An ABCG Transporter Functions in Rab Localization and Lysosome-Related Organelle Biogenesis in *Caenorhabditis elegans*

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ABSTRACT ABC transporters couple ATP hydrolysis to the transport of substrates across cellular membranes. This protein superfamily has diverse activities resulting from differences in their cargo and subcellular localization. Our work investigates the role of the ABCG family member *WHT-2* in the biogenesis of gut granules, a *Caenorhabditis elegans* lysosome-related organelle. In addition to being required for the accumulation of birefringent material within gut granules, *WHT-2* is necessary for the localization of gut granule proteins when trafficking pathways to this organelle are partially disrupted. The role of *WHT-2* in gut granule protein targeting is likely linked to its function in Rab GTPase localization. We show that *WHT-2* promotes the gut granule association of the Rab32 family member *GLO-1* and the endolysosomal *RAB-7*, identifying a novel function for an ABC transporter. *WHT-2* localizes to gut granules where it could play a direct role in controlling Rab localization. Loss of *CCZ-1* and *GLO-3*, which likely function as a guanine nucleotide exchange factor (GEF) for *GLO-1*, lead to similar disruption of *GLO-1* localization. We show that *CCZ-1*, like *GLO-3*, is localized to gut granules. *WHT-2* does not direct the gut granule association of the *GLO-1* GEF and our results point to *WHT-2* functioning differently than *GLO-3* and *CCZ-1*. Point mutations in *WHT-2* that inhibit its transport activity, but not its subcellular localization, lead to the loss of *GLO-1* from gut granules, while other *WHT-2* activities are not completely disrupted, suggesting that *WHT-2* functions in organelle biogenesis through transport-dependent and transport-independent activities.

KEYWORDS lysosome-related organelle; Rab GTPase; ABC transporter; membrane trafficking; *Caenorhabditis elegans*

CELL-type-specific elaboration of the conventional endocytic pathway leads to the generation of lysosome-related organelles (LROs). Well-studied LROs include mammalian melanosomes, mammalian platelet alpha and dense granules, *Drosophila* retinal pigment granules, and *Caenorhabditis elegans* gut granules (Bowman *et al.* 2019). LROs share contents, such as the V-ATPase and LAMPs, with conventional lysosomes. However, they often lack degradative hydrolases and instead harbor factors that endow each type of LRO with specialized functions. Despite having diverse contents,

morphologies, and activities, most LROs utilize the same evolutionarily conserved machinery for their biogenesis. Some of these biogenesis factors have functions that are dedicated to LRO formation, while others also act within the conventional endolysosomal system (Bultema and Di Pietro 2013; Marks *et al.* 2013; Bowman *et al.* 2019). Defects in the factors promoting LRO formation underlie rare human diseases, including Hermansky-Pudlak and Chediak-Higashi syndromes (Huizing *et al.* 2008).

C. elegans gut granules are intestinal cell-restricted LROs that are generated early during embryonic development and persist into adulthood (Laufer *et al.* 1980; Clokey and Jacobson 1986; Bossinger and Schierenberg 1992; Hermann *et al.* 2005). They contain autofluorescent derivatives of anthranilic acid (Coburn *et al.* 2013), as well as rhabditin (Cobb 1914), an optically active birefringent material whose molecular identity is currently unknown. Gut granules function in

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metal and metabolite storage (Roh *et al.* 2012; Lee *et al.* 2015; Chun *et al.* 2017), anabolic reactions (Panda *et al.* 2017), and life span regulation (Folick *et al.* 2015). Gut granule formation requires the function of the Rab32 family member **GLO-1**, and the AP-3 (Hermann *et al.* 2005), BLOC-1 (Hermann *et al.* 2012), and HOPS complexes (Delahaye *et al.* 2014). A BLOC-3-related complex comprising **CCZ-1** and **GLO-3** likely acts as a guanine nucleotide exchange factor (GEF), activating **GLO-1** and promoting its gut granule association (Delahaye *et al.* 2014; Morris *et al.* 2018). In addition to these factors, whose homologs function in the formation of mammalian and *Drosophila* LROs (Bowman *et al.* 2019), we have identified a role for three different ABC transporters in the biogenesis of gut granules (Currie *et al.* 2007; Schroeder *et al.* 2007).

ABC transporters represent a superfamily of conserved proteins that often couple ATP hydrolysis to the movement of ions, lipids, sugars, and peptides across cell membranes (Holland *et al.* 2003). ABC transporters localize to diverse cellular membranes, including conventional lysosomes and LROs (Ridsdale *et al.* 2011; Chapel *et al.* 2013), and there is evidence that they play a role in the formation of both types of organelles. ABC transporters in yeast function in homotypic lysosome fusion and exhibit direct physical interactions with the membrane trafficking machinery, including subunits of the HOPS complex (Paumi *et al.* 2009; Sasser *et al.* 2012, 2013; Snider *et al.* 2013). In *Dictyostelium*, ABC transporters promote trafficking through the conventional endolysosomal pathway (Brazill *et al.* 2001). The biogenesis of lamellar bodies and melanosomes, LROs found within human lung alveolar cells and melanocytes require the function of ABC transporters (Cheong *et al.* 2007; Bergam *et al.* 2018). In *C. elegans*, the **MRP-4**, **PGP-2**, and **WHT-2** ABC transporters function in the formation of gut granules (Currie *et al.* 2007; Schroeder *et al.* 2007). **MRP-4** also affects the size and function of conventional lysosomes (Schaheen *et al.* 2006; Huynh *et al.* 2016).

WHT-2 is an ABCG subfamily member that is homologous to the *Drosophila* White protein (Sheps *et al.* 2004). Similar to White, which transports pigment precursors into retinal pigment granules (Sullivan and Sullivan 1975; O'hare *et al.* 1984), **WHT-2** is required for the accumulation of birefringent material within gut granules (Currie *et al.* 2007). However, genetic interactions between **wht-2** and gut granule biogenesis mutants suggest that **WHT-2** has a broader role in the formation of gut granules (Currie *et al.* 2007; Levitte *et al.* 2010). Here, we show that **WHT-2** has significant functions in gut granule biogenesis that extend beyond the accumulation of birefringent material. Notably, our work indicates that **WHT-2** functions in the localization of Rab GTPases, a novel activity for an ABC transporter.

Materials and Methods

C. elegans strains were cultured on NGM media seeded with *Escherichia coli* strain OP50 at 22° (Stiernagle 1999). All

mutant alleles were generated in N2, which was used as the wild-type strain. The following mutations were used: *apt-7(tm920)*, *ccz-1(ok2182)*, *cup-5(zu223)*, *glo-1(zu437)*, *glo-1(syb1102[gfp::glo-1])*, *glo-1(syb1103[gfp::glo-1])*, *glo-2(tm592)*, *glo-3(gk582755)*, *glo-3(kx38)*, *glo-3(kx94)*, *lyst-1(gk295717)*, *mrp-4(cd8)*, *sand-1(or552)*, *unc-119(ed3)*, *vps-18(tm1125)*, *wht-1(tm688)*, *wht-2(gk891224)*, *wht-2(syb668[K74M])*, *wht-2(syb669[K74M])*, *wht-2(syb745[K74R])*, *wht-2(syb794[K74R])*, *wht-2(ok2775)*, *wht-3(ok927)*, *wht-4(ok1007)*, *wht-5(ok806)*, *wht-6(ok882)*, *wht-7(ok812)*, and *wht-8(ok3112)*. WormBase (www.wormbase.org) hosts descriptions of each allele. The following transgenes were used: *amls4[cdf-2p::cdf-2::gfp; unc-119(+)]* (Davis *et al.* 2009), *jcpEx2[ced-1p::ccz-1::yfp; unc-119(+); myo-2::gfp]* (Nieto *et al.* 2010), *kxEx9[glo-1p::gfp::glo-1; Rol-6^P]* (Hermann *et al.* 2005), *kxEx41[glo-3p::glo-3::gfp; Rol-6^P]* (Rabbitts *et al.* 2008), *kxEx152[asm-1p::asm-1::mCherry; Rol-6^P]* (Levitte *et al.* 2010), *kxEx172[WRM0622AF09; Rol-6^P]* (this work), *kxEx252[vha-6p::gfp::glo-1(D132A); Rol-6^P]* (Delahaye *et al.* 2014), *kxEx272[glo-1p::gfp::glo-1(D132A); Rol-6^P]* (Morris *et al.* 2018), *kxEx286[wht-2(+); Rol-6^P]* (this work), *kxEx287[wht-2(+); Rol-6^P]* (this work), *kxEx289[wht-2(K74R); Rol-6^P]* (this work), *kxEx296[wht-2(K74M); Rol-6^P]* (this work), *kxds5[glo-1p::mans::mCherry; unc-119(+)]* (this work), *kxds15[glo-1p::gfp::glo-1; unc-119(+)]* (this work), *pwl50[lmp-1p::lmp-1::gfp; unc-119(+)]* (Treusch *et al.* 2004), and *sls10428[wht-2p::gfp; dpy-5(+)]* (McKay *et al.* 2003).

Genetic manipulations

Standard genetic tests showed that *wht-2(ok2775)* and *wht-2(gk891224)* led to recessive zygotic effect defects in birefringent gut granule formation. Extrachromosomal (Ex) and integrated (Is) transgenes, present in otherwise wild-type strains, were moved into mutant backgrounds by crossing males homozygous or heterozygous for the mutation with hermaphrodites containing the transgenes. The mutation in the resulting strain was confirmed by the presence of the mutant phenotype, PCR, and/or DNA sequencing. To generate *wht-2(ok2775)* double mutants, the progeny of transheterozygous individuals homozygous for *wht-2(ok2775)*, as evidenced by the number of birefringent gut granules, and heterozygous for the other mutation, were isolated. When generating double mutants between *wht-2(ok2775)* and *apt-7(tm920)*, *glo-2(tm592)*, and *lyst-1(gk295717)*, we used the alteration in autofluorescent gut granule number and morphology exhibited by the *apt-7(-)*, *glo-2(-)*, and *lyst-1(-)* mutants to identify individuals homozygous for *wht-2(-)* and heterozygous for the other mutation. The homozygous double mutants, which exhibited the other mutant phenotype, were then isolated from these strains. In cases where one Glo phenotype masked another, we confirmed the presence of the masked allele by PCR or DNA sequencing. Single and double mutants were homozygous for each mutation except strains containing *cup-5(zu223)* and some strains containing *ccz-1(ok2182)*, which were kept heterozygous due to the maternal effect lethality or severe growth defect caused

by these mutations (Hersh *et al.* 2002; Nieto *et al.* 2010). In strains heterozygous for *ccz-1*, we identified homozygous mutant adults by the presence of large DIC refractile granules within their embryos (Nieto *et al.* 2010). *unc-36(e251)* linked to *cup-5(-)* was used to identify homozygous parents.

wht-2(gk891224) was identified in an ongoing screen of Million Mutation strains for defects in birefringent gut granule number. *gk891224* did not complement the Glo phenotypes of *wht-2(ok2775)* and the *wht-2* mutation was backcrossed three times to N2 before being characterized. CRISPR-Cas9-mediated gene editing was carried out by SunyBiotech to generate *wht-2(syb668)* and *wht-2(syb669)*, which precisely changed lysine 74 to methionine, and *wht-2(syb745)* and *wht-2(syb794)*, which precisely changed lysine 74 to arginine. The presence of the mutations was verified using Sanger sequencing (Genewiz, South Plainfield, NJ). The sequence of the first 55 nucleotides of exon three of the *wht-2* K74M and K74R mutations are shown:

```
TGGCGCCGGAATGACAACCTACTCAACATGCTGCTCTCAC
GTAATTTGcGAGGg
TGGCGCCGGACGTACAACCTACTCAACATGCTGCTCTCAC
GTAATTTGcGAGGg
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The underlined upper case nucleotides denote the point mutations changing K74 and the underlined lower case nucleotides denote synonymous mutations that alter the protospacer adjacent motif site. Both K74M and K74R alleles showed the same effect on the formation of birefringent material and *wht-2(syb668[K74M])* and *wht-2(syb745[K74R])* are used in this study.

The loss of birefringence in *wht-2(ok2775)* embryos was rescued when the *wht-2(+)* containing fosmid WRM0622AF09 was injected at 5 ng/μl with pRF4 at 100 ng/μl. The resulting *kxEx172* array was used to score rescue and other arrays containing the fosmid had the same activity. Birefringent gut granules were also restored by *wht-2(+)* amplified with Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) from N2 genomic DNA, using primers P539 5'AGTGGAC AAGCTACGGATCTCATG3' and P1024 5'TTATAGGCAACAGTC GTAACCTCAG3'. *wht-2(ok2775)* was injected with the resulting PCR product, which contained 2430 bp of promoter sequence 5' to *wht-2(+)*, the *wht-2(+)* coding sequence, and 540 bp of the 3'UTR, at 0.5 ng/μl with pRF4 at 100 ng/μl. The resulting *kxEx286* and *kxEx287* arrays were used to score rescue; however, other arrays had similar activity.

To generate extrachromosomal arrays encoding *WHT-2*(K74M), we similarly amplified two overlapping fragments of *wht-2* from N2 genomic DNA with P1023 5'GCCTACAT AGTTGGTGGTTTTCCG3' and P1025 5'GAGTAGAGTTGTCA TTCCGGCGCCACTGAAATTTAAAAATAATACTG3', and P538 5'ACAGTATTATGTTCTCAGGAGGAG3' and P1026 5'GGC GCCGGAATGACAACCTACTCAACATGCTGCTCTCACGTAA TTTG3'. These were fused with P539 and P1024 as described (Hobert 2002). In P1025 and P1026, the underlined sequences denote the K74M point mutation. To generate extrachromosomal arrays encoding *WHT-2*(K74R), we amplified

two overlapping fragments of *wht-2* from N2 genomic DNA with P1023 and P1027 5'GAGTAGAGTTGTACGTCGGCG CCACTGAAATTTAAAAATAATACTG3' and P538 and P1028 5'GGCGCCGGACGTACAACCTACTCAACATGCTGCTCTCAC GTAATTTG3'. These were fused with P539 and P1024. In P1027 and P1028, the underlined sequences denote the K74R point mutation. *wht-2(ok2775)* was injected with each of the PCR fusion products at 0.5 ng/μl with pRF4 at 100 ng/μl. This resulted in four *wht-2(K74M)* lines (*kxEx294-kxEx297*) and five *wht-2(K74R)* lines (*kxEx289-kxEx293*).

kxEx286[wht-2(+); Rol-6^D], *kxEx289[wht-2(K74R); Rol-6^D]*, and *kxEx296[wht-2(K74M); Rol-6^D]* were crossed into a *wht-2(+)* background to assess their effects on gut granule formation.

To knock down the expression of *wht-2*, *mrp-4*, or *rab-7* we used RNA interference (RNAi) feeding protocol 1, described in (Kamath *et al.* 2001), and clones from the Ahringer RNAi library (Source Bioscience, Nottingham, UK). The effects of RNAi targeting these genes were not seen in embryos treated with *F33E2.4/inert* (RNAi), which targets a gene not required for gut granule biogenesis. In RNAi experiments, inhibition of *wht-2* activity was confirmed by the loss of birefringent granules or the loss of the *WHT-2* protein by immunofluorescence microscopy. Inhibition of *mrp-4* activity was confirmed by the loss of birefringent granules. Inhibition of *rab-7* was confirmed by the appearance of many large refractile bodies with DIC microscopy.

To generate the integrated *kxIs5 mans::mCherry* reporter, we PCR-amplified a 292 bp fragment encoding the first 82 amino acids of *AMAN-2* from N2 genomic DNA with P1061 5'GGGGACAACCTTTGTACAAAAAGTTGTGGGAAAA CGCAATTTCTATATTATCCTATG3' and P1062 5'GGGGACAA CTTTGTACAAGAAAGTTGTTTCTTCATCAAAATCTACCGATT TTTTCAG3' (the italicized sequences hybridize with *aman-2*). A BP Gateway reaction was used to clone this into pDONR221 to generate pCPM5. An LR Gateway reaction was used to insert the mans fragment into VHA-6_mCherry_GTWYB(Asp718) to generate pCPM8 [*vha-6p::mans::mCherry::let-858 3'UTR*]. A PCR fragment containing the *glo-1* promoter was amplified from genomic DNA with P231 5'AACCCAAGCTTCCGTATCTTCTCTCTTATTTTCGAC CG3' and P1063 5'AATATAGAAATTGCGTTTTTCCCATTTT GTTCTGAATATATATTTAAATAG3'. It was fused with a PCR fragment amplified from pCPM8 with P1064 5'ATGGGA AAACGCAATTTCTATATTATCCTATG3' and P570 5'GGATTC TAGACTAGTTTTCTCTCTCC3' using P265 5'ATAATGGGA ACCTGAAATTAGAAGAGG3' and P598 5'TTTATGTTTTCT TTAAACCTTCCTCC3', as described (Hobert 2002). The resulting PCR product was injected into *unc-119(ed3)* at 0.5 ng/μl along with pDP#MM016b at 100 ng/μl. The resulting extrachromosomal array *kxEx307[glo-1p::mans::mCherry::let-858 3'UTR; unc-119(+)]* was integrated with UV to generate *kxIs5* as described (Mariol *et al.* 2013).

To generate the integrated *kxIs15 gfp::glo-1* reporter, PCR fusion was used to generate a *glo-1p::gfp::glo-1* fragment (Hermann *et al.* 2005), which was injected into *unc-119(ed3)*

at 0.5 ng/μl along with pDP#MM016b at 100 ng/μl. The resulting kxEx325 [*glo-1p::gfp::glo-1; unc-119(+)*] extrachromosomal array was integrated with UV to generate *kxds15* as described (Mariol *et al.* 2013). Both integrated transgenes were backcrossed to N2 three times before use.

CRISPR-Cas9-mediated gene editing was carried out by SunyBiotech to generate *glo-1(syb1102[gfp::glo-1])* and *glo-1(syb1103[gfp::glo-1])*, which precisely inserted *gfp* at the 5' end of the *glo-1* coding sequence. The presence of the insertions was verified using Sanger sequencing (Genewiz). The sequence of the 5' end of *gfp::glo-1* postediting is shown:

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ATG(*)GCAGCACTCACAAATAACGATAAAATTCCTAtAAAG  
TGTGGTAATTGGTGAC
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The * denotes where the *gfp* coding sequence was inserted. The underlined lower case nucleotides denote synonymous mutations that alter the protospacer adjacent motif sites. Both *gfp::glo-1* alleles supported gut granule formation and showed the same pattern of GFP::GLO-1 localization. *glo-1(syb1102[gfp::glo-1])* was used in studies of GFP::GLO-1 localization in adult intestinal cells, *mrp-4(RNAi)* experiments, and *wht-2(syb668[K74M])* and *wht-2(syb745[K74R])* point mutants.

Microscopy

A Zeiss AxioImager.M2 widefield microscope equipped for DIC, polarization, and fluorescence imaging was used in some experiments. An AxioCam MRm digital camera controlled by AxioVision 4.8 software (Zeiss, Thornwood, NY) was used to capture images with this instrument. Confocal fluorescence microscopy utilized a Zeiss LSM710. 100x or 63x Plan-Apochromat 1.4 NA objectives were used to image embryos and a 40x Plan-Apochromat 1.3 NA objective was used to image adults.

Adults were immobilized with 10 mM levamisole (Sigma Aldrich, St. Louis, MO) on 3% agarose pads. A Zeiss 38 filter (GFP, excitation, BP 470/40; emission, BP 525/50), a Zeiss 45 filter (mCherry, excitation, BP 560/40; emission, BP 630/75), or a 488 laser line was used to image autofluorescent gut granules. Maximum intensity projections of Z-stacks spanning half of or the entire depth of the intestine are shown. A Zeiss 45 filter was used to visualize gut granules in adults expressing GFP-tagged proteins.

Living embryos were placed in H₂O on 3% agarose pads. To acquire images of immobilized pretzel stage embryos, excess respiring OP50 bacteria was added to induce hypoxia. Polarization optics were used to visualize birefringent material. Maximum intensity projections of Z-stacks of the intestine documenting all of the birefringent material are shown. 405, 488, and 561 laser lines were used to image autofluorescent, GFP, and mCherry markers in living 1.5-fold stage embryos, respectively. To determine the activity of the *wht-2* promoter, we analyzed *gfp* expression of *sls10428[wht-2p::gfp; dpy-5(+)]* in strain BC11858. In this transgene, 2445 bp of promoter 5' to the start codon of *wht-2* drives the expression of *gfp* (McKay *et al.* 2003). GFP was imaged

in living animals using widefield microscopy with a Zeiss 38 fluorescence filter. Nile Red staining of embryos was performed as previously described (Schroeder *et al.* 2007). Acridine Orange staining of acidified organelles was performed as previously described (Hermann *et al.* 2005). The signals from both vital dyes were captured using widefield fluorescence microscopy with a Zeiss 45 filter.

Embryos were freeze-cracked and fixed in -20° methanol as described (Leung *et al.* 1999). The intrinsic fluorescence of GFP or anti-GFP clones 7.1/13.1 (Sigma Aldrich) were used to visualize the distribution of GFP-tagged proteins after fixation. Antibodies to GLO-1 (Hermann *et al.* 2005), LMP-1 (Hadwiger *et al.* 2010), PGP-2 (Schroeder *et al.* 2007), RAB-5 (Audhya *et al.* 2007), RAB-7 (Chen *et al.* 2010), and WHT-2 (this work) were used. Intestinal cells were dissected from adults in 1 × PBS + 10 mM levamisole and then fixed for 10 min in 1 × PBS + 1% paraformaldehyde at room temperature. They were postfixed in -20° 100% methanol for 5 min, blocked in 1 × PBS + 0.1% Tween 20 + 20 μM BSA, and stained with antibodies to GFP (clones 7.1/13.1), PGP-2, and WHT-2 overnight at 4°. Confocal microscopy was used to acquire Z-stacks spanning the intestine of all immunofluorescent samples. Z-stacks through the intestine of fixed LMP-1::GFP expressing embryos were imaged using confocal microscopy. The identity of individual cells can be determined using the plasma membrane localization of LMP-1::GFP. The number of lysosomes located within the four cells that make up Int two and three were manually scored. Zeiss Zen Blue 2012 software was used to measure the diameter of organelles stained with CDF-2::GFP, anti-LMP-1, or anti-PGP-2. Only the diameter of CDF-2::GFP organelles that also contained PGP-2 were measured in *glo-3(kx38)* containing strains, as CDF-2::GFP is mislocalized to non-PGP-2 containing lysosomes in *glo-3(kx38)* mutants (Morris *et al.* 2018). LMP-1 containing organelles that lacked the gut granule marker PGP-2 were measured to determine endolysosome size.

To identify embryos that expressed WHT-2(+) or WHT-2 point mutants from extrachromosomal arrays, which are transmitted to only a subset of progeny, CDF-2::GFP-expressing strains containing the arrays were stained with anti-WHT-2 antibodies. Only embryos that had high levels of WHT-2 signal (much higher than what is seen with anti-WHT-2 staining in wild-type embryos), due to the presence of the array, mislocalized CDF-2::GFP. In anti-PGP-2-staining experiments, the mislocalization of CDF-2::GFP was used as an indicator for the presence of the array.

For colocalization studies, two- or three-channel confocal Z-stacks were acquired and analyzed. In some experiments, randomly selected organelles labeled by one marker were manually scored for the presence of a second marker. Markers were scored as colocalizing when signals overlapped by >50%. The mean colocalization shown in the graphs represents the average percent colocalization per embryo. To computationally score colocalization within entire Z-stacks, SQUASSH software was used to segment and identify every

stained organelle within an embryo. C_{size} represents the fraction of the total volume of one marker that overlapped with the second marker within a single embryo (Rizk *et al.* 2014). For example, C_{size} (PGP-2_{GFP::GLO-1}/PGP-2) refers to the area of PGP-2 that overlapped with GLO-1 divided by the total area of PGP-2, and represents the proportion of PGP-2 that is marked by GFP::GLO-1.

Microsoft Excel for Mac 2011 was used to carry out one-way ANOVAs. Bonferroni or Tukey–Kramer *post hoc* tests were used when making three or more comparisons. Dot plots were made with R (version 3.1.2) and Beeswarm (version 0.1.6) and bar graphs were generated with Excel for Mac 2011. Figures were constructed with Photoshop CS2 and brightness and contrast adjustments were uniformly applied to each panel. Representative images used to determine marker colocalization and organelle presence, number, or size are shown.

WHT-2 antibodies

Affinity-purified rabbit anti-WHT-2 antibodies were generated against cys-321EKSDEGQRPSEHK or cys-599YAGLALRVSKAR peptides by Bethyl Laboratories (Montgomery, TX). Both antisera stained gut granules in *C. elegans* embryos; however, the WHT-2 (321) antibody exhibited less nonspecific signal than the WHT-2 (599) antibody, and therefore was used for the studies presented.

Data availability

Strains, plasmids, and antibodies are available upon request. All of the data necessary for confirming the conclusions of this article are present within the tables and figures. Supplemental material available at figshare: <https://doi.org/10.25386/genetics.11374104>.

Results

Formation of birefringent gut granules in *wht-2*(–) null mutants

We previously identified *wht-2* in a screen for ABC proteins functioning in the formation of birefringent gut granules. *wht-2*(RNAi) causes a reduction in the number of birefringent organelles but does not cause any other obvious defects in gut granule formation (Currie *et al.* 2007), possibly as a result of incomplete penetrance of *wht-2*(RNAi). Since our initial characterization, two different mutations predicted to disrupt *wht-2*(+) activity have been isolated (*C. elegans* Deletion Mutant Consortium 2012; Thompson *et al.* 2013). ABC transporters have two essential functional domains: an ATPase domain that powers membrane transport, and a transmembrane domain, consisting of six membrane spanning alpha helices, that mediates substrate binding and transport (Ford and Beis 2019). The *wht-2*(ok2775) deletion removes a substantial portion of the transmembrane domain of WHT-2, and the *wht-2*(gk891224) point mutation introduces a stop codon prior to both the ATPase and transmembrane domains of WHT-2 (Figure 1A). Both mutations are expected to

abolish WHT-2 function, and thus, likely represent null alleles. Similar to *wht-2*(RNAi), both *wht-2*(–) alleles showed decreased numbers of birefringent granules in pretzel stage embryos (Figure 1B and Table 1). This phenotype was rescued in many *wht-2*(ok2775) mutant embryos by the introduction of extrachromosomal arrays containing *wht-2*(+) on a fosmid, as well as a PCR product containing just *wht-2*(+) and adjacent regulatory sequences (Table 1). The proportion of embryos that were rescued corresponds to the proportion of progeny receiving the array from their parent, indicating that *wht-2* function promotes the formation of birefringent granules.

Pretzel stage *wht-2*(gk891224) and *wht-2*(ok2775) mutant embryos exhibited wide variation in the number of birefringent granules, ranging from none to nearly wild-type levels (Table 1). To investigate what caused this variability, we carried out a time course analysis of birefringent granule number in individual embryos. Our scoring started at the 1.5-fold stage, immediately prior to the pretzel stage, which spans the last 5 hr of embryogenesis. Wild-type embryos always displayed >50 birefringent intestinal granules at each of the time points (Figure 1C). All of the *wht-2*(ok2775) embryos initially lacked birefringent material and the proportion of embryos lacking birefringent granules gradually decreased, such that by the end of embryogenesis, the majority of the embryos contained between 1 and 50 birefringent intestinal granules (Figure 1C). Near the end of embryogenesis, birefringent material often became mislocalized to the intestinal lumen in the *wht-2*(–) mutants (Figure 1, B and C). These results indicate that variation in birefringent gut granule number in pretzel stage *wht-2*(–) embryos is caused by a developmentally linked delay in their formation.

wht-2(–) is the only ABCG transporter mutant that causes defects in the formation of birefringent gut granules

WHT-2 is a member of the ABCG subfamily of ABC transporters (Sheps *et al.* 2004). Like many other ABCG proteins, it is a half-transporter and likely must homo- or heterodimerize with another ABCG protein to function as a transporter (Tarr *et al.* 2009). Strains with deletions predicted to disrupt the function of each of the other seven ABCG genes encoded by the *C. elegans* genome (*wht-1* and *wht-3* to *wht-8*) did not show any alteration in the number of birefringent gut granules resembling *wht-2*(–) (Supplemental Material, Table S1). Although we cannot rule out the possibility that multiple ABCG transporters function redundantly to promote gut granule formation, we think it is unlikely that WHT-2 heterodimerizes with another ABCG transporter to generate birefringent gut granules.

WHT-2 has a partially redundant role in gut granule biogenesis

wht-2(–) mutants accumulate birefringent material within the intestinal lumen (Figure 1B). To date, all of the mutants that mislocalize birefringent material to the intestinal lumen

Figure 1 Birefringent gut granules in *wht-2(-)* mutants. (A) Schematic of WHT-2 showing the location of the conserved K74 amino acid in the Walker A motif, ATPase, and transmembrane spanning alpha helices. Position of the null alleles and the location of peptides used to generate anti-WHT-2 antibodies are shown. The amino acid position of each feature is noted. (B) Pretzel stage embryos were analyzed with polarization microscopy to detect birefringent material. Unlike wild type, *wht-2(-)* mutants had reduced numbers of birefringent compartments in intestinal cells (white arrows) and often misaccumulated birefringent material within the intestinal lumen (between the black arrows). Black arrowheads flank the

intestine and embryos are 50 μ m in length. (C) Individual embryos were examined with polarization microscopy every 60 min, starting at the 1.5-fold stage (460 min postfertilization). The number of birefringent compartments within intestinal cells and the presence of birefringent material within the intestinal lumen were scored at each time point.

have been shown to lack or have significantly altered gut granules (Levitte *et al.* 2010; Hermann *et al.* 2012; Delahaye *et al.* 2014). However, *wht-2(-)* mutants often contain birefringent material within their intestinal cells (Figure 1B and Table 1). To investigate whether this material accumulates within gut granules or within a different organelle, we combined *wht-2(-)* with mutants that do not generate gut granules. Double mutants containing *wht-2(-)* and *ccz-1(-)*, *glo-1(-)*, *glo-3(-)*, or *vps-18(-)* had significantly fewer birefringent granules within embryonic intestinal cells than *wht-2(-)* single mutants (Table 1), strongly suggesting that birefringent material accumulates within gut granules in *wht-2(-)* mutants.

We examined whether gut granules were present within *wht-2(-)* mutant embryos by analyzing the localization of PGP-2, a ABCB subfamily transporter, and CDF-2, a Zn transporter, both of which exclusively associate with gut granule membranes (Schroeder *et al.* 2007; Davis *et al.* 2009). The number and morphology of PGP-2- and CDF-2::GFP-marked compartments and the colocalization of these two proteins was similar in *wht-2(-)* mutants and wild type (Figure 2, A and F). LMP-1, the *C. elegans* LAMP-1 ortholog (Kostich *et al.* 2000), is associated with gut granules and conventional lysosomes (Hermann *et al.* 2012). Its colocalization with PGP-2 was also unaffected by loss of *wht-2* function (Figure 2, B and G). When gut granule protein localization pathways are disrupted, CDF-2::GFP mistrafficks through the endolysosomal pathway and accumulates at conventional lysosomes (Hermann *et al.* 2012). We analyzed the colocalization of CDF-2::GFP with an mCherry-tagged form of ASM-1, a lysosomal acid sphingomyelinase (Lin *et al.* 1998; Guo and Wang 2010; Levitte *et al.* 2010), in *wht-2(-)* mutants and found that CDF-2::GFP was not mislocalized to conventional lysosomes (Figure 2, C and H). Furthermore, CDF-2::GFP did not accumulate on RAB-5 containing early endosomes or RAB-7-labeled endolysosomes in *wht-2(-)* mutants (Figure S1).

Gut granules contain lipases that function in lipid metabolism and gut granules are stained by the lipophilic dye Nile Red (Schroeder *et al.* 2007; O'rourke and Ruvkun 2013). Because of the H⁺ transport activity of the V-ATPase, gut granules are also marked by the acidophilic dye Acridine Orange (Clokey and Jacobson 1986; Oka and Futai 2000; Hermann *et al.* 2005). Staining with these markers of gut granule activity was not disrupted by *wht-2(ok2775)* (Figure 2, D and E).

Together these data indicate that gut granules are generated independently of WHT-2 function, albeit with defects in the formation and localization of birefringent material. However, our results do not rule out the possibility that WHT-2 promotes processes that are complementary to, or redundant with, other pathways mediating gut granule biogenesis. To determine whether WHT-2 makes contributions to LRO biogenesis that are only apparent when gut granule formation is partially disrupted, we analyzed the number of birefringent gut granules in double mutants between *wht-2(ok2775)* and *apt-7(tm920)*, *glo-2(tm592)*, *glo-3(kx38)*, and *lyst-1(gk295717)* mutants. Malformed gut granules containing birefringent material are present in *apt-7(tm920)* mutants (Hermann *et al.* 2012), which lack AP-3 complex function; *glo-2(tm592)* mutants, which partially disrupt the function of the BLOC-1 complex (Hermann *et al.* 2012); *glo-3(kx38)* mutants, which partially disrupt the function of the proposed GEF for the GLO-1 Rab (Morris *et al.* 2018); and *lyst-1(gk295717)* mutants, which disrupt the function of the *C. elegans* homolog of LYST and NBEAL2, and are mutated in Chediak-Higashi and gray platelet syndrome, respectively (Barrett and Hermann 2016) (Table 1). Notably, the addition of *wht-2(-)* to each of these mutants led to significantly fewer birefringent gut granules than the single mutants (Table 1).

To determine whether *wht-2(-)* only altered the formation of birefringent material, we analyzed the distribution of gut granule proteins in the *wht-2(-)* containing double

Table 1 Birefringent gut granules in *wht-2(-)* single and double mutant embryos

Genotype	% of embryos with the specified number of birefringent granules in intestinal cells				n
	0	1–10	11–50	>50	
Wild type	0	0	0	100	52
<i>wht-2(ok2775)</i>	20	38	40	2	121
<i>wht-2(gk891224)</i>	24	43	31	2	161
<i>wht-2(RNAi)</i>	2	21	42	35	43
<i>wht-2(syb668[K74M])^a</i>	0	28	22	50	171
<i>wht-2(syb745[K74R])^a</i>	0	41	23	36	170
<i>wht-2(ok2775) + WRM0622AF09^b</i>	0	11	25	64	47
<i>wht-2(ok2775) + wht-2(+)^c</i>	23	23	14	40	52
<i>apt-7(tm920)</i>	12	60	28	0	50
<i>apt-7(tm920); wht-2(ok2775)^d</i>	49	47	4	0	51
<i>ccz-1(ok2182)</i>	65	35	0	0	86
<i>ccz-1(ok2182); wht-2(ok2775)</i>	59	40	1	0	98
<i>glo-1(zu437)</i>	98	2	0	0	40
<i>glo-1(zu437); wht-2(ok2775)</i>	98	2	0	0	40
<i>glo-2(tm592)</i>	0	0	2	98	47
<i>glo-2(tm592); wht-2(ok2775)^d</i>	86	14	0	0	50
<i>glo-3(kx38)</i>	0	18	82	0	50
<i>glo-3(kx38); wht-2(ok2775)^d</i>	96	4	0	0	50
<i>glo-3(kx38); wht-2(syb668[K74M])^d</i>	71	25	4	0	80
<i>glo-3(kx38); wht-2(syb745[K74R])^d</i>	60	39	1	0	75
<i>glo-3(kx94)</i>	87	13	0	0	170
<i>glo-3(kx94); wht-2(ok2775)</i>	95	5	0	0	40
<i>lyst-1(gk295717)</i>	2	40	52	6	50
<i>lyst-1(gk295717); wht-2(ok2775)^d</i>	98	2	0	0	50
<i>sand-1(or552)</i>	0	0	10	90	50
<i>sand-1(or552); wht-2(RNAi)</i>	12	46	6	36	89
<i>vps-18(tm1125)</i>	96	4	0	0	50
<i>vps-18(tm1125); wht-2(ok2775)</i>	92	6	2	0	50

All strains were grown at 22°C. Threefold and later stage embryos were analyzed using polarization microscopy and scored for the number of birefringent granules in the intestine.

^a The *wht-2* point mutants displayed significantly more birefringent gut granules than the *wht-2(ok2775)* null allele ($P < 0.001$, Fisher's exact test).

^b Embryos scored were the progeny of adults that carried an extrachromosomal array containing the *wht-2(+)* fosmid WRM0622AF09.

^c Embryos scored were the progeny of adults that carried an extrachromosomal array containing the *wht-2(+)* PCR product.

^{b,c} In both cases, the extrachromosomal array will be present in only a fraction of the progeny.

^d The double mutant displays significantly less birefringent gut granules than either of the single mutants used to construct it ($P < 0.001$, Fisher's exact test).

mutants. We found that gut granule proteins were mislocalized when *wht-2(-)* was combined with *lyst-1(-)* and *glo-3(-)*. CDF-2::GFP nearly always colocalized with PGP-2 in *wht-2(-)* and *lyst-1(-)* single mutants (Figure 2, A and F). However, in the double mutant, CDF-2::GFP became mislocalized to the plasma membrane and to organelles lacking PGP-2, which are likely to be conventional lysosomes based on their apical position, morphology, and number (Figure 2A). The localization of LMP-1 to PGP-2-marked gut granules was normal in *wht-2(-)* mutants, partially disrupted in *glo-3(kx38)* mutants, and LMP-1 was rarely detected on PGP-2 compartments in *wht-2(-); glo-3(kx38)* double mutants (Figure 2, B and G). *wht-2(-)* also led to a significant effect on *glo-3(kx38)* gut granule size. Although *glo-3(kx38)*, and to a lesser extent *wht-2(-)*, caused enlarged gut granules, the diameter of gut granules in the double mutant was wild type (Figure 2, B and I).

The restoration of birefringent material in *wht-2(-)* mutants as development proceeds led us to analyze the effects of *wht-2(-)* on gut granules in later stage animals. Gut granule numbers increase during larval development, and gut granules in adult intestinal cells are filled with autofluorescent

material (Clokey and Jacobson 1986; Hermann *et al.* 2005). The number, distribution, and morphology of autofluorescent gut granules in adults were not obviously affected by either *wht-2(-)* mutation (Figure 2J and Table 2). The autofluorescent material present in *wht-2(-)* adults is likely contained within gut granules, as it was lacking when *wht-2(-)* was combined with mutations in *ccz-1(-)*, *glo-1(-)*, and *glo-3(-)*, which are required for the formation of adult gut granules (Table 2). To determine whether WHT-2 plays a role in adult gut granule formation that is masked by the function of other factors, we combined *wht-2(-)* with gut granule biogenesis mutants that retain autofluorescent gut granules. We observed significant reductions in adult autofluorescent gut granule number when *wht-2(ok2775)* was combining with mutations in *lyst-1*, *glo-3*, and the HOPS subunit *vps-18* (Delahaye *et al.* 2014) (Figure 2J and Table 2). For example, while both *wht-2(-)* and *vps-18(-)* single mutants always displayed wild-type numbers of autofluorescent adult gut granules, many *wht-2(-); vps-18(-)* double mutants had few autofluorescent compartments (Figure 2J and Table 2). Based on our analysis of double mutants containing *wht-2(-)*, we conclude that WHT-2 makes important

Figure 2 Effects of *wht-2(-)* on gut granules. (A and B) 1.5-Fold stage embryos were stained with anti-PGP-2 antibodies, anti-LMP-1 antibodies, or ectopically expressed CDF-2::GFP and imaged with confocal microscopy. In insets, white arrows label compartments containing two markers, compartments lacking one of the markers are denoted with white arrowheads, and in A, black arrows denote CDF-2::GFP localized to the cell surface. (C) Living 1.5-fold stage embryos expressing CDF-2::GFP and ASM-1::mCherry were imaged with confocal microscopy. In the insets, CDF-2::GFP containing compartments that lacked the lysosomal hydrolase are marked with white arrowheads. (D and E) Pretzel stage embryos were stained with lipophilic (Nile Red) and acidotropic (Acridine Orange) dyes. Similarly labeled compartments (white arrows) were observed in *wht-2(-)* mutants and wild type. (A–E) In all panels, black arrowheads flank the intestine and single optical sections are shown. (F and G) For each genotype, at least 30 randomly selected CDF-2::GFP or PGP-2 containing organelles in three to five different embryos were scored for the presence of anti-PGP-2 or anti-LMP-1 signals. The mean is plotted and error bars represent the 95% confidence limit. One-way ANOVA comparing mutants to wild type or double mutants to single mutants was used to calculate *P* values (* *P* ≤ 0.05 and ** *P* ≤ 0.001). (H) SQUASSH software was used to calculate *C_{size}* (CDF-2::GFP_{ASM-1::mCherry}/CDF-2::GFP), which represents the proportion of total CDF-2::GFP area in each embryo that also contained the ASM-1::mCherry signal. Five to nine 1.5-fold stage embryos were analyzed. The mean is plotted and error bars represent the 95% confidence limit. One-way ANOVA was used to

compare the mutant to wild type (ns represents *P* > 0.05). (I) Anti-PGP-2 and CDF-2::GFP signals in 1.5-fold stage embryos were acquired using confocal microscopy. The diameters of 15–30 CDF-2::GFP compartments that also contained PGP-2 were measured in at least five embryos. All of the diameters are plotted and the red lines denote the mean and 95% confidence interval. Strains were compared to each other by one-way ANOVA (ns represents *P* > 0.05, * *P* ≤ 0.05, and ** *P* ≤ 0.001). (J) Adults were analyzed for autofluorescent compartments (white arrows) with confocal microscopy. Maximum intensity projections spanning the entire width of the intestine are shown and black arrows mark the location of the intestinal lumen.

contributions to gut granule biogenesis that extend beyond the generation of birefringent material and that *WHT-2* functions overlap with the activity of conserved factors that promote LRO biogenesis.

wht-2(-) does not disrupt conventional endosome biogenesis

Many of the proteins that function in the biogenesis of LROs also act in the formation of conventional lysosomes

(Dell'angelica 2009; Bultema and Di Pietro 2013; Marks *et al.* 2013). We used a number of different assays to investigate whether *WHT-2* had a significant role in the biogenesis of lysosomes. Defects in trafficking to conventional lysosomes lead to an increased number of LMP-1::GFP-marked endolysosomes (Xiao *et al.* 2009; Delahaye *et al.* 2014), which we did not observe in *wht-2(-)* mutants (Figure 3, A and B). Similarly, neither the morphology nor position of RAB-5-labeled early endosomes and RAB-7-labeled endolysosomes

Table 2 Autofluorescent gut granules in *wht-2(-)* single and double mutant adults

Genotype	% of animals with the specified number of autofluorescent organelles in the anterior intestine					n
	0	1–50	51–100	101–200	>200	
Wild type	0	0	0	0	100	45
<i>wht-2(ok2775)</i>	0	0	0	0	100	45
<i>wht-2(gk891224)</i>	0	0	0	0	100	25
<i>wht-2(RNAi)</i>	0	0	0	0	100	20
<i>wht-2(syb668[K74M])</i>	0	0	0	0	100	30
<i>wht-2(syb745[K74R])</i>	0	0	0	0	100	27
<i>apt-7(tm920)</i>	84	16	0	0	0	25
<i>apt-7(tm920); whe-2(ok2775)</i>	4	76	20	0	0	25
<i>ccz-1(ok2182)</i>	67	33	0	0	0	49
<i>ccz-1(ok2182); whe-2(ok2775)</i>	47	53	0	0	0	45
<i>glo-1(zu437)</i>	71	29	0	68	32	20
<i>glo-1(zu437); whe-2(ok2775)</i>	75	25	4	96	0	20
<i>glo-2(tm592)</i>	0	0	0	68	32	25
<i>glo-2(tm592); whe-2(ok2775)</i>	0	0	4	96	0	25
<i>glo-3(kx38)</i>	0	31	61	8	0	26
<i>glo-3(kx38); whe-2(ok2775)</i>	0	0	16	76	8	25
<i>glo-3(kx94)</i>	15	65	20	0	0	20
<i>glo-3(kx94); whe-2(ok2775)^a</i>	100	0	0	0	0	20
<i>lyst-1(gk295717)</i>	0	48	40	12	0	25
<i>lyst-1(gk295717); whe-2(ok2775)^a</i>	64	36	0	0	0	25
<i>sand-1(or552)</i>	0	0	0	9	91	23
<i>sand-1(or552); whe-2(RNAi)</i>	0	0	0	0	100	70
<i>vps-18(tm1125)</i>	0	0	0	0	100	33
<i>vps-18(tm1125); whe-2(ok2775)^a</i>	12	68	20	0	0	25

All strains were grown 22°C. Adult stage animals were analyzed using fluorescence microscopy with a Zeiss 38 filter set and were scored for the number of autofluorescent organelles within the intestine located anterior to the vulva.

^a The double mutant displays significantly less autofluorescent gut granules than either of the single mutants used to make it ($P < 0.001$, Fisher's exact test).

were obviously changed by disrupting *wht-2(+)* function (Figure 3A and Figure S1). *RAB-7* dynamically associates with endolysosomes, and defects in trafficking to lysosomes can alter its steady-state distribution relative to *LMP-1::GFP* (Chotard *et al.* 2010; Poteryaev *et al.* 2010). Approximately 50% of *LMP-1::GFP* containing endolysosomes were marked by *RAB-7* in wild-type embryos, and this was not significantly altered in *wht-2(-)* mutants (Figure 3, A and C). *cup-5(+)*, which encodes a TRPML1 homolog disrupted in mucopolidiosis type IV, promotes the reformation of lysosomes following their fusion with late endosomes (Fares and Greenwald 2001; Hersh *et al.* 2002; Treusch *et al.* 2004). As a result, *cup-5(zu223)* mutants display enlarged *LMP-1*-marked endolysosomes (Figure 3, D and E). Attenuating the delivery of material to lysosomes can suppress *cup-5(zu223)*, leading to smaller endolysosomes (Nakae *et al.* 2010), providing an assay for lysosomal trafficking in embryonic intestinal cells (Morris *et al.* 2018). To further validate the assay, we examined *mrp-4(-)*, which reduces the size of LysoTracker stained organelles in *cup-5(zu223)* mutants (Schaheen *et al.* 2006). *mrp-4*, like *wht-2*, encodes an ABC transporter that is required for the accumulation of birefringent material within gut granules (Currie *et al.* 2007). We found that combining *mrp-4(cd8)* with *cup-5(zu223)* led to a significant reduction in endolysosome diameter (Figure 3, D and E), consistent with a role for *MRP-4* in the formation of conventional lysosomes. In contrast,

endolysosomes remained enlarged in *cup-5(-); whe-2(-)* double mutants, and while being slightly smaller than *cup-5(-)* single mutants (Figure 3, D and E), their diameter was similar to what has been seen in other gut granule biogenesis mutants (Morris *et al.* 2018). Together, these data strongly suggest that *WHT-2* does not make significant contributions to conventional lysosome biogenesis.

WHT-2 is expressed in intestinal cells and localizes to gut granules

Our phenotypic analyses point to a role for *wht-2(+)* in gut granule, but not endolysosome biogenesis. Gut granules are only found in intestinal cells, while endolysosomes are present in most, if not all, *C. elegans* somatic cells. To determine which cells express *wht-2*, we placed *gfp* under the control of the *wht-2* promoter and found that GFP expression in embryos was limited to intestinal cells, first being detected when the intestinal primordium was composed of four cells (E4 stage) (Figure 4A). The activity of the *wht-2* promoter persisted in intestinal cells through embryogenesis into adulthood (Figure 4A). In adults, the *wht-2* promoter was also active in a few unidentified head neurons located near the pharynx (Figure 4A).

To determine how directly *WHT-2* might function in gut granule biogenesis, we generated two different anti-*WHT-2* peptide antibodies and used them to analyze the subcellular distribution of *WHT-2*. Both *WHT-2* antisera labeled gut

Figure 3 Lysosome formation in *wht-2(-)* mutants. (A) Embryos expressing LMP-1::GFP were stained with anti-RAB-7 antibodies and imaged with confocal microscopy. RAB-7 similarly associates with many (white arrows in insets), but not all (white arrowheads in insets), LMP-1::GFP-marked compartments in wild-type and *wht-2(-)* 1.5-fold stage embryos. (B) The total number of LMP-1::GFP compartments was quantified in the four cells that compose Int2 and Int3 (marked with white asterisks) in 10 different 1.5-fold stage embryos of each genotype. (C) Colocalization was scored in five embryos of each genotype by randomly selecting 17–25 LMP-1::GFP organelles in each embryo and assessing the presence the RAB-7 signal. (D) 1.5-Fold stage embryos were stained with anti-LMP-1 and anti-PGP-2 antibodies and imaged with confocal microscopy. Endolysosomes are marked by LMP-1 and lack PGP-2 (white arrows in insets). (E) For each genotype, the diameters of 20 randomly selected endolysosomes were measured in 5–9 1.5-fold stage embryos. In all images, a single optical section is shown and black arrowheads flank the intestine. In all graphs, the mean is plotted and bars represent the 95% confidence limit. One-way ANOVAs were used to compare different genotypes (ns represents $P > 0.05$, $** P \leq 0.001$) and in E, the ANOVA was followed by a Tukey–Kramer *post hoc* test.

granules marked with CDF-2::GFP in embryos (Figure 4, B and C and Figure S2) and WHT-2 was associated with gut granules in adult intestinal cells (Figure S3). The gut granule signal was lacking in both *wht-2(ok2775)* and *wht-2(gk891224)* mutants, indicating the specificity of both antisera toward WHT-2 (Figure 4B and Figure S2).

WHT-2 promotes the gut granule localization of GLO-1

Our double mutant analysis showed that *wht-2* and *glo-3* have partially redundant functions in gut granule biogenesis

(Figure 2, B and G, Table 1, and Table 2). We have recently shown that GLO-3 promotes the association of GLO-1 with gut granules in embryonic intestinal cells, likely by functioning with CCZ-1 as a GLO-1 GEF (Morris *et al.* 2018). We therefore analyzed whether *wht-2(-)* mutants disrupted the subcellular distribution of GLO-1. Rab GTPases cycle on and off membranes, which can lead to changes in their association with organelles. Our localization studies show the steady-state distribution of GLO-1; however, they do not measure GLO-1 dynamics and will not reveal low levels of

Figure 4 WHT-2 expression and localization. (A) GFP was expressed under the control of the 2.4 kbp upstream of *wht-2* and imaged with widefield fluorescence and DIC microscopy. GFP was first detected in the four intestinal precursors (E4 stage) and persisted in just intestinal cells through embryogenesis. In adults, GFP was expressed by the intestine and neurons located near the pharynx (white arrows). (B) Signals from anti-WHT-2 (321) antibodies and CDF-2::GFP in 1.5-fold stage embryos were acquired by confocal microscopy. In insets, white and black arrows denote CDF-2::GFP-marked organelles containing or lacking WHT-2, respectively. In all panels, intestinal cells are located between black arrowheads and single optical sections are shown. (C) The area of WHT-2 organelles that contained CDF-2::GFP was determined for six 1.5-fold stage embryos. The mean is plotted and bars represent the 95% confidence limit. One-way ANOVAs were used to compare the mutants to wild type and $P > 0.05$.

GLO-1 organelle association. PGP-2-marked gut granules were present in *wht-2*(-) (Figure 2, A and B), allowing us to investigate the gut granule localization of GLO-1 in this mutant. In fixed wild-type embryos, ectopically expressed GFP::GLO-1, which can functionally replace GLO-1 (Hermann *et al.* 2005), was enriched on gut granules (Figure

5, A and F). However, in *wht-2(ok2775)* mutants, GFP::GLO-1 was rarely associated with PGP-2 containing gut granules and instead was enriched in the cytoplasm (Figure 5, A and F). A similar effect was seen with the *wht-2(gk891224)* allele (Figure 5, A and F), indicating that loss of *wht-2*(+) function disrupts GFP::GLO-1 localization. Using anti-GLO-1

Figure 5 GLO-1 localization is disrupted in *wht-2(-)* mutants. (A) 1.5-Fold stage embryos expressing GFP::GLO-1 were fixed and stained with antibodies to PGP-2 and imaged with confocal microscopy. White and black arrows in the insets denote PGP-2-marked organelles containing or lacking GFP::GLO-1, respectively. (B) 1.5-Fold stage embryos expressing CDF-2::GFP were fixed and stained with anti-GLO-1 antibodies and imaged with confocal microscopy. White and black arrows in the insets denote CDF-2::GFP-marked organelles containing or lacking GLO-1, respectively. A nucleolar background signal is present in anti-GLO-1 staining of *wht-2(-)*. (C) Confocal microscopy was used to image GLO-1::GFP and autofluorescent gut granules in living 1.5-fold stage embryos. White and black arrows in the insets denote autofluorescent gut granules containing or lacking GFP::GLO-1, respectively. (D) 1.5-Fold stage embryos expressing MANS::mCherry and GFP::GLO-1 were fixed and imaged with confocal microscopy. White and black arrows in the insets denote MANS::mCherry-marked organelles containing or lacking GFP::GLO-1, respectively. (E) Anti-PGP-2 and anti-RAB-7 signals were imaged by confocal microscopy in 1.5-fold stage embryos. In insets, black and white arrows mark PGP-2 containing organelles that lack or contain RAB-7, respectively. In some panels, nonspecific P granule staining by secondary antibodies is marked by a P. In A–E, single optical sections are shown and black arrowheads flank the intestine. Single optical sections are shown. (F) The proportion of total PGP-2 area in each embryo that also contained

antibodies, we found that endogenous **GLO-1** was absent from gut granules in the *wht-2(-)* mutant as well (Figure 5, B and G). To rule out the possibility that fixation used during antibody staining alters the association of **GLO-1** with gut granules in *wht-2(ok2775)* mutants, we examined living embryos and found that GFP::**GLO-1** no longer colocalized with gut granule autofluorescence in *wht-2(-)* mutants (Figure 5, C and H). Disrupting the function of **MRP-4**, another ABC transporter that functions in the accumulation of birefringent material within gut granules (Currie *et al.* 2007), did not result in the loss of GFP::**GLO-1** from gut granules (Figure S4), indicating that the loss of ABC transporter function or birefringent material *per se* does not disrupt the steady-state association of **GLO-1** with gut granules. Together these data indicate that **WHT-2** plays an essential role in the gut granule localization of **GLO-1**.

GFP::**GLO-1**, in addition to being dispersed throughout the cytoplasm, was often localized to small puncta in *wht-2(-)* mutants (Figure 5, A and D). The morphology and distribution of the puncta are reminiscent of the Golgi, which in *C. elegans* intestinal cells are distributed throughout the cytoplasm in ministacks (Chen *et al.* 2006; Grant and Sato 2006). We tested the possibility that **GLO-1** was associating with the Golgi by examining the distribution of GFP::**GLO-1** relative to the Golgi marker **AMAN-2**/Mannosidase (**MANS::mCherry**) (Rolls *et al.* 2002). In wild type, GFP::**GLO-1** rarely colocalized with **MANS::mCherry** (Figure 5, D and I). In contrast, nearly all of the GFP::**GLO-1** puncta overlapped with the Golgi in *wht-2(-)* mutants (Figure 5, D and I). The association of GFP::**GLO-1** with the Golgi was also seen in *glo-3(kx94)* mutants (Figure 5, D and I), which likely lack **GLO-1** GEF activity (Morris *et al.* 2018). These results suggest that the factors promoting the localization of **GLO-1** to gut granules also prevent its enrichment on the Golgi.

wht-2(-) disrupts the accumulation of birefringent material within embryonic gut granules, whereas adult gut granules in *wht-2(-)* mutants do not display any obvious differences from wild type (Figure 1B and Figure 2J). To examine whether this might be linked to differences in **GLO-1** localization, we analyzed the distribution of GFP::**GLO-1** relative to **PGP-2**-marked gut granules in fixed adult intestinal cells. Similar to embryos, GFP::**GLO-1** was enriched on wild-type adult gut granules (Figure S3). However, *wht-2(-)* mutants did not cause the loss of GFP::**GLO-1** from gut granules in adults (Figure S3). Therefore, *wht-2(+)* is not essential for **GLO-1** localization in adults as it is during embryogenesis, possibly due to the expression of factors within adults promoting the association of **GLO-1** with gut granules that function redundantly with **WHT-2**.

WHT-2 promotes the association of RAB-7 with gut granules in *glo-3(kx38)* mutants

The similar loss of **GLO-1** from gut granules in *wht-2(-)* and *glo-3(-)* mutants led us to examine whether they exhibited any other phenotypic similarities. **RAB-7** does not appreciably associate with gut granules in wild type; however, in *glo-3(kx38)* partial loss-of-function mutants, gut granules often misaccumulate **RAB-7** (Morris *et al.* 2018) (Figure 5, E and J). We found that neither endocytic Rab, **RAB-7**, or **RAB-5** associated with gut granules in *wht-2(ok2775)* mutants (Figure 5, E and J and Figure S1). However, **RAB-7** was absent from gut granules in *glo-3(kx38); wht-2(ok2775)* double mutants (Figure 5, E and J), indicating that **WHT-2** promotes the association of **RAB-7** with gut granules when **GLO-3** activity is impaired. Notably, **WHT-2** was associated with gut granules in *glo-3(kx38)* embryos where it could function in localizing **RAB-7** (Figure 4, B and C).

GLO-3 and CCZ-1 localize to gut granules in *wht-2(-)* mutants

GEF localization in large part determines the targeting of a Rab to a specific cellular membrane (Gerondopoulos *et al.* 2012; Blümer *et al.* 2013). **WHT-2** is positioned on gut granules where it could direct the organelle association of **GLO-1** and **RAB-7** by localizing the **GLO-1** and/or **RAB-7** GEFs, respectively. We examined the localization of **GLO-3** and **CCZ-1** tagged with fluorescent proteins, focusing our analysis soon after the reporters are expressed to minimize potential artifacts from overexpression. The embryos analyzed had 4 (E4) or 8 (E8) intestinal precursors and immediately preceded the stage when we have scored Rab localization.

Consistent with our previous work, **GLO-3::GFP**, which functionally substitutes for **GLO-3** (Rabbitts *et al.* 2008), localized to **PGP-2**-marked gut granules at both stages (Figure 6, A, B, and E). When **WHT-2** function was lacking, **GLO-3** remained localized to gut granules, albeit at slightly reduced levels compared to wild type (Figure 6, A, B, and E).

CCZ-1 likely functions as part of the GEF that regulates both **GLO-1** and **RAB-7** (Kinchin and Ravichandran 2010; Nieto *et al.* 2010; Delahaye *et al.* 2014; Morris *et al.* 2018). To determine whether **CCZ-1** associates with gut granules, we examined the distribution of **CCZ-1::YFP**, which functionally replaces **CCZ-1** in both conventional endolysosome and gut granule biogenesis (Nieto *et al.* 2010; Delahaye *et al.* 2014). **CCZ-1** was enriched on gut granules marked by **PGP-2** in both E4 and E8 stage intestinal cells (Figure 6, C, D, and F). In *wht-2(-)* mutants, we did not detect a difference in the level of **CCZ-1::YFP** association with gut granules at either stage (Figure 6, C, D, and F).

GLO-1::GFP is plotted. At least five embryos of each genotype were scored. (G) The proportion of total **CDF-2::GFP** area in each embryo that also contained **GLO-1** is shown. At least eight embryos of each genotype were scored. (H) The proportion of total autofluorescent gut granule area in each embryo that also contained GFP::**GLO-1** is plotted. At least five embryos of each genotype were scored. (I) The proportion of total GFP::**GLO-1** area in each embryo that also contained **MANS::mCherry** is plotted. At least five embryos of each genotype were scored. (J) In five embryos 15–45 randomly selected **PGP-2** containing organelles were scored for the presence of anti-**RAB-7** signals. In F–J, the mean level of colocalization is shown, bars are the 95% confidence intervals, and ** indicates $P \leq 0.001$ by one-way ANOVA, comparing the indicated genotypes.

Figure 6 *WHT-2* is not required for the gut granule association of *CCZ-1* and *GLO-3*. (A–D) E4 or E8 stage embryos expressing *GLO-3::GFP* or *CCZ-1::YFP* were stained with antibodies to GFP/YFP and *PGP-2* and imaged with confocal microscopy. White and black arrows in the insets denote *PGP-2*-marked organelles containing or lacking the GFP/YFP-tagged protein, respectively. In all images, black arrowheads flank the intestine. Single optical sections are shown. (E) The proportion of total *PGP-2* area in each embryo that also contained *GLO-3::GFP* is plotted. At least seven embryos of each genotype were scored. (F) The proportion of total *PGP-2* area in each embryo that also contained *CCZ-1::YFP* is plotted. At least five embryos of each genotype were scored. In E and F, the mean is plotted, bars represent the 95% confidence interval. Mutants were compared to wild type by one-way ANOVA; ** $P \leq 0.001$, * $P \leq 0.05$, and ns indicates $P > 0.05$.

To determine whether the localization of the *GLO-1* GEF is affected by disrupting the function of one of its subunits, we analyzed the distribution of *CCZ-1* in *glo-3(-)* mutants. Strong *glo-3(-)* alleles lack gut granules, so we analyzed *CCZ-1::YFP* localization in *glo-3(kx38)* and *glo-3(gk582755)* partial loss-of-function alleles, which contain gut granules in 1.5-fold stage embryos (Morris *et al.* 2018). *PGP-2*-labeled gut granules were lacking from E4 stage intestinal cells in these mutants and *CCZ-1::YFP* was localized to the cytoplasm. At the E8 stage, gut granules were present, but they lacked *CCZ-1::YFP*. These results show that *GLO-3*, unlike *WHT-2*, is required for the association of *CCZ-1* with gut

granules, which is similar to the role that *SAND-1* plays in localizing *CCZ-1* to activate *RAB-7* (Nieto *et al.* 2010).

GLO-1(D132A) localizes to gut granules independently of *WHT-2*

GLO-3 and *CCZ-1* physically associate and act as the GEF to activate and localize *GLO-1* to gut granules (Morris *et al.* 2018). If *WHT-2* similarly functions in guanine nucleotide exchange, then *GLO-1* would be GDP bound in *wht-2(-)* mutants. The *GLO-1(D132A)* point mutation, which is predicted to weaken the affinity of *GLO-1* for guanine nucleotides and increase its rate of nucleotide exchange, partially

bypasses the requirement of the GLO-1 GEF for the localization of GLO-1 to gut granules; in *glo-3(-)* mutants, GLO-1(+) is lacking from gut granules, whereas GLO-1(D132A) can associate with these organelles (Morris *et al.* 2018). While GLO-1(+) was absent from gut granules in *whit-2(-)*, GLO-1(D132A) often colocalized with PGP-2-marked gut granules in *whit-2(-)* mutants (Figure 7, A and B). This indicates that GLO-1(D132A) expression can partially bypass the requirement of WHT-2 in localizing GLO-1, and suggests that the loss of GLO-1 from gut granules in *whit-2(-)* mutants is due to defective GLO-1 activation. However, the level of association with gut granules was variable, ranging from moderate to very little, possibly due to differences in the amount of GLO-1(D132A), which was expressed from an extrachromosomal array. On average, the localization of GLO-1(D132A) to gut granules in *whit-2(-)* mutants never reached the level seen when GLO-1(D132A) was expressed in *glo-1(-)* mutants (Figure 7, A and B), and instead was comparable to the level of association seen in *glo-3(-)* and *ccz-1(-)* mutants (Figure 7, A and B). These results indicate that while WHT-2 and the GLO-1 GEF are not absolutely necessary for the localization of GLO-1(D132A), they do contribute to its association with gut granules.

The similarities between *whit-2(-)* and the GLO-1 GEF mutants were also seen in the effect of GLO-1(D132A) expression on the gut granule localization of LMP-1. Expression of GLO-1(D132A), similar to GLO-1(+), supported the association of LMP-1 with gut granules in *glo-1(-)* mutants (Figure S5). In contrast, GLO-1(D132A) did not support the steady state association of LMP-1 with gut granules in *glo-3(-)* and *ccz-1(-)* mutants (Morris *et al.* 2018). Similarly, LMP-1 was reduced or lacking from gut granules in *whit-2(-)* mutants expressing GLO-1(D132A) (Figure S5). Although the GLO-1 fast exchange point mutant can functionally replace GLO-1 (Morris *et al.* 2018), it is likely to act differently than its wild-type counterpart. We therefore think that the defect in LMP-1 trafficking in *whit-2(-)* and GLO-1 GEF mutants results from GLO-1(D132A) being expressed in mutant backgrounds that disrupt the association of GLO-1 with gut granules.

WHT-2 functions independently of GLO-3 and CCZ-1

Given the similar roles of WHT-2 and the GLO-1 GEF in GLO-1 localization, we further explored the functional relationships between these factors. GLO-1(D132A) expression bypassed the requirement for *glo-3(+)* and *ccz-1(+)* in the formation of birefringent and PGP-2-marked gut granules in embryos (Figure 7A and Table 3) (Morris *et al.* 2018). Consistent with GLO-3 and CCZ-1 functioning together as GLO-1 GEF to facilitate gut granule biogenesis in embryos, we found that GLO-1(D132A) restored gut granules in *glo-3(-)*; *ccz-1(-)* double mutants (Figure 7A and Table 3). In contrast to the GLO-1 GEF mutants, the biogenesis of birefringent gut granules in *whit-2(-)* embryos was not rescued by GLO-1(D132A) expression (Figure 7C and Table 3). Furthermore, in double mutants, *whit-2(-)* disrupted the restoration of birefringent

organelles by GLO-1(D132A) in *glo-3(-)* and *ccz-1(-)* embryos (Table 3). However, the restoration of PGP-2-marked gut granules in *glo-3(-)* and *ccz-1(-)* mutants by GLO-1(D132A) was not disrupted by the addition of *whit-2(-)* (Figure 7A). These results suggest that WHT-2 functions independently in the formation of birefringent material and GLO-1 localization.

We also investigated the functional relationships between WHT-2 and the GLO-1 GEF subunits in adult gut granule formation. GLO-1(D132A) expression restored autofluorescent gut granules in adult *glo-3(-)* and *ccz-1(-)* single mutants (Figure 7D and Table 4) (Delahaye *et al.* 2014). In contrast, *glo-3(-)*; *ccz-1(-)* double mutant expressing GLO-1(D132A) lacked autofluorescent gut granules (Table 4), suggesting that GLO-3 and CCZ-1 have redundant functions in adults. Adult *whit-2(-)* mutants displayed normal numbers of autofluorescent gut granules (Figure 2J), which were not significantly altered by the expression of the GLO-1 fast exchange mutant (Table 4). GLO-1(D132A) was unable to restore autofluorescent gut granules in *glo-3(-)*; *whit-2(-)* and *ccz-1(-)*; *whit-2(-)* double mutants (Figure 7D and Table 4). *whit-2(gk891224)* showed a similar disruption of the rescuing activity of GLO-1(D132A) in *glo-3(-)* animals (Table 4). Therefore, when CCZ-1 or GLO-3 activity is lacking, *whit-2(+)* becomes necessary for the GLO-1 fast exchange mutant to promote the formation of autofluorescent gut granules. GLO-1(D132A) restored autofluorescent gut granules in *whit-2(ok2775)*; *glo-1(zu437)*, indicating that *whit-2(+)* is dispensable for the activity of the GLO-1 fast exchange mutant to promote gut granule biogenesis in adults (Figure 7D and Table 4). Together these results suggest that WHT-2, GLO-3, and CCZ-1 have some independent functions in adult gut granule biogenesis.

WHT-2 functions redundantly with GLO-3 but not CCZ-1 in the localization of GLO-1 (D132A)

A low, but significant, amount of the GLO-1 fast exchange mutant was found on gut granules in *glo-3(-)* and *ccz-1(-)* mutants (Figure 7, A and B) (Morris *et al.* 2018). As expected, the association of GLO-1(D132A) was not further diminished in the *glo-3(-)*; *ccz-1(-)* double mutant (Figure 7, A and B), which supports the hypothesis that GLO-3 and CCZ-1 function together as the GLO-1 GEF. To investigate whether WHT-2 promotes the association of the GLO-1 fast exchange mutant with gut granules in the GLO-1 GEF mutants, we examined its localization in *whit-2(-)*; *glo-3(-)* and *whit-2(-)*; *ccz-1(-)* double mutant embryos. GLO-1(D132A) was not detected on gut granules in the *glo-3(-)*; *whit-2(-)* double mutant (Figure 7, A and B), showing that GLO-3 and WHT-2 have redundant functions in localizing GFP::GLO-1(D132A). In contrast, GLO-1(D132A) remained associated with gut granules in *whit-2(-)*; *ccz-1(-)* double mutants (Figure 7, A and B). These results reveal a difference between the two GLO-1 GEF subunits in localizing the GLO-1 fast exchange mutant when WHT-2(+) activity is disrupted. Since CCZ-1, unlike GLO-3, also

Figure 7 Effects of *wht-2*(–) on the activity and localization of *GLO-1*(D132A). (A) 1.5-Fold stage embryos expressing GFP::*GLO-1*(D132A) were stained with antibodies to *PGP-2*. Embryos were imaged with confocal microscopy, single optical sections are shown, and white arrows in the insets label organelles containing the gut granule protein *PGP-2*. Black arrowheads flank the intestine. (B) The proportion of total *PGP-2* area in each embryo that also contained *GLO-1*::GFP was calculated for at least five embryos of each genotype. The mean level of colocalization is plotted, bars represent the 95% confidence intervals; * $P \leq 0.05$, ** $P \leq 0.001$, and ns indicates $P > 0.05$, by one-way ANOVA followed by a Bonferroni correction. (C) Pretzel stage embryos were analyzed with polarization microscopy to detect birefringent material. Black arrowheads flank the intestine and embryos are 50 μm in length. (D) Autofluorescent organelles were imaged in young adults using widefield fluorescence microscopy. Maximum intensity projections spanning the entire depth of the anterior intestine are shown. White arrows denote autofluorescent gut granules.

Table 3 Birefringent gut granules in embryos expressing GLO-1 point mutations

Genotype	% of embryos with the specified number of birefringent granules in intestinal cells				n
	0	1–20	21–50	>50	
Wild type	0	0	0	100	190
<i>whit-2(ok2775)</i>	26	41	32	1	214
<i>whit-2(ok2775) + gfp::glo-1(+)</i>	56	35	9	0	65
<i>whit-2(ok2775) + gfp::glo-1(D132A)</i>	50	36	14	0	66
<i>glo-1(zu437)</i>	94	6	0	0	123
<i>glo-1(zu437) + gfp::glo-1(+)</i>	0	0	8	92	103
<i>glo-1(zu437) + gfp::glo-1(D132A)</i>	0	0	1	99	40
<i>glo-1(zu437); whit-2(ok2775)</i>	98	2	0	0	40
<i>glo-1(zu437); whit-2(ok2775) + gfp::glo-1(+)</i>	69	29	2	0	40
<i>glo-1(zu437); whit-2(ok2775) + gfp::glo-1(D132A)</i>	14	76	10	0	29
<i>glo-3(kx94)</i>	98	2	0	0	87
<i>glo-3(kx94) + gfp::glo-1(+)</i>	99	1	0	0	24
<i>glo-3(kx94) + gfp::glo-1(D132A)</i>	0	0	20	80	40
<i>glo-3(kx94); whit-2(ok2775)</i>	95	5	0	0	40
<i>glo-3(kx94); whit-2(ok2775) + gfp::glo-1(+)</i>	90	10	0	0	40
<i>glo-3(kx94); whit-2(ok2775) + gfp::glo-1(D132A)</i>	78	22	0	0	40
<i>ccz-1(ok2182)</i>	57	43	0	0	46
<i>ccz-1(ok2182) + gfp::glo-1(+)</i>	60	40	0	0	40
<i>ccz-1(ok2182) + gfp::glo-1(D132A)</i>	0	3	16	81	38
<i>ccz-1(ok2182); whit-2(ok2775)</i>	91	9	0	0	45
<i>ccz-1(ok2182); whit-2(ok2775) + gfp::glo-1(D132A)</i>	0	13	87	0	40

All strains were grown at 22°C. Threefold and later stage embryos were analyzed using polarization microscopy and scored for the presence and number of birefringent granules in the intestine. The expression of *gfp::glo-1* was assayed with fluorescence microscopy. The expression of *gfp::glo-1* point mutants or *gfp::glo-1(+)* did not alter the number of birefringent gut granules in a wild-type background (not shown).

acts as a GEF to activate **RAB-7** in the conventional endolysosomal pathway (Poteryaev *et al.* 2007; Morris *et al.* 2018), we examined whether the loss of **RAB-7** function in *ccz-1(-)* mutants accounted for the difference in **GLO-1(D132A)** localization between *glo-3(-)*; *whit-2(-)* and *ccz-1(-)*; *whit-2(-)* double mutants. We performed RNAi targeting *rab-7* in *glo-3(-)*, *whit-2(-)*, and *glo-3(-)*; *whit-2(-)* double mutants and did not detect any change in the localization of **GLO-1(D132A)** (Figure S5).

Effects of **WHT-2** ATPase mutants on gut granule biogenesis

WHT-2 is a member of the ABCG subfamily of ABC membrane transporters (Sheps *et al.* 2004). The membrane translocation of substrates by ABCG proteins depends upon ATP binding and hydrolysis (Taylor *et al.* 2017). Mutations changing the conserved lysine in the Walker A motif of ABCG proteins to a methionine or arginine inactivates their ATPase and transport activity (Ozvegy *et al.* 2002; Henriksen *et al.* 2005; Zhang *et al.* 2006). To examine the requirement of ATP hydrolysis by **WHT-2** in gut granule biogenesis, we generated point mutations in the corresponding lysine of **WHT-2** and expressed the point mutants using an extrachromosomal array, as well as using CRISPR/Cas9 genome editing to precisely alter the endogenous *whit-2* locus. The expression of **WHT-2(+)** and the **WHT-2** point mutants from the extrachromosomal array disrupted the formation of the gut granules (Figure S6), possibly due to saturation and disruption of protein trafficking to the gut granule when **WHT-2** is

overexpressed. In contrast, the genome-edited point mutations did not disrupt the localization of **PGP-2** and **CDF-2::GFP** (Figure 8, A and B). We therefore focused our analysis on whether the endogenous *whit-2(K74M)* and *whit-2(K74R)* point mutants affected gut granule biogenesis.

Both ATPase mutants showed a weaker defect than the *whit-2(ok2775)* null mutant in the formation of birefringent gut granules within pretzel stage embryos (Table 1). However, like the null allele, the ATPase mutants lacked birefringent gut granules in twofold stage embryos (Table 1), which immediately precedes the pretzel stage. The loss of birefringent material in twofold stage embryos suggests that the ATPase, and therefore the transport activity, of **WHT-2** contributes to the formation of birefringent gut granules. However, the presence of the birefringent material in pretzel stage embryos significantly differs from the null alleles, indicating that the ATPase mutants retain some activity promoting the formation of birefringent gut granules.

To further explore the function of **WHT-2** ATPase-dependent transport, we combined the point mutants with *glo-3(kx38)*, which revealed roles for *whit-2(+)* in **RAB-7** localization, gut granule size, and gut granule protein trafficking (Figure 2, B, G, and I and Figure 5, E and J). *glo-3(kx38)* alone led to reduced numbers of enlarged gut granules that often lacked **LMP-1** and misaccumulated **RAB-7** (Figure 8, C–G). When the **WHT-2** ATPase mutants were combined with *glo-3(kx38)*, they caused a loss of birefringent organelles similar to *whit-2(-)* null allele (Table 1). The ATPase mutants also led to decreased association of **RAB-7** with gut granules in

Table 4 Autofluorescent gut granules in adults expressing GLO-1 point mutations

Genotype	% of animals with the specified number of autofluorescent organelles in the intestine						n
	0	1–20	21–50	51–100	101–200	>200	
Wild type	0	0	0	0	0	100	60
<i>whit-2(ok2775)</i>	0	0	0	0	0	100	60
<i>whit-2(ok2775) + gfp::glo-1(+)</i>	0	0	0	0	0	100	40
<i>whit-2(ok2775) + gfp::glo-1(D132A)</i>	0	0	0	0	16	84	44
<i>glo-1(zu437)</i>	83	17	0	0	0	0	67
<i>glo-1(zu437) + gfp::glo-1(+)</i>	0	0	0	0	0	100	20
<i>glo-1(zu437) + gfp::glo-1(D132A)</i>	0	0	0	0	5	95	21
<i>glo-1(zu437); whit-2(ok2775)</i>	75	25	0	0	0	0	20
<i>glo-1(zu437); whit-2(ok2775) + gfp::glo-1(+)</i>	0	0	0	0	23	77	22
<i>glo-1(zu437); whit-2(ok2775) + gfp::glo-1(D132A)</i>	0	0	0	0	32	58	22
<i>glo-3(kx94)</i>	37	52	13	0	0	0	65
<i>glo-3(kx94) + gfp::glo-1(+)</i>	60	40	0	0	0	0	25
<i>glo-3(kx94) + gfp::glo-1(D132A)</i>	0	0	0	0	0	100	20
<i>glo-3(kx94); whit-2(ok2775)</i>	100	0	0	0	0	0	20
<i>glo-3(kx94); whit-2(ok2775) + gfp::glo-1(+)</i>	86	14	0	0	0	0	21
<i>glo-3(kx94); whit-2(ok2775) + gfp::glo-1(D132A)</i>	0	71	29	0	0	0	21
<i>glo-3(kx94); whit-2(gk891224)</i>	86	14	0	0	0	0	25
<i>glo-3(kx94); whit-2(gk891224) + gfp::glo-1(+)</i>	48	52	0	0	0	0	25
<i>glo-3(kx94); whit-2(gk891224) + gfp::glo-1(D132A)</i>	24	56	20	0	0	0	25
<i>ccz-1(ok2182)</i>	71	29	0	0	0	0	24
<i>ccz-1(ok2182) + gfp::glo-1(+)</i>	25	70	5	0	0	0	20
<i>ccz-1(ok2182) + gfp::glo-1(D132A)</i>	0	0	0	0	23	77	24
<i>ccz-1(ok2182); whit-2(ok2775)</i>	65	35	0	0	0	0	20
<i>ccz-1(ok2182); whit-2(ok2775) + gfp::glo-1(D132A)</i>	0	39	56	5	0	0	18

All strains were grown 22°C. Adults were analyzed using fluorescence microscopy with a rhodamine filter to score the number of autofluorescent organelles within the intestine located posterior to the vulva and a FITC filter set to assess the expression of GFP::GLO-1.

glo-3(kx38) (Figure 8, D and F); however, they did not cause the complete loss of RAB-7 from gut granules that was seen with the *whit-2(–)* null allele (Figure 5, E and J). Unlike the *whit-2(ok2775)* null mutant, the *whit-2* ATPase mutants did not suppress gut granule enlargement by *glo-3(kx38)* (Figure 2I and Figure 8G). Moreover, they did not cause the loss of LMP-1 from gut granules that was seen in *glo-3(kx38)*; *whit-2(ok2775)* double mutants (Figure 2, B and G and Figure 8, C and E). Together, these observations suggest that the WHT-2(K74M) and WHT-2(K74R) point mutants retain function and that some WHT-2 activities do not require ATP hydrolysis.

Next we determined whether the membrane transport activity of WHT-2 was necessary for the gut granule association of GLO-1. Both WHT-2 ATPase mutants led to a significant reduction in the steady-state levels of GFP::GLO-1 on gut granules (Figure 9, A and B). WHT-2(K74R) lacked gut-granule-localized GFP::GLO-1, resembling *whit-2(ok2775)*. Some GFP::GLO-1 associated with gut granules in the WHT-2(K74M) point mutant, albeit much less than what was seen in wild type (Figure 9, A and B). Both WHT-2(K74M) and WHT-2(K74R) were associated with gut granules (Figure 9, C and D), making it unlikely that the loss or reduction in GLO-1 localization resulted from improper trafficking and loss of WHT-2 from gut granules. These results suggest that the WHT-2 ATPase cycle and its membrane transport activity promote the gut granule association of GLO-1.

Discussion

WHT-2 promotes gut granule biogenesis

Our work shows that the ABCG subfamily protein WHT-2 does not just act in the accumulation of birefringent material within gut granules, but more generally in the biogenesis of gut granules themselves. In support of this function, *whit-2(–)* mutants mislocalize birefringent material into the intestinal lumen, just as in previously described mutants defective in gut granule formation (Figure 1) (Hermann *et al.* 2005, 2012; Delahaye *et al.* 2014). As expected for a gene that promotes the trafficking of lipids and proteins to gut granules, the enlargement of gut granules in *umps-1(–)* and *glo-3(–)* mutants depends upon *whit-2(+)* activity (Figure 2) (Levitte *et al.* 2010). Furthermore, there is a synthetic loss of autofluorescent gut granules and mislocalization of two different gut granule proteins when *whit-2(–)* is combined with weak gut granule biogenesis mutants (Figure 2 and Table 2). Finally, *whit-2(–)* disrupts the gut granule localization of GLO-1 (Figure 5), a key player in gut granule biogenesis (Hermann *et al.* 2005; Morris *et al.* 2018). In contrast to its effects on gut granule formation, loss of *whit-2* function does not significantly alter the biogenesis of conventional endolysosomes (Figure 3 and Figure S1).

WHT-2 is the second ABC transporter implicated in gut granule biogenesis. Previously we found that PGP-2, an ABCB subfamily member, is essential for protein trafficking to gut

Figure 8 WHT-2 transport mutants retain function. (A) 1.5-Fold stage embryos expressing CDF-2::GFP were stained with anti-PGP-2 antibodies and imaged with confocal microscopy. In insets, white arrows label compartments containing both markers. (B) For each genotype, at least 40 randomly selected PGP-2 containing organelles in three to five different embryos were scored for the presence of CDF-2::GFP signals. The mean is plotted and error bars represent the 95% confidence limit. In one-way ANOVAs comparing mutants to wild type, $P > 0.05$. (C and D) 1.5-Fold stage embryos were stained with anti-PGP-2 antibodies and anti-LMP-1 or anti-RAB-7 antibodies, and imaged with confocal microscopy. In insets, white arrows label compartments containing two markers, compartments lacking one of the markers are denoted with black arrows. (E and F) For each genotype, at least 20 randomly selected CDF-2::GFP or PGP-2 containing organelles in 5–10 different embryos were scored for the presence of anti-PGP-2 or anti-LMP-1 signals. The mean is plotted and error bars represent the 95% confidence limit. A one-way ANOVA comparing mutants to wild type or double mutants

granules (Schroeder *et al.* 2007). While *PGP-2* plays an obvious role in the delivery of proteins to gut granules, *WHT-2* does not. Instead, *WHT-2* functions redundantly with important mediators of gut granule formation (Figure 2), suggesting that it has a more specialized or restricted role in gut granule biogenesis.

The role of ABCG proteins in LRO biogenesis is likely not restricted to *C. elegans* gut granules. White, the founding member of the ABCG subfamily, functions in the uptake of pigment precursors by *Drosophila* LROs (Lloyd *et al.* 1998). While *white* mutants are most well known for their defects in eye pigmentation, they also display altered LRO morphology, including retinal pigment granules (Nolte 1961; Shoup 1966; Nickla 1972; Stark and Sapp 1988; Schraermeyer and Dohms 1993) and zinc storage granules in Malpighian tubules (Tejeda-Guzmán *et al.* 2018). The changes in organelle morphology could be due to defects in their formation. Similar to what we have documented with *WHT-2*, White localizes to *Drosophila* pigment granule membranes (Mackenzie *et al.* 2000). Moreover, the *GLO-1* ortholog Lightoid functions in the biogenesis of this organelle (Ma *et al.* 2004). It is possible that *white*, the first *Drosophila* mutant locus (Morgan 1910), promotes the localization of Lightoid, similar to how *WHT-2* supports the localization of *GLO-1*.

***WHT-2* contributes to Rab localization**

Rab GTPases cycle on and off membranes to regulate intracellular cargo transport. The reversible association of Rabs with organelles is coupled with their activity, such that the active GTP-bound form is membrane-associated and the inactive GDP-bound form is typically located in the cytoplasm (Mizuno-Yamasaki *et al.* 2012; Müller and Goody 2018). While GEFs are posited to represent the minimal machinery needed to direct Rab localization, it is likely that each Rab also utilizes a distinct set of interacting factors and mechanisms to ensure its correct spatiotemporal distribution (Barr 2013; Pfeffer 2013). Rab cycling is regulated at multiple levels including membrane targeting, activation by its GEF and the resulting stable association with organelle membranes, as well as inactivation by its GTPase activating protein and subsequent extraction into the cytoplasm. Disrupting the function of factors involved in any of these processes can alter the organelle association of Rabs.

In this work, we show that the ABCG protein *WHT-2* is necessary for the accumulation of *GLO-1* on gut granules when they are first being generated in embryonic intestinal cells. This is demonstrated using GFP::*GLO-1* in both fixed and living embryos as well as with antibodies to endogenous untagged *GLO-1* (Figure 5). Notably, gut granules are present within *wht-2(-)* embryos, and *GLO-1* is the only protein we have found to be absent from these organelles in *wht-2(-)*

single mutants, suggesting that *WHT-2* specifically promotes the gut granule association of *GLO-1*. Consistent with a direct role in this process, *WHT-2* is expressed in intestinal cells and is localized to the gut granule membrane (Figure 4).

The disruption of *GLO-1* localization in *wht-2(-)* mutants could result from decreased *GLO-1* recruitment to gut granules or altered *GLO-1* activation. A heterodimeric complex of *GLO-3* and *CCZ-1* is the likely GEF that activates and promotes the association of *GLO-1* with gut granules (Delahaye *et al.* 2014; Morris *et al.* 2018), which is further supported by our current studies showing that *CCZ-1::YFP* and *GLO-3::GFP* are localized to gut granules (Figure 6). Phenotypic similarities between *wht-2(-)* and *GLO-1* GEF mutants point toward *WHT-2* functioning with the *GLO-1* GEF. Most notably, the loss of *GLO-1* localization to gut granules in *wht-2(-)* closely resembles *glo-3(-)* and *ccz-1(-)* mutants and the *GLO-1*(D132A) fast exchange mutant associates with gut granules in all three mutants (Figure 5 and Figure 7). Finally, like *glo-3(+)* and *ccz-1(+)*, *wht-2(+)* activity is required for the localization of *LMP-1* to gut granules when the *GLO-1* fast exchange mutant is expressed (Figure S5) (Morris *et al.* 2018).

Longin domain-containing GEFs, like *CCZ-1* and *GLO-3*, appear to function as heterodimers (Nordmann *et al.* 2010; Gerondopoulos *et al.* 2012; Kiontke *et al.* 2017), and our results suggest that *WHT-2* is not a third subunit of the *GLO-1* GEF. First, gut granules are present in embryos and adults lacking *wht-2* function (Figure 1, Table 1, and Table 2), whereas they are completely lacking in strong *glo-3(-)* and *ccz-1(-)* mutants (Delahaye *et al.* 2014; Morris *et al.* 2018). Second, while the gut granule association of *CCZ-1* requires *GLO-3*, both *GLO-3* and *CCZ-1* remain associated with gut granules in *wht-2(-)* mutants (Figure 6), suggesting that *WHT-2* is not a gut granule adaptor for the *GLO-1* GEF. Finally, while the *GLO-1* fast nucleotide exchange mutant bypasses GEF function, restoring gut granules in the *glo-3(-)* and *ccz-1(-)* mutants (Figure 7, Table 3, and Table 4) (Delahaye *et al.* 2014; Morris *et al.* 2018), this mutant does not cause the restoration of birefringent material within gut granules in *wht-2(-)* (Figure 7 and Table 3).

The *GLO-1* fast exchange mutant localizes to gut granules in *glo-3(-)* and *ccz-1(-)* single and *glo-3; ccz-1* double mutants, indicating that it can associate with gut granules independent of *GLO-1* GEF activity (Figure 7). In support of the model that *WHT-2* assists in the GEF-independent localization of *GLO-1*, *wht-2(+)* activity is necessary for the association of *GLO-1*(D132A) with gut granules in *glo-3(-)* mutants (Figure 7). However, *wht-2(+)* activity is not similarly required for the association of the *GLO-1* fast exchange mutant with gut granules when *ccz-1* activity is disrupted (Figure 7). If *WHT-2* promotes the *GLO-1* GEF-independent localization

to single mutants was used to calculate *P* values (* *P* ≤ 0.05, ns indicates *P* > 0.05). (G) Anti-*PGP-2* signals in 1.5-fold stage embryos were acquired using confocal microscopy. The diameters of 15–40 *PGP-2* containing compartments were measured in five embryos of each genotype. All of the diameters are plotted and the red lines denote the mean and 95% confidence interval. Strains were compared to each other by one-way ANOVA; * *P* ≤ 0.05, ns indicates *P* > 0.05. In all images, black arrowheads flank the intestine. Single optical sections are shown.

Figure 9 *WHT-2* transport activity promotes the localization of GFP::GLO-1. (A) 1.5-Fold stage embryos expressing GFP::GLO-1 were stained with antibodies to GFP and PGP-2 and imaged with confocal microscopy. White and black arrows in the insets denote PGP-2-marked organelles containing or lacking GFP::GLO-1, respectively. (B) The proportion of total PGP-2 area in each embryo that also contained GFP::GLO-1 is plotted. At least five embryos of each genotype were scored. The mean is plotted, bars represent the 95% confidence interval. Strains were compared to each other by one-way ANOVA; * $P \leq 0.05$, ns indicates $P > 0.05$. (C) 1.5-Fold stage embryos expressing CDF-2::GFP were stained with antibodies to WHT-2 and imaged with confocal microscopy. In insets, white arrows denote CDF-2::GFP-marked organelles containing WHT-2. In all images, black arrowheads flank the intestine. Single optical sections are shown. (D) The proportion of the total CDF-2::GFP area in each embryo that also contained WHT-2 was calculated for five to seven 1.5-fold stage embryos. A one-way ANOVA was used to compare the mutants to wild type and $P > 0.05$.

of GLO-1(D132A), then *wht-2*(−) would be expected cause to the loss of the GLO-1 fast exchange mutant from gut granules in *ccz-1*(−) mutants. As a possible explanation for this difference we considered that CCZ-1, but not GLO-3, functions as part of the GEF for RAB-7 (Nieto *et al.* 2010; Poteryaev *et al.* 2010; Morris *et al.* 2018). Targeting *rab-7* with RNAi in *glo-3*(−); *wht-2*(−) mutants did not enhance the gut granule association of the GLO-1 fast exchange mutant (Figure S5), suggesting that the loss of RAB-7 function is unlikely to underlie the difference between *ccz-1*(−) and *glo-3*(−) mutants. We utilized null alleles of *wht-2* and *glo-3* in this study. While *ccz-1(ok2182)* completely disrupts gut granule formation (Delahaye *et al.* 2014; Morris *et al.* 2018), this strong loss-of-function allele retains a small amount of activity (Nieto *et al.* 2010), which might enable some GLO-1 GEF activity to be present in *ccz-1*(−); *wht-2*(−) mutants. If WHT-2 functions redundantly with the GLO-1 GEF, then a small amount of GEF activity could be sufficient for the gut granule localization of GLO-1(D132A) in this double mutant.

WHT-2 clearly functions in collaboration with other factors to localize GLO-1. Our observation that WHT-2 associates with gut granules that lack GLO-1 in *glo-3(kx38)* mutants indicates that the mere presence of WHT-2 is not sufficient to recruit or stabilize the majority of GLO-1 (Figure 4) (Morris *et al.* 2018). For the most part, gut granules appear to be properly formed in *wht-2*(−) mutants (Figure 2), in contrast to *glo-1*(−) mutants, which lack these organelles

(Hermann *et al.* 2005; Morris *et al.* 2018). In our model, other factors such as the GEF activating GLO-1 are functioning in *wht-2*(−) mutants. These would promote the gut granule localization of a small cohort of GLO-1, which we cannot detect in *wht-2*(−) that supports the biogenesis of gut granules.

There is currently no precedent for how an ABC protein could affect Rab localization. However, the simultaneous localization of WHT-2 and GLO-1 on gut granules suggests a number of possibilities. They include WHT-2 promoting the localization of GLO-1 to gut granules prior to GEF activation; for example, by facilitating the delivery of GLO-1 to membranes from the cytoplasm. WHT-2 might promote interactions between activated GLO-1 and its effectors, which could enhance the association of GLO-1 with gut granules. Finally, WHT-2 could stabilize the association of active GLO-1 with gut granules by inhibiting the inactivation and extraction of GLO-1 from gut granules.

Transport-dependent and -independent functions of WHT-2

ABC transporters have established roles transporting pigments and other optically active molecules into endosomal organelles. For example, the precursor of the red pigment that accumulates in yeast vacuoles of *ade2* mutants is transported by the ABCG protein YCF1 (Chaudhuri *et al.* 1997), the White ABCG protein transports pigments into *Drosophila* LROs

(Sullivan and Sullivan 1975), and the Bmwh3 ABCG transporter transports uric acid into silkworm urate granules (Kômoto *et al.* 2009). The formation of optically active birefringent material within gut granules is delayed in *wht-2(-)* null mutants, suggesting that *WHT-2* functions similarly (Figure 1 and Table 1) (Currie *et al.* 2007). While *WHT-2* clearly affects the appearance of birefringent granules, we favor the interpretation that *WHT-2* does not directly transport this material, or its precursors, into the gut granule lumen. ABC proteins couple ATP binding and hydrolysis with substrate transport (Lewinson and Livnat-Levanon 2017; Ford and Beis 2019). Mutations in the Walker A motif of ABC transporters that disrupt ATP hydrolysis destroy their transport activity, including pigment transport by YCF1 and White (Ewart *et al.* 1994; Falcón-Pérez and Dell'angelica 2002). In contrast, pretzel stage embryos robustly accumulated birefringent material within the gut granules of two different *WHT-2* Walker A motif point mutants predicted to lack transport activity (Table 1). If *WHT-2* functioned to directly transport substrates involved in the generation of birefringent crystals within gut granules, then the *WHT-2* ATPase mutants should more closely resemble *wht-2(ok2775)*. While we cannot rule out the possibility that the *WHT-2* point mutants have some residual ATPase and transport activity, we think it unlikely given the extensive studies of analogous mutations in other ABCG transporters (Azzaria *et al.* 1989; Müller *et al.* 1996; Wemmie and Moyer-Rowley 1997; Hou *et al.* 2000; Ozvegy *et al.* 2002; Henriksen *et al.* 2005; Zhang *et al.* 2006). If *WHT-2* does not directly transport the material that generates birefringent granules, then why is the appearance of birefringent gut granules delayed when the ATPase activity of *WHT-2* is disrupted? We propose that this stems from defective association of *GLO-1* with gut granules in the *WHT-2* point mutants and resulting alterations in gut granule biogenesis, which disrupt the processes creating birefringent granules. Additionally, why is the *GLO-1* fast exchange mutant, which can associate with gut granules, unable to restore birefringent gut granules in *wht-2(-)* null mutants (Figure 7 and Table 3)? This mutant robustly bypasses the requirement for the *GLO-1* GEF in gut granule biogenesis (Figure 7, Table 3, and Table 4), which is expected, given its predicted effects on *GLO-1* nucleotide exchange. However, our data suggest that *WHT-2* functions differently than *GLO-3* and *CCZ-1* to affect *GLO-1* localization, and the *GLO-1*(D132A) that associates with gut granules in *wht-2(-)* mutants could have limited functionality that manifests itself in defective accumulation of birefringent material.

By whatever mechanism *WHT-2* promotes the localization of *GLO-1*, it appears to be dependent upon *WHT-2* membrane transport activity. We found that two different *WHT-2* Walker A point mutations, which inhibit ATPase and membrane transport by homologous ABC proteins (Azzaria *et al.* 1989; Müller *et al.* 1996; Wemmie and Moyer-Rowley 1997; Hou *et al.* 2000; Ozvegy *et al.* 2002; Henriksen *et al.* 2005; Zhang *et al.* 2006), disrupted the steady-state association of

GLO-1 with gut granules (Figure 9). The *WHT-2*(K74R) mutant affected *GLO-1* localization similarly to the *wht-2(-)* null mutant, whereas low levels of *GLO-1* associated with gut granules in the *WHT-2*(K74M) point mutant (Figure 9). Consistent with the point mutants having slight differences in activity, *WHT-2*(K74M) mutants accumulated birefringent material to a greater extent than *WHT-2*(K74R) (Table 1), which correlates with their effects on *GLO-1* localization.

Membrane transport is the best understood function of eukaryotic ABC proteins and the substrate specificity of ABC transporters typically varies by subfamily (Tarling *et al.* 2013; ter Beek *et al.* 2014). Mammalian ABCG proteins transport lipid sterols (ABCG1, ABCG4, ABCG5, and ABCG8) and hundreds of different small molecules with diverse structures (ABCG2) (Kerr *et al.* 2011; Woodward *et al.* 2011; Horsey *et al.* 2016). Phylogenetic analyses point to *WHT-2*, as well as the rest of the *WHT* proteins in *C. elegans*, being most closely related to the *Drosophila* White, Scarlet, and Brown ABCG proteins (Sheps *et al.* 2004). These transporters move guanine- and tryptophan-derived pigments into pigment granules (Sullivan *et al.* 1979; Lloyd *et al.* 1998; Mackenzie *et al.* 1999). We think it unlikely that the localization of a Rab is affected by the presence or absence of small molecules inside the organelle lumen, and favor the possibility that sterols are transported by *WHT-2*. ABC transporters have well-characterized functions removing lipids from the cytoplasmic leaflet of cellular membranes (van Meer *et al.* 2006; Tarling *et al.* 2013). Differences between the lipid composition of cytoplasmic and luminal membrane leaflets regulates vesicle budding, tubule elongation, and membrane fusion (van Meer *et al.* 2008; Johansen *et al.* 2012; Scott *et al.* 2014; Wickner and Rizo 2017), as well as the membrane association of Rabs (Kulakowski *et al.* 2018). *WHT-2* might therefore modify the cytoplasmic leaflet of gut granules to promote the localization of *GLO-1*.

Our results also suggest that *WHT-2* has membrane transport-independent functions. Foremost among these is the robust formation of birefringent granules in both K74 catalytic point mutants (Table 1). *WHT-2* function also affects the localization of *RAB-7* (Figure 5), an endolysosomal Rab GTPase closely related to *GLO-1* (Elias *et al.* 2012; Klöpper *et al.* 2012; Rojas *et al.* 2012). *GLO-3*, likely through its activation of *GLO-1*, prevents the association of *RAB-7* with gut granules (Morris *et al.* 2018). *wht-2(-)* null mutants completely disrupt the mislocalization of *RAB-7* to gut granules in *glo-3(-)* mutants (Figure 5), whereas the *WHT-2* Walker A mutants do not (Figure 8). The *wht-2* point mutants similarly do not cause the reduction in gut granule size and the mislocalization of *LMP-1* seen with the *wht-2(-)* null mutant in *glo-3(kx38)* (Figure 5 and Figure 8). *WHT-2* is not the only ABC protein suggested to have transport-independent activities. The yeast ABCC protein Ycf1p has a membrane transport-dependent function in lysosome membrane fusion and a transport-independent role in SNARE localization (Sasser *et al.* 2013). The transport-independent activity of *WHT-2* and other ABC proteins could result from direct interactions

with other proteins (Paumi *et al.* 2009; Lee *et al.* 2011; Babu *et al.* 2012; Sasser *et al.* 2012, 2013).

ABC superfamily proteins have diverse functions, most of which are focused at cellular membranes (Higgins 1992). They are best known for membrane transport activities that affect a range of important physiological processes (Holland *et al.* 2003). However, it is becoming clear that ABC proteins can play a significant role in organelle formation (Brazill *et al.* 2001; Schaheen *et al.* 2006; Schroeder *et al.* 2007; Kawai *et al.* 2009; Bergam *et al.* 2018). ABC transporters can physically interact with Rabs (Swiatecka-Urban *et al.* 2005, 2007; Saxena and Kaur 2006; Saxena *et al.* 2006; Ferrándiz-Huertas *et al.* 2011), as well as SNAREs and tethers that promote membrane fusion (Naren *et al.* 1997; Cormet-Boyaka *et al.* 2002; Bilan *et al.* 2004; Sasser *et al.* 2013; Snider *et al.* 2013). Our work provides support for an ABC protein in localizing Rab GTPases, key regulators of membrane dynamics (Mizuno-Yamasaki *et al.* 2012; Barr 2013; Pfeffer 2013). Further studies of WHT-2, as well as other ABC proteins that localize to and are involved in the biogenesis of gut granules, will provide insights into the underappreciated roles that these conserved proteins play in membrane and protein trafficking.

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