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The mitochondrial outer membrane protein MDI promotes local protein synthesis and mtDNA replication

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Abstract

Early embryonic development features rapid nuclear DNA replication cycles, but lacks mtDNA replication. To meet the high-energy demands of embryogenesis, mature oocytes are furnished with vast amounts of mitochondria and mtDNA. However, the cellular machinery driving massive mtDNA replication in ovaries remains unknown. Here, we describe a *Drosophila* AKAP protein, MDI that recruits a translation stimulator, La-related protein (Larp), to the mitochondrial outer membrane in ovaries. The MDI-Larp complex promotes the synthesis of a subset of nuclear-encoded mitochondrial proteins by cytosolic ribosomes on the mitochondrial surface. MDI-Larp's targets include mtDNA replication factors, mitochondrial ribosomal proteins, and electron-transport chain subunits. Lack of MDI abolishes mtDNA replication in ovaries, which leads to mtDNA deficiency in mature eggs. Targeting Larp to the mitochondrial outer membrane independently of MDI restores local protein synthesis and rescues the phenotypes of *mdi* mutant flies. Our work suggests that a selective translational boost by the MDI-Larp complex on the outer mitochondrial membrane might be essential for mtDNA replication and mitochondrial biogenesis during oogenesis.

Keywords oogenesis; DNA replication; protein synthesis
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Introduction

Mitochondria contain their own genome, mitochondrial DNA (mtDNA), which encodes 13 key components of the electron-transport chain (ETC) and is therefore vital for life (reviewed in Wallace, 2007). But mtDNA is prone to accumulating mutations because of its close vicinity to damaging reactive species that arise as by-products of oxidative reactions (reviewed in Wallace, 2007). To ensure their reproductive success, metazoans must therefore prevent the transmission of deleterious mtDNA mutations from one generation to the next. Since mitochondria are transmitted exclusively through the female germ line in most metazoans, this task falls to the female germ line (reviewed in Stewart & Larsson, 2014). The process appears linked to the regulation of mtDNA replication during oogenesis. In the *Drosophila* ovary, mtDNA replication commences at the late germarium stage and is dependent on mitochondrial fitness. The selective amplification of wild-type genomes in healthy mitochondria might help limit the transmission of deleterious mtDNA mutations (Hill *et al*, 2014). Selective mtDNA replication may also contribute to the genetic bottleneck that facilitates mtDNA segregation and selection in mammals (Wai *et al*, 2008).

Besides its role in mtDNA quality control, the female germ line is also tasked with providing the massive amounts of mitochondria and mtDNA required to power early embryonic development (Wai *et al*, 2010; Wolff *et al*, 2013). In *Drosophila*, mtDNA replication shuts down completely during early embryogenesis (Rubenstein *et al*, 1977), perhaps as a consequence of the rapid nuclear divisions without gap phases that mark this stage, as mtDNA is preferentially replicated in late G1 and early G2 phases (Zhang *et al*, 2015). However, mitochondria undergo massive mtDNA replication in mid-stage egg chambers (Hill *et al*, 2014), which furnishes the mature oocyte with millions of copies of mtDNA (Wolff *et al*, 2013). Mammalian oocytes also display a burst of mtDNA replication prior to fertilization (St John, 2012). A typical mammalian oocyte contains hundreds of thousands of mtDNA molecules that are essential for post-implantation development (Wai *et al*, 2010). Despite the essential role of mtDNA replication in mtDNA inheritance across species, the mechanisms of its regulation in the female germ line are largely unknown.

Mitochondria are semi-autonomous organelles. The majority of mitochondrial proteins, including all components of the mtDNA replication machinery, are encoded in the nuclear genome, synthesized by cytoplasmic ribosomes, and imported into mitochondria (reviewed in Fox, 2012; reviewed in Moraes, 2001). Massive mitochondrial biogenesis therefore demands that nuclear genes produce vast amounts of mtDNA replication factors and mitochondrial proteins in a short developmental window. Even taking into account

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the fact that each developing oocyte receives the contribution of 16 genomes (from 1 oocyte and 15 accompanying nurse cells), it is perplexing to imagine how single-copy nuclear genes support the production of the millions of mitochondria found in mature oocytes.

We have been using *Drosophila* as a model system to investigate mtDNA inheritance, because of the availability of tools to genetically manipulate mtDNA (Xu *et al*, 2008; Chen *et al*, 2015). Here, we report on a mitochondrial outer membrane protein, MDI that is required for mtDNA replication and mitochondrial biogenesis in the *Drosophila* ovary. *mdi* mutant flies are female semi-sterile and display impaired mtDNA replication in ovaries. MDI recruits Larp, a translation stimulator to the mitochondrial surface, which then catalyzes the synthesis of a subset of nuclear-encoded mitochondrial proteins by cytosolic ribosomes. Constitutively targeting Larp to the mitochondrial surface restores local protein synthesis, mtDNA replication, and fertility of *mdi* mutant female flies. Furthermore, ectopic expression of AKAP1, the human homolog of MDI, in *mdi* mutant flies rescues their fertility defect. Therefore, the translational regulation of mtDNA replication and mitochondrial biogenesis we observe in *Drosophila* might in fact be a conserved mechanism guiding mitochondrial inheritance in metazoan.

Results

mdi is essential for mtDNA replication in the ovary

We identified *CG3249* from an ongoing RNAi screen for genes required for mtDNA replication in *Drosophila* ovaries. RNAi against the *CG3249* locus significantly reduced mtDNA replication, as indicated by a sharp reduction in the number of mitochondria-associated EdU puncta in the germarium (Fig EV1A). *CG3249* is disrupted in a female-sterile mutant, *spoonbill* that was generated by mobilizing a *P*-element near *CG3249* genomic locus (Hadad *et al*, 2011). However, the molecular nature of the chromosomal lesion in *spoonbill* background is uncertain. Another allele of *CG3249*, *yu*, was reported to have learning and memory defects, but normal fertility (Lu *et al*, 2007). Considering the uncertain molecular nature of these alleles and the discrepancies of phenotypes, we generated our own deletion of the *CG3249* locus by CRISPR/Cas9 technology as previously described (Gratz *et al*, 2013). We obtained a 2.4-kb deletion that removed most of the coding region of *CG3249* (Fig 1A). Homozygous flies completely lacked the CG3429 protein product (Fig 1B). Homozygous females were semi-sterile (Fig 2D), but there was no obvious phenotype in male flies. EdU staining showed that 93% of the ovaries carrying the *CG3249* deletion displayed severely reduced mtDNA replication in the germarium, as well as in the egg chambers (Fig 2A and B). The remaining 7% showed normal mtDNA replication in the egg chambers, but reduced replication in the germarium region 2B (Fig 2C). Based on this reduced mtDNA replication phenotype, we named the deletion mutant mdi^1 for mitochondrial DNA insufficient. The mdi^1 mutation also caused mitochondria to clump together in mid-stage egg chambers and in eggs (Fig EV2A–C), a phenotype that, given its later onset, may be a secondary effect of disrupted mtDNA replication.

Despite the mitochondrial deficiencies in their ovaries, mdi^1 female flies produced a similar amount of eggs as wild-type females (251 ± 24 vs. 239 ± 24 per fly). However, only 5% of the eggs

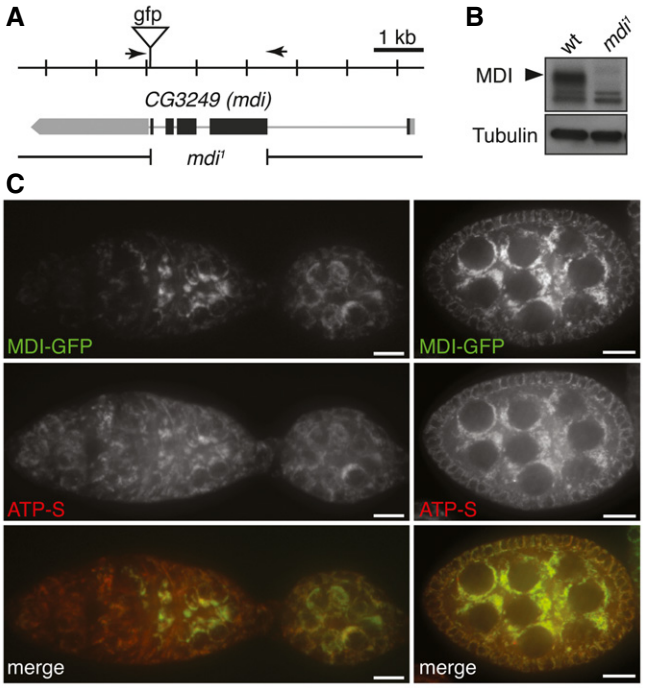

Figure 1. *mdi* gene, protein, and mdi^1 deletion.

A Schematic drawing of the *mdi* genomic locus showing the *CG3249* (*mdi*) transcript (gray box) and coding region (black box), and the location of the gfp insertion generated by CRISPR/Cas9-mediated recombination with two guide RNAs (arrows). The resulting fusion protein is referred to as MDI-GFP. The mdi^1 mutation is a 2.4-kb deletion that removes most of the *mdi* coding region.

B Western blots of MDI protein in wild-type (wt) and mdi^1 flies, showing that mdi^1 is a protein-null mutation. Tubulin was used as a loading control.

C Ovarioles expressing MDI-GFP stained with a mitochondrial marker, ATP synthase (ATP-S). Overlapping signals (merge) indicate that MDI localizes to mitochondria. Note that MDI is highly expressed in germ cells at late germarium stages (left panels) and in egg chambers (right panels). Scale bars, 10 μm.

produced by mdi^1 females crossed with either mdi^1 or wild-type males hatched (Fig 2D), demonstrating the maternal-effect, embryonic lethality of the mdi^1 mutation. Consistent with a reduced level of mtDNA replication during oogenesis, eggs produced by mdi^1 females had only 3% of the mtDNA amount found in the eggs of wild-type females (Fig 2D). Importantly, the expression of an *mdi* cDNA transgene in the female germ line driven by *nanos-gal4* restored the mtDNA level and the hatching rate of mdi^1 eggs (Fig 2D). These observations demonstrate that MDI is required for mtDNA replication during oogenesis.

MDI is a multi-domain protein of the mitochondrial outer membrane

To determine the expression pattern and sub-cellular localization of MDI in ovaries, we inserted a GFP reporter in-frame with the *mdi* ORF at the endogenous locus by CRISPR/Cas9-mediated recombination (Fig 1A). The resulting fusion protein, MDI-GFP, was highly expressed in germ cells, and its expression pattern paralleled the pattern of mtDNA replication in the ovary (Fig 1C): commencing at

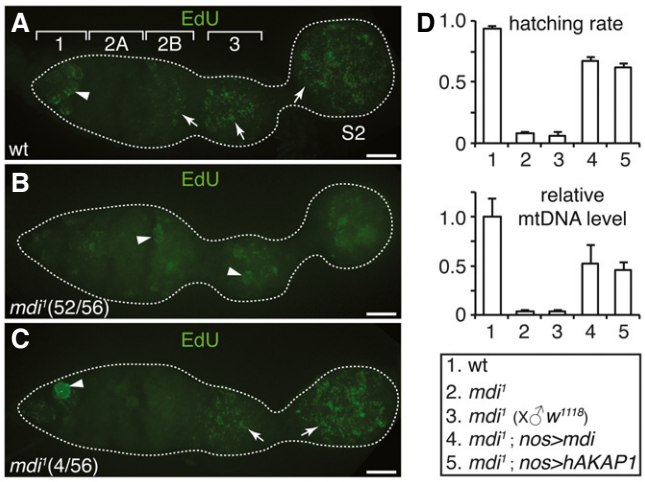

Figure 2. MDI promotes mtDNA replication in the ovary and is essential for female fertility.

A–C mtDNA replication in wt (A) and *mdi*^{1} ovarioles (B, C) as illustrated by EdU incorporation. Arrows point to mitochondrial DNA and arrowheads to nuclei. In wt ovaries (A), mtDNA replication starts at germarium stage 2B and lasts into egg-chamber stage 2 (S2). In most *mdi*^{1} ovarioles (B), mtDNA replication is undetectable at these stages, whereas in some (C), it appears delayed until germarium stage 3. Scale bars, 10 μm.

D Hatching rates and mtDNA content of eggs laid by females of different mutant genotypes relative to eggs laid by wt controls. Each data point represents the mean of three independent replicates. Error bars represent SD. Expression of *mdi* or *hAKAP1* in *mdi*^{1} significantly restored the mtDNA level and hatching rate. $N = 3 \times >100$ eggs/genotype for hatching rate. The relative mtDNA level was determined as the average of three biological repeats. *P*-values of comparing *mdi*^{1}; *nos>mdi* to *mdi*^{1}: hatching rate, $P = 1.1995E^{-05}$; mtDNA, $P = 0.0078$. *P*-values of comparing *mdi*^{1}; *nos>hAKAP1* to *mdi*^{1}: hatching rate, $P = 1.6998E^{-05}$; mtDNA level, $P = 0.0009$.

region 2B germarium and remaining active in mid-stage egg chambers (Hill *et al*, 2014). The concurrence of MDI expression and mtDNA replication is consistent with the observation that MDI is essential for mtDNA replication in the ovary.

MDI contains a putative mitochondrial targeting sequence (MTS) at its N-terminus (Fig 3A). Indeed, an MDI-GFP fusion protein localized exclusively with mitochondria in cultured cells (Fig 3B). In the ovary, MDI-GFP co-localized with a mitochondrial marker, ATP synthase (Fig 1C), confirming that MDI is a mitochondrial protein. Moreover, a truncated MDI lacking the putative MTS, MDI$^{\Delta MTS}$, had a diffuse cytoplasmic localization (Fig 3B) and failed to rescue the mtDNA levels and the hatching rate of *mdi*^{1} eggs (Fig 3A). Therefore, mitochondrial localization appears essential for MDI's function.

To determine the sub-mitochondrial localization of MDI, we purified mitochondria from cultured cells stably expressing an MDI-Myc fusion protein and digested them with protease K. MDI-Myc was completely removed by protease K treatment (Fig 3C), as was Tom20, a marker of the outer membrane protein. By contrast, cytochrome C and SOD2, which reside at the inter-membrane space and the matrix, respectively, were resistant to protease K treatment (Fig 3C), confirming the integrity of the purified mitochondria. We thus conclude that MDI localizes to the mitochondrial outer membrane, with its C-terminus facing the cytoplasm.

Since mtDNA replication takes place in the matrix, it is puzzling that MDI, an outer membrane protein, should have such profound impact on mtDNA replication. Besides its MTS, MDI is predicted to have a RNA-binding KH domain, a protein phosphatase 1-interacting domain (PP1), a R domain that might bind to protein kinase A (PKA) and a Tudor domain (Hadad *et al*, 2011; Fig 3A). We generated a series of MDI mutants: MDI$^{\Delta KH}$, MDI$^{\Delta PP1}$, MDI$^{\Delta R}$, and MDI$^{\Delta Tudor}$ with deletion in each of these domains (Fig 3A). All of these mutants localized to mitochondria properly (Fig 3B). The expression of MDI$^{\Delta KH}$ or MDI$^{\Delta PP1}$ in the *mdi*^{1} female germ line rescued the hatching rate and mtDNA level of their eggs, but the expression of MDI$^{\Delta R}$ and MDI$^{\Delta Tudor}$ did not (Fig 3A). This observation suggests that the R domain and Tudor domain are essential for MDI's function. MDI's mammalian homolog, AKAP1, recruits PKA through its R domain to the mitochondrial surface and regulates mitochondrial metabolism and dynamics (Wong & Scott, 2004). However, PKA is believed to localize on the plasma membrane, not the mitochondria, in ovaries (Lane & Kalderon, 1995). Additionally, mtDNA replication was normal in *PKA* mutant ovaries (Fig EV1B), indicating that MDI does not need to interact with PKA to regulate mtDNA replication. Some Tudor domain proteins localize to the mitochondrial outer membrane and regulate piRNA biogenesis (Honda *et al*, 2013). However, piRNA level was not affected in the *CG3249 (mdi)* knockdown ovaries (Handler *et al*, 2011). It is therefore unlikely that MDI regulates mtDNA replication via a piRNA-related process.

Recruitment of Larp to mitochondria by MDI is essential for mtDNA replication

To identify potential interacting partners of MDI, we expressed MDI-myc fusion protein in S2 cells, purified MDI complexes using anti-myc antibody, and subjected them to mass-spectrum (MS) analysis. We arbitrarily set the threshold of twofold enrichment in MDI-myc immunoprecipitates compared to the control immunoprecipitates from non-transfected S2 cells. To increase the confidence of the MS analysis, we also filtered out hits with less than 25% sequence coverage. Among the 15 proteins that met these criteria were SesB, the mitochondrial ATP:ADP antiporter, and Bor, a mitochondrial AAA protein (Table EV1). The remaining 13 are either ribosomal proteins or proteins involved in translational regulation (Table EV1). The top three candidates (those with the most peptide counts) are eukaryotic translation initiation factor 4G (eIF4G), Larp, and poly-A binding protein (PABP). Mammalian homologs of these three proteins are all involved in initiating or boosting protein translation (Kahvejian *et al*, 2005; Sonenberg & Hinnebusch, 2009; Tcherkezian *et al*, 2014). A previous high-throughput proteomic study also found that MDI (CG3249) interacts with CG7414 (Guruharsha *et al*, 2011), which encodes the translation initiation factor 2A. All these observations suggest that MDI might regulate protein translation.

We decided to focus on Larp for two reasons: (i) most *bona fide* ribosomal proteins are essential for cell viability, which would complicate the genetic analyses; (ii) mutations in *larp*, like the loss of function of *mdi*, cause maternal-effect lethality (Blagden *et al*, 2009). Importantly, mtDNA replication was severely impaired in *larp* mutant ovaries (Fig 4B), implying that MDI and Larp might function in the same pathway. We confirmed the MDI-Larp

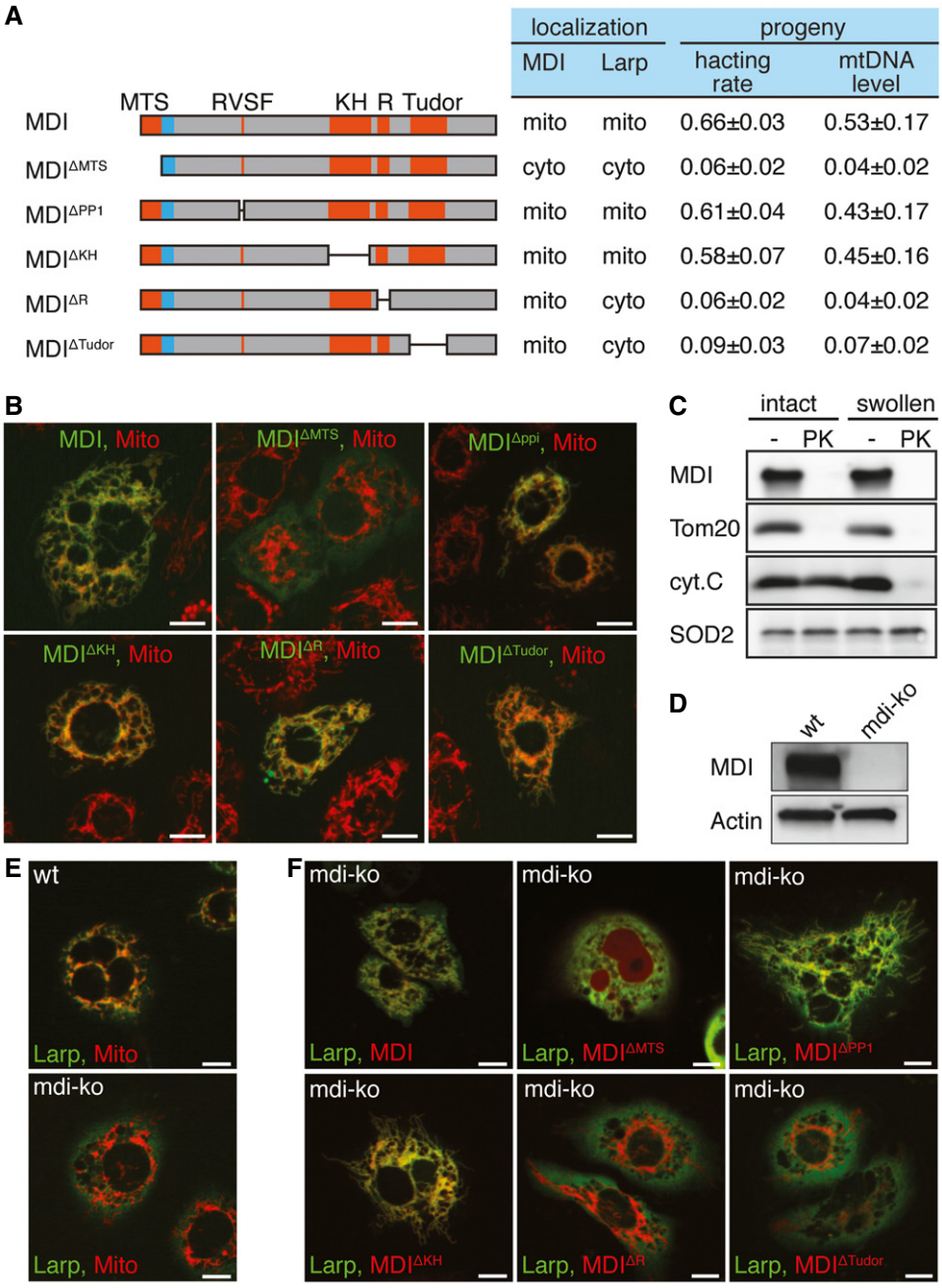

Figure 3. Functional-genetic analyses of MDI and its interaction with Larp.

A Schematic drawing of MDI protein and deletion mutants. These proteins were expressed under the *nanos-gal4* driver and tested for their ability to rescue the hatching rate and mtDNA level of eggs from *mdi*^{1} females (progeny column) or for localization and recruitment of partner protein Larp to mitochondria in cultured cells (localization column). $N = 3 \times >100$ eggs/genotype for hatching rate. The relative mtDNA level was the average of three biological repeats.

B Representative images of S2 cells expressing GFP fusions (green) of the MDI proteins diagramed in (A). Staining with MitoTracker Red (red) to label mitochondria shows an overlapping signal (yellow), indicating proper localization to mitochondria for all fusion proteins except MDI$^{\Delta MTS}$.

C Submitochondrial localization of MDI. Mitochondria from cultured cells expressing an MDI-myc fusion protein were kept intact or subjected to swelling to disrupt their outer membranes and digested or not with protease K (PK). Protein extracts were subjected to Western blot analysis. Tom20, cytochrome C (cyt.C), and SOD2 were used as markers of the outer membrane, inter-membrane space, and matrix, respectively. MDI behaved like an outer membrane protein.

D Western blots of MDI protein in wt and *mdi* knockout (mdi-ko) cells confirming the mdi-ko cells completely lacked MDI protein. Actin was used as a loading control.

E Representative images of wt or mdi-ko cells expressing a Larp-GFP fusion protein and stained with MitoTracker Red to label mitochondria. The majority of Larp localized to mitochondria in wt cells (overlapping red and green signal), but diffused into the cytoplasm of mdi-ko cells.

F Representative images of mdi-ko cells co-expressing Larp-GFP with MDI or the deletion mutants diagramed in (A) fused to the mCherry. Of the 4 MDI deletions that localized to mitochondria, 2 (MDI$^{\Delta R}$ and MDI$^{\Delta Tudor}$) did not allow Larp-GFP to localize to mitochondria. The localization of Larp in *mdi*^{1} flies expressing MDI or MDI deletion mutants, and the hatching rate and mtDNA level in their eggs were determined. The result is summarized in (A).

Data information: Scale bars in (B, E, F), 10 μm.

interaction by co-immunoprecipitation from ovary extracts (Fig 4A). Although Larp is not annotated as a mitochondrial protein, it associates with mitochondria in spermatocytes (Ichihara *et al*, 2007). We also found that the majority of Larp coalesced around mitochondria in wild-type ovaries (Figs 4C and EV3A), but had a diffuse cytoplasmic localization in the *mdi*^{1} ovaries (Figs 4C and EV3A). This observation suggests that MDI is necessary for recruiting Larp to mitochondria.

We next examined whether Larp's association with mitochondria was required for MDI's function. Larp localized to the mitochondria in wild-type S2 cells, but diffused into the cytoplasm of mdi-ko S2 cells (Fig 3E), in which the endogenous *mdi* locus was disrupted by CRISPR/Cas9 technology (Fig 3D). We introduced a series of MDI deletion mutants into mdi-ko cells and tested their ability to recruit Larp to mitochondria (Fig 3A and F). The same set of MDI mutants were also expressed in the *mdi*^{1} background to test whether they could rescue the fertility of female *mdi*^{1} flies. MDI mutants that failed to recruit Larp to mitochondria also failed to restore the fertility of female *mdi*^{1} flies (Fig 3A and F). By contrast, the MDI mutants that were able to recruit Larp to mitochondria significantly restored the fertility of female *mdi*^{1} flies and the amount of mtDNA in their eggs (Fig 3A and F). Taken together, these results demonstrate that MDI promotes mtDNA replication in ovaries by recruiting Larp to mitochondria.

Cytosolic ribosomes synthesize proteins on the mitochondrial surface

Many mRNAs encoded by the nuclear genome are associated with the mitochondrial outer membrane in yeast and animal cells (Sylvestre *et al*, 2003; Fox, 2012). This localization is thought to facilitate the import of their protein products into mitochondria. Interestingly, Larp interacts with PABP and eIF4G (Tcherkezian *et al*, 2014), through which it may stabilize mRNAs and stimulate protein synthesis. Given the interaction between Larp and MDI and their mitochondrial localization, we hypothesized that MDI-Larp might promote protein synthesis on the mitochondrial surface in the ovary. To our knowledge, protein synthesis on the mitochondrial surface has not been demonstrated previously.

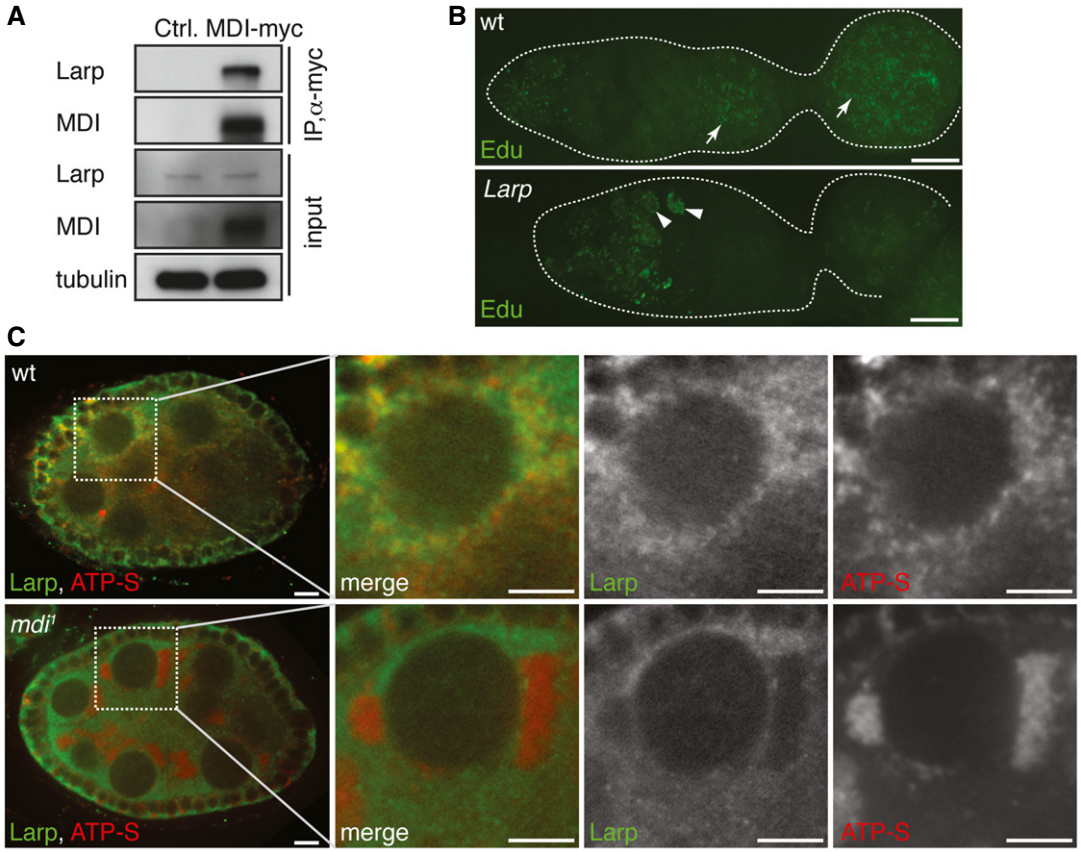

Figure 4. MDI recruits Larp to mitochondria.

A Co-immunoprecipitation of Larp with MDI-myc in transfected S2 cells. Tubulin was used as a loading control.

B mtDNA replication, as illustrated by EdU incorporation, in wt and *larp* mutant (*larp*) ovarioles. mtDNA replication is dramatically reduced in the *larp* ovariole. Arrows: mtDNA; arrowheads: nuclei.

C wt and *mdi*^{1} egg chambers stained for Larp (green) and ATP-S (red) to reveal mitochondria. Larp closely associates with mitochondria in wt egg chambers. Mitochondria in *mdi*^{1} flies are clumped together and completely lack Larp staining.

Data information: Scale bars in (B, C), 10 μm.

We thus visualized nascent protein synthesis by incorporation of a methionine analog homopropargylglycine (HpG) and subsequent fluorescence-click chemistry (Dieterich *et al*, 2010). After incubating dissected ovaries with HpG for 30 min, we observed strong HpG labeling in germaria and egg chambers of wild-type ovaries (Fig EV4A). Both ER and mitochondria are tightly associated with the fusome in the late germarium stages (Cox & Spradling, 2003; Snapp *et al*, 2004), which makes it difficult to clearly distinguish ER labeling from mitochondrial labeling. By contrast, mitochondria and ER are distinct from each other except for a few contacting sites, in the mid-stage egg chambers (Fig 5B). We thus focused on the mid-stage egg chambers to assess the potential association of protein synthesis with mitochondria.

In mid-stage egg chambers, strong HpG signal was detected in the perinuclear region where rough ER, the main site of cytoplasmic protein synthesis, is located (Figs 5A and EV4A). There were also many HpG puncta closely associated with the mitochondrial marker Tom20-mCherry in the cytoplasm (Figs 5A and EV4A). HpG labeling associated with mitochondria could have resulted from the translation of mtDNA-encoded mRNAs inside the matrix or the translation of cytoplasmic RNAs at the mitochondrial surface. To distinguish these two possibilities, we treated the ovaries with two ribosomal inhibitors, chloramphenicol, or cycloheximide. Chloramphenicol inhibits mitochondrial ribosomes, but not cytosolic ribosomes (Millis & Suyama, 1972). Chloramphenicol treatment had little effect on HpG labeling in mid-stage egg chambers (Fig EV4B). In contrast, cycloheximide, which inhibits cytoplasmic ribosomes (Millis & Suyama, 1972), greatly abolished HpG labeling (Fig EV4C). These results demonstrate that the HpG puncta associated with mitochondria are mainly derived from proteins synthesized by cytosolic ribosomes on the mitochondrial surface.

MDI promotes the synthesis of specific proteins on the mitochondrial surface

mdi^{1} egg chambers also displayed strong HpG labeling in the perinuclear region and on many cytoplasmic clumps that perfectly overlapped with Tom20-mCherry (Fig 5A). Meanwhile, the ER structure appeared normal in *mdi*^{1} ovary (Fig 5B). These results further prove that protein synthesis by cytosolic ribosomes occurs on the mitochondrial surface. They also demonstrate that protein synthesis at the mitochondrial surface is still present in *mdi* mutants.

Since mitochondria clump together in *mdi*^{1} ovaries (Figs 5A and EV2), it is difficult to normalize and quantify the level of HpG incorporation from fluorescence microscopy images. We thus used Western blotting to achieve some quantification of *de novo* protein synthesis. We incubated the isolated ovaries for 4 h with another methionine analog, L-azidohomoalanine (AHA), which was subsequently labeled with biotin by click chemistry. The newly synthesized proteins can then be probed with an anti-biotin antibody on the blot. We first confirmed that most biotin-reactive bands on the blot were indeed proteins newly synthesized by cytosolic ribosomes, as co-incubation with cycloheximide greatly blocked the AHA incorporation (Fig EV5A). We next separated the mitochondrial fraction from cytosolic fraction by differential centrifugation. The AHA signal in the cytosolic fractions was comparable between wild-type and *mdi* mutant extracts (Fig EV5B), suggesting that the overall protein synthesis by cytoplasmic ribosomes was not affected in *mdi*^{1} ovary. However, the AHA signal associated with the mitochondria fraction was reduced in *mdi*^{1} ovary (Fig 6A), even though not all bands were affected. This observation suggests that MDI promotes the *de novo* synthesis of a subset of proteins.

Given MDI's impact on mtDNA replication, we expected that the proteins affected in *mdi* mutant would be involved in mtDNA replication. We checked the steady-state protein levels of mitochondrial DNA polymerase (Tamas) and TFAM on Western blots probed with antibodies against endogenous proteins (Matsuda *et al*, 2013; Zhang *et al*, 2015). For two other replication factors, mtSSB and mitochondrial RNA polymerase (mtRNAP), against which there are no effective antibodies, we generated GFP fusion proteins by inserting GFP reporter into the genomic loci. We then crossed these transgenes into *mdi* mutant background and probed mtSSB-GFP and mtRNAP-GFP using a GFP antibody. We found that the amounts of both TFAM and Tamas were markedly reduced in *mdi*^{1} compared to wild-type ovaries (Fig 6B), while those of mtSSB-GFP and mtRNAP-GFP remained unchanged (Fig EV5B).

To test whether the reduced steady-state protein levels are truly caused by reduced protein synthesis, we performed polysome profiling on several candidates. We prepared ribosomal fractions by gradient sedimentation of ovary extracts and quantified the relative abundance of mRNA in each ribosomal fraction by real-time PCR analyses. Overall, only a small fraction of ribosomes were assembled into polysome complexes (Fig 6D). This is consistent with a previous work showing a lack of protein translation in mature oocytes (Kronja *et al*, 2014). Additionally, the ribosome peaks in wt and *mdi*^{1} ovaries overlapped almost perfectly (Fig 6D), suggesting that overall translational activity is not impaired in *mdi*^{1}. Of primary significance, the levels of *tfam* and *tamas* mRNAs in polysome fractions were significantly reduced in *mdi*^{1} compared to wild-type extracts (Fig 6E), suggesting a reduced translation of these specific mRNAs in *mdi*^{1} ovary.

To further confirm that *de novo* synthesis of these proteins was reduced, we directly assessed the AHA incorporation after metabolic labeling. Because the endogenous antibodies of TFAM and Tamas did not work effectively for immunopurification, we generated BAC clone transgenes expressing TFAM-GFP and Tamas-GFP in both wild type and *mdi* mutant. We purified these GFP fusion proteins using a GFP antibody from AHA-labeled ovaries and probed with an anti-biotin antibody to visualize the newly synthesized proteins. We found that AHA labeling on both Tamas and TFAM was markedly reduced in *mdi* mutant (Fig 6C), which reflects the reduced synthesis and/or import of these two proteins.

The MDI-Larp complex promotes mitochondrial biogenesis

To systematically identify the targets of the MDI-Larp complex, we compared the proteomes of mature eggs produced by wild-type and *mdi*^{1} mothers using quantitative mass-spectrum analysis. Of 2,182 proteins detected in both extracts, 406 were nuclear-encoded mitochondrial proteins (Tables EV2, EV3 and EV4). There were a total of 65 proteins reduced more than twofold in *mdi*^{1} compared to wild-type eggs. Among these, 64 proteins were nuclear-encoded mitochondrial proteins including 21 mitochondrial ribosomal proteins and 23 electron-transport chain (ETC) subunits (Table EV3). To validate the proteomics results, we probed two

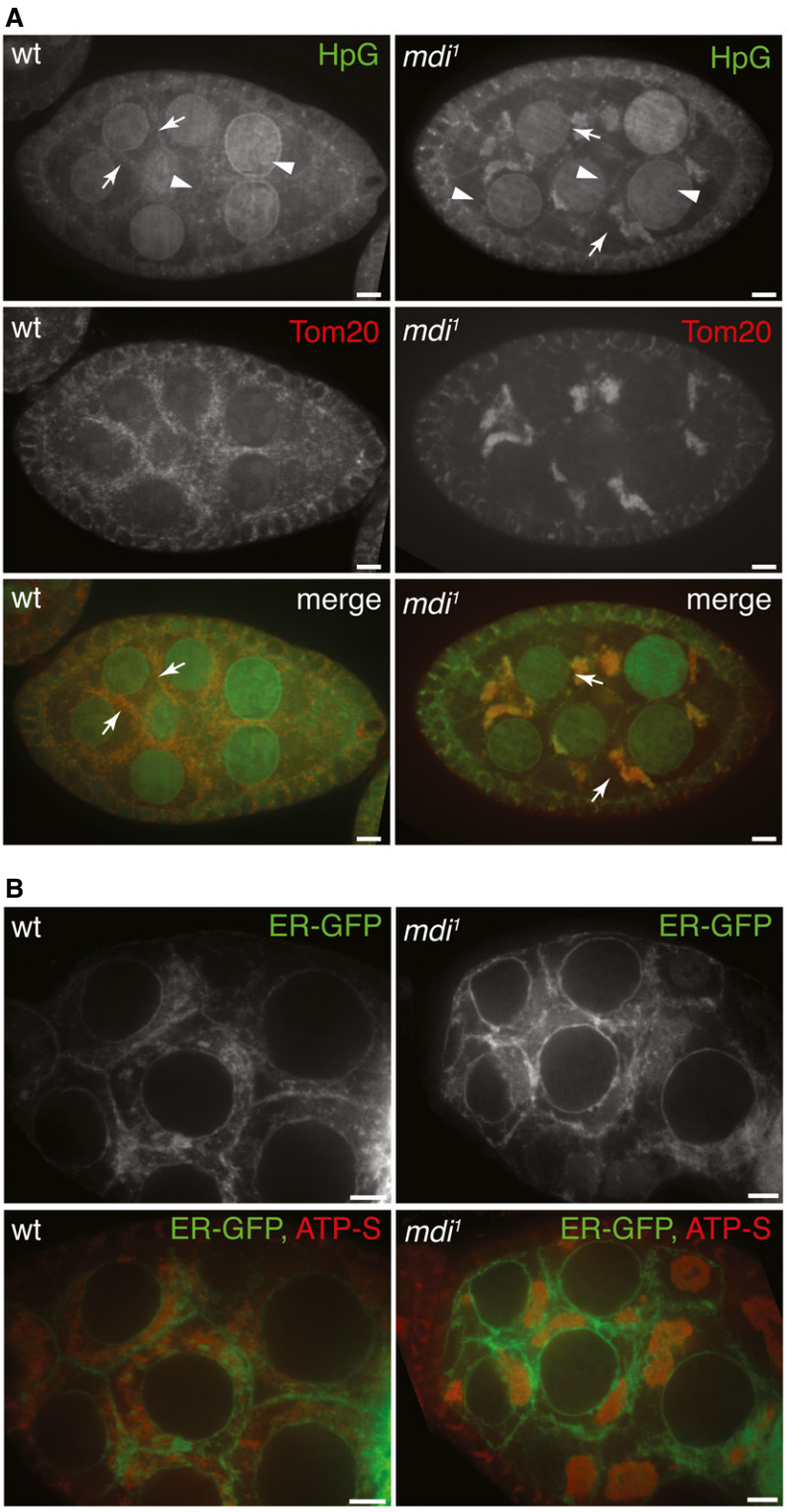

Figure 5. Protein synthesis on the mitochondrial surface.

A Nascent protein synthesis revealed by HpG incorporation (green) in wt and *mdi*^{1} egg chambers. Arrowheads point to the HpG signal on the ER in the perinuclear region and cell periphery. Arrows point to the HpG signal associated with mitochondria. Mitochondria are marked by a Tom20-mCherry (red) created by inserting mCherry at the endogenous *Tom20* locus.

B ER location is unaltered in *mdi*^{1} egg chambers. An ER marker (ER-GFP) was expressed in wt and *mdi*^{1} egg chambers that were co-stained with ATP-S to mark mitochondria. ER localizes to the perinuclear region, cytoplasm, and cell periphery in both wt and *mdi*^{1} egg chambers.

Data information: Scale bars in (A, B), 10 μm.

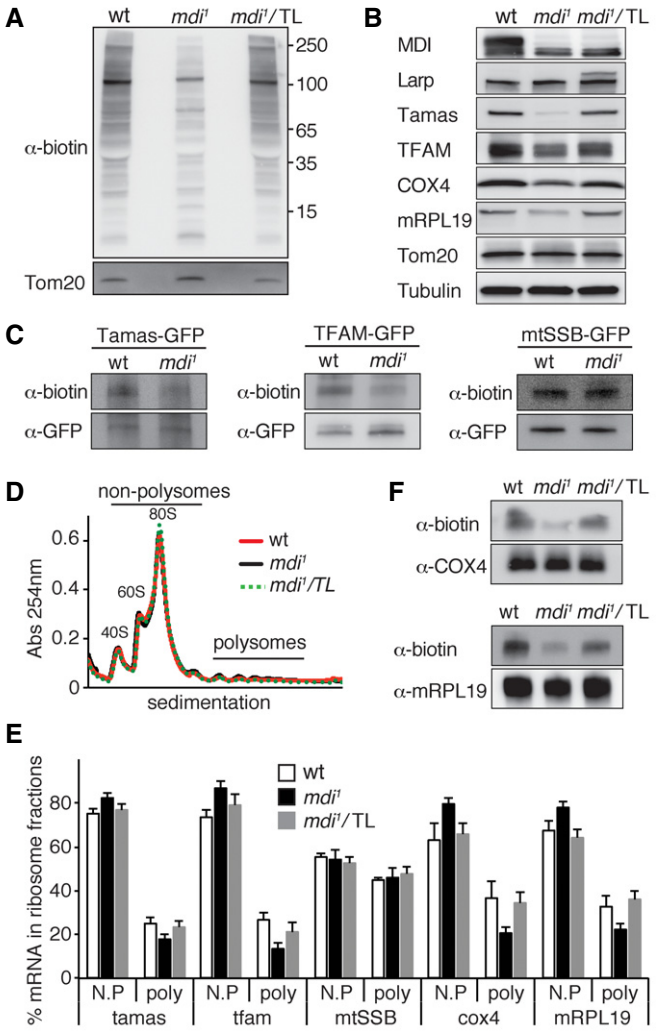

Figure 6. MDI-Larp complex promotes the *de novo* synthesis or import of a subset of nuclear-encoded mitochondrial proteins.

A Detection of nascent protein synthesis in the mitochondrial fraction of the ovary. Nascent protein synthesis was monitored by AHA incorporation and detected by anti-biotin antibody. Tom20 served as a loading control. Note that the synthesis of nuclear-encoded mitochondrial proteins was decreased in the mdi^1 background and was restored by overexpressing Tom20-Larp (mdi^1/TL).

B Western blots of mitochondrial proteins in ovarioles of wt, mdi^1, and mdi^1 flies expressing Tom20-Larp (mdi^1/TL) fusion protein. Note that levels of Tamas, TFAM, mRpL19, and COX4 were reduced in mdi^1 flies, but restored in mdi^1/TL flies.

C Nascent protein synthesis of Tamas and TFAM was decreased in mdi^1 ovary, whereas mtSSB was not affected. Tfamgfp, Tamasgfp, and mtSSBgfp were expressed in wt or mdi^1 background. Nascent proteins were labeled by AHA incorporation, and then, the GFP-tagged protein was immunopurified with a GFP antibody and the nascent protein synthesis was detected by anti-biotin antibody.

D Representative profile of 254 nm absorbance of wt, mdi^1, and mdi^1/TL ovary extracts.

E Polysome mRNA profiling for *tamas, tfam, mtSSB, cox4,* and *mRPL19* in wt, mdi^1, and mdi^1/TL ovary. The percentage of mRNA for each gene in non-polysomal fractions (N.P, including ribosomal subunits and monosome-associated) and polysomal fractions (poly) was calculated and plotted. The fractions of *tamas, tfam, cox4,* and *mRPL19* mRNAs in the polysomal fractions were significantly decreased in mdi^1 compared to wt, but were restored in mdi^1/TL flies. $N = 4$ for all samples. *P*-values of comparing wt to mdi^1: *tamas*, $P = 0.0055$; *tfam*, $P = 0.001$; *cox4*, $P = 0.0066$; *mRPL19*, $P = 0.0097$. *P*-values of comparing mdi^1/TL to mdi^1: *tamas*, $P = 0.0206$; *tfam*, $P = 0.0346$; *cox4*, $P = 0.0036$; *mRPL19*, $P = 0.0016$.

F Nascent protein synthesis of COX4 and mRPL19 was decreased in mdi^1 ovary, but restored by overexpressing Tom20-Larp (mdi^1/TL). The proteins were immunopurified with antibodies against the endogenous proteins.

candidates, a mitochondrial ribosomal protein, mRPL19, and cytochrome C oxidase subunit 4 (COX4), by Western blot. Indeed, the abundance of both proteins was decreased in mdi^1 compared to wild-type eggs (Fig 6B).

mtDNA encodes the 16S and 12S rRNAs of the mitochondrial ribosomes and 13 core components of the ETC complexes. Given the reduced amount of mtDNA in mdi^1 ovary and embryo, the reduced steady-state amounts of nuclear-encoded mitochondrial ribosomal proteins and ETC subunits could result from a lack of mtDNA-encoded partners to assemble full complexes, or reduced *de novo* synthesis. To distinguish between these possibilities, we performed the same ribosome profiling on these two genes as described above. We found that *cox4* and *mRPL19* mRNAs were less abundant in the polysome fractions of mdi^1 ovaries than of wild-type ovaries (Fig 6E). Furthermore, AHA labeling confirmed that newly synthesized COX4 and mRPL19 proteins were both markedly reduced in mutant ovaries (Fig 6F).

We next examined whether Larp mediated MDI's role in mitochondrial protein synthesis. A Tom20-Larp fusion protein that constitutively targeted Larp to the mitochondria outer membrane independently of MDI (Figs 7A and EV3B) restored the steady-state levels and the *de novo* protein synthesis of Tamas, TFAM, mRPL19, and COX4 (Figs 6B, C and F) in mdi^1 ovary. Tom20-Larp also rescued the mtDNA level and the hatching rate of mdi^1 eggs (Fig 7B). These results suggest that Larp mediates most, and perhaps all, of MDI's roles in mtDNA replication and mitochondrial biogenesis in the ovary.

Discussion

Here, we demonstrate that MDI-Larp complex on mitochondrial surface promotes the translation of a subset of nuclear-encoded mitochondrial proteins and is required for mtDNA replication and mitochondrial biogenesis in *Drosophila* ovaries. Our evidence that MDI and Larp work as a complex is multifold: They have similar loss-of-function phenotypes, they interact physically, and mitochondrially targeted Larp overexpression can rescue most of the *mdi* mutant phenotypes that we tested. However, the mechanisms by which they promote the translation of a subset of mitochondrial proteins remain puzzling. Although MDI contains an RNA-binding KH domain, it is dispensable for MDI's function and thus unlikely to contribute to the specificity of MDI-Larp complex (Fig 3A). The Larp homolog in yeast binds to a subset of cytosolic mRNAs including many nuclear-encoded mitochondrial proteins (Schenk *et al*, 2012; Kershaw *et al*, 2015). This suggests that Larp might dictate the specificity of the MDI-Larp complex by binding to a subset of mRNAs and promoting their translation. We also note that the list of MDI-Larp's targets derived from our proteomic analyses appears incomplete. Many mitochondrial proteins, including most mtDNA replication factors, were not recovered, presumably because their

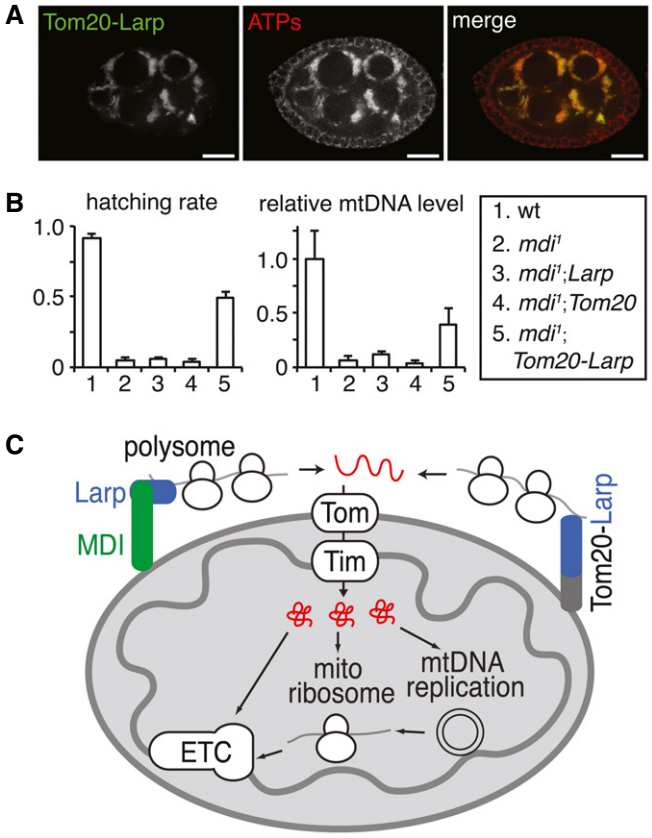

Figure 7. Targeting Larp to the mitochondrial surface partially rescues the *mdi*[1] phenotype.

A Mitochondrial localization of a Tom20-LarpGFP (Tom20-Larp) fusion protein in an *mdi*[1] egg chamber stained with ATP-S to reveal mitochondria. Scale bars, 20 μm.

B Hatching rates and mtDNA contents of eggs produced by flies with different genotypes relative to wt control; each data point represents the mean of three independent replicates. Error bars represent SD. Expression of Tom20-Larp in *mdi*[1] (*mdi*[1]; *Tom20-Larp*) significantly restored the mtDNA level and hatching rate. $N = 3 \times >100$ eggs/genotype for hatching rate. The relative mtDNA level was the average of three biological repeats. *P*-values of comparing *mdi*[1]; *Tom20-Larp* to *mdi*[1]: for mtDNA level, $P = 0.0209$; for hatching rate, $P = 3.4758E^{-05}$.

C Model of the role of MDI and Larp in mitochondrial biogenesis. MDI (green rectangle) localizes at the mitochondrial outer membrane and recruits Larp (blue rectangle) to the mitochondrial surface. Larp interacts with polysome (blobs) and translation stimulators and stimulates the translation of a subset of nuclear-encoded mRNAs. Proteins synthesized on the mitochondrial surface are in close vicinity of the Tom–Tim mitochondrial transporter complexes, which would facilitate their rapid transport into the matrix. The targets of MDI-Larp complexes include most nuclear-encoded ETC subunits, mitochondrial ribosomal proteins, TFAM, and mtDNA polymerase (Tamas) (collectively represented in red). Mitochondrial ribosomes are required for the biogenesis of proteins encoded by mtDNA, all of which are ETC subunits. Thus, MDI-Larp complex seem to coordinate the expression of both nuclear and mitochondrial genome to promote the biogenesis of ETC complexes.

low abundance makes them difficult to detect in our proteomics analyses. Nonetheless, our work demonstrates that the MDI-Larp complex promotes the biogenesis of a subset of nuclear-encoded mitochondria proteins including ETC subunits, mitochondrial ribosomal proteins, and mtDNA replication factors.

The mitochondrial ribosomes are responsible for synthesizing the ETC subunits encoded on the mtDNA. Thus, MDI-Larp participates in ETC biogenesis by both promoting mtDNA replication and coordinating the expression of the nuclear- and mitochondria-encoded components of the ETC complexes. In *mdi*[1] ovary, reduced biogenesis of ETC should render mitochondria less active, which, together with the reduced supply of mitochondrial DNA replication factors, would impair mtDNA replication (Fig 7C). We recognize that the full spectrum of MDI-Larp's impact on mitochondrial biogenesis remains to be explored. Nonetheless, the fact that a mitochondrially targeted Larp fusion protein (Tom20-Larp) can restore local protein synthesis and mtDNA levels in *mdi*[1] ovaries suggests that local protein synthesis on the mitochondrial surface is likely essential to drive mitochondrial DNA replication and biogenesis during oogenesis.

Our data suggest that post-transcriptional regulation of gene expression is the major driver of massive mitochondrial biogenesis in the late stages of oogenesis.

Massive mtDNA replication demands vast amounts of replication factors (Moraes, 2001). In fact, several of these factors were found to be more abundant in the female germ line than in somatic tissues (Fig EV3C). However, the mRNA levels of mtDNA replication factor are either unchanged, or only slightly increased in the *Drosophila* ovary relative to somatic tissues (Chintapalli *et al*, 2007). Thus, the boost in mitochondrial biogenesis in the ovary must rely primarily on post-transcriptional mechanisms. Moreover, this post-transcriptional regulation appears localized to the mitochondrial surface. Localized protein synthesis within a cell has been demonstrated in various biological systems (reviewed in Lesnik *et al*, 2015). The local translation of specific mRNAs may help to compartmentalize proteins at their active sites (Besse & Ephrussi, 2008), provide fast responses to local needs, and enable protein synthesis under repressive conditions (reviewed in Lesnik *et al*, 2015). In *Drosophila* oogenesis, translational control is prevalent during oogenesis and confines the temporal and spatial pattern of various developmental factors (reviewed in Lasko, 2012). Several key factors of germ cell development, including Nanos, are translational inhibitors. Interestingly, there is massive mitochondrial proliferation in a *nanos* mutant ovary (Bhat, 1999), implying an inhibitory role of Nanos on mitochondrial biogenesis. It is possible that local protein synthesis on mitochondrial surface mediated by MDI-Larp might relieve the translational inhibition by Nanos.

Protein synthesis on the mitochondrial outer membrane has been proposed as an effective way to couple translation and import of nuclear-encoded mitochondrial proteins (reviewed in Fox, 2012). Such coupling might be particularly crucial for the rapid biogenesis of mitochondria during oogenesis and might be another important function of the MDI-Larp complex. Many mRNAs encoding mitochondrial proteins are localized to the mitochondrial surface (Marc *et al*, 2002; Sylvestre *et al*, 2003), and cytoplasmic ribosomes have been known to associate with mitochondria for a long time (Kellems *et al*, 1974). Recent work on proximity-specific ribosome profiling identified over 100 mitochondrial proteins that are translated at the vicinity of the outer mitochondrial membrane (Williams *et al*, 2014). In animal cells, mRNAs encoding subunits of the ETC are recruited to the mitochondrial surface through a PINK1/Parkin-regulated process (Gehrke *et al*, 2015). PINK1 is also suggested to promote the translation of these mRNAs. Despite increased

evidences demonstrating the association of mRNAs with mitochondria and its proposed implication in co-translational import, direct evidence for protein synthesis at the mitochondrial surface has so far been lacking. Moreover, the physiological significance of coupled translation–import has yet to be explored. Our experiments applying metabolic labeling to visualize protein synthesis demonstrate that protein synthesis does in fact take place on the mitochondrial surface. The locally synthesized proteins including mtDNA replication factors would be perfectly poised for efficient translocation into the mitochondria to drive massive mitochondrial biogenesis (Fig 7C).

Aside from its association with Larp, MDI appears to interact with other proteins that may mediate other functions. For instance, we found that MDI associates with many proteins involved in ribosomal biogenesis or translational control (Table EV1). MDI appears to be a scaffold protein that recruits ribosomes and translation regulators to the mitochondrial surface. We found that Bor, the *Drosophila* ATAD3 protein, also co-purified with MDI (Table EV1). ATAD3, a nucleoid protein, is thought to tether mtDNA to cholesterol-rich membrane structures at the ER–mitochondria contacting sites in mammalian cells (Gerhold *et al*, 2015). Interestingly, two outer membrane proteins, Mmm1-p and Mmm2-p, are required for mtDNA maintenance in yeast (Hobbs *et al*, 2001; Youngman *et al*, 2004). Mmm1-p associates with the nucleoid protein Mgm101P and forms two-membrane spanning structure where actively replicating nucleoids localize (Meeusen & Nunnari, 2003). It is an intriguing idea that MDI may also complex with ATAD3 or other unidentified nucleoid proteins and interact with mtDNA directly. However, loss of MDI impairs mtDNA replication in ovary specifically, and this impairment can be rescued by mitochondrially targeted Larp (Tom20-Larp). Thus, the physiological significance of the potential association between MDI and nucleoid proteins remains to be explored, even if the potential association was confirmed.

MDI belongs to a family of AKAP proteins that are highly conserved among metazoans (reviewed in Wong & Scott, 2004). Some AKAPs function as scaffold to tether PKA and its downstream effectors on the mitochondrial outer membrane, regulating diverse mitochondrial processes including mitochondrial protein import (Schmidt *et al*, 2011), mitochondrial fission, and apoptosis (Cribbs & Strack, 2007). However, PKA is not required for mtDNA replication in the *Drosophila* ovary, arguing against the idea that MDI regulates mtDNA replication via a cAMP-PKA pathway. AKAP1, the mammalian homolog of MDI, also localizes to the mitochondria. Interestingly, AKAP1 knockout mice are female semi-sterile (Newhall *et al*, 2006), which is similar to the phenotype of *mdi* mutant flies. Interaction between PKA and AKAP1 is essential for maintaining meiotic arrest of developing oocytes. However, whether AKAP1 is required for mtDNA replication and mitochondrial biogenesis in mammals has not been examined. In the final stages of mammalian oogenesis prior to fertilization, mtDNA undergoes a burst of replication that significantly increases mtDNA copy number (reviewed in St John, 2012). This massive replication would demand a large amount of mtDNA replication factors, which, in mammals like in flies, are encoded in the nucleus. Interestingly, ectopic expression of human AKAP1 rescues the fertility of mdi^1 female flies (Fig 2F). Thus, the post-transcriptional regulation on mitochondrial biogenesis by MDI/AKAP1 likely represents an evolutionarily conserved mechanism that may be crucial for mitochondria inheritance across species, even though the details of oogenesis differ between mammals and flies.

Materials and Methods

Fly genetics and husbandry

All flies were maintained on cornmeal medium at 25°C. w^{1118} was used as the wild-type control. MDI-GFP and Tom20-mCherry were constructed by inserting GFP or mCherry cDNA into endogenous loci by CRISPR/Cas9-mediated recombination. Tfam-gfp, tamas-gfp, mtSSB-gfp, and mtRNApol-gfp reporter lines were generated by integrating BAC clones carrying gfp cDNA to landing sites of VK37(2L) 22A3 (tfam and mtSSB) or VK31(3L)62E1(tamas and mtRNApol) using phi-C31-mediated transgenesis (Venken *et al*, 2009). The $larp^{mtr\text{-}null}$ and PKA^{E95} were described previously (Blagden *et al*, 2009; Xia *et al*, 2012). Fecundity test and the embryo-hatching test were carried out as previously described (Von Stetina *et al*, 2011; Chen & Wagner, 2012).

CRISPR/Cas9 in flies and cells

To generate *mdi* deletion, two chiRNA plasmids containing targeting sequences GAGGTAGAGTAGAGGACGAC and GCTAGTTGAGTTGT TCACTA were injected into *PBac{y[+mDint2]=vas-Cas9}VK00027* embryos. The genomic DNA of G1 adults flies was prepared and screened for deletion by PCR using oligos: AACGCATAACCCAGCT GATCCCTA; GCGAAGTTGTTGTGCCCTTATCTTAC.

To insert GFP or mCherry into the endogenous loci of *mdi* and *Tom20*, respectively, chiRNA plasmids containing targeting sequences GCTAGTTGAGTTGTTCACTA (*mdi*) or GTCCGGCTAGA ACATGGCAT (*Tom20*) and a donor plasmid were injected into the embryos of *PBac{y[+mDint2]=vas-Cas9}VK00027* (*mdi)*, or *M{vas-Cas9}ZH2A (Tom20)*. Donor plasmids contain a 1-kb upstream and a 1-kb downstream fragment flanking stop codons of target genes in POT2 vectors. The GFP or mCherry was inserted in front of stop codons. G1 adults were screened for insertion events by PCR using primers for *mdi*: GATTTCATGAATGTGCCCTTCCA, TTACTT GTACAGCTCGTCCATG; and *Tom20:* CTTATTGCCTCAGGCATCTA, TTACTTGTACAGCTCGTCCATG.

To generate *mdi* knockout cell line, S2 cells were transfected with an *mdi* chiRNA plasmid containing targeting sequence GAG GTAGAGTAGAGGACGAC, a Cas9 expression plasmid (Addgene #42230) and pCoBlast (Invitrogen). The cells were seeded to 96-well plate 36 h after the transfection and selected with blasticidin (100 μg/ml) for 7 days. The knockout clones were screened using Western blot for the loss of MDI protein.

Molecular biology

BAC clones carrying gfp reporters in the genomic loci were constructed by recombineering (Venken *et al*, 2009). cDNAs or gene fragments of *mdi*, *mdi* truncations, *hAKAP1*, *Tom20,* and *Tom20-Larp* were cloned into pA-myc, pA-GFP, pA-mCherry, and pUASp-GFP expression vectors using *Drosophila* Gateway Cloning system (T. Murphy laboratory, Carnegie Institute of Washington).

Quantitative real-time PCR analysis of mtDNA level was performed as previously described (Zhang *et al*, 2015).

Immunohistochemistry

EdU incorporation was preformed as previously described (Hill *et al*, 2014). For detecting nascent protein synthesis, ovaries (5–10 pairs) from 4- to 5-day-old female flies were dissected in methionine-free media (MFM) and washed three times with MFM. The ovaries were equilibrated in MFM for 45 min and then incubated with or without 20 μM of chloramphenicol or cycloheximide in MFM for 30 min. The ovaries were then incubated in MFM containing 50 μM HPG with or without 20 μM chloramphenicol or cycloheximide for 30 min. Click-iT HpG labeling was performed according to the manufacturer's instructions (Life Technology). All images were collected on a Perkin Elmer Ultraview system and processed with Volocity software. Antibodies used in this study were as follows: mouse α-ATP synthase subunit α (Abcam, 15H4C4, 1:1,000), rabbit α-Larp (provided by David Glover, 1:500), Alexa Fluor 488 goat α-rabbit IgG (Invitrogen, 1:200), Alexa Fluor 568 goat α-mouse IgG (Invitrogen, 1:200).

Biochemistry

Primary antibodies used for Western blot in this study were as follows: α-Cox4 (ab16056, Abcam), α-β tubulin (E7, DHSB), α-actin (C4, Millipore), α-SOD2 (NB100-1992, Novus Biologicals), α-ATP synthase 5α (15H4C4, Abcam), α-Tom20 (#13929, CST), α-biotin (#7075, CST), α-mRPL19 (PA5-31240, Life Technologies), α-cytochrome c (7H8.2C12, Novus Biologicals), α-GFP (11814460001, Roche), α-Tamas (Zhang *et al*, 2015), and α-TFAM (Matsuda *et al*, 2013). A rabbit polyclonal α-MDI antibody was raised against a GST-tagged MDI truncation containing residues 100–585.

Mitochondrial isolation was performed as previously described (Zhang *et al*, 2015). For protease protection assay, intact mitochondria (200 μg of protein) were resuspended in 20 mM HEPES-KOH, pH 7.4, 0.6 M sorbitol. Swollen mitochondria were prepared by incubating mitochondria (200 μg of protein) in 20 mM HEPES-KOH, pH 7.4 for 10 min. Protease K (100 μg/ml) was added to mitochondria preparation and kept on ice for 20 min in protease K treatment experiments. The reaction was stopped by the addition of 2 mM phenylmethylsulfonyl fluoride. Mitochondria were collected by centrifugation at 16,000 *g* for 5 min at 4°C and followed by SDS–PAGE and Western blot analyses.

To identify MDI interacting proteins, cells expressing MDI-myc were lysed in 150 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl (pH 8.0) and incubated on ice for 30 min with occasional mixing. Cell lysates were centrifuged for 10 min at 10,000 *g* at 4°C. Supernatants were collected and incubated with 50 μl α-Tag MicroBeads (Miltenyi Biotec) for 2 h at 4°C. The beads were washed four times with 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 8.0), and one time with 20 mM Tris–HCl (pH 7.5). The proteins were eluted with 50 mM Tris–HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromphenol blue, 10% glycerol and subjected to SDS–PAGE. Coomassie-stained bands were excised from the gel, destained with acetonitrile, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin overnight. For embryonic mass spectrometry, triplicates of wt and mutant embryo lysates were sequentially reduced, alkylated, digested overnight with trypsin, and labeled with 6-plex Tandem Mass Tag (TMT) reagents (Thermo Fisher Scientific; Dayon *et al*, 2008). Six labeled protein digests were pooled and then separated into 12 fractions using high-pH reverse-phase liquid chromatography (Wang *et al*, 2011). All fractions and protein digests were analyzed using a nanoLCMS system equipped with an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptide and protein IDs were assigned by searching LCMS raw data against Uniprot Drome database (http://www.uniprot.org) using Sequest HT algorithm on Proteome Discoverer 1.4 platform (Thermo Fisher Scientific). The results were compiled and quantitatively compared using Scaffold 4.0 software (Proteome Software, Inc, Portland OR). The relative protein abundance in corresponding bands from IP pull-down samples was quantified using spectral counting method. TMT-labeled samples were normalized using the total reported ion intensities of their corresponding channels, and then, individual proteins were compared using the normalized report ion intensities.

Polysomal profiling

The polysome profiling was performed as previously described (Baird *et al*, 2014). Briefly, sucrose gradients ranging from 10 to 50% in 10 mM Tris–HCl (pH 7.5), 75 mM KCl, 1.5 mM $MgCl_2$, and 50 μg/ml cycloheximide were prepared with a tilted tube rotation method on a gradient station (BioComp). Fifty pairs of ovaries were dissected in PBS containing 100 μg/ml cycloheximide and then incubated with 100 μg/ml cycloheximide in PBS for 10 min on ice. The ovaries were homogenized in 10 mM Tris–HCl, pH 7.5, 75 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 1% deoxycholate, 2% Tween-20, 100 μg/ml cycloheximide, 1 mg/ml heparin, and 50 units/ml RNasin and incubated on ice for 10 min. The debris were removed by centrifugation at 12,000 *g* for 10 min at 4°C, and supernatants were loaded onto 10–50% sucrose gradients and subjected to centrifugation in a Beckman SW41Ti rotor at 200,000 *g* for 2 h at 4°C. Sucrose fractions and the resulting polysome profiles for each sample were then collected using a Piston Gradient Fractionator and a 254-nm ultraviolet monitor with Data Quest software. Samples were then immediately mixed with 750 μl of TRIzol Reagent LS. About 5 ng/ml firefly luciferase control RNA (Promega) was added to each pooled sample before RNA isolation, allowing for normalizing the transcript of interest to an exogenous RNA control. RNA isolation and quantitative RT–PCR were performed as described previously (Zhang *et al*, 2015). Oligonucleotides used for qPCR: *Cox4*: GGGCGTTTCACTCCTCTTC, GTGCTCCTCATCGAAGG TAAC; *mRPL19*: TTGTGACCTTCTCCACCAAA, GGAATGATTGTCT TCCGGTT; *Tfam*: CTCCGAGAAGGAGGTCTACAT, GGATCATCTTC TCCTCCCAAAC; *Tamas*: CCCTGCTCCGTCAGTTTAAT, CTCCTCTC GCAATCGATACAC; *mtSSB*: TGCTACACACACCAACTACAA, CGCT GTCCCTTCTTCAAGTAT; firefly luciferase: ATCCGGAAGCGACCA ACGCC, GTCGGGAAGACCTGCCACGC.

Detection of nascent protein synthesis by Western blot

AHA labeling of nascent protein synthesis in ovaries was carried out using the same protocol as HpG labeling described above, except that HpG was replaced with AHA in the media and incubated for

4 h. After labeling, ovaries were homogenized in 20 mM HEPES-KOH, pH 7.4, 0.25 M sucrose, and then centrifuged at 150 *g* for 10 min at 4°C to remove tissue debris. Supernatants were centrifuged at 9,000 *g* for 15 min at 4°C to separate mitochondrial pellets from soluble cytosolic fractions. The mitochondrial fraction was resuspended in 1% SDS in 50 mM Tris–HCl (pH 8.0). The cytosolic fraction was first precipitated by methanol and then solubilized in 1% SDS, 50 mM Tris–HCl (pH 8.0). To pull down a specific protein, the ovaries were homogenized in 150 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl (pH 8.0), incubated with a specific antibody conjugated with magnetic beads for 2 h, and then washed with 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 8.0). The proteins were eluted with 1% SDS in 50 mM Tris–HCl (pH 8.0). AHA was labeled with biotin according to the manufacturer's instructions (Life Technology) and probed with α–biotin (#7075, CST).

Statistical analysis

Error bars represent standard deviations in all the charts. Data were analyzed using two-tailed Student's *t*-test. The difference was considered statistically significant when $P < 0.05$.

Expanded View for this article is available online.

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Author contributions
HX and YZ conceived the idea and designed the research; YZ and YC performed the experiments; YZ, YC, MG, and HX analyzed data; HX and YZ wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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