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RESEARCH ARTICLE

A conserved retromer-independent function for RAB-6.2 in *C. elegans* epidermis integrity

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ABSTRACT

Rab proteins are conserved small GTPases that coordinate intracellular trafficking essential to cellular function and homeostasis. RAB-6.2 is a highly conserved *C. elegans* ortholog of human RAB6 proteins. RAB-6.2 is expressed in most tissues in *C. elegans* and is known to function in neurons and in the intestine to mediate retrograde trafficking. Here, we show that RAB-6.2 is necessary for cuticle integrity and impermeability in *C. elegans*. RAB-6.2 functions in the epidermis to instruct skin integrity. Significantly, we show that expression of a mouse RAB6A cDNA can rescue defects in *C. elegans* epidermis caused by lack of RAB-6.2, suggesting functional conservation across phyla. We also show that the novel function of RAB-6.2 in *C. elegans* cuticle development is distinct from its previously described function in neurons. Exocyst mutants partially phenocopy *rab-6.2*-null animals, and *rab-6.2*-null animals phenocopy mutants that have defective surface glycosylation. These results suggest that RAB-6.2 may mediate the trafficking of one or many secreted glycosylated cuticle proteins directly, or might act indirectly by trafficking glycosylation enzymes to their correct intracellular localization.

KEY WORDS: RAB-6.2, Epidermis, Retromer, RAB6, Exocyst

INTRODUCTION

Intracellular trafficking is crucial for development, cellular function and homeostasis, and is regulated by many families of proteins that interact with lipid membranes. The Rab family of small GTPases act as essential molecular switches to coordinate trafficking (Stenmark, 2009). To date, over 60 Rab proteins have been identified in humans and 30 in *C. elegans*, and their importance in the regulation of physiological processes, regeneration and disease has recently gained more recognition (Hutagalung and Novick, 2011; Zeng et al., 2018 preprint; Sekine et al., 2018).

Mammalian RAB6 isoforms coordinate intra-Golgi and ER-Golgi trafficking, and have links to the secretory pathway (Goud et al., 1994; Martinez et al., 1997; White et al., 1999; Bardin et al., 2015; Patwardhan et al., 2017).

C. elegans has emerged as a powerful genetic model for the study of intracellular trafficking because of its high amenability to genetic manipulation and microscopy (Sato et al., 2014). Genetic screens in *C. elegans* coelomocytes, intestinal cells and oocytes have identified many genes involved in intracellular trafficking, and genetic analysis has uncovered novel mechanisms of action (Balklava et al., 2007; Fares and Greenwald, 2001; Grant and Hirsh, 1999; Hermann et al., 2005). More recently, an RNAi screen for genes that affect cuticle structure revealed that members of the exocyst complex are necessary for alae formation, suggesting that the *C. elegans* epidermis is another tissue that can be used as a model for the genetic analysis of pathways that regulate intracellular trafficking (Hyenne et al., 2016). While the *C. elegans* epidermis is molecularly and structurally distinct from mammalian skin, some aspects of its cell biology are conserved, making it a good model to study epidermis development (reviewed in Chisholm and Hsiao, 2012; Chisholm and Xu, 2012; Page and Johnstone, 2007).

In *C. elegans*, RAB-6.2 is best known for its role in retrograde trafficking in neurons and for its role in grinder development (Straud et al., 2013; Zhang et al., 2012, 2016). So far, no studies have focused on the function of RAB-6.2 in the epidermis, a tissue that is affected in patients with mutations in RAB6-interacting proteins (Freeze and Ng, 2011; Hennies et al., 2008; Egerer et al., 2015; Wu et al., 2004; Sun et al., 2007).

C. elegans RAB-6.2 shares 81% amino acid identity with human RAB6A (Blast2p $E=3\times10^{-119}$) suggesting that uncovering the cell biology of RAB-6.2 function in *C. elegans* epidermis may yield valuable information about the functional role of this conserved protein in general and in epidermis development in particular. Significantly, mutations in RAB6-interacting proteins cause severe inherited diseases with strong skin defects such as gerodermia osteodysplastica and congenital disorders of glycosylation (CDG) in humans (Freeze and Ng, 2011; Hennies et al., 2008; Egerer et al., 2015; Wu et al., 2004; Sun et al., 2007), suggesting that uncovering the mechanisms of action of RAB-6.2 in *C. elegans* epidermis may also yield information that could be important for future translational studies to develop disease management strategies.

While characterizing a *rab-6.2*-null *C. elegans* strain for another study, we observed that *rab-6.2(ok2254)* animals were fragile. We set out to analyze the fragile-skin phenotype in *rab-6.2(ok2254)* animals genetically. Here, we show that *rab-6.2*-null mutants rupture at significantly higher levels than wild-type worms. This phenotype is observed spontaneously and is more pronounced under hypotonic stress. We also show that, *rab-6.2(ok2254)* animals are hypersensitive to exogenously added drugs and staining procedures, making this mutant an attractive sensitized background for drug assays in *C. elegans*. RAB-6.2 functions in the skin, and the mechanism of function in *C. elegans* epidermis integrity is distinct from mechanisms of function of RAB-6.2 previously identified in neurons (Zhang et al., 2012, 2016). Significantly, *rab-6.2(ok2254)* affects the sensitivity of *C. elegans* to *Microbacterium nematophilum* infection, a phenotype that has previously been linked to defects that affect surface glycosylation,

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suggesting that RAB-6.2 may mediate its effect on cuticle integrity, at least partially, by affecting the trafficking of glycosylated cuticle proteins. Finally, we show that mouse RAB6A cDNA is sufficient for the rescue of cuticle integrity when ectopically expressed in the skin of *rab-6.2*-null worms suggesting that RAB-6.2 function, and its mechanism of action in *C. elegans* epidermis are conserved across phyla.

RESULTS

rab-6.2-null animals have fragile cuticles

The genetic lesion in the allele *ok2254* is a deletion that eliminates part of the promoter and all of exons 1–4 of the gene *rab-6.2* suggesting that *rab-6.2(ok2254)* is a null strain. RAB-6.2 is expressed in multiple tissues including the nervous system and epidermis (Zhang et al., 2012, 2016). We observed several ruptured animals on our *rab-6.2(ok2254)* culture plates during normal maintenance, a phenotype very rarely observed in wild-type cultures (Fig. 1A,B). We hypothesized that RAB-6.2 is required for skin integrity. We first outcrossed the *rab-6.2(ok2254)* strain six times to minimize background mutations. To quantify the spontaneous rupture phenotype, we selected L4 animals on a new plate and monitored rupture after 24 h. In our outcrossed strain, called REB16, ∼70% of L4 stage *rab-6.2(ok2254)* animals developed to adults and 30% spontaneously ruptured on the plate overnight (Fig. 1C). No wild-type animals ruptured overnight.

Mutations that affect vulval development often result in spontaneous rupture at the vulva. We did not observe any vulval defects in surviving *rab-6.2(ok2254)* animals, and observed that *rab-6.2* nulls rupture at random locations along the body rather than specifically at the vulva, suggesting that cuticle integrity in *rab-6.2(ok2254)* animals may be compromised (see Fig. 6D). To test the mechanical strength of the worm cuticle, we assessed resistance to hypotonic shock. We suspended 1-day-old adult hermaphrodites in deionized water and quantified rupture after 15 min. Hypotonic shock results in a strong rupture phenotype in *rab-6.2(ok2254)* but not in age matched wild-type worms (Fig. 1D,E). A similar assay has been previously used to describe other fragile skin mutants (Kage-Nakadai et al., 2010). Most *rab-6.2(ok2254)* animals ruptured after 15 min in water (Fig. 1F) (∼80% rupture, N=166 from nine independent experiments), in contrast, few N2 wild-type adults of the same age ruptured after 15 min in water (∼10% rupture N=177 from nine independent experiments) (P<0.0001). These data support our hypothesis that the cuticle in *rab-6.2* null animals is fragile. We use the sensitivity to hypotonic-shock phenotype as a measure of cuticle mechanical integrity in the rest of the paper.

rab-6.2(ok2254) adults are hypersensitive to neurotransmitter agonists in a non-specific manner

C. elegans locomotion is a well-described model to study neurotransmission. Hypersensitivity or resistance to paralysis upon treatment with neurotransmitter agonists has been used to identify and describe genes involved in acetylcholine and GABA neurotransmission (Jorgensen, 2005; Rand, 2007). Specifically, if a mutant strain is hypersensitive to a GABA agonist, it is usually resistant to acetylcholine agonists and vice versa. If *rab-6.2(ok2254)* animals rupture under hypotonic shock because of a fragile cuticle, *rab-6.2*-null animals may also show generally increased permeability to exogenous chemicals because of a compromised cuticular barrier, resulting in non-specific hypersensitivity.

We moved 1-day-old adult animals onto plates containing tetramisole as an acetylcholine agonist and quantified paralysis after 15 and 20 min. We observed that all *rab-6.2* animals became

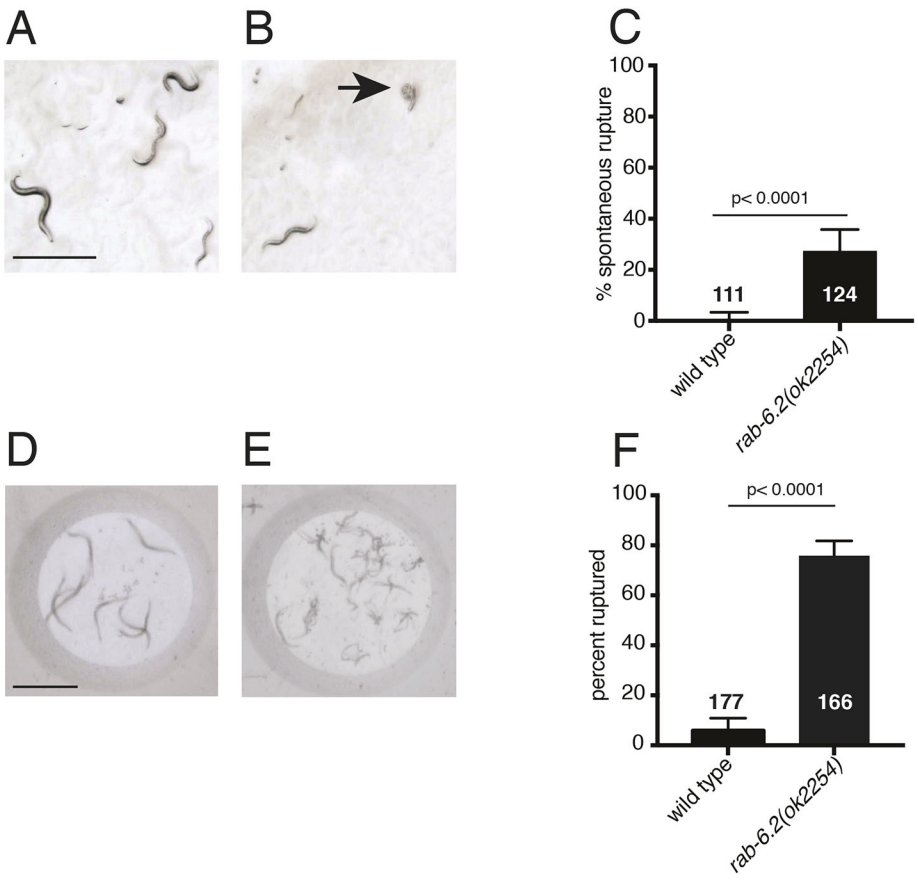

Fig. 1. *rab-6.2(ok2254)* animals rupture at higher rates than wild type. (A,B). Images of wild-type (A) and *rab-6.2(ok2254)* (B) animals feeding on a bacterial lawn. Spontaneously ruptured animals are visible in *rab-6.2(ok2254)* cultures (arrow in B). (C) Quantification of the percentage of animals showing spontaneous rupture overnight for wild-type and *rab-6.2(ok2254)*. (D,E) Images of wild type (D) and *rab-6.2(ok2254)* (E) animals after 15 min in dH_2O. (F) Quantification of rupture after 15 min in dH_2O. P<0.0001, Fisher's exact test. Error bars are 95% c.i. The total numbers of animals counted are shown for each data point. The water rupture experiment is compiled data from nine independent experiments. Scale bars: 1 mm.

Journal of Cell Science (2019) 132, jcs223586. doi:10.1242/jcs.223586

paralyzed and could not initiate escape as early as 15 min after being moved to nematode growth medium (NGM) tetramisole plates. These data are in striking contrast to the effect of the same concentration of tetramisole on wild-type animals. Almost no wild-type adults were paralyzed after 15 min of treatment and ∼30% became paralyzed after 20 min on plates containing 150 µM of tetramisole (Fig. 2A,B).

Because hypersensitivity to an acetylcholine agonist could result from a defect in GABA neurotransmission that would lead to resistance to GABA agonists, we compared paralysis of *rab-6.2(ok2254)* worms to wild-type animals in the presence of the GABA agonist piperazine (Delcastillo et al., 1963). We observed that, similar to the effect of tetramisole, *rab-6.2(ok2254)* animals are hypersensitive to piperazine; ∼70% of *rab-6.2(ok2254)* animals became paralyzed after 15 min of piperazine treatment and could not initiate escape movement compared to ∼10% of wild-type worms (Fig. 2C, $P<0.0001$). This effect is consistent with a mutation that does not specifically affect acetylcholine or GABA neurotransmission individually but rather affects sensitivity to drugs in general because of compromised cuticular permeability. We did not observe a stronger increased paralysis after 30 min suggesting a ceiling effect for piperazine in NGM plates at the concentration used (Fig. 2D).

rab-6.2 expression is sufficient for rescue of the skin integrity defect

To determine whether the cuticle integrity/permeability defect observed in *rab-6.2(ok2254)* animals is only due to the deletion in question and not to tightly linked mutations not removed by outcrossing to wild type, we PCR-amplified a ∼6.1 kb genomic locus containing 4 kb of sequence upstream of the START codon, the coding region and 400 bp downstream of the STOP codon from wild-type worms (Table S1). We found that this PCR amplicon is sufficient to fully rescue the rupture phenotype in two independently generated extrachromosomal transgenic lines suggesting that *ok2254* is the only mutation responsible for the skin integrity phenotype observed in our outcrossed strain (Fig. 3A). One of the transgenic lines that is rescued for the rupture phenotype was also tested for hypersensitivity to tetramisole. We show that the same transgene also rescues sensitivity to tetramisole back to wild-type levels (Fig. 3B).

The *C. elegans* skin is a simple syncytial epidermis covered by a collagenous cuticle, an epicuticle and a surface coat, all secreted by

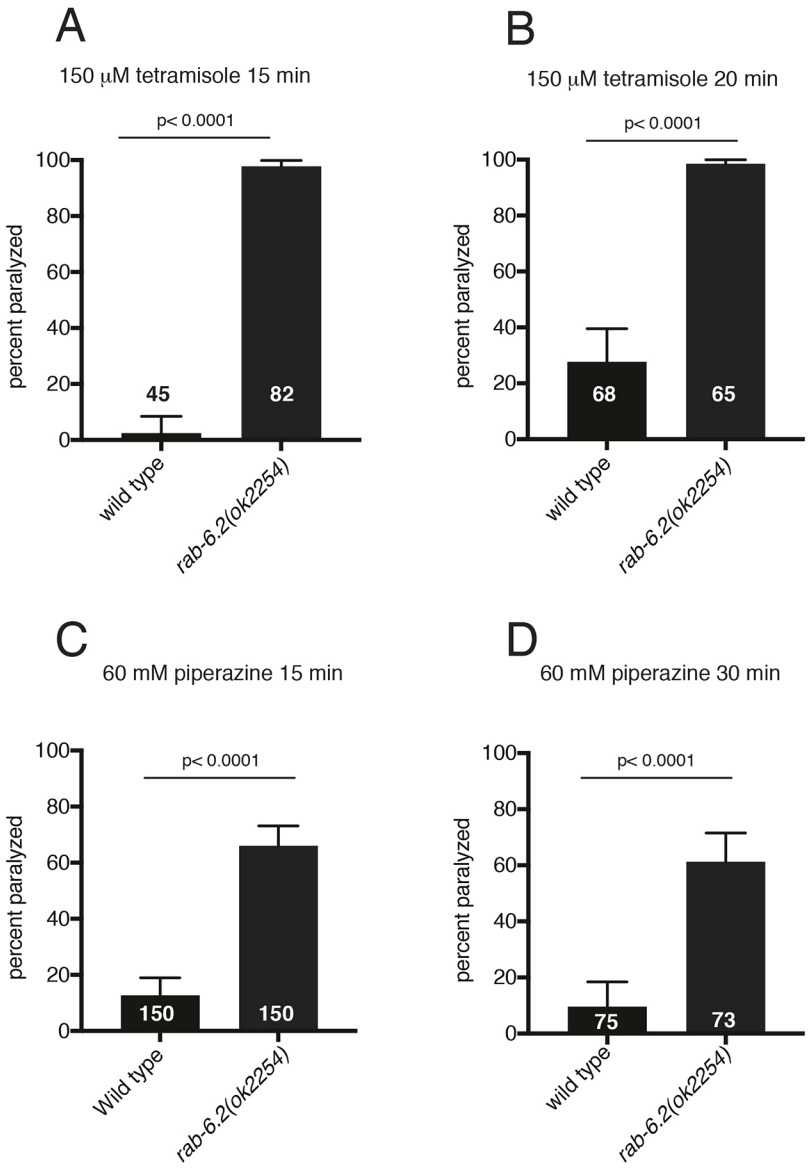

Fig. 2. *rab-6.2(ok2254)* animals are hypersensitive to neurotransmitter agonists in a non-specific manner. (A,B) Quantification of paralysis on NGM plates containing 150 µM tetramisole after 15 and 20 min respectively. (C,D) Quantification of paralysis on NGM plates containing 60 mM piperazine after 15 and 20 min respectively. $P<0.0001$, Fisher's exact test. Error bars are 95% c.i. Total numbers of animals counted from at least two independent experiments are shown.

Journal of Cell Science (2019) 132, jcs223586. doi:10.1242/jcs.223586

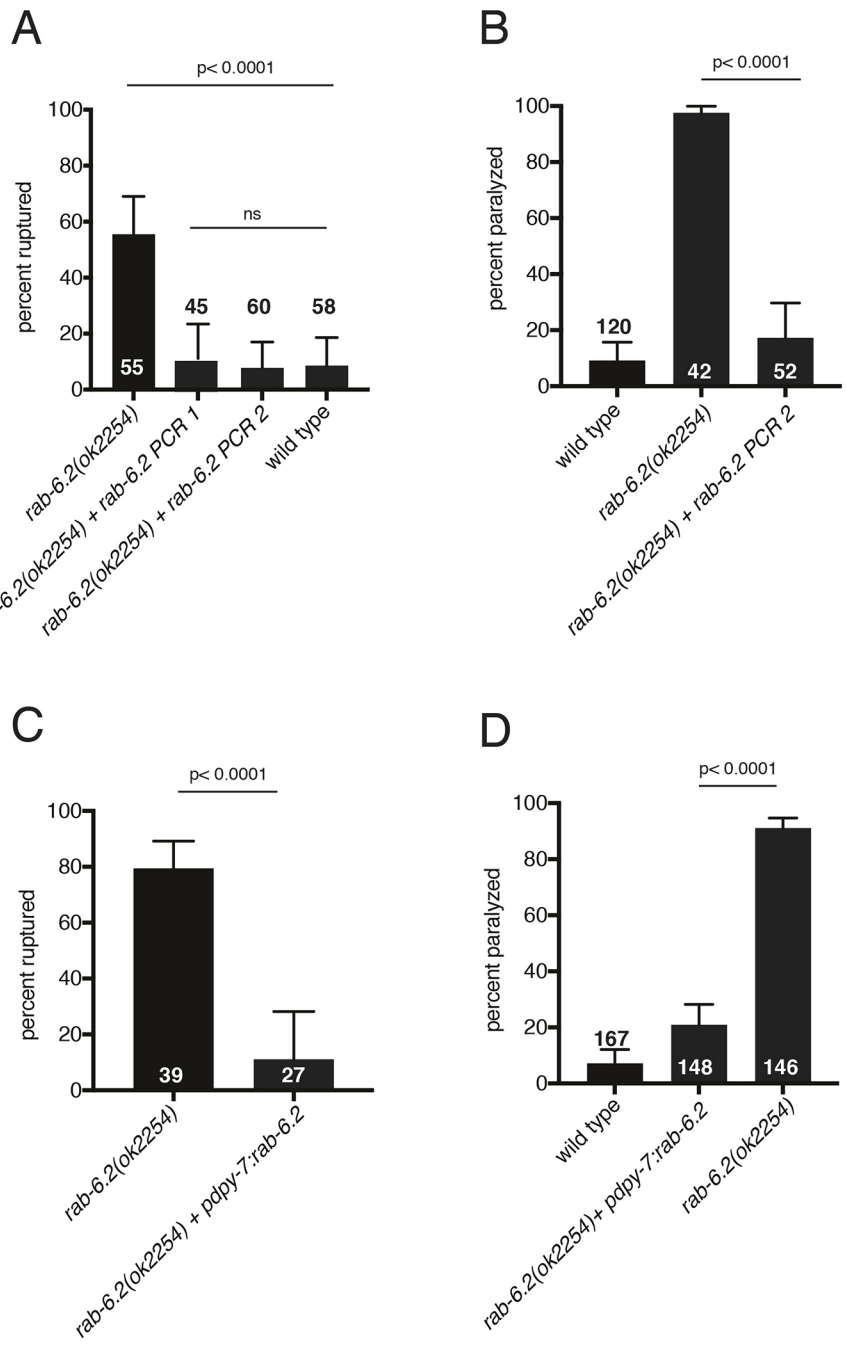

Fig. 3. *rab-6.2* genomic locus and skin-specific expression transgenes rescue the hypotonic stress and paralytic agent hypersensitivity phenotypes. (A) Quantification of rupture after 15 min in dH_2O in wild type, *rab-6.2(ok2254)* and two independent transgenic lines carrying an extrachromosomal array containing a PCR product of the wild-type *rab-6.2* locus in the *rab-6.2(ok2254)* background. (B) Quantification of paralysis on NGM plates containing 150µM tetramisole after 15 min in wild type, *rab-6.2(ok2254)* and a transgenic line carrying an extrachromosomal array containing a PCR product of the wild-type *rab-6.2* locus in the *rab-6.2(ok2254)* background. (C) Quantification of rupture after 15 min in dH_2O in *rab-6.2(ok2254)* and *rab-6.2(ok2254)* carrying an epidermis-specific RAB-6.2 from an extrachromosomal array containing *Pdpy-7:rab-6.2 cDNA*. (D) Quantification of paralysis on NGM plates containing 150 µM tetramisole after 15 min in wild type, *rab-6.2(ok2254)* and *rab-6.2(ok2254)* carrying an epidermis-specific RAB-6.2 from an extrachromosomal array containing *Pdpy-7:rab-6.2 cDNA*. $P<0.0001$, Fisher's exact test. Error bars are 95% c.i. Total numbers of animals counted from at least three independent experiments are shown.

the hypodermal syncytium and the seam cells (Chisholm and Hsiao, 2012; Chisholm and Xu, 2012; Page and Johnstone, 2007). To determine whether RAB-6.2 functions in the epidermis to promote skin strength, we expressed a *rab-6.2* cDNA under the control of the promoter of the epidermally expressed collagen, *dpy-7*, in *rab-6.2(ok2254)* mutants using a previously described regulatory sequence (Gilleard et al., 1997). We confirmed the specificity of the expression pattern driven by the above described regulatory region by independently generating transgenic animals containing an extrachromosomal array driving soluble mCherry with the same promoter (*Pdpy-7:mCh*), from three independent extrachromosomal transgenic lines (Fig. S2A,B).

We find that tissue-specific expression of RAB-6.2 in the epidermis rescues the rupture phenotype of *rab-6.2(ok2254)* animals (Fig. 3C). Furthermore, we show that skin-specific expression of *rab-6.2* cDNA also rescues tetramisole hypersensitivity to levels comparable to those of wild-type animals (Fig. 3D). These data suggest that RAB-6.2 functions in the epidermis to promote skin integrity and impermeability.

rab-6.2 mutants are permeable to Hoechst staining

Previous studies have used the DNA stain Hoechst 33258 to directly image permeability to chemicals in *C. elegans* (Kage-Nakadai et al., 2010). We stained live N2 and *rab-6.2(ok2254)* animals using the same protocol to image any increased permeability. We focused our imaging of animals in the pharynx and anal area to avoid background autofluorescence from intestinal granules (dashed line). Our results show that Hoechst 33258 penetrates the skin and stains the nuclei of cells in the pharynx and anal areas in non-permeabilized, live *rab-6.2(ok2254)* animals to a significantly

Journal of Cell Science (2019) 132, jcs223586. doi:10.1242/jcs.223586

stronger degree than seen in wild-type animals stained in parallel with the same protocol and imaged using the same exposure time (Fig. 4). We also observed nuclear staining along the body in *rab-6.2(ok2254)* animals but not in wild-type animals (data not shown). Importantly, an extrachromosomal array containing a skin-specific promoter upstream of *rab-6.2* cDNA (*Pdpy-7:rab-6.2* cDNA) fully rescues impermeability to Hoechst 33258 stain in *rab-6.2(ok2254)* animals, confirming that RAB-6.2 expression in *C. elegans* epidermis is sufficient to promote the impermeability of the *C. elegans* cuticle barrier.

RAB-6.2 promotes skin integrity independently of retromer-dependent retrograde trafficking or the GARP complex subunit VPS-52

RAB-6.2 function is best described in neurons, where it mediates retrograde trafficking of the GLR-1 AMPA receptor subunit, and the scaffolding protein LIN-10 via the retromer complex and RME-8 (Zhang et al., 2016, 2012). We hypothesized that RAB-6.2 function in skin integrity may also require retromer complex, RME-8 and LIN-10 function as it does for the previously described GLR-1 trafficking in neurons. We tested mutants in the core protein of the retromer complex, VPS-35, using our hypotonic medium hypersensitivity assay to uncover any role(s) the retromer may be playing in skin integrity. To our surprise, *vps-35(hu68)* animals ruptured at rates that were indistinguishable from those of wild-type animals (Fig. 5A).

RAB-6.2, LIN-10 and RME-8 are also required for retrograde trafficking of GLR-1 in neurons. We tested hypersensitivity to hypotonic shock in the *rme-8(b1023)* temperature-sensitive mutant. We did not observe a difference in sensitivity to hypotonic shock relative to wild-type animals regardless of whether L4 animals were kept at 20°C or 25°C overnight before the assay (Fig. 5B; Fig. S1B). We also tested *lin-10(e1439)* animals (null for LIN-10) and did not observe any hypersensitivity to hypotonic shock (Fig. 5C). Taken together, these data suggest that RAB-6.2 promotes skin integrity through a different mechanism than its well-described role in retromer-dependent retrograde trafficking of GLR-1 in neurons.

RAB-6.2 has also been shown to directly interact with the Golgi-associated retrograde transport complex (GARP) in *C. elegans* (Luo et al., 2011). GARP complex proteins are ubiquitously expressed, suggesting that RAB-6.2 may mediate its role in the epidermis via its previously identified association with the GARP. We tested sensitivity to hypotonic stress in the GARP protein mutant *vps-52(ok853)*. We show that loss of VPS-52 does not affect skin integrity, suggesting that RAB-6.2 function in the epidermis may be also independent of its interaction with the GARP (Fig. 5D).

Mutants that affect the exocyst do not phenocopy *rab-6.2*-null mutants

The exocyst is an octameric protein complex that regulates many physiological processes mainly by controlling exocytosis (Wu and Guo, 2015). A recent RNAi screen identified multiple genes that affect cuticular alae formation in *C. elegans* and showed that components of the exocyst complex are necessary for alae formation (Hyenne et al., 2015). We hypothesized that *rab-6.2(ok2254)* may lead to a defect in secretion in alae, rendering animals fragile at these malformed structures. We tested putative null mutants in the exocyst genes *sec-8(ok2187), exoc-8(ok2523),* and *exoc-7(ok2006).* These mutations delete a large part of the proteins in question and put the rest of the sequence out of frame suggesting that they are putative null alleles. *exoc-7(ok2006)* adults ruptured at rates indistinguishable from wild-type (Fig. 6A). However, we observed a significant increase in rupture rates in *sec-8(ok2187)* and *exoc-8(ok2523)* adults (Fig. 6B,C). Significantly, *sec-8(ok2187)* and *exoc-8(ok2523)* adults ruptured at their vulva, in contrast to *rab-6.2(ok2254)* animals that rupture at random positions along the body (Fig. 6D), suggesting distinct mechanisms to mediate epidermal integrity.

To further investigate the potential for distinct mechanisms in skin integrity, we sought to determine whether the increase in rupture in *sec-8(ok2187)* and *exoc-8(ok2523)* also correlates with increased drug sensitivity, as observed in *rab-6.2 (ok2254)* mutants. We treated wild-type, *rab-6.2(ok2254)*, and *sec-8(ok2187)* worms with 150 µM of tetramisole and counted the numbers of paralyzed animals after 15 min as previously described. We determined that, unlike *rab-6.2(ok2254)* animals, *sec-8(ok2187)* and *exoc-8(ok2523)* animals of the same age do not exhibit hypersensitivity to tetramisole (Fig. 6E,F). We then imaged the alae in *rab-6.2(ok2254)* and wild-type adults and were not able to distinguish differences between the two genotypes in experiments where researchers were

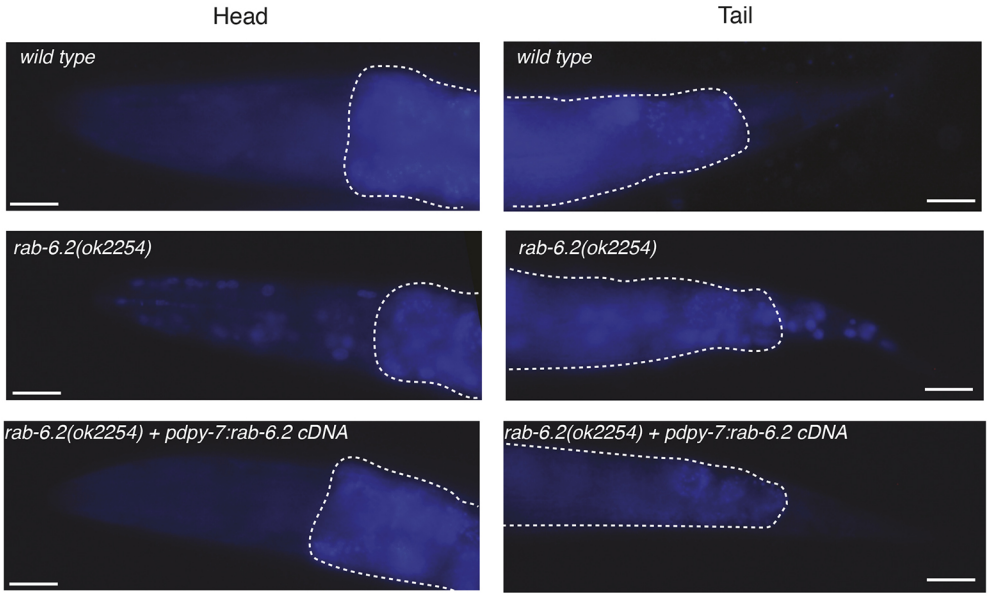

Fig. 4. *rab-6.2(ok2254)* are easily stained by Hoechst DNA dye. Representative image of the pharynx (left) and tail (right) in animals stained with Hoechst 33258. Dashed line separates autofluorescence in the intestine from the pharynx and tail cells. The top to bottom row show: wild type, *rab-6.2(ok2254)*, and *rab-6.2(ok2254)* carrying an epidermis-specific RAB-6.2 from an extrachromosomal transgene containing *Pdpy-7:rab-6.2 cDNA*. Images are representative of at least 20 animals from each genotype. Scale bars: 20 µm.

Journal of Cell Science (2019) 132, jcs223586. doi:10.1242/jcs.223586

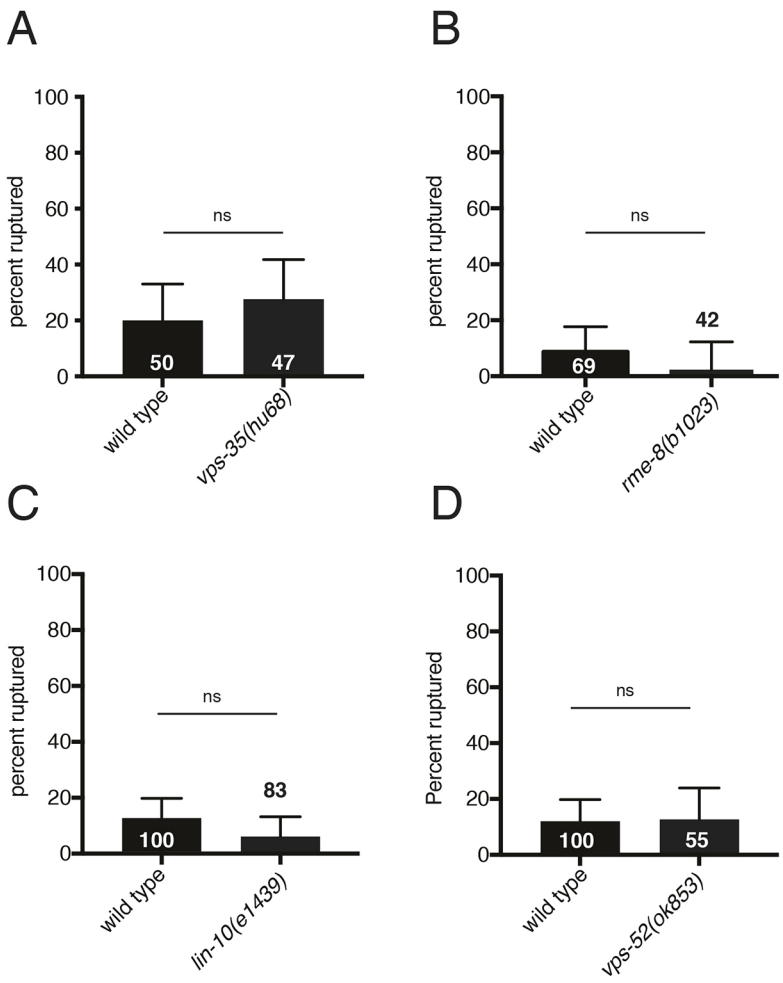

Fig. 5. Mutations in retromer and retromer-associated genes do not affect skin integrity. Quantification of the percentage of animals showing rupture after 15 min in dH_2O (A) in wild type versus *vps-35(hu68)*, (B) wild type versus *rme-8(b1023)* at 20°C, (C) wild type versus *lin-10(e1439)*, and (D) wild type versus *vps-52(ok853)*. ns, not significant ($P>0.05$), Fisher's exact test. Error bars are 95% c.i. Total numbers of animals counted from at least three independent experiments are shown.

blind to the genotypes (data not shown), in contrast to previously published alae defects in exocyst mutants (Hyenne et al., 2015). Taken together, our data suggest that, while a possible interaction between the exocyst and RAB-6.2 during cuticle secretion may be involved, additional intracellular trafficking mechanisms defective in *rab-6.2(ok2254)* animals play an essential role in mediating cuticle integrity in general, and specifically, in promoting cuticle impermeability.

RAB-6.2 is necessary for *M. nematophilum* infection

In an effort to elucidate the mechanism of action of RAB-6.2 in the cuticle and to determine whether the top layer of glycosylation on the cuticle is affected by the lack of RAB-6.2, we examined susceptibility of the *rab-6.2(ok2254)* mutant to the bacterial pathogen *M. nematophilum* (Hodgkin et al., 2000). It has been previously shown that *M. nematophilum*, when present in bacterial food on NGM plates, causes a specific *dar* (deformity of the anal region) phenotype in wild-type worms (Fig. 7A). Several mutants that are resistant to *M. nematophilum* have been isolated and cloned to genes associated with glycosylation of the cuticular surface, suggesting that failure to exhibit a *dar* phenotype in response to exposure to *M. nematophilum* is a good assay for cuticular glycosylation defects (Gravato-Nobre et al., 2005, 2011; Hodgkin et al., 2000; Höflich et al., 2004). Significantly, some of these mutants also affect cuticle integrity as assayed by resistance to sodium hypochlorite treatment (Xiong et al., 2017; Gravato-Nobre et al., 2005). We set out to investigate whether *rab-6.2(ok2254)* affects the *dar* phenotype in response to *M. nematophilum* infection as an indirect assay for surface glycosylation defects.

We obtained *M. nematophilum* from the *C. elegans* Genetics Center and added it to *C. elegans* food bacteria, *E. coli* OP50, as previously described (Gravato-Nobre et al., 2005). Significantly, whereas wild-type animals showed a *dar* phenotype at a highly penetrant rate, *rab-6.2(ok2254)* are not susceptible to *M. nematophilum.* Susceptibility to *M. nematophilum* is rescued by a transgene containing a previously described PCR amplicon of the wild-type *rab-6.2* locus (Fig. 7A–D). Furthermore, we tested the cuticular integrity in known glycosylation mutants in our hypotonic shock and tetramisole hypersensitivity assays. Importantly, we show that distinct mutations affect rupture and permeability differently. Specifically, the *srf-3(e2689)* mutation in the nucleotide sugar transporter gene increases fragility but does not affect sensitivity to tetramisole, in contrast, the *bus-17(e2800)* mutation in the glycosyltransferase gene results in fragility and hypersensitivity to tetramisole (Fig. 7E,F) (Xiong et al., 2017). Taken together, these data suggest that RAB-6.2 may be necessary for cuticular surface glycosylation, as has been shown for many genes that, when mutated, confer resistance to *M. nematophilum* infection.

RAB-6.2 function in epidermis integrity is conserved across phyla

C. elegans RAB-6.2 shares 81% amino acid identity with human RAB6A (Blast2p $E=3\times10^{-119}$). To determine whether the function of RAB-6.2 in the epidermis is also conserved, we cloned a mouse

Journal of Cell Science (2019) 132, jcs223586. doi:10.1242/jcs.223586

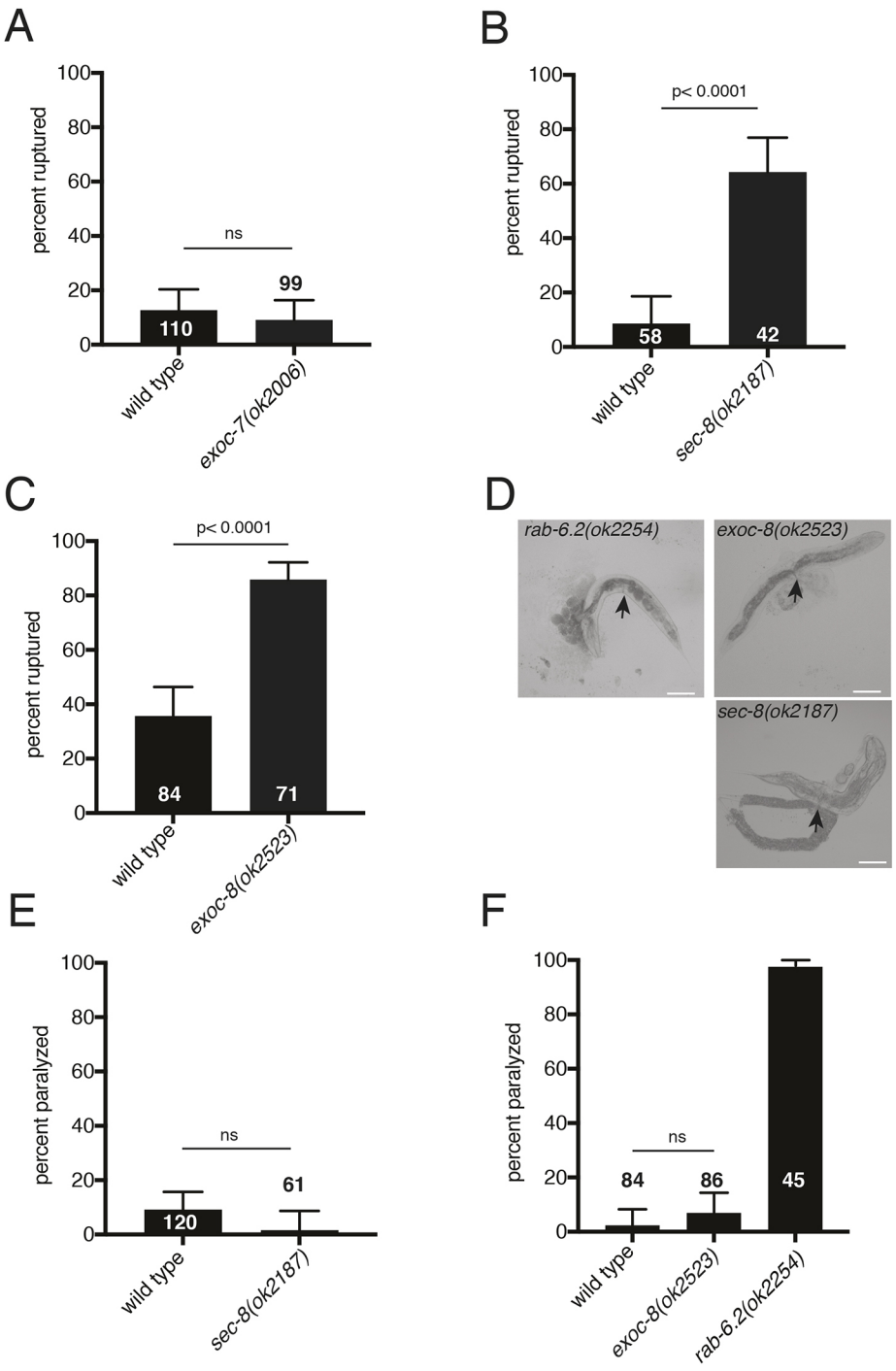

Fig. 6. Exocyst mutants rupture at their vulva in hypotonic medium but do not show drug hypersensitivity. Quantification of the percentage of animals showing rupture after 15 min in dH_2O (A) in wild type versus *exoc-7(ok2006)*, (B) wild type versus *sec-8(ok2187)*, and (C) wild type versus *exoc-8(ok2523)*. (D) Images of *rab-6.2(ok2254)* and exocyst mutants ruptured at different locations along the body during hypotonic shock. Arrows show the intact vulva in a ruptured *rab-6.2(ok2254)* animal and ruptured vulvas in *exoc-8(ok2523)* and *sec-8(ok2187)* animals. Scale bars: 100 µm. (E) Paralysis after 15 min in 150 µM tetramisole in wild type versus *sec-8(ok2187)*. (F) Paralysis after 15 min in 150 µM tetramisole in wild type versus *exoc-8(ok2523)*. ns, not significant ($P>0.05$), Fisher's exact test. Error bars are 95% c.i. Total numbers of animals counted from at least three independent experiments are shown.

RAB6A cDNA driven by the *Pdpy-7 C. elegans* skin promoter for use in tissue-specific rescue experiments (*Pdpy-7:Mouse rab6a*). We confirmed the specific expression driven by *Pdpy-7* by generating *Pdpy-7:mCherry* transgenic animals and imaging red fluorescence in three independent transgenic lines. We observe strong mCherry expression in the hypodermal syncytium (old terminology for *C. elegans* epidermis; Chisholm and Hsiao, 2012) but not in other cells, including seam cells (Fig. S2A,B). Significantly, tissue-specific expression of a mouse RAB6A cDNA driven by the above described promoter in *C. elegans* skin (*Pdpy-7:Mouse rab6a*) rescues cuticular strength and impermeability to wild-type levels in the *rab-6.2(ok2254)* mutant (Fig. 8A,B). This suggests that RAB-6.2 function in *C. elegans* skin is conserved across phyla.

DISCUSSION

RAB-6.2 is a highly conserved small GTPase of the Rab family. The amino acid sequence of *C. elegans* RAB-6.2 is 81% identical to human RAB6A. In *C. elegans*, RAB-6.2 has previously been identified as being involved in retrograde transport (Zhang et al., 2012, 2016). These studies converge on a retromer-dependent role for RAB-6.2 in *C. elegans* neurons and intestine. We set out to describe and analyze a novel fragile skin phenotype in *rab-6.2*-null mutant *C. elegans*.

We show that RAB-6.2 is required for the structural integrity and impermeability of the *C. elegans* skin. These phenotypes are rescued by expression of the wild-type *rab-6.2* locus and by tissue-specific expression of *rab-6.2* cDNA in the epidermis using the

Journal of Cell Science (2019) 132, jcs223586. doi:10.1242/jcs.223586

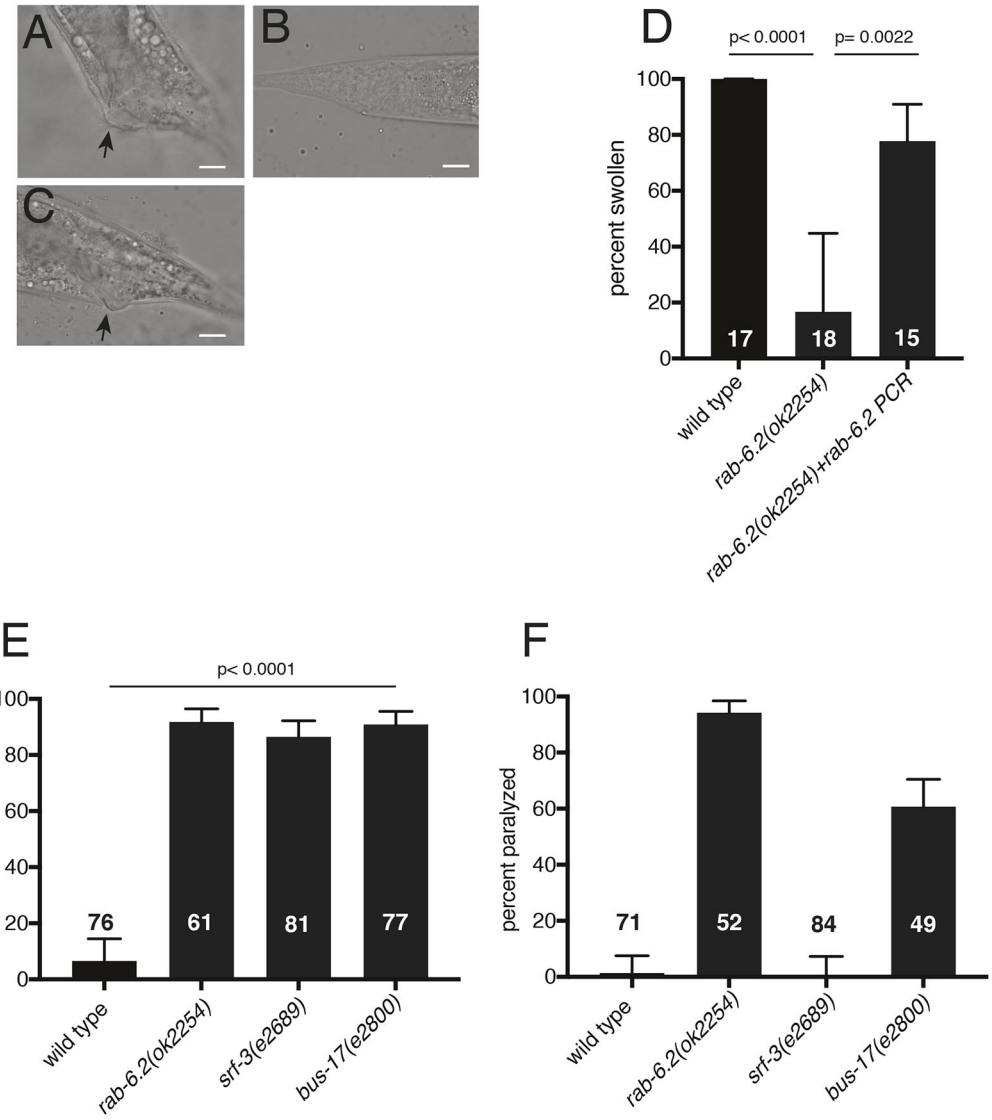

Fig. 7. *rab-6.2 (ok2254)* is resistant to *M. Nematophilum* infection. (A) Wild-type animals show the *dar* phenotype (deformity at the anal region, arrow) in response to *M. nematophilum* infection. (B) *rab-6.2(ok2254)* animals exposed to *M. nematophilum* do not show the *dar* phenotype. (C) Rescue of sensitivity to *M. nematophilum* upon expression of a *rab-6.2* genomic locus (arrow shows *dar* phenotype). Scale bars: 10 µm. (D) Quantification of the percentage of animals with *a dar* phenotype in the strains pictured in A–C. (E) Quantification of rupture after 15 min in dH_2O in wild type, *rab-6.2(ok2254)*, and known glycosylation mutants. (F) Paralysis after 15 min in 150 µM tetramisole in wild type and known glycosylation mutants. $P<0.0001$, Fisher's exact test. Error bars are 95% c.i. Total numbers of animals counted from at least three independent experiments are shown.

dpy-7 tissue-specific promoter. The previously described mechanism of action of RAB-6.2 in neurons requires the retromer complex, RME-8 and the scaffolding protein LIN-10.

We show that the mechanism of action of RAB-6.2 in cuticle integrity is distinct from the well-described mechanism of its action in neurons (Fig. 5A–C). RAB-6.2 is also known to associate with the GARP complex involved in linking endosomes to the late Golgi (Luo et al., 2011). Significantly, a mutation in the GARP complex protein VPS-52 does not affect skin integrity (Fig. 5D). Taken together, our data suggest that the mechanism of action of RAB-6.2 in mediating *C. elegans* epidermis integrity is distinct from its well-described functions in other processes.

Because the fragility and increased permeability of *rab-6.2*-null animals may be caused by intracellular trafficking defects affecting secretion of a component of the cuticle, we investigated genes known to be involved in this process. The exocyst complex is associated with the regulation of exocytosis (Wu and Guo, 2015). Recent studies showed that the exocyst complex is necessary for the development of visible collagenous ridges, alae, that run along the entire *C. elegans* cuticle (Hyenne et al., 2016; Liégeois et al., 2006). We hypothesized that mutations in exocyst genes may cause animals to rupture because of the alae defect, suggesting that RAB-6.2 and exocyst may both control cuticle integrity, and therefore may interact genetically and molecularly. We found that the phenotypes of *rab-6.2* null and exocyst mutants are similar but separable. Specifically, while a deletion in the exocyst genes *sec-8* and *exoc-8* significantly increases sensitivity to hypotonic stress (Fig. 6B,C), neither of the mutants we tested fully replicated the permeability defect observed in the *rab-6.2*-null animals (Fig. 6E,F). These data suggest that, while the exocyst may play a partial role in the function of RAB-6.2 in the skin, the mechanisms of action of RAB-6.2 likely include additional mechanisms. We also show that extracellular vesicles that are increased by a *tat-5* mutation (Beer et al., 2018; Wehman et al., 2011) do not affect cuticle integrity (Fig. S1C).

In order to uncover the nature of any additional mechanisms that may mediate RAB-6.2 function in the epidermis, we searched for other *C. elegans* mutants that show fragility and a compromised skin. Mutations that affect cuticle protein glycosylation are known to affect *C. elegans* cuticle integrity and susceptibility to infection, suggesting that RAB-6.2 could mediate cuticle integrity, at least in part by regulating the intracellular trafficking of a glycosylated secreted protein (Gravato-Nobre et al., 2011; Partridge et al., 2008).

Several genes that affect surface glycosylation were initially identified as mutations that confer resistance to the nematode

Journal of Cell Science (2019) 132, jcs223586. doi:10.1242/jcs.223586

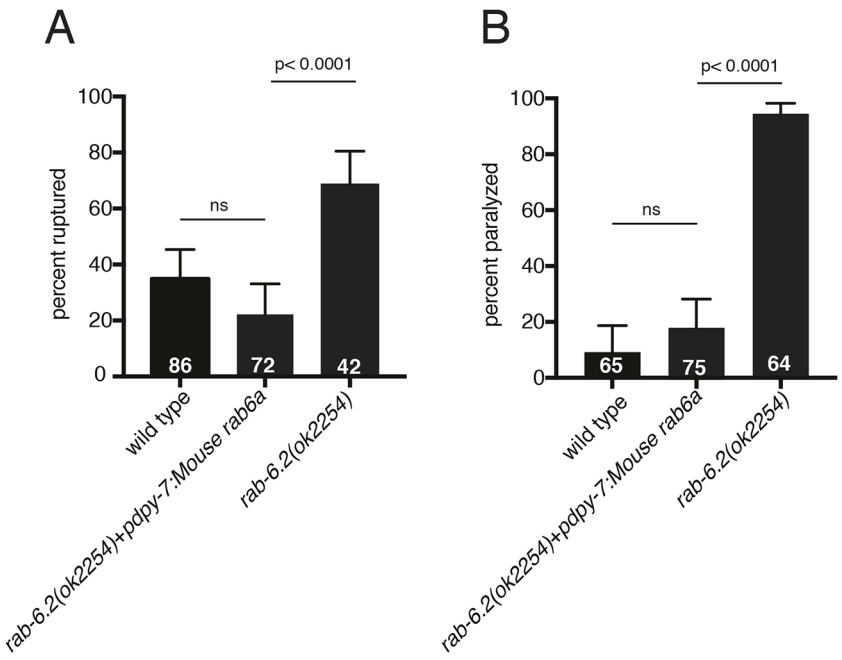

Fig. 8. *rab-6.2 (ok2254)* function in skin integrity and permeability is conserved across phyla.
(A) Quantification of rupture after 15 min in dH_2O in *rab-6.2(ok2254)* and *rab-6.2(ok2254)* carrying a mouse *Rab6a* cDNA expressed in the worm epidermis from an extrachromosomal transgene containing *Pdpy-7:Mouse rab6a cDNA*. (B) Quantification of paralysis on NGM plates containing 150 µM tetramisole after 15 min in the same strains as in A. Error bars are 95% c.i. Total numbers of animals counted from at least three independent experiments are shown.

pathogen *M. nematophilum* (Gravato-Nobre et al., 2005, 2011; Hodgkin et al., 2000; Höflich et al., 2004). We show that *rab-6.2*-null animals are resistant to infection in a similar manner to glycosylation mutants and in strong contrast to wild-type worms. Sensitivity to *M. nematophilum* infection is rescued by a transgene containing a genomic copy of wild-type *rab-6.2* (Fig. 7A–D). Significantly, a mutant allele in the glycosyltransferase *bus-17* phenocopies the fragility and permeability of *rab-6.2(ok2254)* (Fig. 7E,F). *rab-6.2(ok2254)* animals also show poor traction on plates (the 'skiddy' phenotype) similarly to *bus-17* mutants. We also tested *srf-2(e2171)* animals and showed that, as described for other glycosylation mutants, these animals are also resistant to *M. nematophilum* infection (Fig. S1D). Our results suggest that RAB-6.2 may regulate surface glycosylation, as was previously shown for other *M. nematophilum*-resistant mutants. The mechanism by which RAB-6.2 may regulate glycosylation is likely indirect, resulting from the role of RAB-6.2 in Golgi trafficking of client proteins and/or glycosylation enzymes like BUS-17 (Fisher and Ungar, 2016). Significantly, Golgi-related glycosylation defects are also linked to many inherited diseases, some of which affect the skin and bones, and mutations in RAB6-interacting proteins cause severe inherited diseases with strong skin defects such as gerodermia osteodysplastica and congenital disorders of glycosylation (CDG) in humans (Chan et al., 2018; Freeze and Ng, 2011; Hennies et al., 2008; Egerer et al., 2015; Wu et al., 2004; Sun et al., 2007).

The high level of sequence conservation between RAB-6.2 and human RAB6A and the role of RAB6 and glycosylation in human diseases led us to hypothesize that the cell biology of RAB-6.2 in *C. elegans* skin may also be conserved. We show that a mouse RAB6A cDNA rescues skin integrity and impermeability when ectopically expressed in in *C. elegans* skin (Fig. 8A,B). Thus, a mouse RAB6A copy can fully rescue the defects caused by *rab-6.2(ok2254)* in a tissue-specific manner. We hypothesize that studying the molecular function of RAB-6.2 in *C. elegans* skin may help further characterize conserved cell biological mechanisms pertinent to human development and disease.

Additionally, the increased permeability of *rab-6.2* animals shown here could also be advantageous when using *C. elegans* in high-throughput pharmacological screens. These screens are usually difficult to perform because of poor bioavailability of the drug due to the impermeability of the wild-type worm cuticle (Burns et al., 2010). Thus, *rab-6.2* null animals could be used as a genetic background to enhance high-throughput pharmacogenetics in *C. elegans* (Zheng et al., 2013).

MATERIALS AND METHODS

C. elegans strains

Animals were maintained on NGM agar plates with *E. coli* OP50 as a source of food (Stiernagle, 2006). Temperature was controlled at 20°C unless otherwise stated. Detailed descriptions of worm strains, genotypes, transgenes and PCR primers can be found in the supplementary information (Tables S1–S4). Some mutant strains were provided by the *C. elegans* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

Spontaneous rupture and water rupture

For the quantification of spontaneous rupture, L4 stage animals were picked and placed on NGM plates with OP50 bacteria food source 24 h before counting using a Meiji EMZ13 stereomicroscope. At 24 h after picking, ruptured animals (arrows in Fig. 1C,D) and live animals were counted. For the water rupture assay, L4 animals were picked 1 day before the experiment and allowed to mature to day 1 adults before the experiment. During the experiment, 10–20 animals were transferred into the cap of a microfuge tube filled with 200 µl of deionized water. After 15 min in water, the numbers of ruptured animals were counted. Controlled experiments were performed nine times. *N* is the total number of animals counted. Animals were picked and counted using a Meiji EMZ13 stereomicroscope.

For the detailed image of rupture, 1-day-old adult hermaphrodites were mounted in a slurry of 0.05 µm diameter polystyrene beads (Polysciences Inc) to immobilize the animals. Animals were visualized with a Nikon Eclipse Ci L microscope and a Nikon plan 10×/0.25 NA objective and captured with a Hamamatsu Orca 05-G camera.

Paralysis assays

One-day-old adults were transferred to unseeded NGM plates containing 150 µM tetramisole hydrochloride (Sigma) or 60 mM piperazine (Sigma-Aldrich). Animals remained on the drug plates for 15 or 20 min for tetramisole treatment and 15 or 30 min for piperazine treatment. Paralysis was defined as the inability to move when prodded three times consecutively

with a platinum wire pick. These experiments were repeated at least twice independently for each time point.

Molecular biology

Plasmids were assembled using Gateway recombination (Invitrogen). A Rab-6.2 PCR-amplified rescue fragment was injected as a linear double-stranded (ds)DNA. All PCR reactions for entry clones and rescue were performed using Phusion DNA polymerase (Thermo Scientific) or Q5 high fidelity polymerase (New England Biolabs). The *rab6A* cDNA was cloned as described above from a mouse thymus cDNA library graciously donated by Dr Sophia Sarafova (Davidson College. Davidson, NC). Primer sequences, templates, and plasmid names are listed in the supplementary information (Table S1).

Transgenics

Transgenic animals were obtained by microinjection on a MINJ-1000 microinjection system (Tritech research) as described previously (Mello et al., 1991). Transgene name, content and concentrations are listed in the supplementary information (Table S3). For most strains, stable transgenic lines were selected based on GFP expression in the pharyngeal muscles from a P*myo-2*:GFP co-injection marker.

Hoechst staining

Hoechst 33258 pentahydrate was purchased from Invitrogen (Molecular Probes). Animals were stained according to the protocol by Kage-Nakadai et al. (2010). Images were captured using an Olympus BX60 microscope and an Olympus UPlanFl 40×0.75 NA Ph2 objective and an Olympus DP80 camera. All compared images were captured using the same exposure time.

Microbacterium nematophilum infection

Microbacterium nematophilum (strain CBX102) was obtained from the *C. elegans* Genetics Center and added to *E. Coli* feeding bacteria OP50 according to the protocol by Gravato-Nobre et al. (2005). Tails of animals of different genotypes were captured using a Nikon Eclipse Ci L microscope and a Nikon plan 100X/0.4 NA objective and images were captured with a Hamamatsu Orca 05-G camera. The images were then scored blindly for the *dar* phenotype.

Statistics

The investigator was blinded to the genotype when performing the assay. All experiments were repeated on different days by at least two investigators. The total numbers of worms from independent experiments were pooled and compared. The total number is displayed on each graph. All experiments are adequately powered as calculated G*power software. No samples were excluded. Error bars represent 95% confidence intervals as calculated by the Wald method, and two-tailed *P*-values were calculated using Fisher's exact test (http://www.graphpad.com/quickcalcs/).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.D.K., A.Y.C., R.J.M., B.M.P., R.E.B.; Methodology: J.D.K., A.Y.C., R.J.M., H.D., R.E.B.; Validation: R.E.B.; Formal analysis: R.E.B.; Investigation: J.D.K., A.Y.C., R.J.M., G.B., H.D., A.L., R.E.B.; Resources: B.M.P., R.E.B.; Data curation: J.D.K., A.Y.C., G.B., B.M.P., A.L., R.E.B.; Writing - original draft: J.D.K., R.E.B., A.Y.C.; Writing - review & editing: J.D.K., A.Y.C., R.J.M., B.M.P., R.E.B.; Visualization: R.E.B.; Supervision: R.E.B.; Project administration: R.E.B.; Funding acquisition: A.Y.C., R.E.B.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.223586.supplemental

References

Balklava, Z., Pant, S., Fares, H. and Grant, B. D. (2007). Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic. *Nat. Cell Biol.* **9**, 1066-1073.

Bardin, S., Miserey-Lenkei, S., Hurbain, I., Garcia-Castillo, D., Raposo, G. and Goud, B. (2015). Phenotypic characterisation of RAB6A knockout mouse embryonic fibroblasts. *Biol. Cell* **107**, 427-439.

Beer, K. B., Rivas-Castillo, J., Kuhn, K., Fazeli, G., Karmann, B., Nance, J. F., Stigloher, C. and Wehman, A. M. (2018). Extracellular vesicle budding is inhibited by redundant regulators of TAT-5 flippase localization and phospholipid asymmetry. *Proc. Natl. Acad. Sci. USA* **115**, E1127-E1136.

Burns, A. R., Wallace, I. M., Wildenhain, J., Tyers, M., Giaever, G., Bader, G. D., Nislow, C., Cutler, S. R. and Roy, P. J. (2010). A predictive model for drug bioaccumulation and bioactivity in Caenorhabditis elegans. *Nat. Chem. Biol.* **6**, 549-557.

Chan, W. L., Steiner, M., Witkos, T., Egerer, J., Busse, B., Mizumoto, S., Pestka, J. M., Zhang, H., Hausser, I., Khayal, L. A. et al. (2018). Impaired proteoglycan glycosylation, elevated TGF-beta signaling, and abnormal osteoblast differentiation as the basis for bone fragility in a mouse model for gerodermia osteodysplastica. *PLoS Genet.* **14**, e1007242.

Chisholm, A. D. and Hsiao, T. I. (2012). The Caenorhabditis elegans epidermis as a model skin. I: development, patterning, and growth. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 861-878.

Chisholm, A. D. and Xu, S. (2012). The Caenorhabditis elegans epidermis as a model skin. II: differentiation and physiological roles. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 879-902.

Delcastillo, J., Morales, T. A. and Sanchez, V. (1963). Action of Piperazine on the Neuromuscular System of Ascaris Lumbricoides. *Nature* **200**, 706-707.

Egerer, J., Emmerich, D., Fischer-Zirnsak, B., Chan, W. L., Meierhofer, D., Tuysuz, B., Marschner, K., Sauer, S., Barr, F. A., Mundlos, S. et al. (2015). GORAB missense mutations disrupt RAB6 and ARF5 binding and golgi targeting. *J. Invest. Dermatol.* **135**, 2368-2376.

Fares, H. and Greenwald, I. (2001). Genetic analysis of endocytosis in Caenorhabditis elegans: coelomocyte uptake defective mutants. *Genetics* **159**, 133-145.

Fisher, P. and Ungar, D. (2016). Bridging the gap between glycosylation and vesicle traffic. *Front. Cell Dev. Biol.* **4**, 15.

Freeze, H. H. and Ng, B. G. (2011). Golgi glycosylation and human inherited diseases. *Cold Spring Harb. Perspect Biol.* **3**, a005371.

Gilleard, J. S., Barry, J. D. and Johnstone, I. L. (1997). cis regulatory requirements for hypodermal cell-specific expression of the *Caenorhabditis elegans* cuticle collagen gene dpy-7. *Mol. Cell. Biol.* **17**, 2301-2311.

Goud, B., Yang, C., Roa, M., Martinez, O. and Mayau, V. (1994). Study of rab6, a ras-like GTP-binding protein associated with the Golgi complex. *Ann. N. Y. Acad. Sci.* **710**, 192-195.

Grant, B. and Hirsh, D. (1999). Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell* **10**, 4311-4326.

Gravato-Nobre, M. J., Nicholas, H. R., Nijland, R., O'rourke, D., Whittington, D. E., Yook, K. J. and Hodgkin, J. (2005). Multiple genes affect sensitivity of *Caenorhabditis elegans* to the bacterial pathogen Microbacterium nematophilum. *Genetics* **171**, 1033-1045.

Gravato-Nobre, M. J., Stroud, D., O'rourke, D., Darby, C. and Hodgkin, J. (2011). Glycosylation genes expressed in seam cells determine complex surface properties and bacterial adhesion to the cuticle of *Caenorhabditis elegans*. *Genetics* **187**, 141-155.

Hennies, H. C., Kornak, U., Zhang, H., Egerer, J., Zhang, X., Seifert, W., Kuhnisch, J., Budde, B., Natebus, M., Brancati, F. et al. (2008). Gerodermia osteodysplastica is caused by mutations in SCYL1BP1, a Rab-6 interacting golgin. *Nat. Genet.* **40**, 1410-1412.

Hermann, G. J., Schroeder, L. K., Hieb, C. A., Kershner, A. M., Rabbitts, B. M., Fonarev, P., Grant, B. D. and Priess, J. R. (2005). Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*. *Mol. Biol. Cell* **16**, 3273-3288.

Hodgkin, J., Kuwabara, P. E. and Corneliussen, B. (2000). A novel bacterial pathogen, Microbacterium nematophilum, induces morphological change in the nematode *C. elegans*. *Curr. Biol.* **10**, 1615-1618.

Höflich, J., Berninsone, P., Göbel, C., Gravato-Nobre, M. J., Libby, B. J., Darby, C., Politz, S. M., Hodgkin, J., Hirschberg, C. B. and Baumeister, R. (2004). Loss of srf-3-encoded nucleotide sugar transporter activity in *Caenorhabditis*

elegans alters surface antigenicity and prevents bacterial adherence. *J. Biol. Chem.* **279**, 30440-30448.
Hutagalung, A. H. and Novick, P. J. (2011). Role of Rab GTPases in membrane traffic and cell physiology. *Physiol. Rev.* **91**, 119-149.
Hyenne, V., Apaydin, A., Rodriguez, D., Spiegelhalter, C., Hoff-Yoessle, S., Diem, M., Tak, S., Lefebvre, O., Schwab, Y., Goetz, J. G. et al. (2015). RAL-1 controls multivesicular body biogenesis and exosome secretion. *J. Cell Biol.* **211**, 27-37.
Hyenne, V., Labouesse, M. and Goetz, J. G. (2016). The Small GTPase Ral orchestrates MVB biogenesis and exosome secretion. *Small GTPases* **9**, 445-451.
Jorgensen, E. M. (2005). Gaba. *WormBook* 1-13. http://www.wormbook.org/chapters/www_gaba/gaba.html
Kage-Nakadai, E., Kobuna, H., Kimura, M., Gengyo-Ando, K., Inoue, T., Arai, H. and Mitani, S. (2010). Two very long chain fatty acid acyl-CoA synthetase genes, acs-20 and acs-22, have roles in the cuticle surface barrier in *Caenorhabditis elegans*. *PLoS ONE* **5**, e8857.
Liégeois, S., Benedetto, A., Garnier, J.-M., Schwab, Y. and Labouesse, M. (2006). The V0-ATPase mediates apical secretion of exosomes containing Hedgehog-related proteins in *Caenorhabditis elegans*. *J. Cell Biol.* **173**, 949-961.
Luo, L., Hannemann, M., Koenig, S., Hegermann, J., Ailion, M., Cho, M.-K., Sasidharan, N., Zweckstetter, M., Rensing, S. A. and Eimer, S. (2011). The *Caenorhabditis elegans* GARP complex contains the conserved Vps51 subunit and is required to maintain lysosomal morphology. *Mol. Biol. Cell* **22**, 2564-2578.
Martinez, O., Antony, C., Pehau-Arnaudet, G., Berger, E. G., Salamero, J. and Goud, B. (1997). GTP-bound forms of rab6 induce the redistribution of Golgi proteins into the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **94**, 1828-1833.
Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959-3970.
Page, A. P. and Johnstone, I. L. (2007). The cuticle. *WormBook* 1-15.
Partridge, F. A., Tearle, A. W., Gravato-Nobre, M. J., Schafer, W. R. and Hodgkin, J. (2008). The *C. elegans* glycosyltransferase BUS-8 has two distinct and essential roles in epidermal morphogenesis. *Dev. Biol.* **317**, 549-559.
Patwardhan, A., Bardin, S., Miserey-Lenkei, S., Larue, L., Goud, B., Raposo, G. and Delevoye, C. (2017). Routing of the RAB6 secretory pathway towards the lysosome related organelle of melanocytes. *Nat. Commun.* **8**, 15835.
Rand, J. B. (2007). Acetylcholine. *WormBook* 1-21.
Sato, K., Norris, A., Sato, M. and Grant, B. D. (2014). *C. elegans* as a model for membrane traffic. *WormBook* 1-47.
Sekine, Y., Lin-Moore, A., Chenette, D. M., Wang, X., Jiang, Z., Cafferty, W. B., Hammarlund, M. and Strittmatter, S. M. (2018). Functional genome-wide screen identifies pathways restricting central nervous system axonal regeneration. *Cell Rep.* **23**, 415-428.
Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell Biol.* **10**, 513-525.
Stiernagle, T. (2006). Maintenance of *C. elegans*. *WormBook* 1-11.
Straud, S., Lee, I., Song, B., Avery, L. and You, Y.-J. (2013). The jaw of the worm: GTPase-activating protein EAT-17 regulates grinder formation in *Caenorhabditis elegans*. *Genetics* **195**, 115-125.
Sun, Y., Shestakova, A., Hunt, L., Sehgal, S., Lupashin, V. and Storrie, B. (2007). Rab6 regulates both ZW10/RINT-1 and conserved oligomeric Golgi complex-dependent Golgi trafficking and homeostasis. *Mol. Biol. Cell* **18**, 4129-4142.
Wehman, A. M., Poggioli, C., Schweinsberg, P., Grant, B. D. and Nance, J. (2011). The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in *C. elegans* embryos. *Curr. Biol.* **21**, 1951-1959.
White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Tzschaschel, B., Echard, A., Goud, B. and et al. (1999). Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J. Cell Biol.* **147**, 743-760.
Wu, B. and Guo, W. (2015). The exocyst at a glance. *J. Cell Sci.* **128**, 2957-2964.
Wu, X., Steet, R. A., Bohorov, O., Bakker, J., Newell, J., Krieger, M., Spaapen, L., Kornfeld, S. and Freeze, H. H. (2004). Mutation of the COG complex subunit gene COG7 causes a lethal congenital disorder. *Nat. Med.* **10**, 518-523.
Xiong, H., Pears, C. and Woollard, A. (2017). An enhanced *C. elegans* based platform for toxicity assessment. *Sci. Rep.* **7**, 9839.
Zeng, L., El Bejjani, R. and Hammarlund, M. (2018). The amyloid precursor-like protein APL-1 regulates axon regeneration. *bioRxiv*, 305284.
Zhang, D., Isack, N. R., Glodowski, D. R., Liu, J., Chen, C. C.-H., Xu, X. Z., Grant, B. D. and Rongo, C. (2012). RAB-6.2 and the retromer regulate glutamate receptor recycling through a retrograde pathway. *J. Cell Biol.* **196**, 85-101.
Zhang, D., Dubey, J., Koushika, S. P. and Rongo, C. (2016). RAB-6.1 and RAB-6.2 promote retrograde transport in *C. elegans*. *PLoS ONE* **11**, e0149314.
Zheng, S.-Q., Ding, A.-J., Li, G.-P., Wu, G.-S. and Luo, H.-R. (2013). Drug absorption efficiency in Caenorhbditis elegans delivered by different methods. *PLoS ONE* **8**, e56877.