrosomal spindles with a bipolar orientation are often assembled around the chromosomes of polar bodies in embryos from E\textit{lys} mutant females. (G) A bifurcated configuration of the dual spindle. The tandemly oriented two small spindles are connected at the central poles with an aster organized around individualized sister centrosomes. One of the distal poles is astral and the other anastral. A subset of the polar bodies with the normal, circular configuration of microtubules is shown at the lower left. (H) The embryo contains two groups of chromosomes that are distantly located in the cytosol and are encompassed by microtubule arrays of high density. Among four individual centrosomes, three are present as free asters, whereas the remaining one is attached to one of the spindles. Arrows indicate the centrosomes. The scale bars represent 10 \mu m.
**Figure 3** A comparison of *Elys* gene sequences among *Drosophila* species. (A) $K_a/K_s$ test (180-bp sliding widow) between *D. melanogaster* and *D. simulans* (exons are separated by vertical dashed lines). The horizontal line ($K_a/K_s = 1$) indicates neutral evolution. (B) Replacement (R) vs. synonymous (S) substitutions in the ELYS-like domain.

**Figure S1** Sperm were visualized by *dj-GFP* in the eggs from *Elys* mutant females. *Elys$^2$/Elys$^2$* females were crossed with *dj-GFP/CyO* males. Left, confocal fluorescence image; right, phase contrast image.

**Figure S2** Mating scheme to determine the lethal stage of *Elys/Y; Df-Nup160/+*. Green box represents a GFP-marked balancer chromosome. *Elys/FM7c, GFP* females were crossed with *Df(2L)Nup160M190/CyO, GFP* males, and third instar larvae not carrying the green balancers were selected among the offspring, because GFP on *CyO* was difficult to detect in pupae. Larvae were illuminated by Handy Blue Pro Plus, and GFP fluorescence was detected by using the
Yellow 2 goggles (Relyon). Flies that emerged were counted and dead pupae were dissected to determine the lethal stage.

Table 1 Hatchability of eggs from females crossed with wild-type (OR) males

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Number of eggs</th>
<th>Hatchability, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collected</td>
<td>Hatched</td>
</tr>
<tr>
<td>Elys(^2)/FM7c, Elys(^+) (control)</td>
<td>222</td>
<td>184</td>
</tr>
<tr>
<td>Elys(^5)/FM7c, Elys(^+) (control)</td>
<td>208</td>
<td>177</td>
</tr>
<tr>
<td>Elys(^2)/Elys(^2)</td>
<td>204</td>
<td>0</td>
</tr>
<tr>
<td>Elys(^5)/Elys(^5)</td>
<td>203</td>
<td>0</td>
</tr>
<tr>
<td>Elys(^2)/Elys(^5)</td>
<td>1,068</td>
<td>0</td>
</tr>
<tr>
<td>Elys(^2)/Df(1)ED7620, Elys(^-)</td>
<td>219</td>
<td>0</td>
</tr>
<tr>
<td>Elys(^5)/Df(1)ED7620, Elys(^-)</td>
<td>209</td>
<td>0</td>
</tr>
<tr>
<td>Elys(^2)/Df(1)BSC871, Elys(^-)</td>
<td>573</td>
<td>0</td>
</tr>
<tr>
<td>Elys(^5)/Df(1)BSC871, Elys(^-)</td>
<td>209</td>
<td>0</td>
</tr>
<tr>
<td>Elys(^2)/Elys(^2); Dp(1;3)DC365, Elys(^+)/TM6C</td>
<td>240</td>
<td>220</td>
</tr>
<tr>
<td>Elys(^5)/Elys(^5); Dp(1;3)DC365, Elys(^+)/TM6C</td>
<td>237</td>
<td>222</td>
</tr>
</tbody>
</table>
To obtain Elys hemizygotes, *Df(1)ED7620/FM7h* or *Df(1)BSC871/FM7h* females were crossed with *Elys/Y* males. *Elys/Df(1)ED7620* females exhibited etched abdominal tergites.

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Number of embryos observed</th>
<th>Stages of embryos: Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Meiosis or pronuclear stages</td>
<td>1st cycle</td>
</tr>
<tr>
<td>Elys^2^ /FM7c, Elys^+^ (control)</td>
<td>89</td>
<td>1.1</td>
</tr>
<tr>
<td>Elys^5^ /FM7c, Elys^+^ (control)</td>
<td>67</td>
<td>9.0</td>
</tr>
<tr>
<td>Elys^2^ /Elys^2^</td>
<td>50</td>
<td>4.0</td>
</tr>
<tr>
<td>Elys^5^ /Elys^5^</td>
<td>55</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Females were crossed with wild-type (OR) males.
<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Number of embryos observed</th>
<th>Prophase</th>
<th>Prometaphase–metaphase</th>
<th>Anaphase–telophase</th>
<th>Unidentified</th>
<th>Embryos with free asters (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elys/FM7c, Elys+ (control)⁹</td>
<td>15</td>
<td>6.7</td>
<td>40.0</td>
<td>46.7</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>Elys²/Elys²</td>
<td>48</td>
<td>0</td>
<td>95.8</td>
<td>0</td>
<td>4.2</td>
<td>70.8</td>
</tr>
<tr>
<td>Elys⁵/Elys⁵</td>
<td>54</td>
<td>0</td>
<td>88.9</td>
<td>0</td>
<td>11.1</td>
<td>79.6</td>
</tr>
</tbody>
</table>

⁹Embryos are from the 1st cycle column of Table 2.  
¹The Elys mutation is Elys² or Elys⁵.
Table 4 Interaction between *Elys* and *Nups*  

<table>
<thead>
<tr>
<th>Locus examined</th>
<th>Paternal genotype</th>
<th>Elys allele</th>
<th>Elys/+</th>
<th>+/-</th>
<th>Elys/Y</th>
<th>+/-</th>
<th>+/-/Y</th>
<th>+/-/O</th>
<th>Exceptional males</th>
<th>Ambiguous males</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nap98–96</td>
<td>Df(3R)BSC489/TM6C</td>
<td>2</td>
<td>144</td>
<td>169</td>
<td>114</td>
<td>130</td>
<td>145</td>
<td>0</td>
<td>121</td>
<td>110</td>
<td>1 1 1 2</td>
</tr>
<tr>
<td>Nap96</td>
<td>Nup98–96/+/TM3</td>
<td>2</td>
<td>167</td>
<td>204</td>
<td>125</td>
<td>144</td>
<td>128</td>
<td>0</td>
<td>100</td>
<td>128</td>
<td>1 1 2 3</td>
</tr>
<tr>
<td>Nap160</td>
<td>Df(2L)Nups160M190/CyO</td>
<td>2</td>
<td>120</td>
<td>115</td>
<td>88</td>
<td>96</td>
<td>101</td>
<td>3</td>
<td>86</td>
<td>69</td>
<td>1 2 0 0</td>
</tr>
<tr>
<td>Nap37</td>
<td>Df(3R)ED1053/TM6C</td>
<td>2</td>
<td>283</td>
<td>293</td>
<td>222</td>
<td>231</td>
<td>250</td>
<td>27</td>
<td>212</td>
<td>163</td>
<td>1 0 4 2</td>
</tr>
<tr>
<td>Nap133</td>
<td>Df(3R)ED6901/TM6C</td>
<td>2</td>
<td>285</td>
<td>263</td>
<td>221</td>
<td>224</td>
<td>251</td>
<td>157</td>
<td>199</td>
<td>190</td>
<td>1 1 2 2</td>
</tr>
<tr>
<td>Nap44A</td>
<td>Df(2R)Exel6055/CyO</td>
<td>2</td>
<td>268</td>
<td>263</td>
<td>211</td>
<td>199</td>
<td>299</td>
<td>278</td>
<td>172</td>
<td>174</td>
<td>4 2 1 3</td>
</tr>
<tr>
<td>Nap43</td>
<td>Df(3R)ED5815/TM6C</td>
<td>2</td>
<td>264d</td>
<td>236</td>
<td>238</td>
<td>222</td>
<td>214a</td>
<td>112</td>
<td>190</td>
<td>125</td>
<td>0 2 0 1</td>
</tr>
<tr>
<td>Nap107</td>
<td>Df(2L)Exel6026/CyO</td>
<td>2</td>
<td>198</td>
<td>198</td>
<td>165</td>
<td>157</td>
<td>170</td>
<td>179</td>
<td>125</td>
<td>158</td>
<td>2 0 4 4</td>
</tr>
<tr>
<td>Nap75</td>
<td>Df(2R)ED3601/CyO</td>
<td>2</td>
<td>207</td>
<td>223</td>
<td>186</td>
<td>185</td>
<td>237</td>
<td>225</td>
<td>164</td>
<td>148</td>
<td>1 2 1 3</td>
</tr>
<tr>
<td>Sec13</td>
<td>Df(3R)BSC56/TM6C</td>
<td>2</td>
<td>239</td>
<td>227</td>
<td>215</td>
<td>198</td>
<td>215</td>
<td>223</td>
<td>210</td>
<td>133</td>
<td>1 5 2 1</td>
</tr>
</tbody>
</table>

1 Males were crossed with *Elys/FM7c* females. The replicates that produced maternal nondisjunctional flies at high frequency were not included in the data, because some of the mothers must have been XXY.
2 Full genotypes are available upon request. *Df(3R)ED10953* exhibits a slight Minute phenotype, because the *Nap37* locus is close to *RpS27*.
3 Presumably produced by the break in *FM7c* (see Hutter, 1990).  
4 The presence of the chromosome 2 balancer could not be determined.  
5 Calculated as (class II × class III)/(class I × class IV).  
6 One was Minute, presumably haplo-4.  
7 One was a gynandromorph.  
8 One was apparently paternal nondisjunctional XO.  
9 Two were apparently paternal nondisjunctional XO.
Fig. 1

- seven-bladed beta propeller repeats
- ELYS-like domain
- coiled coil
- Glu-rich

5'UTR

\[ E_{ys^2} \text{ (1 bp del)} \]

\[ E_{ys^5} \text{ (3475 bp del + 8 bp ins)} \]

3'UTR

1000 bp