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RESEARCH ARTICLE

The Arf family G protein Arl1 is required for secretory granule biogenesis in *Drosophila*

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ABSTRACT

The small G protein Arf like 1 (Arl1) is found at the Golgi complex, and its GTP-bound form recruits several effectors to the Golgi including GRIP-domain-containing coiled-coil proteins, and the Arf1 exchange factors Big1 and Big2. To investigate the role of Arl1, we have characterised a loss-of-function mutant of the *Drosophila* Arl1 orthologue. The gene is essential, and examination of clones of cells lacking Arl1 shows that it is required for recruitment of three of the four GRIP domain golgins to the Golgi, with *Drosophila* GCC185 being less dependent on Arl1. At a functional level, Arl1 is essential for formation of secretory granules in the larval salivary gland. When Arl1 is missing, Golgi are still present but there is a dispersal of adaptor protein 1 (AP-1), a clathrin adaptor that requires Arf1 for its membrane recruitment and which is known to be required for secretory granule biogenesis. Arl1 does not appear to be required for AP-1 recruitment in all tissues, suggesting that it is crucially required to enhance Arf1 activation at the trans-Golgi in particular tissues.

KEY WORDS: Secretory granule, Small G protein, Arf family

INTRODUCTION

The members of the Arf family of small G proteins are key regulators of membrane and cytoskeletal organisation, and several are present in all eukaryotic kingdoms (Gillingham and Munro, 2007; Donaldson and Jackson, 2011). The Arf family G proteins are characterised by being anchored to the bilayer in their GTP-bound state by an N-terminal amphipathic helix rather than the C-terminal lipid anchor used by most other members of the Ras superfamily of small G proteins (Pasqualato et al., 2002). The prototypic member of the family is Arf1, which recruits the COPI and adaptor protein 1 (AP-1)-containing clathrin vesicle coats to the cis and trans sides of the Golgi complex, respectively. In addition, there are several Arf-like (Arl) proteins, which appear to share sequence and mechanistic features with Arf1 (Burd et al., 2004; Kahn et al., 2006). Although several of the Arls are present in all eukaryotic kingdoms, less is known about their regulation and precise function. Where functional roles have been identified, they are in membrane trafficking or cilia formation. One of the best conserved Arls is Arl1, which is present in protozoa, plants, yeasts and mammals and appears to play a major role in trans-Golgi function (Munro, 2005; Price et al., 2005; Stefano et al., 2006). Removal of Arl1 by gene deletion in yeast, or by small interfering RNA (siRNA) in mammalian cells, results in defects in trafficking between endosomes and the Golgi complex (Lee et al., 1997; Lu et al., 2001; Benjamin et al., 2011). In both species, Arl1 has been found to bind directly to particular long coiled-coil proteins through their C-terminal GRIP domains (Van Valkenburgh et al., 2001; Gangi Setty et al., 2003; Lu and Hong, 2003; Panic et al., 2003a). These 'golgins' have been suggested to capture incoming carriers from endosomes, although their precise role is unclear (Chia and Gleeson, 2011; Munro, 2011). In yeast and plants, there is a single GRIP domain golgin, which binds directly to Arl1 (Gangi Setty et al., 2003; Panic et al., 2003b; Latijnhouwers et al., 2005). In metazoans, there are four GRIP domain golgins, and although it seems certain that mammalian Arl1 binds to three of them (golgin-97, golgin-245 and GCC88, encoded in humans by *GOLGA1*, *GOLGA4* and *GCC1*, respectively), there is controversy over whether it is required for the Golgi localisation of the fourth (GCC185, encoded in humans by *GCC2*) (Burguete et al., 2008; Houghton et al., 2009). GCC185 has been found to interact with the microtubule regulator CLASP and so might have distinct or additional roles compared to the other GRIP domain golgins (Efimov et al., 2007). In addition, Arl1 has been recently shown to bind to two further trans-Golgi proteins, the BAR domain protein Arfaptin, and the Arf1 exchange factor Sec71 (the *Drosophila* orthologue of Big1 and Big2) (Man et al., 2011; Christis and Munro, 2012).

Although Arl1 has been studied in tissue culture cells and single-celled eukaryotes, there has been little characterisation of its role in the context of the diverse tissues of a multicellular metazoan. In this paper, we report a characterisation of an Arl1-null mutant in *Drosophila melanogaster*. *Drosophila* has a single Arl1 gene and single orthologues of all four of the metazoan GRIP domain proteins (Sinka et al., 2008). We examine the effect of removing Arl1 on GRIP domain recruitment to the Golgi and the sorting of proteins in various tissues. We find a striking defect in secretory granule biogenesis in the salivary gland, suggesting that there is a key role for Arl1 in tissues with an elevated secretory load.

RESULTS

Drosophila arl1-null mutants

Two independent mutations in the *Drosophila* Arl1 orthologue were isolated serendipitously in a screen to find mutants in the adjacent *Brahma* locus, and both of these mutations were reported to be lethal (Tamkun et al., 1991). The gene has been referred to as *arl1*, *arflike* (*arl*) or *Arflike at 72A* (*Arf72A*), but for simplicity we will refer to it as *arl1*. We sequenced these two *arl1* alleles and found that $arl1^{1}$ has a point mutation that causes an amino acid mutation of glycine 2 to aspartate (Fig. 1A). Like most other Arf family members, Arl1 is N-terminally myristoylated, and this modification requires a glycine following the initiator methionine, with the latter being cleaved off prior to the

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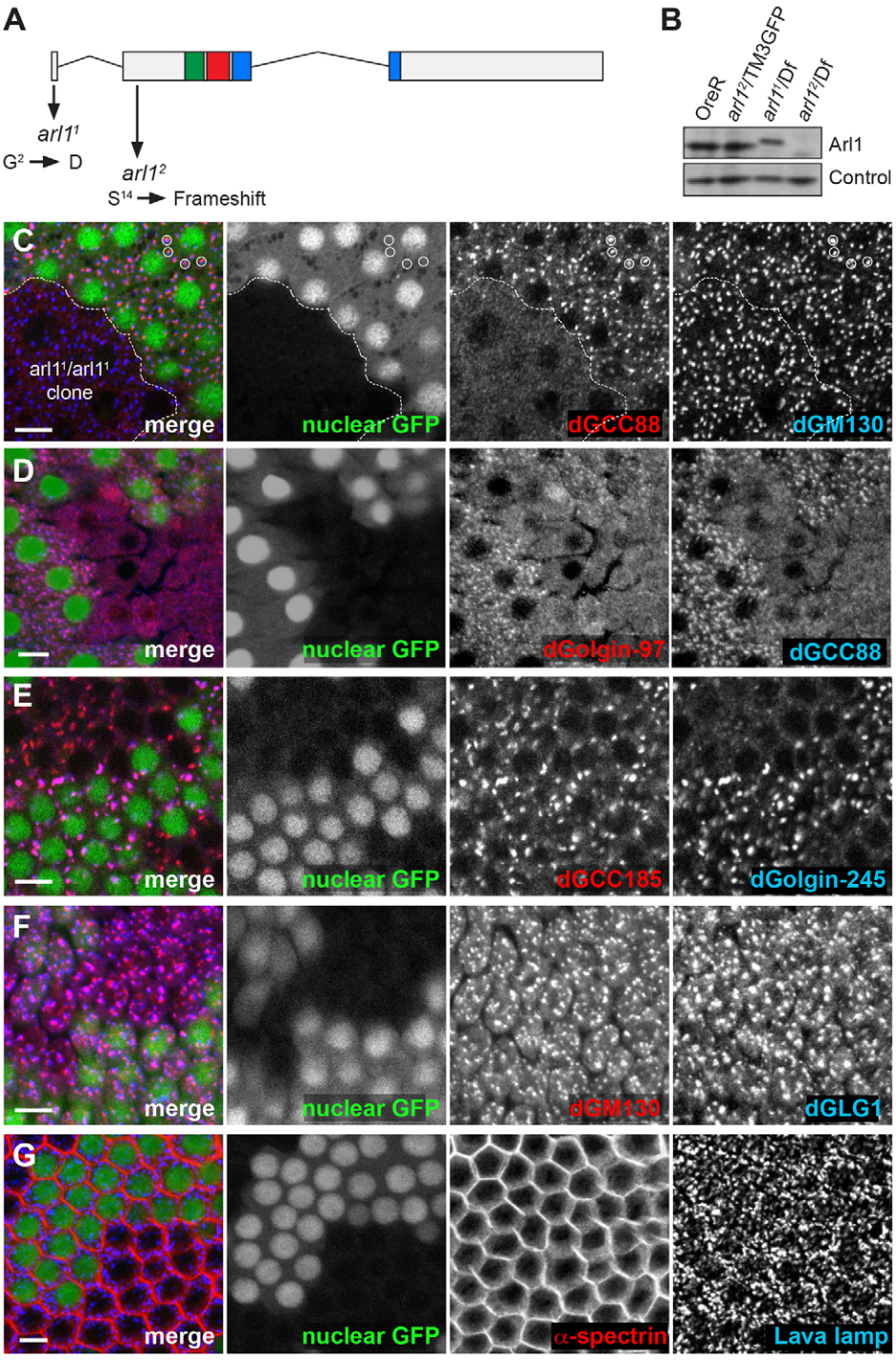

Fig. 1. Arl1 is required to recruit dGolgin-245, dGolgin-97 and dGCC88 to the Golgi, but not dGCC185. (A) Schematic representation of the *Drosophila arl1* gene. Colours indicate the switch I (green), interswitch (red) and switch II (blue) regions. Boxes represent exons and lines introns. (B) Protein blot of L3 larval head lysates from OregonR (OreR), *arl1*^{2}/TM3GFP, *arl1*^{1}/Df(3L)Brahma and *arl1*^{2}/Df(3L)Brahma, probed with an anti-Arl1 polyclonal antibody. The loading control is a background band. (C–F) Cross-section view of epithelial follicle cells with *arl1*^{1}/*arl1*^{1} mitotic clones marked by the absence of nuclear GFP and stained with the indicated antibodies. (C) An *arl1*^{1}/*arl1*^{1} mitotic clone is indicated, with circles around four representative Golgi stacks that are typically scattered throughout the cytoplasm of each cell. (C,D) dGCC88 and dGolgin-97 are displaced from the Golgi in *arl1*^{1} mutant cells, but the cis-Golgi protein GM130 remains well localised. (E) In *arl1*^{1} mutant cells, dGolgin-245 but not dGCC185 is displaced from the Golgi. (F) The structure of the Golgi is not affected in *arl1*^{1} mutant cells, as indicated by the cis- and medial-Golgi markers dGM130 and dGLG1, respectively. (G) The apical targeting of α-spectrin and the medial Golgi protein Lava lamp are not affected in *arl1*^{1} mutant cells. Scale bars: 10 μm.

addition of myristate (Lee et al., 1997; Farazi et al., 2001). Myristoylation of Arl1 and other Arfs has been shown to be essential for membrane targeting and function, explaining the severity of this missense allele, which we expected to be a null (Kahn et al., 1995; Lu et al., 2001).

We found that the second *arl1* allele, *arl1*^{2}, has a frameshift mutation that truncates the protein after only 14 amino acids and so it seems certain to be a null allele (Fig. 1A). To test our predictions on the nature of these two *arl1* alleles, we raised an antibody to Arl1 and performed a western blot on larval extracts from both alleles crossed to a deficiency that uncovers the *arl1* locus. The gel mobility of the mutant Arl1 protein from the *arl1*^{1}/Df mutant larvae was reduced, as would be expected for a non-cleaved, non-myristoylated form (Franco et al., 1995). The *arl1*^{2}/*Df* mutant larvae had no detectable Arl1 protein confirming that this allele is a protein null (Fig. 1B). Both mutant alleles are lethal in late L3 larval stages or pupae, indicating that *arl1*^{1} is as severe as *arl1*^{2} and so is likely to also be a loss-of-function allele.

Arl1 is required for the Golgi localisation of *Drosophila* Golgin-245, Golgin-97 and GCC88, but not GCC185

In order to investigate the function of Arl1, we recombined *arl1*^{1} and *arl1*^{2} onto an FRT chromosome to generate mitotic mutant clones using the FLP/FRT technique. It transpired that *arl1*^{2} has a

second lethal mutation very close to the *arl1* gene that we were unable to recombine away, and so we used $arl1^{1}$ throughout this study. We first tested the requirement of Arl1 to recruit the GRIP domain proteins *Drosophila* (d)Golgin-245, dGolgin-97 (also known as Cbs), dGCC88 and dGCC185 to the Golgi in epithelial follicle cells. As previously described in *Drosophila* S2 cells (Sinka et al., 2008), in wild-type epithelial cells the GRIP proteins localise close to the cis-Golgi marker dGM130 but are somewhat displaced as they are present at the trans-Golgi (Fig. 1C). In clones of $arl1^{1}$ mutant cells, the Golgi labelling of dGCC88, dGolgin-245 and dGolgin-97 are greatly reduced (Fig. 1C,D; data not shown). The trans-Golgi network (TGN) recruitment of dGolgin-245 and dGCC88 was restored by expression of Arl1 from a genomic rescue transgene (supplementary material Fig. S1A). To test whether these proteins fail to localise in the mutant because the entire Golgi complex is disrupted, we examined Golgi integrity using other markers. In $arl1^{1}$ mutant cells, the cis-Golgi proteins dGM130 and dGMAP210, and the medial-Golgi proteins dGLG1 and Lava lamp, localise as in wild-type cells, indicating that Arl1 is not required for Golgi integrity but rather has a more specific role in GRIP domain protein recruitment (Fig. 1C–G; data not shown). However, the fourth GRIP domain protein, dGCC185 was clearly less affected than the other three proteins, with the Golgi levels being, at most, only slightly reduced (Fig. 1E; supplementary material Fig. S1B).

To verify that these effects on the GRIP domain proteins are not unique to follicle cells, we analysed the distribution of the proteins and other Golgi markers in further *Drosophila* cell types. In all tissues analysed, including wing imaginal discs (data not shown), salivary glands (see below) and DMel *Drosophila* cells (supplementary material Fig. S1C,D), loss of Arl1 affected dGolgin-245, dGolgin-97 and dGCC88, but not dGCC185, whereas other Golgi markers appeared unaffected.

Arl1 is not required for E-cadherin traffic or for cell polarity

It has previously been reported that golgin-97 is required for trafficking of E-cadherin in HeLa cells (Lock et al., 2005). This result suggested that Arl1 effectors could have a role in polarised secretion and/or establishment of cell polarity, and indeed this might explain the expansion of the GRIP domain family in metazoans. To test this, we analysed the distribution of cell polarity markers in *arl1* epithelial cell clones. In $arl1^{1}$ mutant follicle cells, α-spectrin localised to the apical domain as in the wild type (Fig. 1G), and E-cadherin was correctly targeted to the apical membrane and adherens junctions of $arl1^{1}$ mutant epithelial cells in wing imaginal discs and salivary glands (see below; data not shown). The septate junction markers Discs-large (Dlg) and Fasciclin III, and the basally secreted protein Perlecan were also correctly localised in *arl1* mutant clones (data not shown). These results show that, at least in *Drosophila*, Arl1 is not required for apical delivery of E-cadherin or for the establishment of cell polarity.

Targeting of dGCC185 in the absence of Arl1 does not require Rab6

It has been reported that in mammalian cells Arl1 and Rab6 cooperate to recruit GCC185 to the TGN (Burguete et al., 2008). However, it has also been reported that siRNA against Arl1 or Rab6A in HeLa cells singly or in combination had no effect on the localisation of GCC185 (Houghton et al., 2009). Given that our results show that Arl1 is not essential for dGCC185 TGN targeting in *Drosophila*, we tested the possibility that there is cooperation between Arl1 and Rab6. To this end, we expressed UAS-RNAi transgenes in epithelial follicle cells with an actin-Gal4 promoter in mitotic clones marked with GFP using the 'flip-out' system. When Arl1 levels are reduced by RNA interference (RNAi), dGolgin-245 was displaced to the cytoplasm but dGCC185 remained primarily localised to the TGN, consistent with the results obtained with the $arl1^{1}$ mutant (supplementary material Fig. S2A). However, *rab6* knockdown did not affect the targeting of dGolgin-245 or dGCC185 (supplementary material Fig. S2B). To confirm this result, we generated mitotic clones in follicle cells of the Rab6-null allele $rab6^{D23D}$ and found no obvious difference in the distribution of the GRIP domain proteins between *rab6* mutant and wild-type cells (supplementary material Fig. S2C). Finally, when we knocked down *arl1* and *rab6* simultaneously, Golgi targeting of dGolgin-245 was again reduced, whereas the localisation of dGCC185 appeared unchanged (supplementary material Fig. S2D). Likewise, in $rab6^{D23D}$ (null) mutant clones generated in follicle cells that also expressed *arl1* RNAi there was a clear reduction of Golgi-targeted dGCC88, but at most only a small effect on dGCC185 (supplementary material Fig. S2E).

dGCC185 binds to Arl1 and Rab2, but not to Rab6

Given that neither Arl1 nor Rab6 are essential for targeting of dGCC185 to the TGN, we used a yeast two-hybrid assay to test a panel of 15 known or putative Golgi Rabs against either full-length dGCC185, dGCC185 without the GRIP domain (N6 1–1057), or just the GRIP domain with an adjacent N-terminal coiled-coil region corresponding to the Rab6-binding site reported in mammals (GRIP 979–1135). As expected from our previous work, full-length dGCC185 and the GRIP domain on its own bound to Arl1 in its GTP-bound state, but there was no binding when the GRIP domain was absent (supplementary material Fig. S3A,B) (Panic et al., 2003a; Sinka et al., 2008). Of all the Rabs tested, only Rab2 bound strongly to dGCC185 in a GTP-dependent manner (consistent with previous biochemical studies; Sinka et al., 2008), and the Rab2-binding site could be mapped to the second coiled-coil domain of dGCC185 (residues 379–676, supplementary material Fig. S3B).

These results show that dGCC185 binds directly to Arl1 and Rab2 in a GTP-dependent manner, but not to Rab6 or any other Golgi Rab tested. We thus performed RNAi for either *arl1* or *rab2* or for both simultaneously using the UAS-GAL4 flip-out system in follicle cells. However, knockdown of *rab2*, or *arl1* and *rab2* together, did not result in substantial displacement of dGCC185 from the TGN (supplementary material Fig. S3C,D). In addition, truncations of dGCC185 that remove the Rab2-binding region were still recruited to the Golgi when expressed in DMel cells (supplementary material Fig. S3E–H). These results indicate that although dGCC185 binds to both Arl1 and Rab2, there must be further interactions that target dGCC185 to the *Drosophila* Golgi.

Arl1 is required for normal wing development

To investigate the function of Arl1 in tissue development, we induced *arl1* RNAi in the wing using the UAS/GAL4 system. When we expressed the *arl1* RNAi transgene in the whole wing with *scalloped*-GAL4, the wings were smaller than wild type and accumulated extra vein material (Fig. 2A–C). We also tested the *arl1* RNAi transgene with an *engrailed*-GAL4 driver, which is expressed only in the posterior compartment of the wing. Wings

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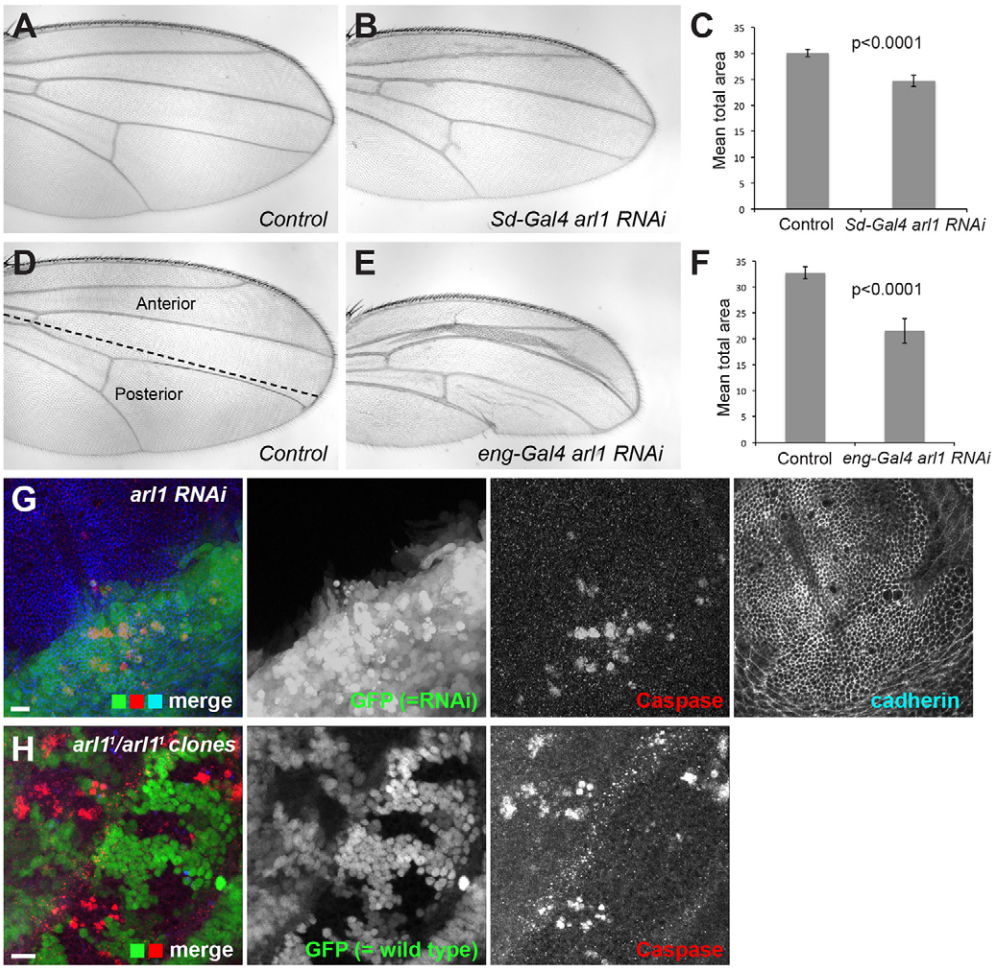

Fig. 2. Loss of Arl1 in imaginal discs induces cell death. (A–F) Knockdown of *arl1* in the wing gives rise to smaller wings with extra vein material. (A) Control female wing (*sd-GAL4*). (B) Wings of females expressing an *arl1* UAS-RNAi transgene with *sd-GAL4* are smaller than the wild type and have extra vein material. (C) Quantification of the wing size of control (*sd-GAL4*) and *arl1*-knockdown wings with *sd-GAL4*. $P<0.0001$ (Student's *t*-test). (D) Control female wing (OregonR) showing the boundary between the anterior and posterior compartments (dashed line). (E) Wings of females expressing an *arl1* UAS-RNAi transgene with *engrailed-GAL4* have a smaller posterior compartment and extra vein material. (F) Quantification of the wing size of control (*OreR*) and *arl1*-knockdown wings with *engrailed-GAL4*. $P<0.0001$ (Student's *t*-test). In both C and F at least 10 wings each from different flies of each genotype were measured with ImageJ software. (G) Projection of a wing imaginal disc of L3 larvae expressing a UAS-RNAi transgene with *engrailed-GAL4* and stained with active caspase 3 and E-cadherin (the later is a single image plane near the apical surface included to reveal the position of the disc cells by their boundaries). The *arl1*-knockdown cells are marked by the UAS-driven expression of nuclear GFP. (H) Projection of a wing imaginal disc with $arl1^1$ mutant clones marked by the absence of GFP and stained with the indicated antibodies. There is increased apoptosis in *arl1* mutant cells. Scale bars: 10 μm.

with reduced levels of Arl1 in the posterior compartment were again smaller than wild-type wings and the L3 vein curved towards the posterior, typical of posterior compartment growth defects (Fig. 2D–F).

We next tested whether *arl1* RNAi increases apoptosis in wing discs, and found that reduced Arl1 levels resulted in increased number of cells expressing cleaved (i.e. active) Caspase-3 (Fig. 2G). We also generated $arl1^1$ mitotic clones the L3 larval wing discs and found apoptosis in the $arl1^1$ mutant clones (Fig. 2H). The triggering of apoptosis in the absence of *arl1* could be due to the missorting of a signalling receptor or secreted protein essential for cell viability, but the distribution of several candidate proteins (Notch, Wntless, Synaptobrevin, Fat, Roughest, Eiger and Perlecan) appeared unaffected in $arl1^1$ mutant wing imaginal discs cells (data not shown). Interestingly, the cell death phenotype of $arl1^1$ mutants seen in imaginal disks was not seen in other tissues such as follicle cells or salivary gland (data not shown), suggesting that $arl1^1$ mutant cells might be outcompeted by wild-type cells in wing discs (Díaz and Moreno, 2005).

Arl1 is required for secretory granule maturation

The *arl1* gene is expressed in all tested *Drosophila* tissues and developmental stages, but it shows the highest relative expression in the larval salivary gland [4.9-fold elevation, FlyAtlas (Chintapalli et al., 2007)]. The larval salivary glands comprise large epithelial cells, which form secretory granules that contain mucin-like glue proteins (Rizki, 1967; Beckendorf and Kafatos, 1976; Burgess et al., 2011). Recent work has shown the *Drosophila* salivary gland to be an excellent model system for studying the role of proteins in regulated and constitutive secretion (Abrams and Andrew, 2005; Wendler et al., 2010; Burgess et al., 2011; Burgess et al., 2012). We thus analysed sections of glands from wild-type and *arl1* mutant L3 larvae by electron microscopy, and found a striking difference in the size of the secretory granules between *arl1* mutants and wild type

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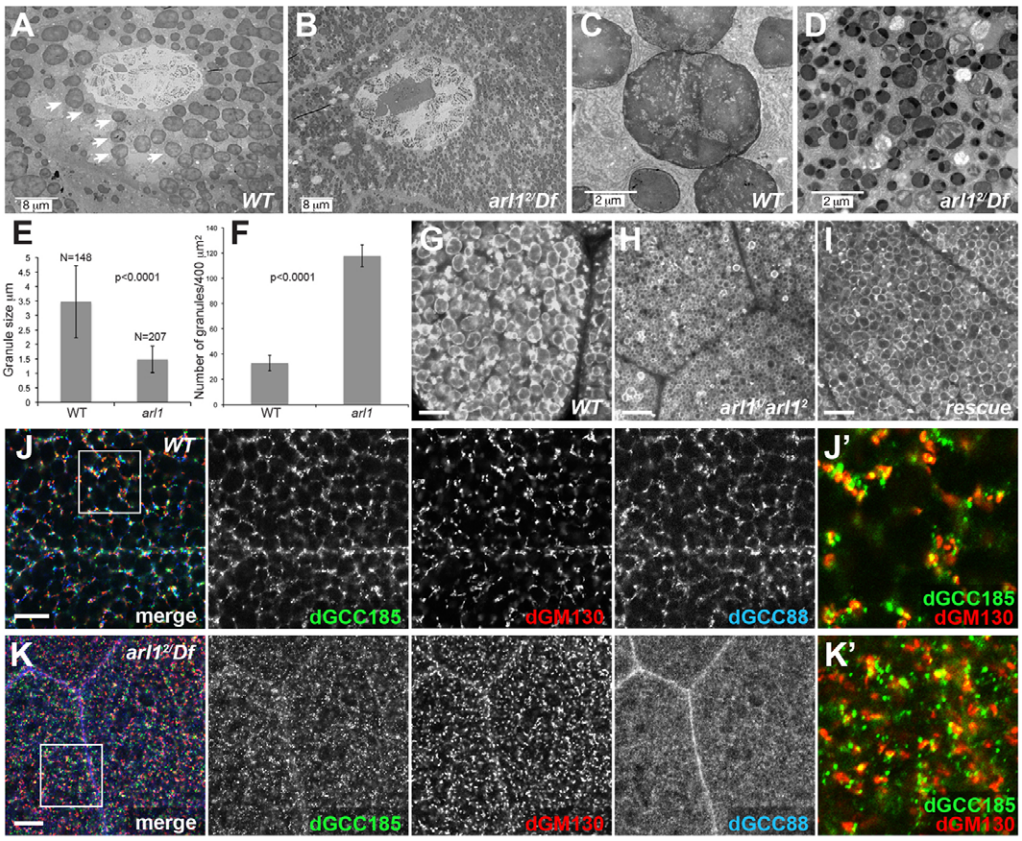

Fig. 3. Arl1 is required for secretory granule maturation. (A–D) Transmission electron microscope (TEM) images of secretory cells from the salivary glands of late L3 larvae. (A) Secretory cells from wild-type (OregonR) salivary glands; the secretory granules are dark grey and fill most of the cytoplasm (arrows). (B) In *arl1*^{2}*/Df(3L)brahma* salivary glands the secretory granules are smaller and more numerous than in wild type. (C) Magnified images of the secretory granules from wild-type OregonR (C) and *arl1*^{2}*/Df(3L)brahma* (D) salivary glands. (E) Measurement of the secretory granule diameter in wild-type (TM6B balancer) and *arl1* (*arl1*^{1}*/arl1*^{2}) mutant salivary glands from mid-to-late L3 larvae. Quantification was done using ImageJ software and at least four different larvae per genotype. $P<0.0001$ (Student's *t*-test). The *arl1*^{2}*/Df(3L)brahma* and *arl1*^{1}*/arl1*^{2} provided two independent means of generating a homozygous loss of arl1, and their phenotypes appeared indistinguishable. (F) Quantification of the number of secretory granules within a square of 20 µm per side in wild-type (TM6B balancer) and *arl1* (*arl1*^{1}*/arl1*^{2}) mutant salivary glands from mid-to-late L3 larvae. $P<0.0001$ (Student's *t*-test in four different larvae per genotype). (G–I) Salivary glands of wild-type (G), *arl1*^{1}*/arl1*^{2} (H) and rescue Pw^{+}2.5BP-1 *arl1*^{1}*/arl1*^{2} (I) mid-to-late L3 larvae stained with LysoTracker which outlines the granules. (J,K) Confocal micrographs of wild-type Oregon R (J) or *arl1*^{2}*/Df(3L)brahma* (K) salivary glands of mid-to-late L3 larvae stained for dGCC185 (green), dGM130 (red) and dGCC88 (blue). J′ and K′ are magnifications of the indicated areas, showing the green and red channels only. dGCC88, but not dGCC185 is displaced from the Golgi in the absence of Arl1. Scale bars: 10 µm (G–K).

(Fig. 3A–E). In the wild type, secretory granules were on average 3.5 µm in diameter (Fig. 3A,C,E), consistent with previous studies (Burgess et al., 2011). In the mutant, the granules averaged 1.5 µm in diameter (Fig. 3B,D,E). In addition, the *arl1* mutants have about three times as many granules as wild type at the same larval stage (Fig. 3F). Expression of an *arl1* genomic rescue transgene in the salivary glands of *arl1*^{1}*/arl1*^{2} mutants restored the appearance of large granules (Fig. 3G–I). These results suggest that Arl1 is required for the formation of full-sized mature secretory granules. Studies in mammalian cells have shown that secretory granules form in several steps: (1) budding of small immature granules from the TGN; (2) fusion of immature granules to form larger granules; and (3) granule remodelling by extraction of proteins for recycling, with both the first and third steps being proposed to require AP-1 and clathrin (Borgonovo et al., 2006). These steps require a Golgi complex. In the *arl1* mutant salivary gland, the cis-Golgi marker dGM130 localises as in wild type, indicating that the Golgi are still present. As expected, removal of Arl1 results in dGCC88 being displaced from the TGN and, as before, dGCC185 is less affected (Fig. 3J,K)

Arl1 is required for AP-1 recruitment

It has been recently reported that the clathrin adaptor AP-1 is required for the formation and maturation of granules in the *Drosophila* salivary gland (Burgess et al., 2011). Our recent studies have shown that Arl1 binds to the Arf1 exchange factor Sec71 which is localised to the TGN (Christis and Munro, 2012). In mammalian cells, AP-1 recruitment to the TGN is known to be mediated by binding to Arf1, and *Drosophila* AP-1 has been found to bind to GTP-bound *Drosophila* Arfs (Zhu et al., 1999; Christis and Munro, 2012). Thus, it seemed plausible that AP-1 recruitment might be perturbed in the *arl1* mutant salivary glands, which would provide an explanation for the defect in granulogenesis. Anti-AP-1 staining showed that, as described previously, AP-1 accumulates around secretory granules in wild-type mid-to-late L3 instar larvae either on the granules or on Golgi structures squashed between the granules (Fig. 4A). However, in *arl1* mutants, AP-1 was dispersed with only a few puncta remaining (Fig. 4B; supplementary material Fig. S4A,B). This phenotype could be rescued with an *arl1* genomic rescue construct, which restored the accumulation of AP-1 around secretory granules (supplementary material Fig. S4C). In late L3

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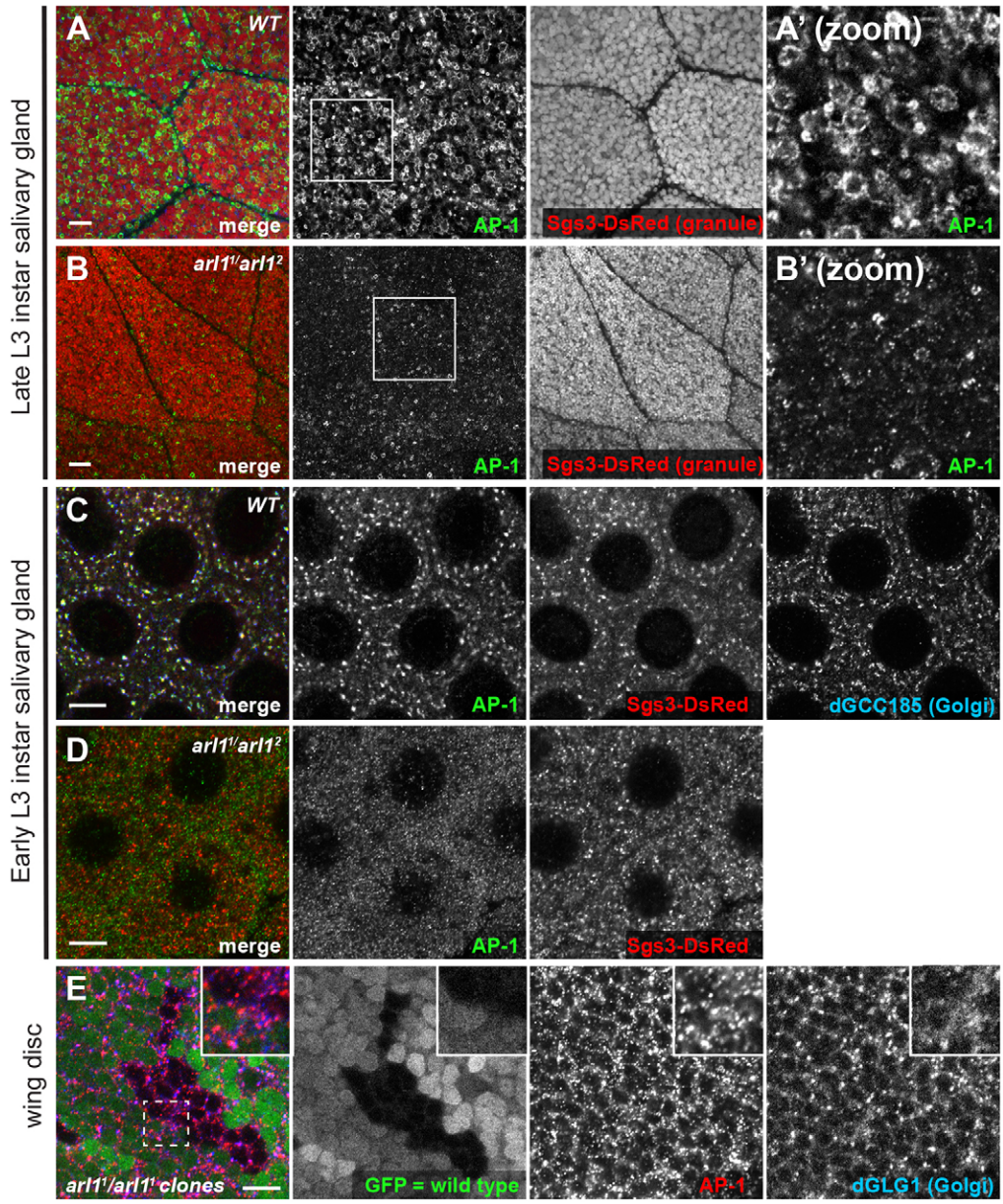

Fig. 4. Arl1 recruits AP-1 to the Golgi and immature secretory granules. (A,B) Salivary glands from mid-to-late L3 larvae expressing *Sgs3-dsRed* to mark the secretory granules and stained with an AP-1 antibody (green). In wild-type (TM6 balancer) AP-1 accumulates around secretory granules (A, A′ shows a magnification of the boxed area). In $arl1^{1}/arl1^{2}$ mutant larvae, AP-1 is rarely found around the secretory granules, with some residual staining of small puncta in the cytoplasm (B, B′ shows a magnification of the boxed area). (C,D) Salivary glands from early L3 larvae expressing *Sgs3-DsRed* to mark the nascent glue proteins that are being packed into forming secretory granules and stained with AP-1 (green) and the Golgi marker dGCC185 (blue in C) antibodies. In wild-type (TM6 balancer) larvae, AP-1 is recruited to the TGN where it colocalises with Sgs3–DsRed (C). In $arl1^{1}/arl1^{2}$ mutant larvae, AP-1 does not colocalise with nascent glue proteins present in the Golgi (D). (E) Wing imaginal disc with $arl1^{1}$ mutant clones marked by the absence of GFP (green) and stained for AP-1 (red) and dGLG1 (blue) to mark the Golgi. The box shows a magnification of the clonal border. Scale bars: 10 μm (A–D); 5 μm (E).

instar stages, there was less AP-1 around the granules both in the wild-type and in the rescued larvae, probably because at this stage most granules are in their mature form (supplementary material Fig. S4D). We also examined AP-1 in salivary glands earlier in development, at the stage in early L3 larvae when the glue proteins are being produced and packaged into forming secretory granules. In the wild type, the glue protein Sgs3 colocalised with AP-1 and the TGN (Fig. 4C). However, in *arl1* mutants AP-1 was again dispersed from the TGN and nascent secretory granules (Fig. 4D). These results show that Arl1 is required to recruit AP-1 to the TGN and immature secretory granules in salivary glands.

Arl1 is not essential to recruit AP-1 to the TGN in all tissues

To investigate whether Arl1 is generally required to recruit AP-1 to the TGN, we analysed the distribution of AP-1 in the absence of Arl1 in several tissues. We first induced $arl1^{1}$ mutant clones in wing imaginal discs and follicle cells and in both cases we found no striking difference in AP-1 targeting to the Golgi between *arl1* mutant and wild-type cells, although in some cases the amount of AP-1 at the Golgi seemed slightly reduced (Fig. 4E; data not shown). However, when we looked at AP-1 in the duct cells of the salivary gland, which do not make secretory granules, we found that AP-1 is not recruited to the Golgi in the *arl1* mutant, and this phenotype is rescued by an *arl1* genomic transgene (supplementary material Fig. S4E–G). These results suggest that the contribution of Arl1 to activation of Arf1 at the trans-Golgi varies between different cell types.

Sec71 is required for AP-1 recruitment and the formation of normal granules

We have recently found that Sec71, the *Drosophila* orthologue of the mammalian Big1 and Big2 Arf exchange factors, is an effector of Arl1 (Christis and Munro, 2012). To test whether Sec71 is required for normal granule formation and AP-1 recruitment, we induced RNAi for *Sec71* in the salivary glands

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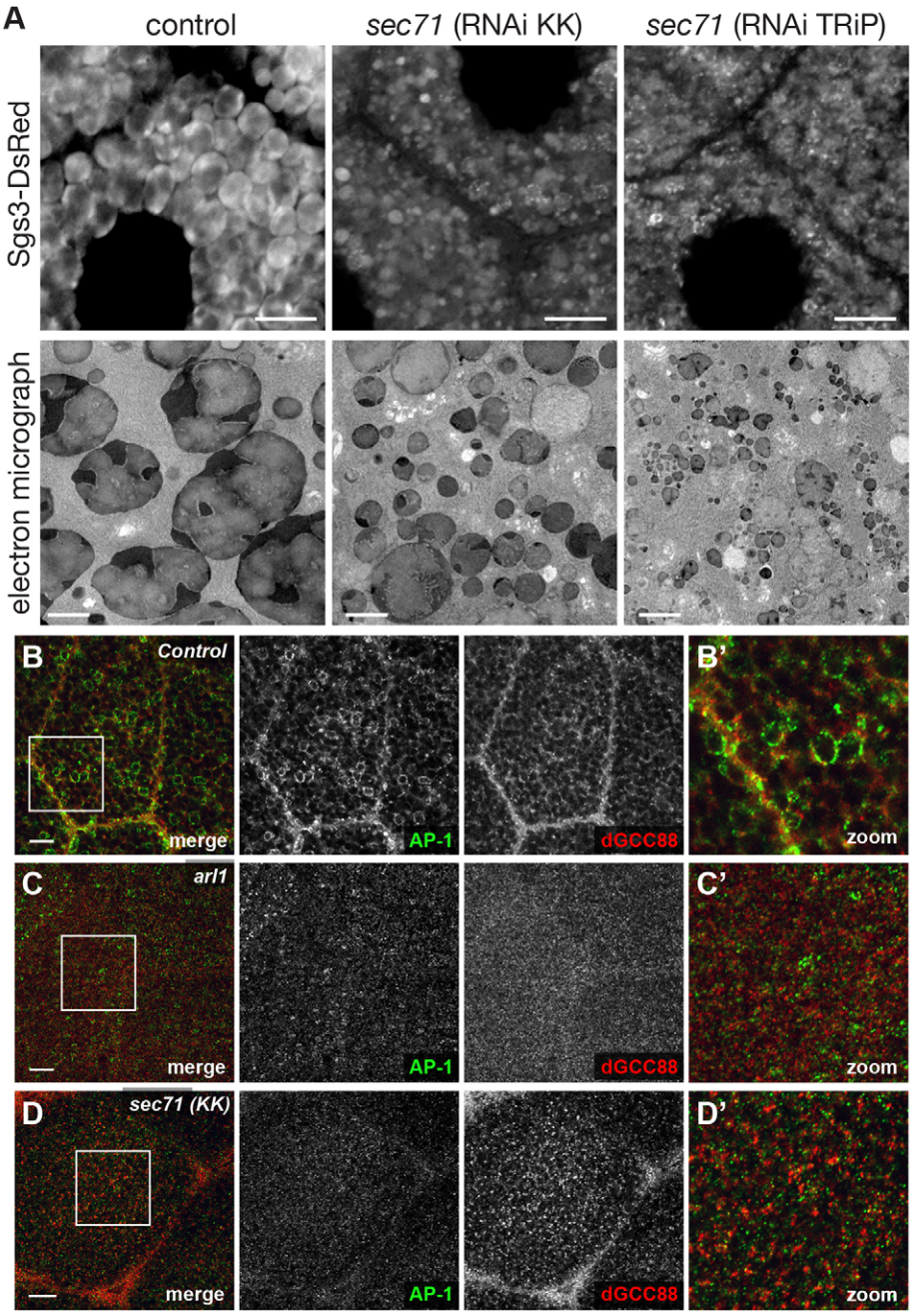

Fig. 5. Sec71 is required for AP-1 recruitment and normal salivary granule formation. (A) Salivary glands from late L3 larvae expressing the salivary gland driver *forkhead-GAL4* at 29°C and either no RNAi (control) or two different UAS-RNAi transgenes against Sec71. The secretory granules are visualised by either confocal imaging of Sgs3-DsRed, or by transmission electron microscopy. In both *sec71* knockdowns, the secretory granules are smaller than in the wild type. The stronger phenotype with the TRiP RNAi might reflect the degree of knockdown, or the different genetic background of the stock. (B–D) Salivary glands from mid-to-late L3 larvae expressing a UAS-RNAi transgene driven by the salivary gland driver *forkhead-GAL4* at 29°C and stained for AP-1 (green) and dGCC88 (red). With both *arl1* and *sec71* knockdown the secretory granules are smaller and AP-1 does not accumulate around them, but the GRIP domain protein dGCC88 is only affected by *arl1* knockdown. B′ to D′ are magnifications of the boxed areas. Scale bars: 10 μm (A, Sgs3–DsRed; B–D); 2 μm (A, electron micrographs).

of mid L3 larvae and compared the results to RNAi of *Arl1*. Fig. 5A shows that two non-overlapping RNAi constructs directed against *Sec71* induced a reduction in granule size as seen in *arl1* mutants and with *Arl1* RNAi. In addition, in *sec71*-knockdown salivary glands AP-1 is also dispersed from its normal location on the Golgi and secretory granules (Fig. 5B–D). However, in contrast to the situation with *Arl1* RNAi, the GRIP domain protein dGCC88 was still targeted to the Golgi (Fig. 5D). Finally, in both the Arl1 mutant and Sec71 depletion we observed the appearance of vacuolated structures containing material that was less dense than normal granule contents (Fig. 3D; Fig. 5D), with similar structures being reported to appear following depletion of AP1 (Burgess et al., 2011). Taken together, these results are consistent with the model that AP-1 is recruited to the TGN and immature secretory granules by Sec71-dependent activation of Arf1 downstream of Arl1.

DISCUSSION

Arl1 is almost certain to have been present in the last eukaryotic common ancestor, and thus it evolved to play a fundamental role in the working of the eukaryotic cell. However, it has not been previously investigated how this is manifested in multicellular metazoans, and in this paper we have used *Drosophila* to start to address this question. Previous expression profiling studies indicate that the protein is likely to act in most if not all tissues, but given the different membrane trafficking requirements of different cell types it seems possible that it precise role varies between cell types (Chintapalli et al., 2007).

Recruitment of GCC185 might require several interaction partners

Defining the role of a G protein at a molecular level requires knowledge of its effectors, and our work shows that Arl1 is

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required in *Drosophila* for recruitment to the Golgi of three of the four GRIP domain golgins. The exception is dGCC185 whose recruitment to the Golgi was clearly less sensitive to removal of Arl1. The importance of mammalian Arl1 for recruiting GCC185 has been debated, with one report saying that it does bind, but that recruitment is augmented by a second interaction with Rab6, whereas another report says that neither Arl1 nor Rab6 is required for GCC185 to be localised to the Golgi (Burguete et al., 2008; Houghton et al., 2009). Our results provide a partial resolution of this issue in that we find that, although Arl1 does bind to dGCC185, it is not required for dGCC185 to be localised to the Golgi. In our view, the most plausible explanation is that other interactions made by dGCC185 are sufficient for Golgi targeting, with Arl1 acting to augment this or to hold dGCC185 in a functional orientation once it is on the Golgi. We did not find a role for Rab6 in dGCC185 recruitment, but then we also did not find binding of Rab6 to dGCC185 by yeast two-hybrid or by biochemistry (Sinka et al., 2008). Of course there might be differences in Rab requirements between mammals and *Drosophila*, but, at least in flies, the only Rab we could find binding to dGCC185 was Rab2, and this did not seem required for recruitment, at least as judged by RNAi. Analysis of truncated forms of GCC185 indicated that the first 379 residues contain some Golgi-targeting information, this region being N-terminal to the Rab2- and Arl1-binding sites. Further work will be needed to identify the factors that act with Arl1, and possibly Rab2, to recruit dGCC185 to the Golgi. This requirement for additional factors might reflect the fact that, at least in mammalian cells, GCC185 interacts with the microtubule regulator CLASP and has been proposed to contribute to microtubule organisation (Efimov et al., 2007).

A new role for Arl1 in secretory granule formation

When we examined the functional phenotype of removing Arl1 we found a clear defect in secretory granule formation in the salivary gland. This observation fits very well with three recently reported findings: firstly, the finding that Arl1 binds Sec71, an Arf1 exchange factor of the TGN; secondly, that *Drosophila* Arf1 binds the AP-1 clathrin adaptor as is known for the mammalian proteins; and, thirdly, the finding that secretory granule formation in the salivary gland requires the AP-1 adaptor (Burgess et al., 2011; Christis and Munro, 2012). Sec71 is the *Drosophila* orthologue of the mammalian Arf exchange factors Big1 and Big2, which are known to act on the TGN and to be required for AP-1 recruitment (Ishizaki et al., 2008; Boal and Stephens, 2010). AP-1 is thought to contribute to secretory granule formation either during budding of the nascent granule from the TGN and/or in the recycling of components from newly formed granules as they mature and coalescence into larger granules (Dittie et al., 1996; Austin et al., 2000; Lui-Roberts et al., 2005; Borgonovo et al., 2006; Burgess et al., 2011). It is not clear whether both of these are direct requirements, as components might need to recycle to sustain the budding of immature granules from the Golgi and their subsequent fusion. In our view, the most likely interpretation of our data is that when Arl1 is absent, insufficient Arf1 is activated on the TGN and thus insufficient AP-1 is recruited to form normal granules, although we cannot exclude the possibility that displacement of GRIP domain proteins also contributes to the phenotype.

The small granule phenotype seen with the $arl1^{1}$ mutant is somewhat milder than the substantial loss of granule formation seen when the AP-1 subunit AP-47 is absent, but is quite similar to the phenotype seen with AP-47 RNAi (Burgess et al., 2011). This might reflect residual targeting of AP-1 in the absence of Arl1. In yeast, Arf1 itself can bind to the orthologue of Sec71, and it seems likely that Arl1 is not the only factor involved in recruiting this large protein to the Golgi (Richardson et al., 2012). Alternatively, Sec71 is not the only Arf exchange factor on the Golgi, as a related protein called Gartenzwerg (Garz) is present on the cis-Golgi. Garz is the orthologue of mammalian GBF1, and for both proteins it has been shown that their removal results in the Golgi being severely disrupted with a concomitant block in secretory pathway function (Manolea et al., 2008; Sáenz et al., 2009; Boal et al., 2010; Szul et al., 2011; Armbruster and Luschnig, 2012; Wang et al., 2012). Thus some Arf1 activated by Garz/GBF1 on the early Golgi might linger as the cisternae mature. Either of these hypotheses could explain the observation that the effect of loss of Arl1 on AP-1 recruitment was not seen in all tissues, even though GRIP domain golgin recruitment was affected in all cases. Indeed, it seems plausible that the reason Arl1 helps to recruit Sec71 is to boost activation of Arf1 on the TGN to enhance recruitment of AP-1 and other effectors to a compartment that is downstream of Garz/GBF1 action. Given that Arl1 also recruits the golgins, which are required for tethering of carriers returning from endosomes, it seems plausible that Arl1 is as a key coordinator of the arrival and departure of membrane traffic at the TGN.

MATERIALS AND METHODS

Drosophila genetics

For FRT/FLP recombination we used *abxUbxFLP* (gift from Alexandre Djiane, Department of Physiology, Development and Neuroscience, Cambridge, UK) and *e22cGAL4 UASFLP* (Bloomington Stock Center, Indiana, USA) in combination with FRT2AUbi-GFP (Bloomington) for clones in the wing disc and follicle cells respectively, and $hsFLP^{22}$; FRT40AUbi-GFP (gift from Daniel St Johnston, The Gurdon Institute, Cambridge, UK) for clones in the wing disc and follicle cells. $arl1^{1}$ and $arl1^{2}$ (Tamkun et al., 1991) were recombined to FRT2A (Bloomington), FRT40A $rab6^{D23D}$ was a gift from Anne Ephrussi (EMBL, Heidelberg, Germany). Other stocks were Oregon R (Bloomington), *Df(3L)brm11* (Bloomington), *scalloped-Gal4* (gift from Alexandre Djiane), *engrailed-GAL4 UAS-dicer* (Bloomington), *forkhead-GAL4* (gift from Katja Röper, MRC LMB, Cambridge, UK), *Sgs3-DsRed* (gift from Andrew Andres, University of Nevada, Las Vegas, NV), *arl1* genomic rescue $Pw^{+}2.5$ BP-1 (Tamkun et al., 1991). For RNAi with the flip-out system we used *Ay-GAL4 UAS-nlsGFP* (Bloomington) in combination with $hsFLP^{22}$ (Bloomington). The RNAi lines used were *Arl1* (Arf72A, TRiP.JF02378 Bloomington or KK.106474 VDRC, Vienna, Austria), *Rab2* (TRiP.HMS01271 Bloomington or KK.105358 VDRC), *Rab6* (KK.100774 VDRC) and *Sec71* (TRiP.HMS00357 Bloomington or KK.107637 VDRC, which recognise nucleotides 4110–4130 and 800–1350 of the Sec71-PA coding region, respectively).

Cell culture and RNAi

Drosophila DMel cells were grown at 25°C in Express Five SFM medium (Invitrogen). For RNAi DMel cells were plated in 6-well plates and 30 µg of dsRNA was added after 1 h. Plasmids were transfected after 3 days with FugeneHD (Promega), and cells were fixed or lysed in SDS-PAGE sample buffer 24 h after transfection. dsRNAs were amplified using T7 Ribomax Express (Promega) against *Drosophila* Arl1 (bp 344–541) or *P. pyralis* luciferase (bp 161–878) as control. Cells were fixed with 4% formaldehyde in PBS for 15 min and blocked for 1 h in PBS, 0.1% Triton X-100 and 10% FCS. Primary and secondary (Alexa Fluor; Invitrogen) antibodies were applied for 1 h, and cells were washed five times with PBS and mounted in Vectashield (Vector Laboratories). Images were obtained with Zeiss LSM710 or LSM780 confocal microscopes.

Antibodies and vital staining

Antibody staining of wing imaginal discs, ovaries or salivary glands was performed using standard protocols. *Drosophila* primary antibodies were rabbit anti-GM130 (ab30637, Abcam), anti-GRIP-domain-containing proteins (Sinka et al., 2008), rabbit anti-dGMAP210 (Friggi-Grelin et al., 2006), rat anti-E-Cad (DCAD2; DSHB, Iowa City, USA), mouse anti-Fasciclin III (7G10 DSHB), mouse anti-Discs large (4F3 DSHB), mouse anti-Notch (C17.9C6 DSHB), mouse anti-α-spectrin (3A9 DSHB), rabbit anti-cleaved Caspase3 (9661 Cell Signalling), rabbit anti-Lava lamp (Sisson et al., 2000), rabbit anti-Perlecan (gift from Stefan Baumgartner, Lund University, Sweden), rabbit anti-Wntless (gift from Jean-Paul Vincent, MRC NIMR, London, UK), rabbit anti-Synaptobrevin (gift from Cahir O'Kane, Department of Genetics, Cambridge, UK), rabbit anti-Eiger (gift from Tian Xu, Yale University, New Haven, CT), rat anti-Fat (gift from Sarah Bray, Department of Physiology, Development and Neuroscience, Cambridge, UK), mouse anti-Roughest (gift from Sarah Bray), chicken anti-Avalanche (gift from David Bilder, University of California Berkeley, CA), rabbit anti-AP-1 (Hirst et al., 2009), mouse anti-dGLG1/p120 (DG13; Stanley et al., 1997)). Secondary antibodies were conjugated to Alexa Fluor 488, Alexa Fluor 555, Alexa Fluor 647 (Invitrogen) and horseradish peroxidase (HRP) (Dako). Antibodies against GST-tagged *Drosophila* Arl1 (residues 15–180) were raised in chicken (Eurogentec) and affinity purified. For LysoTracker staining, larvae were dissected in cold PBS and salivary glands were incubated in the same buffer with 2.5 µM LysoTracker Red DND-99 (Molecular Probes) at room temperature for 5 to 15 min. After two washes in PBS, salivary glands were imaged live in the same buffer. This approach outlined the salivary granules for reasons that are unclear, but was somewhat variable and so was not used routinely.

Plasmids for expression and yeast two-hybrid analysis

Full-length *Drosophila* Arl1 was cloned into pUAST, and transgenics made by BestGene (Chino Hills, USA). GTP- and GDP-locked forms of *Drosophila* Arl1 and Rabs 1, 2, 3, 6, 8, 9, 10, 11, 14, 18, 19, 21, 23, 30 and 44 were cloned into the bait vector pGBDU-C1 for yeast two-hybrid assays (James et al., 1996). *Drosophila* full-length GCC185 and GCC185 truncations were PCR amplified (EST clone RE20855) and cloned into pDEST22 (Invitrogen) for yeast two-hybrid assays or pAGW for transfection in S2 cells.

Electron microscopy

Late L3 instar larvae were identified by visual inspection (i.e. had exited from wandering stage), with the state of the gland providing confirmation of staging because substantial and widespread glue granule formation does not occur until late L3 (Burgess et al., 2011). Whole salivary glands were dissected out in PBS, and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate pH 7.4, at room temperature, then placed in fresh fixative overnight at 4℃. Following several buffer washes, tissues were post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4℃, washed in water and stained *en bloc* with 2% uranyl acetate in 30% ethanol and further dehydrated in an ascending ethanol series followed by infiltration with propylene oxide and embedding in CY212 resin. Ultrathin sections were counterstained with Reynold's lead citrate and viewed on a Philips 208 transmission electron microscope operated at 80 kV.

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Competing interests

The authors declare no competing interests.

Author contributions

I.L.T. and S.M. planned the experiments. I.L.T. and C.R. performed the experiments. I.L.T. and S.M. wrote the manuscript.

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Supplementary material

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