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The ABC Transporter Eato Promotes Cell Clearance in the Drosophila melanogaster Ovary

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ABSTRACT The clearance of dead cells is a fundamental process in the maintenance of tissue homeostasis. Genetic studies in Drosophila melanogaster, Caenorhabditis elegans, and mammals have identified two evolutionarily conserved signaling pathways that act redundantly to regulate this engulfment process: the ced-1/-6/-7 and ced-2/-5/-12 pathways. Of these engulfment genes, only the ced-7/ABCA1 ortholog remains to be identified in D. melanogaster. Homology searches have revealed a family of putative ced-7/ABCA1 homologs encoding ATP-binding cassette (ABC) transporters in D. melanogaster. To determine which of these genes functions similarly to ced-7/ABCA1, we analyzed mutants for engulfment phenotypes in oogenesis, during which nurse cells (NCs) in each egg chamber undergo programmed cell death (PCD) and are removed by neighboring phagocytic follicle cells (FCs). Our genetic analyses indicate that one of the ABC transporter genes, which we have named Eato (Engulfment ABC Transporter in the ovary), is required for NC clearance in the ovary and acts in the same pathways as drpr, the ced-1 ortholog, and in parallel to Ced-12 in the FCs. Additionally, we show that Eato acts in the FCs to promote accumulation of the transmembrane receptor Drpr, and promote membrane extensions around the NCs for their clearance. Since ABCA class transporters, such as CED-7 and ABCA1, are known to be involved in lipid trafficking, we propose that Eato acts to transport membrane material to the growing phagocytic cup for cell corpse clearance. Our work presented here identifies Eato as the ced-7/ABCA1 ortholog in D. melanogaster, and demonstrates a role for Eato in Drpr accumulation and phagocytic membrane extensions during NC clearance in the ovary.

KEYWORDS cell death engulfment ABC transporter ced-7 Eato

PCD is a fundamental biological process in animal development and tissue homeostasis. Cells undergoing PCD are selectively cleared by phagocytes in a multi-step engulfment process involving recognition followed by internalization of the dying cell (Arandjelovic and Ravichandran 2015; Green et al. 2016). In some instances, phagocytes can promote the death of their target cells (Reddien et al. 2001; Brown and Neher 2012; Timmons et al. 2016). Abnormal regulation of the engulfment process has been implicated in several human diseases, including developmental malformations, physiological disorders, autoimmunity, neurodegeneration, and cancer (Arandjelovic and Ravichandran 2015; Green et al. 2016).

Engulfment is generally performed by “professional” phagocytes, such as mammalian macrophages, whose primary function is the phagocytosis of cellular debris. In tissues where professional phagocytes have little to no access, resident cells can function as “nonprofessional” phagocytes to remove dead cells (Arandjelovic and Ravichandran 2015; Green et al. 2016). For example, in the Drosophila ovary, a system closed to circulating cells, clearance of dying NCs is accomplished by neighboring epithelial cells called FCs (Giorgi and Deri 1976; Etchegaray et al. 2012). Current evidence suggests that engulfment by professional and nonprofessional phagocytes is regulated similarly (Arandjelovic and Ravichandran 2015; Green et al. 2016). Extensive genetic studies in Caenorhabditis elegans have identified two parallel but partially redundant signaling pathways, CED-1/-6/-7.
and CED-2/5/-12, which regulate the engulfment process (Ellis et al. 1991; Kinchen et al. 2005). These pathways appear to be conserved in mammals as MEGF10/GULP/ABCA1 and Crk/DOCK180/ELMO, and in Drosophila melanogaster as Drpr/Ced-6 and Crk/Myoblast city/Ced-12, respectively (Mangahas and Zhou 2005). However, the D. melanogaster ortholog for CED-7/ABCA1 has not been identified.

The genes ced-7 and ABCA1 encode members of the ABCA subfamily of ABC transporters (Luciani and Chiminil 1996; Wu and Horvitz 1998). ABC transporters are important in a wide range of physiological processes and can translocate a variety of substrates, including sugars, ions, lipids, and proteins (Rees et al. 2009; ter Beek et al. 2014; Wilkens 2015). Mutations that abolish the ATP-binding function of CED-7 or ABCA1 cause engulfment defects that lead to the accumulation of cell corpses in vivo (Luciani and Chiminil 1996; Wu and Horvitz 1998; Hamon et al. 2000).

In C. elegans, CED-7 has been shown to be required in both the phagocytic and the dying cell for efficient engulfment (Wu and Horvitz 1998). In mammals, ABCA1 is clearly required in phagocytic cells (Hamon et al. 2000), but whether the protein is required in dying cells in vivo has not been determined. In vitro studies in mouse cell culture have demonstrated a role for ABCA1 in phosphatidylserine (PtdSer) exposure following apoptotic stimuli (Hamon et al. 2000), suggesting that ABCA1 may act in dying cells to promote cell corpse recognition. In contrast, PtdSer was clearly detected on the surface of cell corpses in vivo in ced-7 mutants (Mapes et al. 2012), indicating that CED-7 is not required for PtdSer exposure in C. elegans.

Multiple reports have speculated whether CED-7/ABCA1 acts as a lipid transporter. Indeed, most ABCA-type transporters appear to be involved in lipid trafficking (Vassiliou et al. 2009; Quazi and Molday 2011). In mammals, ABCA1 has been shown to promote the export of lipids from the Golgi to the plasma membrane, and the efflux of lipids to form high-density lipoproteins (HDLs) (Hamon et al. 2000; Orsó et al. 2000). In humans, deficiency for ABCA1 is implicated in Tangier disease, a recessive disorder of lipid metabolism characterized by the lack of HDLs due to defective translocation of membrane lipids (Hamon et al. 2000; Orsó et al. 2000; Vassiliou et al. 2009).

In C. elegans, CED-7 has been shown to play a role in both intracellular and extracellular lipid trafficking during engulfment. CED-7 was shown to act with CED-1, CED-6, and DYN-1 to promote the intracellular delivery of vesicles to the phagocytic cup, presumably to provide lipid and protein materials to the growing membrane for pseudopod extensions (Yu et al. 2006). CED-7 has also been shown to be required for the presence of extracellular vesicles and is proposed to mediate the exocytosis of vesicles containing engulfment signals, such as the bridging molecule TTR-52, which facilitates CED-1 recognition of PtdSer (Mapes et al. 2012). However, because CED-7 activity appears to be required in both the phagocytic and dying cells for engulfment in C. elegans (Wu and Horvitz 1998), it has been complicated to determine exactly where CED-7 acts in the signaling pathway.

Downstream of CED-7/ABCA1, a prominent feature observed during engulfment, is the clustering of the transmembrane receptors CED-1/MEGF10 at the phagocytic cup (Zhou et al. 2001). In vivo studies in C. elegans and in vitro studies in mouse cell culture, respectively, show that CED-1/MEGF10 clusters around the cell corpse and facilitates cell clearance in a manner dependent on CED-7/ABCA1 (Zhou et al. 2001; Hamon et al. 2006). Given its putative role in lipid transport, it is tempting to speculate that CED-7/ABCA1 may function at the phagocytic cup to remodel the local lipid composition, and perhaps generate domains such as lipid rafts to which CED-1/MEGF10 can be recruited.

To identify and characterize the CED-7/ABCA1 ortholog in D. melanogaster, we used the D. melanogaster ovary as an in vivo model system to study cell death and engulfment. Two distinct germline PCD events, developmental PCD in late oogenesis and stress-induced PCD in midoogenesis, have been well characterized in the ovary (Jenkins et al. 2013; Peterson et al. 2015). The ovary is comprised of a bundle of 15–20 ovarioles, sheaths of progressively developing egg chambers through 14 stages of oogenesis. Each egg chamber contains 16 interconnected germline-derived cells, composed of a single oocyte and 15 NCs, surrounded by a layer of somatically-derived FCs (King 1970; Spradling, 1993). As each oocyte reaches maturation, the 15 NCs undergo PCD and are cleared by the neighboring FCs. We have found that phagocytosis genes including dpr and Ced-12 are required for NC clearance, as their loss-of-function results in stage 14 egg chambers that exhibit persistent NC nuclei (Timmons et al. 2016). Additionally, genetically inducing death in a small subset of the phagocytic FCs inhibits the death and removal of the NCs, suggesting that the FCs nonautonomously promote the death and removal of the NCs via phagoptosis.

During midoogenesis, PCD of the germline can occur in response to stress, such as protein starvation. PCD in midoogenesis requires active caspases, including death caspase 1 (Dcp-1), and autophagy genes, suggesting that death is executed via apoptotic and autophagic cell death pathways (Jenkins et al. 2013). As the NCs degenerate, the surrounding FCs synchronously enlarge to engulf the germline debris. Similar to clearance in late oogenesis, this process in midoogenesis is regulated by dpr and Ced-12 (Etchegaray et al. 2012; Meehan et al. 2015a). dpr and Ced-12 mutants produce egg chambers that exhibit dying NCs, with FCs that fail to enlarge or take up the germline material.

The engulfment pathways first defined in C. elegans are highly conserved in D. melanogaster, but a ced-7/ABCA1 ortholog has not been reported. The D. melanogaster genome contains 56 ABC genes, of which 10 encode ABCA type transporters similar to CED-7/ABCA1. Only two of the ABCA genes, CG31731 and CG1718, are expressed at appreciable levels in the ovary (FlyBase). We functionally analyzed these two genes in the D. melanogaster ovary and found that CG31731 mutants show profound defects in NC clearance. Moreover, CG31731 appears to play a similar role to ced-7/ABCA1 in the engulfment process. Thus, CG31731 likely serves a CED-7/ABCA1 role in engulfment in the D. melanogaster ovary, and hereafter will be referred to as Eato.
To make germline clones, we generated an Eato\textsuperscript{FRT} 40A stock by recombination and used the FLP \textit{ovo}\textsuperscript{D} system (Chou and Perrimon 1996). RNAi knockdown lines were generated using the GAL4-UAS binary system, with \textit{GAL4} expressed under control of an endogenous tissue-specific enhancer, specifically \	extit{GR1}, which is expressed in all FCS after stage 3 including the stretch FCS (Goentoro et al. 2006), and \textit{nanoS}, which is expressed in the NCS (Rorth 1998).

All strains were reared on standard cornmeal molasses yeast media at 25°. Prior to dissection, adult males and females were transferred to a vial containing fresh media and a teaspoon of yeast paste, and conditioned for ~2 d. To induce cell death in midstage egg chambers, adults were conditioned with yeast paste for ~1 d then transferred to apple juice agar vials and starved of yeast for the last 16–20 hr period prior to dissection.

Staining and microscopy

Ovaries were dissected in Grace’s Insect Media (Fisher) and then processed as previously described (Meehan et al. 2015b). Primary antibodies used were: \textit{\alpha-Drpr} [1:50; Developmental Studies Hybridoma Bank (DSHB)], \textit{\alpha-Dlg} (1:100; DSHB), and \textit{\alpha-cleaved Dcp1} (1:100; Cell Signaling). Secondary antibodies were used: goat-\textit{\alpha}-rabbit Cy3, goat-\textit{\alpha}-mouse Cy3, and goat-\textit{\alpha}-rabbit Alexa Fluor 647 (1:200; Jackson ImmunoResearch).

Ovaries were mounted in Vectashield with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories) and slides were stored at 4°. Egg chambers were imaged on an Olympus BX60 upright fluorescence microscope or an Olympus FV10i confocal microscope, and images were processed in ImageJ.

Quantitative RT-PCR

RNA samples were extracted from pooled ovaries using the QIAGEN RNeasy Mini Kit, and then converted to cDNA using the Thermo Scientific Maxima First Stand cDNA Synthesis Kit. qPCR was performed following the Promega GoTaq qPCR Master Mix protocol with two primer sets, one flanking the fourth and fifth exons and another flanking the 14th and 15th exons of \textit{Eato}. The results were normalized to \textit{Rpl32} as an internal control.

Results

Eato encodes an ABC transporter similar to ced-7/ABCA1

A search of databases [FlyBase, UniProt, and the \textit{Drosophila} RNAi Screening Center Interactive Ortholog Prediction Tool (DIOPTR)] revealed that the predicted amino acid sequence of \textit{Eato} encodes an ABC transporter of the ABCA subfamily, which includes CED-7 and ABCA1. Like other ABC transporters, \textit{Eato} encodes a protein with two transmembrane domains (TMDs) and two cytosolic nucleotide-binding domains (NBDs) (Figure 1A) with predicted ATP-binding and catalytic capability. Each NBD was found to contain an “A-loop” (aromatic), “Walker A motif” (GxxGxG/K/S/T), “Q-loop” (glutamine, Q), “ABC signature motif” (L/YSGGQ/M), “Walker B motif” (\textit{\phi\phi\phi\phi\phi\phi\phi\phi\phi}), “D-loop” (aspartate, D), and “H-loop” (histidine, H), in highly conserved sequential and spatial organization (Figure 1B), classifying the protein as an ABC transporter (Rees et al. 2009; ter Beek et al. 2014). Additional structural analyses of the predicted amino acid sequence indicated that each Eato TMD contains six hydrophobic \textit{\alpha}-helical segments, generating a 12-pass transporter. In comparison, both CED-7 and ABCA1 each contain 15 transmembrane segments.

Using Basic Local Alignment Search Tool (BLAST) algorithms to compare the predicted amino acid sequences of \textit{Eato} with that of CED-7 and ABCA1, a substantial amount of similarity and identity were found throughout their entire length, most notably in the catalytic NBD regions. Overall, \textit{Eato} was found to be 20% identical to CED-7 and 18% identical to ABCA1. More specifically, the NBDs of \textit{Eato} were found to be 35% identical and 36% similar to those of CED-7, and 40% identical and 43% similar to those of ABCA1.

Additional homology searches revealed that the predicted amino acid sequence of \textit{Eato} with that of ABCA1 is the best predicted ortholog of ced-7 and also a predicted ortholog of ABCA1. Alignment of the proteins provided by DIOPTR revealed a 24% identity and 42% similarity overall between \textit{Eato} and CED-7, and a 26% identity and 43% similarity between \textit{Eato} and ABCA1.

To quantify engulfment, the number of PN in each stage 14 egg chamber was counted. The criteria for a stage 14 egg chamber was fully developed dorsal appendages (Jia et al. 2016). The egg chambers were then grouped into bins of 0 PN, 1–3 PN, 4–6 PN, 7–9 PN, 10–12 PN, or 13–15 PN, and each bin was presented as a percentage of all stage 14 egg chambers quantified per genotype. Alternatively, the average number of PN in stage 14 egg chambers from each genotype was presented. “n” represents the total number of stage 14 egg chambers quantified.

To quantify \textit{Drpr} accumulation or stretch FC (SFC) membrane extensions around the NCs, using ImageJ, the length around each NC nucleus that was \textit{\alpha-Drpr}- or GFP-positive was measured as a percentage of the circumference around each NC nucleus. Each NC nucleus was then grouped into bins of 0–10, 11–30, 31–50, 51–70, 71–90, or 91–100%, surrounded by \textit{\alpha-Drpr} or GFP, and each bin was presented as a percentage of all NC nuclei quantified per genotype. “n” represents the total number of NC nuclei quantified.

All quantifications were performed blind and statistical analyses were performed in Graphpad Prism.
Eato mutants have persisting nurse cell (NC) corpses in the ovary

To determine whether Eato can act as a functional equivalent for ced-7/ABCA1 during PCD, we obtained several transposon-induced alleles of Eato (Figure 2, A and B) and analyzed them for phenotypes in oogenesis. We focused our initial analysis on late oogenesis because defective clearance is directly quantifiable in late oogenesis compared to mid-oogenesis. Ovaries from control and Eato mutant strains were dissected and then stained with DAPI to label DNA, and the number of NC nuclei persisting in stage 14 egg chambers were counted. The presence of PN indicated a failure in NC clearance. In wild-type (WT) egg chambers, the 15 germline-derived NCs underwent PCD and were cleared normally, leaving only the mature oocyte by stage 14 (Figure 2C). However, in several Eato mutant allelic combinations, we observed significant clearance defects characterized by the presence of PN in stage 14 egg chambers (Figure 2, C–E).

Homozygous Eato^{PBac/PBac} mutants displayed an average of 2–3 PN, while hemizygous Eato^{PBac/Df}, trans-heterozygous Eato^{PBac/Mi}, and homozygous Eato^{Mi/Mi} mutants displayed an average of 6 PN per stage 14 egg chamber. The strongest phenotype was observed in hemizygous Eato^{Mi/Df} mutants, with stage 14 egg chambers exhibiting an average of 8 PN (Figure 2, D and E). Flies heterozygous for a WT Eato allele did not exhibit any notable defects in NC clearance, suggesting that Eato is not haploinsufficient.

The PBac and Mi{MIC} constructs are inserted at the end of the first coding exon and in the third intron of the Eato gene, respectively (Figure 2A). The weaker phenotype observed in Eato^{PBac/PBac} egg chambers relative to the other alleles suggests that the Eato^{PBac} allele is a weak hypomorph. The Mi{MIC} insertion provides a gene trap and a protein trap (Figure 2B) (Venken et al. 2011), which in theory should generate a null allele. However, the more severe persisting phenotype
observed in hemizygous Eato<sup>M027</sup> mutants compared to homozygous Eato<sup>M027</sup> mutants. The Eato<sup>M027</sup> allele is a strong hypomorph of Eato. RT-qPCR analysis indicated that the Eato<sup>M027</sup> allele is not a null, but is instead a strong hypomorph with a 16.6-fold decrease in transcript levels, while the Eato<sup>PHO</sup> allele is a weaker hypomorph with a 3.2-fold decrease in mRNA transcript expression in the ovary relative to WT (w<sup>1118</sup>) (Figure 2F).

**Eato is required in the FCs for NC clearance during developmental PCD**

We next wanted to discern in which cell type Eato function is required to facilitate removal of the NCs. Studies in C. elegans showed that CED-7 is required in both the phagocytic cell and the dying cell for efficient engulfment of cell corpses (Wu and Horvitz 1998), while studies in mammals showed that ABCA1 expression in phagocytic cells is sufficient for engulfment (Hamon et al. 2000). To determine in which cell type Eato acts during engulfment of the NCs, we used tissue-specific drivers to express Eato RNAi constructs and knock down Eato expression specifically in the FCs or NCs.

**Eato** knockdown with three different RNAi constructs (Figure 2A) using a FC-specific driver, GR1-GAL4 (GR1-GAL4 > UAS-Eato<sup>RNAi</sup>), resulted in stage 14 egg chambers that exhibited PN (Figure 3, A–C). Egg chambers expressing the GD1133, KK104197, or HMC06027 RNAi constructs in FCs displayed an average of 7, 5, and 4–5 PN, respectively (Figure 3C). All three RNAi constructs exhibited stronger phenotypes than the Eato<sup>PHO</sup>/PHO mutants. The GD1133 RNAi construct also exhibited a stronger phenotype than the Eato<sup>M027</sup> mutants, suggesting that PN observed in these mutants can be primarily attributed to loss of Eato function in the FCs. Sibling controls (Eato<sup>RNAi</sup>/TM6B) containing the RNAi constructs without the driver showed no defects in NC clearance (Figure 3, B and C).

To determine whether Eato also acts in dying cells, we knocked down Eato specifically in the NCs (namos-GALA > UAS-Eato<sup>RNAi</sup>). We did not observe a requirement for Eato in the NCs for their clearance. Egg chambers expressing any of the three Eato<sup>RNAi</sup> constructs specifically in the NCs did not show any engulfment defects and were able to clear all 15 NCs normally (Figure 3D). While GD and KK RNAi libraries generate long hairpin RNA sequences, which are typically ineffective for RNAi-mediated knockdown in the germline (Ni et al. 2011), the HMC06027 allele encodes a short hairpin RNA sequence that should competently knock down Eato expression in the germline-derived NCs (Ni et al. 2011). The lack of persisting NCs in these Eato RNAi-expressing stage 14 egg chambers suggests that Eato is not required in the dying cells for their clearance. To confirm that Eato was not required in the dying NCs, we generated Eato<sup>M027</sup> germline clones. Indeed, stage 14 egg chambers from Eato<sup>M027</sup> germline clones did not show a significant persisting NC phenotype (Figure 3A). However, ~3% of stage 14 egg chambers exhibited ≥ 4 PN (Figure 3D). We suspect that these egg chambers may have FC clones in addition to germline clones, which can occur in the process of generating germline clones (Peterson and McCall 2013).

**Eato acts in parallel to Ced-12, likely in the same pathway as Drpr**

In C. elegans, the engulfment mechanism is primarily regulated by two parallel signaling pathways, CED-1/-6/-7 and CED-2/-5/-12 (Ellis et al. 1991; Kinchen et al. 2005). We have shown that, similar to C. elegans,
Figure 4 Eato acts in the same pathway as drpr and in parallel to Ced-12. (A) Stage 14 egg chambers, stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (white) to label DNA, from drpr<sup>Δ5/Δ5</sup> and Ced12<sup>RNAi</sup> expressed specifically in the follicle cells (FCs) (GR1-GAL4 > UAS-Ced12<sup>RNAi</sup>, single and double mutants with Eato<sup>Mi/Mi</sup>). (B) The percentage of stage 14 egg chambers exhibiting 0 persisting nurse cell (NC) nuclei (PN), 1–3 PN, 4–6 PN, 7–9 PN, 10–12 PN, and 13–15 PN per indicated genotype. (C) The average number of persisting NC nuclei in stage 14 egg chambers from each indicated genotype. Error bars indicate ± SEM. Unpaired t-tests were performed: ** P < 0.001 and *** P < 0.0001.

drpr, the ced-1 ortholog, and Ced-12 act in parallel to regulate clearance of the NCs during D. melanogaster oogenesis (Timmons et al. 2016). Double knockdowns expressing Ced-12<sup>RNAi</sup> and drpr<sup>RNAi</sup>, or the null allele drpr<sup>Δ5</sup>, in the FCs exhibit a significantly more severe persisting NC phenotype compared to either single knockdown alone. In C. elegans and mammals, ced-7/ABCA1 acts in the same pathway as ced-1/MEGF10, in parallel to ced-12/ELMO. The similarities that we have observed between Eato and ced-7/ABCA1 suggest that Eato may act in the same pathway as drpr and in parallel to Ced-12. To ascertain which pathway Eato is involved in during engulfment, we generated Eato<sup>Δ5/Δ5</sup> and Eato<sup>+/+</sup>+Ced-12<sup>Δ5/Δ5</sup> double mutants and analyzed the severity of their engulfment defects in NC clearance.

To determine whether Eato acts in the same pathway as drpr, we analyzed Eato<sup>Mi/Mi</sup>, drpr<sup>Δ5/Δ5</sup> double mutants compared to drpr<sup>Δ5/Δ5</sup> single mutants. We found that the double mutant did not show a stronger phenotype than the single mutant (Figure 4, A–C), and that both drpr<sup>Δ5/Δ5</sup> and the double mutants displayed an average of ~9 PN (Figure 4C). The similar clearance defects between the single and double mutants indicate that Eato acts in the same pathway as drpr.

To clarify whether Eato acts in parallel to Ced-12, we needed to avoid the lethality of Ced-12 mutants and thus we knocked down Ced-12 expression only in the phagocytic FGCs (GR1 > Ced-12<sup>RNAi</sup>). In these Ced-12 knockdowns, stage 14 egg chambers displayed an average of 7 PN. Impressively, in the Eato<sup>Mi/Mi</sup>, GR1 > Ced-12<sup>RNAi</sup> double mutants, stage 14 egg chambers displayed an average of 11 PN (Figure 4C). The Eato<sup>Mi/Mi</sup>, GR1 > Ced-12<sup>RNAi</sup> double mutants displayed a much more severe engulfment defect than either single mutant alone, and a stronger phenotype than the Eato<sup>Mi/Mi</sup>, drpr<sup>Δ5/Δ5</sup> double mutants. Moreover, a considerable percentage of these double mutants completely failed to clear any of the NCs (Figure 4B), indicating that the engulfment machinery had been severely impaired. These findings suggest that Eato acts in parallel to Ced-12, and that its function is important for clearance of the NCs.

Eato promotes Drpr enrichment and stretch FC membrane extensions surrounding the NCs

During engulfment, CED-1/MEGF10 has been shown to accumulate at the phagocytic cup (Hamon et al. 2000; Zhou et al. 2001). In vivo and in vitro studies in C. elegans and mouse cell culture, respectively, show that the uniform clustering of CED-1/MEGF10 around cell corpses occurs in a manner dependent on CED-7/ABCA1 activity. To examine whether Eato may function orthologously to CED-7/ABCA1 and be required for Drpr accumulation around the NCs, we analyzed late-stage egg chambers from WT and Eato<sup>Mi/Mi</sup> mutants with DAPI to label DNA and anti-Drpr antibody to label Drpr.

In late-stage egg chambers, a subset of anterior FCs, known as the SFCs, associate with the NCs as a squamous epithelium (Wu et al. 2008). In WT stage 12–14 egg chambers, Drpr staining becomes enriched in the SFCs and clearly surrounds each NC (Figure 5A) (Timmons et al. 2016). However, in Eato<sup>Mi/Mi</sup> egg chambers, Drpr staining appeared unevenly enriched and scattered around the NCs. Strikingly, in some areas, Drpr staining was completely absent in the SFCs surrounding the NCs (Figure 5, B and E), indicating that Drpr failed to properly accumulate. These observations suggest a requirement for Eato in Drpr accumulation during NC clearance.

We considered two possible mechanisms that could produce the lack of Drpr staining: either Drpr fails to accumulate at the SFC membrane or the SFC membrane fails to extend and surround the NC. To observe any defects in SFC membrane extensions, we expressed the mCD8-GFP transgene, which encodes a membrane-tethered GFP fusion protein, to label FC membranes. In both control (GR1-GAL4 > UAS-mCD8-GFP) and Eato knockout egg chambers (GR1-GAL4 > UAS-mCD8-GFP, UAS-Eato<sup>RNAi</sup>-GFP) we observed an overlap of the presence or absence of GFP expression and Drpr staining in the SFCs (Figure 5, C and D). However, in Eato knockout egg chambers, some areas exhibiting GFP did not exhibit Drpr accumulation (Figure 5, D–F). Altogether, these observations indicate a requirement for Eato in both SFC membrane extension and Drpr enrichment around the NCs.

CG1718 may act with Eato in the FCs for NC clearance during developmental PCD

We also examined the engulfment function of CG1718 in NC clearance during oogenesis. CG1718 encodes another ABCA protein that is expressed in the ovary, and has been proposed as the D. melanogaster homolog of ABCA1 for its role in lipid and cholesterol homeostasis (Bujold et al. 2010), and the homolog of ced-7 in cell clearance.
Additionally, while studies in engulfing glia did not find a role for CG1718 in neuronal corpse or axonal debris clearance (Ziegenfuss 2012), pan-neuronal-specific knockdown of CG1718 resulted in synaptic bouton overgrowth at the neuromuscular junction (Ueoka et al. 2018). To determine whether CG1718 plays a role in NC clearance, we quantified PN in CG1718 knockdowns and mutants.

Egg chambers expressing one of five CG1718RNAi constructs (Figure 6A) specifically in the NCs (nanos > CG1718RNAi) did not exhibit any engulfment defects (data not shown). Intriguingly, while egg chambers expressing any one of four CG1718RNAi constructs specifically in the FCS (GR1 > CG1718RNAi) did not exhibit any engulfment defects, expression of the HMS01821 RNAi construct specifically in the FCS resulted in moderate engulfment defects as 45% of these stage 14 egg chambers exhibited PN (Figure 6B). Unique from the other CG1718 RNAi constructs, the HMS01821 RNAi sequence targets the 3’ untranslated region (3’UTR) of CG1718 transcripts (Figure 6A).

We also examined a miR-1007 deletion strain (CG1718miR/miR), in which the expression of CG1718 was reported to be reduced (Chen et al. 2014). The miR-1007 gene is nestled in one of the introns of CG1718 (Figure 6A), and a systematic study of Drosophila microRNA (miR) functions previously generated the CG1718miR/miR strain. Stage 14 egg chambers from these mutants did not exhibit any pronounced NC clearance defects (Figure 6, C–E).

ABC transporters of the ABCA family share a common function in lipid transport (Vasiliou et al. 2009; Quazi and Molday 2011), and we wondered whether CG1718 and Eato could provide compensatory or redundant roles for each other in NC clearance. We generated CG1718+Eato double mutants carrying homozygous CG1718miR/miR and EatoMi/Mi, or EatoPBac/Pbac, hypomorphic alleles. Interestingly, egg chambers from these mutants did not exhibit any pronounced NC clearance defects (Figure 6, C–E).

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phenotype than either single mutant alone, with an average of 6 PN per stage 14 egg chamber (Figure 6E). While the less severe phenotype observed in CG1718miR/miR;EatoMi/Mi double mutants remains bewildering, the much more severe phenotype observed in CG1718miR/miR;EatoPBac/PBac double mutants suggests a functional relationship between the two ABCA transporters. In mammals, ABCA1 and ABCA7 have been demonstrated to provide redundant functions (Wang et al. 2003; Abe-Dohmae et al. 2004; Kim et al. 2005). Perhaps in D. melanogaster, CG1718 could provide a compensatory or redundant function for Eato.

Eato is required in the FCs for engulfment of the germline during stress-induced PCD

To determine if Eato is required for clearance in other forms of cell death, we investigated whether Eato plays a role during starvation-induced PCD in midoogenesis. In response to starvation, NGs die via apoptosis during midoogenesis (Jenkins et al. 2013) and are engulfed by surrounding epithelial FCs. The cell death of the NGs in midoogenesis proceeds through five morphologically distinct phases defined by changes in the NC chromatin (Etchegaray et al. 2012). In phase 0 healthy egg chambers, the 15 NCs exhibit dispersed chromatin; in phase 1 dying egg chambers, the NC chromatin becomes disordered and begins to condense; by phase 3 the NC chromatin becomes highly condensed in single large balls and the FCs appear enlarged; and by phase 5 the FCs have phagocytosed the germline material and constitute almost the entire egg chamber.

From our studies in late oogenesis, we selected the Eato mutants that exhibited the most severe phenotypes and looked for engulfment defects in midoogenesis. We selected EatoMi/Mi and FC knockdown...
EatoRNAi-GD mutants and starved the adults for a 16–20 hr period to induce PCD in midstage egg chambers. Subsequently, we dissected and stained the ovaries with DAPI to label DNA, anti-Dlg to label FC membranes, anti-cleaved Dcp-1 antibody (yellow) to label active caspases and pyknotic FC nuclei (arrowheads) without membrane markers. (A) Phase 0 healthy egg chambers. (B) Phase 1 dying egg chambers of EatoRNAi-GD mutants, or when EatoRNAi-GD1133 is expressed specifically in the FCs (GR1-GAL4 > UAS-EatoRNAi-GD1133), resemble the control (UAS-EatoRNAi-GD1133/TM6B) dying egg chambers. (C) Phase 3 dying egg chambers of Eato mutants exhibit enlarged FCs; however, they show fewer Dcp-1-positive vesicles inside the FCs (arrows). (D) Phase 5 dying egg chambers of Eato mutants show severe engulfment defects with completely unengulfed germline and pyknotic FC nuclei (arrowheads) without membrane markers.

**DISCUSSION**

Here, we report the characterization of Eato, which encodes an ABC transporter that is structurally and functionally similar to ced-7/ABCA1. Like ced-7/ABCA1, Eato encodes an ABCA type transporter and presents substantial sequence similarity and identity to that of CED-7 and ABCA1. Moreover, our genetic analyses identify a role for Eato in cell clearance during PCD, demonstrating functional conservation. To our knowledge, prior to this study there have been no reports of a D. melanogaster functional equivalent for CED-7/ABCA1.

Using the D. melanogaster ovary as an in vivo model system, we observed cell clearance defects in Eato mutants in both developmental PCD, which proceeds via phagoptosis (Timmons et al. 2016), and starvation-induced PCD, which proceeds via apoptosis (Jenkins et al. 2013). Unlike in C. elegans, where CED-7 was reported to be required both in the dying cell and the phagocytic cell for corpse clearance (Wu and Horvitz 1998), our investigation indicates a requirement for Eato only in the phagocytic FCs and not in the dying NCs. Genetically knocking down Eato specifically in the phagocytic FCs resulted in engulfment defects, while knocking down Eato expression specifically in the germline did not affect clearance. These findings demonstrate a conserved role for Eato in two distinct PCD modalities, specifically in the phagocytic cells for cell clearance.

In C. elegans and mammals, ced-7/ABCA1 acts in the same pathway as ced-1/MEGF10, in parallel to ced-12/ELMO (Ellis et al. 1991; Kinchen et al. 2005; Mangahas and Zhou 2005). Correspondingly, our double mutant analyses of EatoRNAi-GD with drprRNAi or Ced-12RNAi show that Eato acts in the same pathway as drpr and in parallel to Ced-12, strongly suggesting that Eato provides a ced-7/ABCA1-like role in this conserved engulfment mechanism. Indeed, we observed a requirement for Eato in Drpr enrichment around the NCs, similar to the requirement for CED-7/ABCA1 in CED-1/MEGF10 clustering at the phagocytic cup (Zhou et al. 2001; Hamon et al. 2006).

Multiple investigations in C. elegans and mammals have reported a role for CED-7/ABCA1 in lipid transport (Hamon et al. 2000; Wang et al. 2004; Yang et al. 2006). Our discovery of the conserved role for Eato in engulfment suggests a conserved function in lipid transport during cell death.

In this study, we show that Eato plays a key role in lipid transport during engulfment defects. These results have implications for understanding lipid transport in other developmental and disease processes.
et al. 2010; Mapes et al. 2012). Importantly, CED-7 has been implicated in intracellular vesicle delivery to the phagocytic cup to provide membrane material (Yu et al. 2006). Our investigation showed that in Eato mutants, phagocytic membrane extensions around the NCs are disrupted. In both late-stage and dying midstage egg chambers from Eato mutants, the FCs fail to extend and complete engulfment of the germline debris. Thus, we speculate that Eato may function as a lipid transporter to deliver vesicles containing membrane material and other proteins, such as Drpr, to the growing FC membrane. This would explain the defects observed in SFC membrane extensions and Drpr accumulation in late oogenesis, and the FC enlargement and germline uptake in midoogenesis in Eato mutants.

Transporters of the ABCA family are commonly involved in lipid trafficking, though the specific substrates transported by CED-7/ABCA1 remain to be identified. Cell culture experiments have suggested that ABCA1 may act as a translocase to translocate the “eat-me” signal PtdSer from the inner leaflet to the outer leaflet (Hamon et al. 2000; Smith et al. 2002; Albrecht et al. 2005). However, in vivo PtdSer exposure on the surface of dying cells does not require CED-7 in C. elegans. Instead, CED-7 was shown to be required for the transfer of PtdSer-containing vesicles from the surface of dying cells to the surface of phagocytic cells (Mapes et al. 2012), suggesting that CED-7 can efflux PtdSer and potentially other phospholipids. In the context of HDL formation, ABCA1 has also been speculated to preferentially transport phosphatidylcholine (Takahashi et al. 2006). Our identification of Eato provides another system to elucidate the transport activities of this unique class of proteins in engulfment.

In mammals, ABCA1 and ABCA7 have been observed to provide homologous functions, especially in lipid homeostasis (Wang et al. 2003; Abe-Dohmae et al. 2004; Hamon et al. 2006). Like ABCA1, ABCA7 has been demonstrated to mediate phospholipid and cholesterol release to form HDLs, and even compensate for ABCA1 in certain conditions (Wang et al. 2003; Abe-Dohmae et al. 2004; Kim et al. 2005). Similarly, we observed a role for another ABCA-encoding gene, CG1718, in NC clearance. Most noticeably, in an EatoPbacPbac hypomorphic background, CG1718 may provide a compensatory or redundant function for Eato during NC clearance. Since both genes encode ABC transporters of the same family, which are known to share a functional relationship in lipid trafficking, the proteins may be able to provide similar if not the same functions. Thus, as in the case of ABCA1 and ABCA7, CG1718 may share redundant functions or possibly provide compensatory mechanisms in the absence of Eato.

CG1718 pan-neuronal knockdown flies were recently established as a model for autism spectrum disorder. These flies exhibited behavioral characteristics similar to those observed in human autism spectrum disorder patients, and showed excessive synaptic satellitebouton outgrowths (Ueoka et al. 2018), similar to those in Fmr1 mutants (Zhang et al. 2001). The Fmr1 gene has been reported to play a role in glial phagocytosis of neuronal and axonal debris, and in hemorrhage, phagocytosis of bacteria (Logan 2017; O’Connor et al. 2017), and thus, by analogy, CG1718 may similarly play a phagocytic role in cell clearance, though likely not in engulfing glia (Ziegenfuss 2012).

We also looked for a role for Eato in glial phagocytosis of neuronal debris. EatoWtM mutants did not show any accumulation of uncleared neuronal corpses, suggesting that Eato does not play a prominent role during corpse clearance in the brain. We speculate that perhaps there is another ABCA gene that provides a ced-7/ABCA1-like role during PCD events in engulfing glia, perhaps CG34120, which appears to be the most appreciably expressed ABCA transporter in the head (FlyBase).

Eato may also be involved in salivary gland clearance during development. Eato is expressed at high levels in the salivary glands (FlyBase), which undergo autophagic cell death and are cleared during larval development. E93 mutants, which exhibit persisting salivary glands (Lee and Baehrecke 2001), show decreased expression of Eato (Dutta 2008). Additionally, Drpr was also found to be enriched in the salivary glands and to be required for salivary gland clearance (McPhee and Baehrecke 2010). As our study indicates a relationship between drpr and Eato, it seems likely that Eato may have a role in degradation and clearance of the salivary glands.

In conclusion, our findings provide insight into the molecular activities that occur during engulfment in PCD, with specific attention to the role of ABCA transporters. We have identified Eato, a ced-7/ABCA1-like ABCA transporter gene that is required during engulfment in the D. melanogaster ovary. To our knowledge, this is the first report of a role for ABCA transporters in PCD in Drosophila. Further characterization of this ced-7/ABCA1 ortholog in D. melanogaster will help elucidate the functions and mechanisms of this unique class of transporters during PCD.

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