RESEARCH ARTICLE

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clueless, a conserved Drosophila gene required for mitochondrial subcellular localization, interacts genetically with *parkin*

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SUMMARY

Parkinson's disease has been linked to altered mitochondrial function. Mutations in *parkin (park),* the Drosophila ortholog of a human gene that is responsible for many familial cases of Parkinson's disease, shorten life span, abolish fertility and disrupt mitochondrial structure. However, the role played by Park in mitochondrial function remains unclear. Here, we describe a novel Drosophila gene, *clueless* (*clu*), which encodes a highly conserved tetratricopeptide repeat protein that is related closely to the CluA protein of Dictyostelium, Clu1 of *Saccharomyces cerevisiae* and to similar proteins in diverse metazoan eukaryotes from Arabidopsis to humans. Like its orthologs, loss of Drosophila *clu* causes mitochondria to cluster within cells. We find that strong *clu* mutations resemble *park* mutations in their effects on mitochondrial function and that the two genes interact genetically. Conversely, mitochondria in *park* homozygotes become highly clustered. We propose that Clu functions in a novel pathway that positions mitochondria within the cell based on their physiological state. Disruption of the Clu pathway may enhance oxidative damage, alter gene expression, cause mitochondria to cluster at microtubule plus ends, and lead eventually to mitochondrial failure.

INTRODUCTION

Parkinson's disease results from the catastrophic loss of dopaminergic neurons within the substantia nigra of the brain. Significant declines in mitochondrial function are now recognized as a frequent feature of both heritable and sporadic Parkinson's disease (reviewed by Abou-Sleiman et al., 2006). Mutations or environmental factors associated with the disease are thought to increase oxidative stress within susceptible neurons by a wide variety of mechanisms, leading eventually to mitochondrial failure, accelerated cell death and the onset of symptoms. Mutations in *parkin* (also known as *PARK2*) (Kitada et al., 1998) are found in about 50% of heritable cases of autosomal recessive juvenile Parkinsonism (Betarbet et al., 2005), and the protein is found within lesions in the affected neurons of patients with sporadic disease (Schlossmacher et al., 2002). Although less frequent, *PINK1* mutations (Valente et al., 2004) are also responsible for a significant number of cases of familial Parkinson's disease.

Studies of the Drosophila *parkin* (*park*) and *pink1* orthologs (Greene et al., 2003; Wang et al., 2006) (reviewed by Pallanck and Greenamyre, 2006) strongly support the view that mitochondrial defects are central to Parkinson's etiology. Flies that are mutant for *park* contain defective mitochondria in many tissues and prematurely lose dopaminergic neurons in the brain. Park is predicted to function as an E3 ubiquitin ligase and might act to remove damaged mitochondrial proteins through ubiquitylation and degradation in the proteasome. Pink1, a mitochondrially targeted serine/threonine kinase, is also required for normal mitochondrial morphology and function, possibly by facilitating mitochondrial fission/fusion (Clark et al., 2006; Wang et al., 2006; Yang et al., 2008). Overexpression of Park can rescue *pink1* mutant flies, suggesting that these genes function in a common pathway (Clark et al., 2006; Park et al., 2006). Interactions of *pink1* and *park* with genes controlling mitochondrial dynamics suggest that the mitochondrial fission pathway may be involved (Poole et al., 2008). However, the specific molecular mechanisms connecting Park and Pink1 to mitochondrial function remain imperfectly understood.

The Dictyostelium *cluA* gene encodes a highly conserved protein that affects mitochondrial localization within cells, but its relationship to other mitochondrial pathways is unclear (Zhu et al., 1997). Mitochondria aggregate into a single cluster in *cluA* mutant cells, and similar clustering occurs in Baker's yeast (*Saccharomyces cerevisiae*) that is mutant for *clu1*, an orthologous gene that can functionally substitute for *cluA* (Fields et al., 1998; Dimmer et al., 2002). Metazoan eukaryotes contain CluA orthologs, such as *friendly mitochondria* (*fmt*) in Arabidopsis, a gene that is also required to prevent mitochondria from clustering (Logan et al., 2003). The *C. elegans* gene *clu-1* and a human ortholog, KIAA0664 ('human Clu'), exist but little information is currently available on their function.

Drosophila oogenesis (for a review, see Spradling, 1993) represents a highly favorable system for studying the functional importance of mitochondrial subcellular localization. Developing follicles grow extensively, facilitating the visualization of organelles within the oocyte and its 15 interconnected nurse cells. Moreover, mitochondria undergo a series of developmentally regulated behaviors as germ cells progress from stem cell to completed egg (Cox and Spradling, 2003). These include programmed fission after the stem cell stage, Balbiani body formation at the time of follicle formation, and extensive replication within growing follicles. Microtubule-based transport mediated by Dynein (Dhc), Kinesin (Khc) and the adaptor protein Milton (Milt) plays a crucial role in these events (Cox and Spradling, 2006). By studying how

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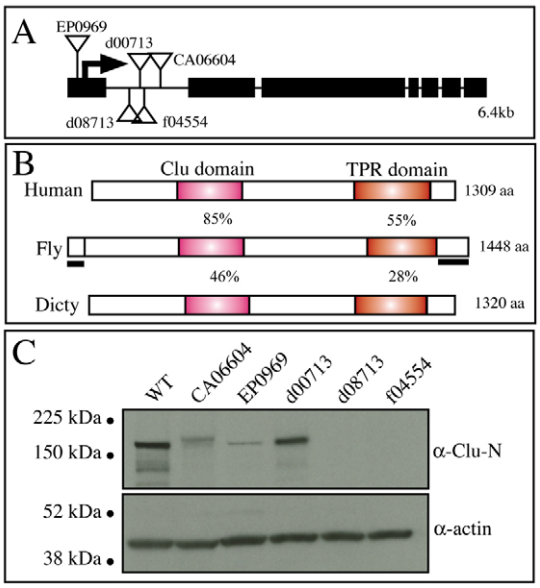

Fig. 1. *clu* structure and expression. (A) Map of the *clu* locus showing sites of insertion mutation (triangles) and the translation start (arrow). Black boxes are exons. (B) Drosophila Clu compared with human Clu and Dictyostelium (Dicty) CluA. The position and percentage of amino acid identity of the Clu and TPR domains are shown. The segments used to prepare N-terminal- and C-terminal-specific antibodies are indicated by solid black bars. (C) Western blot of adult extracts probed with N-terminal anti-Clu antibody showing the effect of *clu* mutations (listed at the top of the lanes) on the 160 kD Clu protein.

mitochondria redistribute in ovaries bearing *Dhc*, *Khc* and *milt* mutations, the predominant orientation of the microtubules used to transport mitochondria could be deduced (Cox and Spradling, 2006).

Here, we describe the gene '*clueless* (*clu*)' encoding a Drosophila CluA ortholog that is 53% identical to human Clu, and show that *clu* mutations cause mitochondrial dysfunction and clustering. Similar to *parkin* mutants, *clu* null mutant adults display a shortened life span, male and female sterility, as well as severe mitochondrial abnormalities in flight muscle. Furthermore, *park* mutations cause clustering of mitochondria in follicle cells and nurse cells during oogenesis. *clu* interacts genetically with *park* because *park*/+; *clu*/+ trans-heterozygotes show enhanced mitochondrial clustering. Based on these findings, we propose that Clu and Park participate in a physiological and oxidative damage control pathway(s) that links mitochondrial localization and function.

RESULTS

clu encodes a Drosophila CluA ortholog

The Drosophila gene CG8443 produces a single annotated transcript encoding a 1448-amino acid (aa) predicted protein that is related closely to CluA from Dictyostelium and its orthologs (Fig. 1A,B). Consequently, we named this gene *clueless* (*clu*). Clu shows 53% identity to human Clu (KIAA0664) across the entire protein. Both proteins contain a region of tetratricopeptide repeats (TPR), as well as a putative 'Clu domain' between amino acids 424-666 of Drosophila Clu, that is even more strongly conserved in metazoan

Table 1. *clueless* alleles

Allele	Adult viability	Fertility	Adult phenotype
d08713	4%	Sterile	Short lived; small, uncoordinated, wings up/down
f04554	n.d.	Sterile	Short lived; small, uncoordinated, wings up/down
d00713	44%	Male semi-sterile	WT
EP0969	39%	Male sterile	WT
CA06604	Viable	Fertile	WT

Clu proteins (85% identity between Drosophila Clu and KIAA0664) (supplementary material Fig. S1). N- and C-termini protein segments of Drosophila Clu were expressed and used to raise antibodies in guinea pigs (Fig. 1B) (see Methods). Both antibodies recognize a single band of apparent molecular weight 160 kD on western blots (Fig. 1C) whose abundance is reduced in *clu* mutants (see below).

Flies that are mutant for *clu* display abnormal mitochondrial structure and function

To analyze *clu* function, we identified multiple strains bearing single insertions located within the *clu* transcription unit (Table 1; Fig. 1A). Flies that are homozygous for clu^{d08713} or clu^{f04554} lack detectable Clu protein on western blots, whereas clu^{d00713} or clu^{EP0969} homozygotes contain reduced amounts (Fig. 1C). These *clu* mutant flies showed striking defects. Putative null clu^{d08713} (Fig. 2A) or clu^{f04554} homozygous flies (data not shown) are small, uncoordinated, sterile and live for only 3-7 days. They move slowly, do not fly, and frequently hold their wings up or down, a sign of flight muscle defects (Fig. 2A,B). The larval fat body fails to turn over within the first 2 days of adult life, another indication of a metabolic defect (Fig. 2C). Mutant flies bearing the weaker clu^{d00713} or clu^{EP0969} alleles are viable but the males are sterile. Flies bearing combinations of *clu* alleles, or trans-heterozygotes with a deficiency for the *clu* region, failed to complement, indicating that these defects are the result of disruption of *clu* expression. Finally, the Clu protein trap line CA06604 (Buszczak et al., 2007) produces a Clu-fusion protein of the expected size (Fig. 1C) that is likely to be functional because CA06604 homozygotes are viable and fertile.

Flies that are mutant for *park*, *pink1* or *rho7*, which encode mitochondrial proteins, exhibit many similar defects (Greene et al., 2003; Wang et al., 2006; McQuibban et al., 2006). To determine whether changes in mitochondrial function underlie the abnormalities in *clu* mutant flies, we carried out electron microscopy on the flight muscles of 3-day-old clu^{d08713} adults. Nearly all the flight muscle mitochondria were swollen and showed severe abnormalities, especially vesiculation/loss of cristae (Fig. 2D,E). Mitochondria in the ovary showed similar but less severe effects, including reduced cristae and swelling (Fig. 2F,G). Mature sperm from sterile *clu* males were completely immobile (data not shown). In addition, the mitochondrial derivative in elongated spermatids was irregular and swollen (Fig. 2I) when compared with wild-type flies (Fig. 2H). We conclude that *clu* is required for normal mitochondrial structure and function.

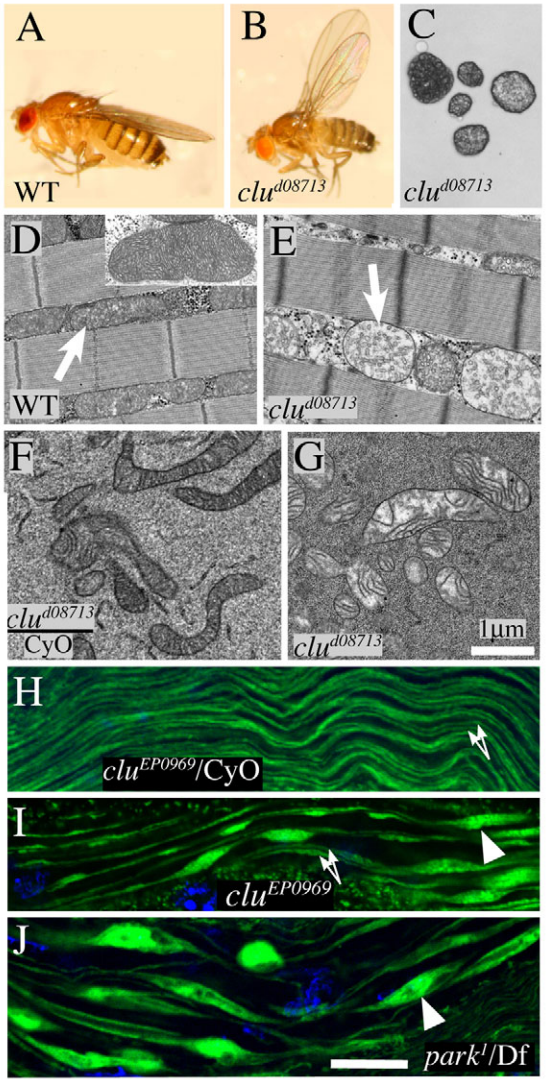

Fig. 2. Mitochondrial defects in *clu* mutants. (A) Wild-type adult female. (B) *clu*^{d08713} adult female showing reduced size and abnormal wing position. (C) Light micrograph showing persistent larval fat body cells from the abdominal hemolymph of a 4-day-old *clu*^{d08713} mutant female. (D) Electron micrograph of normal flight muscle showing mitochondria (arrow, inset). (E) *clu*^{d08713} flight muscle showing abnormal, swollen mitochondria with extensive vacuolization of the inner membrane (arrow). (F) Normal mitochondria from *clu*^{d08713}/CyO ovarian germ cells. (G) Abnormal mitochondria from *clu*^{d08713} ovaries showing partial vacuolization of the inner membrane. (H-J) Complex V-alpha subunit (CVα) expression (green) labeling mitochondrial derivatives in elongating spermatids from *clu*^{EP0969}/CyO (H), *clu*^{EP0969} (I) and *park*^{1}*/Df* (J) flies. In contrast to the uniform, paired mitochondrial derivatives that are seen normally (H, arrows), the mitochondria in both mutant animals (I,J) are uneven with swollen regions (arrowheads). Nuclei are stained with DAPI (blue). Bars, 1 μm (D-G); 5 μm (H-J).

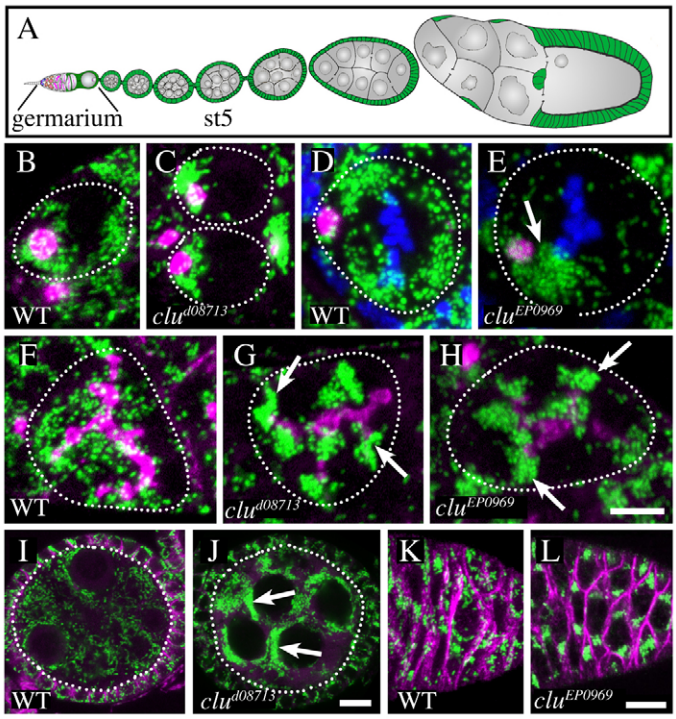

Fig. 3. *clu* mutations cause mitochondrial aggregation. (A) Diagram of a Drosophila ovariole, showing the nurse cells (gray), follicle cells (green) and progressively maturing follicles. The location of the germarium and a stage 5 (st5) follicle are indicated. In the germarium, germline stem cells (GSCs) contain a specialized organelle called the spectrosome that grows and interconnects the 16-cell germline cysts, where it is called the fusome (magenta). (B) Wild-type (WT) GSC showing dense mitochondria (green) at the anterior near the spectrosome (magenta circle), as well as throughout the cytoplasm. Note: the nucleus occupies the center of the cell. (C) *clu*^{d08713} GSCs showing increased mitochondrial clustering around spectrosomes. (D,E) Mitochondria disperse across the spindle of a wild-type single germ cell (D), but remain clustered in a similar mitotic *clu*^{EP0969} germ cell (E, arrow). DAPI (blue) stains the metaphase plate. (F-H) Mitochondria disperse throughout the cytoplasm of early wild-type cysts (F), but cluster at the ends of the fusome in *clu*^{d08713} (G) and *clu*^{EP0969} (H) mutant cysts. (I,J) Mitochondria are dispersed throughout the cytoplasm of stage 5 nurse cells in a wild-type cyst (I), but cluster (arrows) in *clu*^{d08713} nurse cells (J). (K,L) Mitochondria in wild-type germarial follicle cells are dispersed (K), but clump extensively in *clu*^{EP0969} germarial follicle cells (L). White dashed line outlines indicate germ cells (B-E) or germline cysts (F-J). Fusome and lateral membranes are stained magenta (1B1); mitochondria are stained green (CVα). Bars, 10 μm.

To examine whether Drosophila *clu* is also needed for mitochondrial positioning, we analyzed mitochondria during oogenesis. Drosophila ovarian follicles arise from stem cells in the germarium of each ovariole and subsequently develop in sequence (Fig. 3A). Changes in mitochondrial localization were observed in *clu* mutant germ cells beginning as early as the germline stem cells (GSCs). Normally, mitochondria in GSCs are enriched anteriorly, but are also found throughout the cytoplasm (Fig. 3B). In *clu*^{d08713} mutant GSCs, nearly all mitochondria occupy the anterior region of the cell (Fig. 3C). This increased clustering raises the question of whether, during GSC division, the daughter cystoblast and newly formed GSC inherit equal amounts of mitochondria. At mitosis in wild-type GSCs and cystoblasts, mitochondria spread uniformly across the outer spindle and are inherited equally by the daughter cells (Fig. 3D) (Cox and Spradling, 2003). By contrast, mitochondria remained clustered during mitosis in *clu*^{EP0969} early germ cells (Fig. 3E, arrow), suggesting that inheritance may be unequal and that one cell may have to make up for a deficit of mitochondria by increased mitochondrial replication (Cox and Spradling, 2006).

Enhanced clustering was also observed in older germ cells in the absence of normal Clu function. Mitochondria are dispersed in young wild-type germline cysts (Fig. 3F) but were highly clustered in similarly aged cysts that were homozygous for *clu*^{d08713} or *clu*^{EP0969}, where they associate with the tips of the fusome (Fig. 3G,H), an organelle that interconnects all the germ cells in a cyst. In growing ovarian follicles, mitochondria normally spread evenly throughout the nurse cell cytoplasm (Fig. 3I), but in similarly aged *clu*^{d08713} follicles nearly all mitochondria cluster in a giant aggregate in each nurse cell (Fig. 3J). *clu* mutations also caused mitochondrial clustering in some somatic follicle cells in the germarium (Fig. 3K,L).

Clu is cytoplasmic and may mediate mitochondrial movement along microtubules

We next examined the distribution of Clu protein to gain insight into the requirement for Clu in mitochondrial positioning and membrane integrity. Both the N-terminal and C-terminal antibodies, and the Clu-GFP protein trap gave indistinguishable results. Clu protein is found exclusively in the cytoplasm and is often present in discrete bodies or aggregates, as well as throughout the cytoplasm (Fig. 4A,B). Lower labeling is observed in somatic cells. Antibody reactivity is abolished in *clu* null mutations in all cells (Fig. 4C). Double labeling with anti-Clu and a mitochondrial antibody reveals that multiple mitochondria associate with Clu particles (Fig. 4D); however, Clu protein is only observed outside of mitochondria (Fig. 4E,E').

Ovarian mitochondria frequently move along microtubules as a result of tethering to both Dhc and Khc motors via linker proteins such as Milt (Cox and Spradling, 2006). Examining ovaries that were stained for Clu and microtubules revealed that Clu particles sometimes associate with microtubules (Fig. 4F). The distribution of microtubules in the ovary did not change appreciably in *clu*^{d08713} nurse cells where mitochondria are highly clustered (Fig. 4G,H).

These observations argue that mitochondrial clustering in *clu* mutants is not caused by changes in microtubule organization. However, comparing the location of mitochondrial clusters in *clu* mutants with their location in follicles that are mutant for microtubule motor protein genes or for the *milton* linker (Cox and Spradling, 2006) strongly suggested that Clu affects the movement of mitochondria along microtubules. In particular, sites of mitochondrial accumulation in *clu* mutants, such as the proximal side of GSCs (Fig. 3C) and adjacent to the fusome in early cysts (Fig. 3G,H), correspond to sites of microtubule plus ends in these cells (Cox and Spradling, 2006). Consequently, *clu* mutations cause mitochondria to cluster at the sites of microtubule plus ends in several stages where the polarity of the microtubules that transport mitochondria is known. In other situations, such as in nurse cells, mitochondria are less affected by microtubule motor mutations, and the systems controlling their location, as well as microtubule polarity, have yet to be determined.

Mitochondria cluster in *park* mutant cells

Many of the effects of *clu* mutation that we observed resembled those described following disruption of *park* (Greene et al., 2003). Consequently, we examined mitochondria in *park* mutant follicles to see whether they also form clusters. *park*^{1}/*Df* females are sterile, but follicles develop far enough to reveal strong mitochondrial clustering in the nurse cells of previtellogenic follicles (Fig. 5A,B). As follicles age, mitochondrial clustering worsens (Fig. 5C,D). Examining these clustered mitochondria using light and electron microscopy revealed that some were doughnut shaped (Fig. 5E,F) and some grew larger than normal (Fig. 5F; compare Fig. 2F). *park* mutants also induced clustering in somatic follicle cells (Fig. 5G,H). During spermatogenesis, only one mitochondrial derivative forms in *park*^{1}/*Df* mutant germ cells (Fig. 5P), as reported previously (Riparbelli and Callaini, 2007). *clu*^{EP0969} forms two derivatives but they appear frayed (Fig. 5N,O). Despite this initial difference, during spermatid elongation the *park*^{1}/*Df* mutant mitochondrial derivative becomes irregular and swollen like those in *clu*^{EP0969} spermatids (Fig. 2I,J).

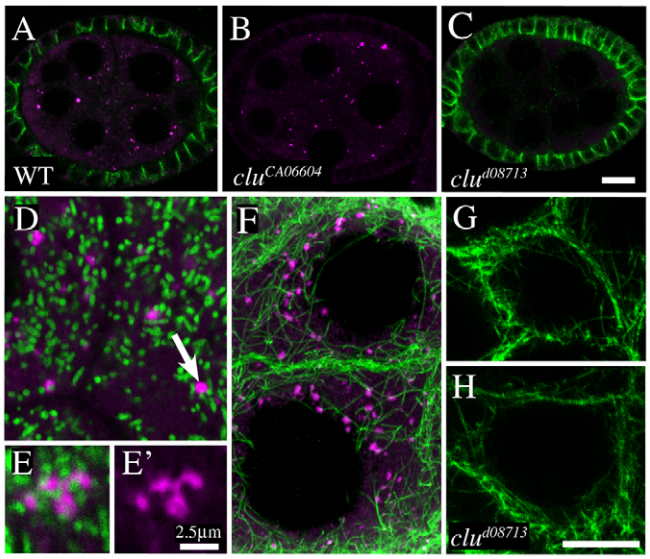

Fig. 4. Clu is a cytoplasmic, not mitochondrial, protein. (A-C) Stage 5 follicles: in wild-type follicles (A), Clu antibody (magenta) stains the nurse cell cytoplasm including some particles. Lateral membranes are stained green (1B1). The distribution of Clu-GFP in the protein trap line CA06604 (B), visualized with anti-GFP antibody (magenta), was indistinguishable from the distribution in wild-type follicles (A). In *clu*^{d08713}, Clu antibody staining is absent (C). (D-H) Stage 9 follicles: double label of Clu (magenta, arrow) and mitochondria (green) shows that Clu particles associate with one or more mitochondria (D). Juxtaposition of mitochondria (E, green) and Clu protein (E,E′, magenta) can be seen more clearly at higher magnification. (F) Association of microtubules (green) with some Clu bodies (magenta). (G,H) Microtubules in *clu*^{d08713} stage 9 nurse cells (H) appear normal compared with a wild-type control (G). Bars, 10 µm (A-D,F-H); 2.5 µm (E′,E).

clu and *park* interact

Because of the similarities between *clu* and *park*, we investigated whether these two genes interact genetically. *park* mutant ovaries still contained normal amounts of Clu protein (Fig. 5I,J), so the clustering observed in these animals is not simply the result of reduced Clu expression. The only difference appeared to be a reduction in the number of Clu particles in the *park* mutant cells. Females that were heterozygous for either *clu* or *park* alone were phenotypically wild type and mitochondria did not cluster in nurse cells from stage 5 follicles (Fig. 5K,L). By contrast, mitochondria were noticeably clustered in all similarly aged

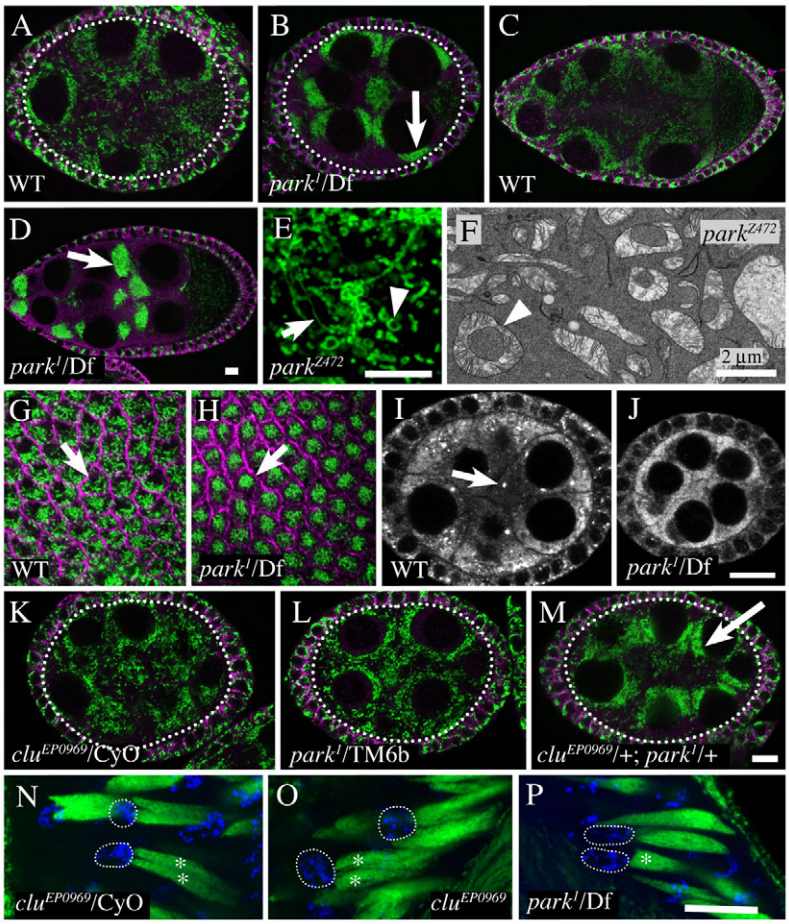

Fig. 5. *park* mutations cause mitochondrial clustering and interact genetically with *clu*. (A) Wild-type stage 5 follicles lack clustered mitochondria (green). Lateral membranes are stained magenta (1B1). (B) Severe mitochondrial clustering in a stage 5 $park^{1}/Df$ follicle. (C) Wild-type stage 9 follicles lack clustered mitochondria. (D) Extreme mitochondrial clustering in a stage 9 $park^{1}/Df$ follicle. (E) The mitochondria in $park^{Z472}$ follicles are clustered, long (arrow) and frequently form ring structures (arrowhead). (F) Electron micrograph of a $park^{Z472}$ mitochondrial cluster showing doughnut-shaped mitochondria (arrowhead). (G) Wild-type stage 9 follicle cells mostly lack clustered mitochondria (arrow). (H) Mitochondria cluster in the cell center in most $park^{1}/Df$ stage 9 follicle cells (arrow). (I,J) Clu protein (white) is present at similar levels in wild-type (I) and $park^{1}/Df$ (J) follicles, but large Clu particles are absent in the mutant. (K-M) Normal dispersed mitochondrial distribution in clu^{EP0969}/CyO (K) and $park^{1}$/TM6b (L) stage 5 follicles. (M) Clustered mitochondria in a stage 5 follicle from a clu^{EP0969}/+; $park^{1}$/+ female. (N-P) Mitochondrial derivatives in clu^{EP0969}/CyO (N), clu^{EP0969} (O) and $park^{1}/Df$ (P) male germ cells at the leaf blade stage. In wild-type spermatids (N), the Nebenkern unfurls revealing two mitochondria (asterisks) elongating below the nucleus (dotted circle). These mitochondria are less compact and appear frayed in clu^{EP0969} mutants (O). $park^{1}/Df$ mutants (P) have only one mitochondrion (asterisk) that looks smooth as in N. Mitochondria are stained green (CVα); membranes are stained magenta (1B1); nuclei are stained blue (DAPI). Bars, 10 μm, (A-D,G-M); 5 μm (E,N-P); 2 μm (F).

follicles from $park^{1}$/+; clu^{EP0969}/+ females (Fig. 5M). However, evidence of severe mitochondrial dysfunction was not observed, and both male and female trans-heterozygotes were fertile. We conclude that *clu* and *park* interact genetically to promote mitochondrial clustering.

Gene expression changes in *clu* mutant cells

To further analyze the roles played by Clu and Park we investigated whether *clu* or *park* mutant animals contain differences in the major mitochondrial proteins that are encoded in the cell nucleus. Western blots revealed that both Pyruvate dehydrogenase and Complex V-alpha (CVα) chain levels were reduced significantly in null clu^{d08713} homozygotes (Fig. 6). This decrease was not observed in clu^{EP0969} homozygotes, which retain a small amount of Clu protein. The observed reduction indicates that clu^{d08713} mitochondria contain reduced amounts of these major mitochondrial proteins, or that the total number of mitochondria is reduced in clu^{d08713} homozygotes.

To try and identify genes that are impacted directly by *clu* mutation, we used microarrays to analyze stage 10B ovarian follicles bearing the weaker clu^{EP0969} allele. Such follicles appear normal and successfully complete oogenesis, however, Clu protein is depleted significantly (Fig. 1C) and mitochondria cluster early in germ cell development. These studies revealed a small number of high-level changes in gene expression between wild-type and clu^{EP0969} follicles (Table 2).

Several of the most strongly altered genes protect cells from reactive oxygen species that are generated during mitochondrial metabolism. For example, both CG12896 and *GstE1*, which are genes that are thought to counteract reactive oxygen damage to thiols and to lipids, respectively (Sawicki et al., 2003), are strongly decreased by *clu* mutation. Several putative DNA repair proteins, including CG10354, a 5′-3′ exonuclease, CG5181 (SSB1), a single-stranded DNA binding protein, and phr6-4, a (6-4)-photolyase, are all strongly lowered. Apoptosis regulators such as the Rep1 caspase inhibitor (Mukae et al., 2000) and the putative SALL4-homolog CG18446 are reduced as much as tenfold. Several genes involved in mitochondrial metabolism are also changed, including *SCAP*, which regulates fatty acid production (reviewed by Rawson, 2003). In Drosophila, SCAP and its partner SREBP sense 16-carbon fatty acids and control the transcription of target genes involved in fatty acid biosynthesis in mitochondria. Perhaps in response to altered lipid metabolism, transcripts of several other candidate metabolic genes are also altered, including CG3424 (*path*), which encodes an amino acid/fatty acid transporter; CG11880, which encodes a putative choline transporter; *U26*, which encodes a putative aminoadipate-semialdehyde dehydrogenase; and others. Thus, the changes in transcript levels that we observe in relatively mild *clu* mutant follicles suggest that multiple cellular functions that are linked to mitochondria have been compromised. The results suggest that *clu* mutation reduces mitochondrial function, enhances oxidative damage, and decreases repair or apoptosis.

Table 2. Gene expression changes in *clu*^{EP0969} stage 10B follicles

Gene	-fold change	Comment
Top downreglated genes (<0.15 of control)		
CG14036	0.013	Related to gametocyte specific factor 1
CG18446	0.014	SALL4 related; regulates survival and apoptosis in mammals
U26	0.019	Putative aminoadipate-semialdehyde dehydrogenase
CG5181	0.022	Single-stranded DNA binding protein/DNA repair
SCAP	0.028	Sterol regulatory element binding protein cleavage-activating protein
CG12896	0.051	Thiol-specific antioxidant
GLaz	0.068	Lipid metabolic process
Pde11	0.083	cGMP-specific phosphodiesterase
GstE1	0.097	Metabolizes products of lipid peroxidation
CG10962	0.148	Oxidoreductase activity
Rep1	0.132	Inhibits caspase-activated DNase (CAD), apoptosis
CG10354	0.135	5′-3′ exoribonuclease activity
phr6-4	0.149	(6-4)-photolyase
Top upregulated genes (>5× control)		
CG32912	18.0	N-acetylmuramoyl-L-alanine amidase activity
CG12708	17.9	Ovary-enriched protein
CG7650	11.1	Phosducin-like protein; thioredoxin-like superfamily
path	10.7	PAT-class amino acid/fatty acid transporter
CG8964	10.3	IgG domain protein resembling human PTK7
CG11880	9.70	Putative choline transporter/solute carrier protein
GstE10	8.33	Glutathione transferase
CG32364	8.27	Gonad-enriched protein, function unknown
CG14636	6.0	Function unknown
Cyp6a17	5.7	Cytochrome P450 protein
Cyp6d4	5.6	Cytochrome P450 protein

DISCUSSION

clu encodes a highly conserved protein required for mitochondrial localization and function

Our studies identify a novel Drosophila gene, *clu*, that is essential for the normal cytoplasmic localization and function of cellular mitochondria. Clu-related proteins are highly conserved, especially within a novel 243-amino acid region (the 'Clu domain'), which is 85% identical (0 gaps) between Drosophila and human Clu. Clu function also appears to be remarkably conserved, because mutations in orthologs in Dictyostelium, Saccharomyces and Arabidopsis all cause mitochondria to cluster into a restricted cytoplasmic region (Zhu et al., 1997; Fields et al., 1998; Dimmer et al., 2002; Logan et al., 2003). In Drosophila, the Clu pathway is also essential to maintain mitochondrial integrity, whereas Dictyostelium and yeast cells that lack CluA or Clu1, respectively, have no detected functional defects.

park interacts with *clu*

Disruptions in a conserved process of mitochondrial maintenance involving the *park* and *pink1* genes underlies many cases of Parkinson's disease (Abou-Sleiman et al., 2006). We find that Drosophila *clu* mutations cause phenotypes that strongly resemble those caused by *park* mutations. Moreover, we showed that mitochondria in *park* mutants cluster in ovarian cells in a very similar manner to their behavior in *clu* mutants. In addition, *park* and *clu* interact genetically; compound heterozygotes have a mitochondrial clustering phenotype that is not seen in either heterozygote alone. These observations argue that Clu and Park function in the same or closely related pathways, and that the human Clu ortholog KIAA0664 might play a role in Parkinson's

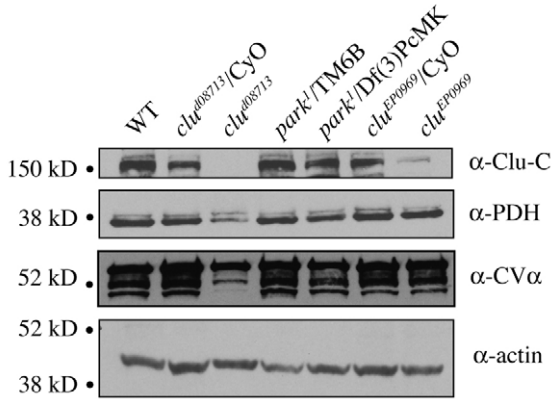

Fig. 6. *clu* adults contain reduced amounts of mitochondrial protein. (A) Western blot of whole fly extracts probed for Clu, Pyruvate dehydrogenase (PDH), CVα and Actin. The level of the two mitochondrial proteins PDH and CVα were unchanged from wild-type levels in heterozygotes that were mutant for *clu* or *park*. However, both PDH and CVα levels were reduced significantly relative to the Actin control in strong *clu*^{d08713} mutants (but not in *clu*^{EP0969}).

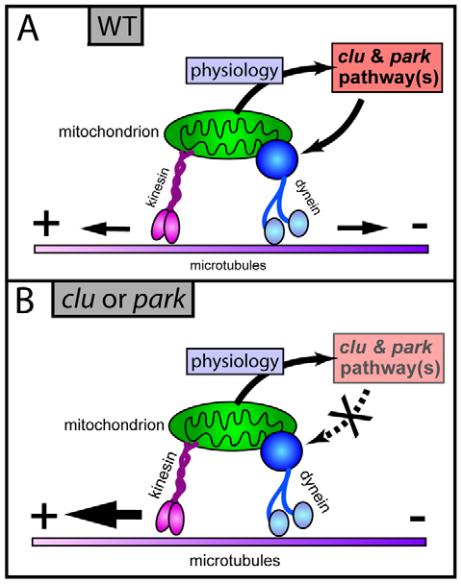

Fig. 7. Model for *clu* and *park* function. The figure shows a model for the role of the *clu* and *park* pathway(s) in cells (such as early ovarian germ cells) in which mitochondrial movement is controlled by the regulated engagement of plus-end-directed Kinesin motors (pink) and minus-end-directed Dynein motors (blue). The *clu* and *park* pathway(s) are located in the cytoplasm where they are proposed to sense the physiological state of a mitochondrion and modify its movement accordingly. (A) Under normal conditions, modulating the level of Dynein engagement relative to plus-end-directed motor activity allows the *clu* and *park* pathway(s) to cause a mitochondrion to move in either direction (arrows). (B) When the functioning of the *clu* and *park* pathway(s) is defective, or when levels of reactive oxygen species are elevated, minus end microtubule movement is blocked, causing mitochondria to move preferentially toward microtubule plus ends (large arrow). Note that in other cells, the *clu* and *park* pathway(s) might regulate mitochondrial movement by linking to different motors and transport systems.

disease. Furthermore, these findings suggest that changes in mitochondrial localization may be of significance in this condition.

Mitochondria cluster at microtubule plus ends in the early germ cells of *clu* mutants

Mitochondria are often positioned within cells by motor-dependent movement along microtubules (reviewed by Hollenbeck and Saxton, 2005). Clu protein appears to contact mitochondria, especially in the large Clu particles, many of which are located adjacent to microtubules. Our experiments suggest that *clu*-induced mitochondrial aggregates are caused by changes in microtubule-based mitochondrial transport. In both the Drosophila brain and ovary, mitochondria associate with transport complexes containing the adaptor protein Milt that are linked to both plus-end-directed motors such as Khc and minus-end-directed motors such as Dhc (Glater et al., 2006; Cox and Spradling, 2006). During oogenesis, loss of Khc, or of one of the Milt isoforms that interacts with Khc, cause mitochondria to cluster in cellular regions that are rich in microtubule minus ends, presumably because countervailing movement toward plus ends has been lost [see fig. 5 in Cox and Spradling (Cox and Spradling, 2006)]. Strikingly, mitochondria in *clu* mutant ovaries accumulate at predicted sites of microtuble plus ends; these include the proximal region of GSCs (Fig. 3C) and against the fusome in early cysts (Fig. 3G,H). Thus, in some situations, *clu* mutations may act by interfering with minus-end-directed mitochondrial movement along microtubules. Whether Clu particles correspond to mitochondrial transport complexes that mediate these changes, or whether Clu acts indirectly on mitochondrial positioning could not be determined with certainty from our studies.

In other cells, such as nurse cells, *clu* mutations also cause pronounced mitochondrial clustering (Fig. 3J), but that clustering is not mimicked by mutations in genes encoding microtubule motor proteins or Milt (Cox and Spradling, 2006). Other systems of mitochondrial localization may predominate in such cells but still be subject to regulation by the Clu pathway. For example, in plant cells, Clu was postulated to control mitochondrial localization by regulating the choice between microtubule-dependent and microfilament-dependent transport (Logan et al., 2003). In particular, the TPR domain of Clu was proposed to repress the interaction of mitochondria with microtubules by competing for binding with the TPR region of Kinesin light chain, thereby allowing transport along actin to predominate. The loss of this postulated interaction through *clu* mutation predicts that mitochondria would accumulate at microtubule minus ends in these cells. Although the default position where mitochondria accumulate in the absence of Clu might vary depending on the particular cell type and transport systems involved, the use of a Clu-dependent pathway to control mitochondrial subcellular location appears to be widespread.

Clu and Park may function in a novel pathway affecting mitochondrial physiology

Our experiments show that mitochondria require Clu to maintain their subcellular location and structural integrity. In the presence of even a relatively mild *clu* mutation, transcripts from genes involved in mitochondrial function and in protection from oxidative damage are reduced. Consequently, we propose that the normal role of Clu is to function in a pathway that controls the location and activity of mitochondria within the cell. Frequently, mitochondria may need to move to a different subcellular location in order to maximize access to substrates or to mitigate damage from oxidative metabolism. For example, in neurons, microtubule-dependent mitochondrial transport is modulated based on the level of respiratory activity (Miller and Sheetz, 2004). Clu protein would participate in a pathway that senses the internal physiological state of individual mitochondria and transduces this information into homeostatic changes in their positions and metabolic activities. When the Clu pathway is impaired, mitochondria would not move or operate normally, and might consequently suffer damage. This scenario is consistent with the changes that we observed in the inner mitochondrial membranes of Clu mutants, with their reduced levels of mitochondrial enzymes and the observed changes in nuclear gene expression. The greater severity of *clu* mutations in Drosophila compared with Dictyostelium *cluA* and yeast *clu1* might be because of an intrinsically greater requirement for dynamic mitochondrial positioning in the specialized cells of complex metazoans. This model may provide a rationale for the unexpected effects of microtubule inhibitors on mitochondrial function that were reported recently (Wagner et al., 2008).

Fig. 7 illustrates how the *clu* and *park* pathway(s) might link microtubule-based mitochondrial transport, mitochondrial physiology and oxidative damage. Under normal conditions, the

clu and *park* pathway(s) would sense the physiological state of mitochondria and activate appropriate levels of minus-end-directed mitochondrial movement along microtubules. For example, mitochondria that are low in respiratory substrates might move to cellular regions where these substrates are abundant. Mitochondria in need of repair might move close to the nucleus where appropriate repair genes would be induced. In conjunction with active plus-end-directed motors, the result would be that mitochondria move dynamically to locations throughout the cell that are appropriate to their physiological state. However, without functional *clu* and *park* pathway(s), or if the local level of toxic metabolic products exceeded a threshold, the mitochondria in question would cease minus-end-directed transport and undergo concerted plus-end-directed movement. In many cells, the major locus of microtubule minus ends is found near the nucleus so that plus-end-directed movement would increase the distance between reactive oxygen production and the nuclear DNA.

Clu and mitochondrial inheritance

During early germ cell development in many organisms, a subset of mitochondria within the germ cell cyst is transported and accumulates in a concerted fashion to form the Balbiani body (Cox and Spradling, 2003; Kloc et al., 2004). It has been postulated that Balbiani body formation is a selective process that rids the germline of mitochondria with defective mitochondrial genomes (Pepling et al., 1999; Cox and Spradling, 2003). In Drosophila, mitochondrial movement leading up to Balbiani body formation takes place by minus-end-directed transport along microtubules (Cox and Spradling, 2003; Cox and Spradling, 2006). Consequently, the Clu pathway might contribute to mitochondrial screening. However, Balbiani bodies of normal size were observed to form in *clu* mutants, suggesting that Clu function is not utilized, or is redundant, at this crucial stage of germ cell development.

The Clu pathway and Parkinson's disease

Our studies have several implications for understanding Parkinson's disease. According to our model, the Clu pathway would contribute strongly to the ability of mitochondria to remain functional during aging, despite the high metabolic requirements and environmental stresses experienced by many tissues. This may be particularly important in neural cells such as those that are compromised in Parkinson's disease. In a cell whose Clu pathway is compromised, mitochondria would operate less efficiently, suffer more damage and wear out faster because they would spend more time in cellular locations that are not appropriate to their metabolic state. The number of mitochondria producing elevated levels of reactive oxygen species would rise, increasing reactive oxygen damage to the nucleus and other cellular components, thereby leading to greater cell death. Consequently, the level of Clu pathway function within an individual might influence their susceptibility to sporadic Parkinson's disease and to other late-onset neurological disorders.

Mitochondrial mutations may fall into two classes based on their phenotype and on their relationship to the Clu pathway. Some mutations, such as those affecting mitochondrial ribosomal proteins (Royden et al., 1987) or certain metabolic pathways (Zhang et al., 1999), cause bang sensitivity, but differ phenotypically from *clu* mutations (Fergestad et al., 2006). By contrast, mutations in *park* (Greene et al., 2003), *pink1* (Wang et al., 2006), *rho7* (McQuibban et al., 2006), mitochondrial *ATP6* (Celotto et al., 2006) and mitochondrial *cytochrome oxidase* (Xu et al., 2008) resemble *clu* mutations in causing general defects in movement, flight, muscle or nerve degeneration, male fertility, and longevity. The effects of this latter class of mutations may be too severe to be compensated for by Clu pathway operation, leading to mitochondrial mis-positioning and increased production of reactive oxygen species. Consistent with this, expression of anti-oxidants partially suppresses the effects of *pink1* on neurodegeneration (Wang et al., 2006). Mutations in some of these genes, in addition to *park*, may also cause mitochondrial clustering and interact genetically with *clu*. Clearly, further studies of the Clu pathway will deepen our understanding of how mitochondria are maintained in cells and why they sometimes become damaged with age.

METHODS

Drosophila strains

All symbols are as described in FlyBase. $park^{1}$ (Cha et al., 2005) was obtained from Dr Kyoung Sang Cho, and $park^{Z472}$ (Greene et al., 2003) was obtained from Leo Pallanck. Wild-type flies were CantonS, except for those used in Fig. 3C, which were OregonR. All other stocks were obtained from Bloomington or Harvard. All stocks were cultured at 22-25°C on standard food.

Antibody production

Antibodies were raised to amino acids 1-104 and 1301-1448 of Clu using the protocol described in Buszczak and Spradling (Buszczak and Spradling, 2006).

Immunofluorescence microscopy

Fattened females and males were dissected and fixed following the protocol in Cox and Spradling (Cox and Spradling, 2003). Primary antibodies were diluted as follows: mouse Complex V, α-subunit (1:500, MitoSciences); mouse 1B1 (1:100, Developmental Studies Hybridoma Bank); and guinea pig Clu-C (1:2000, this work). The following secondary antibodies were used: goat anti-mouse IgG2b and goat anti-mouse Alexa Fluor 488; goat anti-mouse IgG1 Alexa Fluor 568; goat anti-guinea pig Alexa Fluor 568 (1:400, Molecular Probes). For microtubule labeling, fattened ovaries were fixed for 15 minutes in 4% paraformaldehyde in Grace's medium with 1 mM EGTA. Primary antibodies were a 1:1:1 mix of E7, AA 12.1 and AA 4.3 (1:50, Developmental Studies Hybridoma Bank). For DNA labeling, DAPI was added at 5 μg/ml for 10 minutes before VectoShield (Vector Labs) was added. Confocal analysis was performed using a 63× (NA 1.32) PlanApo lens and Leica TCS SP2 or SP5 confocal microscopes, or a Zeiss LSM510 Pascal confocal microscope.

Electron microscopy

Adult flight muscles and fattened ovaries were dissected in Grace's media (Sigma), then processed and imaged as described previously (Cox and Spradling, 2006).

Western blotting

Western blotting was done as described in Wilhelm et al. (Wilhelm et al., 2000) with the following modifications: to isolate protein, adults were ground with a pestle in Laemmli buffer with β-

TRANSLATIONAL IMPACT

Clinical issue

Parkinson's disease (PD) is a chronic neurodegenerative disease of the brain and affects approximately one million people in the USA. PD patients lose motor control, balance and coordination following loss of dopamine-releasing neurons in the substantia nigra region of the brain. Current PD therapies attempt to alleviate symptoms by augmenting dopaminergic signaling, but there presently is no cure for PD.

The majority of PD cases (85-90%) are sporadic, but 10-15% result from inherited mutations. Three genes associated with inherited forms of the disease, *parkin*, *pink1* and *DJ-1*, are involved in mitochondrial function, supporting the idea that oxidative damage and/or defective mitochondria may cause the loss of dopaminergic neurons seen in PD.

Results

In this study, the authors identified the previously uncharacterized gene *clueless* (*clu*). Flies that are mutant for *clu* are short-lived, uncoordinated, and male and female sterile. They exhibit structurally abnormal mitochondria in muscle and germ cells, and mislocalized and clustered mitochondria in germ cells. These phenotypes are very similar to those observed in *parkin* mutants, a Drosophila model for PD. Additionally, *clu* and *parkin* interact genetically, thus suggesting that they similarly affect mitochondrial function and movement. The authors hypothesize that both *clu* and *parkin* influence the subcellular localization of mitochondria in response to the physiological state of the cell. Mutant *clu* and the absence of dynamic positioning may therefore lead to impaired mitochondrial function, increased reactive oxygen production, and eventual cell apoptosis owing to mitochondrial damage.

Implications and future directions

This work identifies a new mechanism linking mitochondrial subcellular localization to mitochondrial function, repair and reactive oxygen output. Since *Clu* orthologs have highly conserved functions, *clu*-mediated mitochondrial regulation may have implications for neuronal health and PD in humans. Mutation or misregulation of the human Clu ortholog, KIAA0664, may underlie some heritable and sporadic cases of PD. Further studies of *clu* are likely to advance our understanding of how mitochondrial dynamics contributes to oxidative damage control. This work also supports the previous notion that altered mitochondrial function may be important in the onset of some cases of PD, and suggests new approaches for therapeutic intervention of disease progression.

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mercaptoethanol (BME), and the sample was boiled for 7 minutes before loading on a 4-15% acrylamide gel. The following primary antibodies were used: goat anti-Clu-N (1:15,000, this work), mouse anti-actin (JLA-20, 1:200, Sigma), mouse anti-Pyruvate dehydrogenase E1 α-subunit (PDF, 1:500, Mitosciences) and mouse anti-Complex V, α-subunit (CVα, 1:10,000, Mitosciences).

Microarray analyses

RNA was extracted from 100 stage 10B ovarian follicles as described previously (Allen and Spradling, 2008). Microarray analyses were performed in duplicate with RNA samples isolated on separate days, and greater than 90% of the genes that changed significantly were reproducible.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

R.T.C. and A.C.S. designed research, analyzed data, and wrote the paper. R.T.C. performed research.

SUPPLEMENTARY MATERIAL

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