Investigation of Isoform Specific Functions of the V-ATPase a Subunit During Drosophila Wing Development

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The vacuolar ATPases (V-ATPases) are ATP-dependent proton pumps that play vital roles in eukaryotic cells. Insect V-ATPases are required in nearly all epithelial tissues to regulate a multiplicity of processes including receptor-mediated endocytosis, protein degradation, fluid secretion, and neurotransmission. Composed of fourteen different subunits, several V-ATPase subunits exist in distinct isoforms to perform cell type specific functions. The 100 kD a subunit (Vha100) of V-ATPases are encoded by a family of five genes in Drosophila, but their assignments are not fully understood. Here we report an experimental survey of the Vha100 gene family during Drosophila wing development. A combination of CRISPR-Cas9 mutagenesis, somatic clonal analysis and in vivo RNAi assays is used to characterize the requirement of Vha100 isoforms, and mutants of Vha100-2, Vha100-3, Vha100-4, and Vha100-5 genes were generated. We show that Vha100-3 and Vha100-5 are dispensable for fly development, while Vha100-1 is not critically required in the wing. As for the other two isoforms, we find that Vha100-2 regulates wing cuticle maturation, while Vha100-4 is the single isoform involved in developmental patterning. More specifically, Vha100-4 is required for proper activation of the Wingless signaling pathway during fly wing development. Interestingly, we also find a specific genetic interaction between Vha100-1 and Vha100-4 during wing development. Our results revealed the distinct roles of Vha100 isoforms during insect wing development, providing a rationale for understanding the diverse roles of V-ATPases.

Keywords: V-ATPase a subunit, wing development, Drosophila melanogaster, V-ATPase isoform, V100-2

INTRODUCTION

The vacuolar ATPases (V-ATPases) are ubiquitous proton pumps which play important roles in eukaryotic cells (Forgac, 2007). V-ATPases transport proton into intracellular compartments, and are therefore crucial for pH homeostasis in organelles such as endosomes, secretory vesicles, synaptic vesicles, and lysosomes (Hinton et al., 2009). The V-ATPases are generally required for a broad spectrum of cellular processes, including endosomal trafficking, lysosomal degradation, and exocytosis (Forgac, 2007). Insect V-ATPases are expressed in nearly all epithelial tissues and are well-known for their roles in physiological activities such as secretion of K + and...
Na+ and formation of fluid (Wieczorek et al., 2009). Moreover, recent studies have illuminated the importance of V-ATPases for insect development. In Drosophila melanogaster, mutations that dampen the V-ATPase activity are reported to disrupt the formation of eye, wing and egg chambers (Yan et al., 2009; Buechling et al., 2010; Herrme et al., 2010; Vaccari et al., 2010). Further studies demonstrated that V-ATPases are involved in regulation of cell proliferation, cell fate determination and tissue patterning through modification of key developmental signaling pathways (Kobia et al., 2013; Gleixner et al., 2014; Portela et al., 2018).

The V-ATPase is a large protein complex composed of 14 different subunits that are organized into the cytosolic V1 region and the membrane-bound V0 region (Nelson, 2003). Region V1 hydrolyzes ATP and provides energy to pump protons through the protein lipid pores formed in region V0 (Jianhua et al., 2015). The V1 region contains eight subunits while the V0 domain is assembled by six different subunits (Forgac, 2007). The regulatory C subunit is located in the V1 domain and interacts with subunit a in the V0 domain (Wilkens et al., 2004; Inoue and Forgac, 2005; Cipriano et al., 2008). Therefore, the C subunit is well positioned to control the reversible dissociation of the V-ATPase complex (Inoue et al., 2005; Forgac, 2007). The C subunit is encoded by Vha44 in Drosophila, which is required for endolysosomal acidification and regulates elimination of nurse cells in the ovary (Mondragon et al., 2019), cell competition in the eye imaginal disk (Nagata et al., 2019), and apical endocytosis in the wing disk epithelial cells (Gleixner et al., 2014). Ectopic Vha44 expression is shown to impair endolysosomal degradation and induce invasive cell behavior in the developing wing disk (Petzoldt et al., 2013) as well as differentiation defects in the eye disk (Portela et al., 2018).

Many of the V-ATPase subunits exist in multiple isoforms which are often expressed in a cell type specific manner (Toei et al., 2010). In Drosophila, the V-ATPase multigene family consists of 33 different genes (Julian, 1999; Allan et al., 2005). Apart from five subunits in the V1 region and the accessory subunits VhaAC45 and VhaM8.9, other V-ATPase subunits are encoded by two to five genes (Allan et al., 2005). In vertebrates, isoforms of subunit a in the V0 domain contain information necessary for targeting the V-ATPase complexes to the appropriate plasma membrane (Toyomura et al., 2003; Qi et al., 2007; Saw et al., 2011). In Drosophila, the V-ATPase a subunit is encoded by Vha100-1, Vha100-2, Vha100-3, Vha100-4, and Vha100-5 with specific tissue distribution patterns (Toei et al., 2010). Previous studies have suggested that Vha100-1 is an isoform required for synaptic vesicle exocytosis in the nervous system (Hiesinger et al., 2005). Loss of Vha100-1 leads to vesicle accumulation in synaptic terminals (Wang et al., 2014), neuronal degeneration (Williamson et al., 2010a), and defects in brain wiring (Williamson et al., 2010b). RNAi knock-down experiments indicate that Vha100-2 is involved in regulation of neural stem cells proliferation (Wissel et al., 2018), acid generation of the midgut (Overend et al., 2016), elimination of nurse cells in the ovary (Mondragon et al., 2019), and cell competition in the eye disk (Nagata et al., 2019). Similar as Vha100-2, knock-down of Vha100-4 also leads to acidification defect in the larval midgut (Overend et al., 2016). The roles of Vha100-3 and Vha100-5 are still unclear, and our understanding of whether and how Vha100 isoforms collaboratively regulate the development of specific tissue is incomplete.

In order to further investigate the functional diversity of the V-ATPase a subunit isoforms, we generated and characterized mutants of Vha100-2, -3, -4, and -5. We found that among the five isoforms, Vha100-3 and Vha100-5 are dispensable for fly development. We further demonstrated that Vha100-2 is specifically required for wing cuticle formation, while Vha100-4 is involved in Wingless signaling activation. Comparative studies revealed that Vha100-1 and Vha100-4 execute both independent and redundant function during fly wing development. Our studies uncovered the isoform specific functions of the V-ATPase a subunit during Drosophila wing development.

MATERIALS AND METHODS

Fly Genetics

Fly stocks and all fly crosses were maintained at 25°C on standard fly food. The following fly stocks were used: hh-Gal4, UAS-mCD8-gfp/TM6B (Ren et al., 2018); Vha100-2 RNAi (TH04790.N; TsingHua Fly Center); FRT42D, Vha44KG0915/Cyo (#111534; Kyoto Stock Center); FRT42D, Vha44K05440/Cyo (#111081; Kyoto Stock Center); FRT82B, Vha100-1/TM3, Sb (#39669; Bloomington Drosophila Stock Center); w;Sco/Cyo (#2555; Bloomington Drosophila Stock Center); and w;TM3/TM6B (#2537; Bloomington Drosophila Stock Center). The Ubx-FLP, FRT82B, Ubi-RFP/TM6B, Ubx-FLP, FRT42D, Ubi-RFP/Cyo, and Ubx-FLP, FRT42D, Ubi-GFP/Cyo stocks were used to generate mosaic mutant clones in the wing disks (Ren et al., 2018). The sens-GFP reporter was described before (Sarov et al., 2016) and obtained from Bloomington Drosophila Stock Center (#38666). The fz-LacZ reporter was described before (Sato et al., 1999).

CRISPR-Cas9 Mediated Mutagenesis

The sgRNA targets were designed against the genomic sequences of Vha100-2, Vha100-3, Vha100-4, and Vha100-5 with CRISPR Optimal Target Finder1 (Gratz et al., 2014). Templates for sgRNA transcription were generated by annealing of two DNA oligonucleotides and subsequent PCR amplification (Bassett et al., 2013). In vitro transcription was performed with the T7 RiboMAX™ Kit (Promega, P1320) and the sgRNAs were purified by phenol-chloroform extraction and isopropanol precipitation. Cas9 mRNA was transcribed with the mMESSAGE mMACHINE® T7 Transcription Kit (Ambion), using a linearized plasmid containing the Cas9 cDNA (Addgene plasmid 42251) as template. The Cas9 mRNA were polyadenylated with the Escherichia coli Poly(A) polymerase Kit (NEB), and purified with the RNasy Mini Kit (QIAGEN). 15 µg of Cas9 mRNA and 7.5 µg sgRNA were mixed with DEPC water in a 30 µl volume for embryo injection. Fly embryos of the w1118 strain (#5905; Bloomington Drosophila Stock Center) were injected using standard protocols by Fungene Biotech (Beijing, China).

1http://tools.flycrispr.molbio.wisc.edu/targetFinder/
FIGURE 1 | Continued
Males developed from the injected embryos (G0) were outcrossed to virgin females of TM3/TM6B (for Vha100-2 and Vha100-4) or Sco/Cyo (for Vha100-3 and Vha100-5). Single G1 males were each crossed to 4–5 females of the corresponding balancer stocks and the progenies (G2) bearing the same balancer chromosome were maintained as an independent stock. About 50 G2 stocks were established from each injection, and mutations were screened by PCR test of genomic DNAs. Primer sequences (Vha100-1, Vha100-2, Vha100-3, Vha100-4, and Vha100-5) is shown. Identical residues are indicated by blue shades and similar residues are labeled by light purple shades. The seven putative transmembrane helices predicted from topographical analysis are shown with a red bar. The residues whose mutation has a significant effect on activity or assembly of the V-ATPase are indicated by asterisks. GenBank accession numbers assigned to Vha100-1, Vha100-2, Vha100-3, Vha100-4, and Vha100-5 are AAF56861, AAF55551, AAM68427, AAF55550, and AAF53116, respectively.

TABLE 1 | The sequence identity (and similarity) between pairs of Drosophila a subunit isoforms.

| Pairs of isoforms | Identity (and similarity) |
|-------------------|---------------------------|
| Vha100-1 and Vha100-2 | 58% (71%) |
| Vha100-1 and Vha100-3 | 36% (51%) |
| Vha100-1 and Vha100-4 | 53% (67%) |
| Vha100-1 and Vha100-5 | 57% (71%) |
| Vha100-2 and Vha100-3 | 35% (54%) |

TABLE 2 | Summary of CRISPR-Cas9 sgRNA sequences.

| Gene symbol | sgRNA-1 sequence | sgRNA-2 sequence |
|-------------|------------------|------------------|
| Vha100-2    | GGCGTGGCTCTCGCCGAC | GTATACCTGGATCTCTGC |
| Vha100-3    | GGTTTGCAAGCTGTCAGT | GCAGACCGCTGCTTGGAG |
| Vha100-4    | GCTCGAGGTCGCGCCGAC | GGTGCGGGCTTGGGCGAG |
| Vha100-5    | GGCGTAGCTGCTTGGAGG | GTTCGCAGTCTGAG |

Eosin Y and FB28 Staining and Microscopy of the Adult Wing

Adult flies with correct genotypes were collected and fixed overnight in isopropanol. Dissected adult wings were mounted in Euparal mounting media (BioQuip). For Eosin Y and FB28 staining experiments, 2 days old files were fixed in formaldehyde phosphate buffer, washed several times with PBS, and stained with FB28 solution (1 mg/ml; Sigma-Aldrich) at room temperature for 1 h or 0.5% Eosin Y at 55°C for 35 min. Adult wings were then dissected and mounted in 80% glycerol. The images were acquired with a Leica SP8 confocal microscope and processed in Photoshop and ImageJ.

RESULTS

Vha100 Isoforms Are Differentially Required for Fly Development

The a subunit of the V-ATPase is encoded by five genes in Drosophila, which are spread at different locations throughout...
FIGURE 2

A

Vha100-1<sup>I</sup>
Vha100-2<sup>o2</sup>
Vha100-3<sup>o3</sup>
Vha100-4<sup>o4</sup>
Vha100-5<sup>o5</sup>

- 59bp deletion
- 27bp deletion, premature-stop
- 38bp deletion
- 72bp deletion

B

Vha100-2 mRNA

WT

C

Vha100-4 mRNA

WT

D

Vha100-2 mRNA
Vha100-2<sup>o2</sup> clones

D’

GFP

D”

Merge

E

Vha100-4 mRNA
Vha100-4<sup>o4</sup> clones

E’

GFP

E”

Merge

FIGURE 2 | Continued
homology reveals that Vha100-3 is the most diverse member of the family, while the other isoforms share a similarity about 70% between each other (Table 1). A number of residues of the α subunit have been experimentally demonstrated to be important for the activity or assembly of V-ATPase complex in yeast (Leng et al., 1996, 1998), which were later found to be conserved in mouse orthologs (Nishi and Forgac, 2000; Toyomura et al., 2000). All of these residues are conserved in the fly isoforms, with the exception of L800 (the Vha100-1 numbering), which is conserved in -3 and -5 but is a phenylalanine in -2 and -4 (Figure 1). It is likely that the less conserved regions may render different functions of Vha100 isoforms. Construction of a phylogenetic tree (Minh et al., 2013; Trifinopoulos et al., 2016) using the mouse, fly and yeast sequences reveals that the development of multiple isoforms of the α subunit appears to have occurred independently in these species (Supplementary Figure S1E). Previous studies have isolated several Vha100-1 mutant alleles and revealed a neuronal specific role of Vha100-1 during fly development (Hiesinger et al., 2005). For the other four isoforms, mutagenesis analyses have not been reported yet. To better understand how Vha100 isoforms function in different developmental contexts, we generated Vha100-2, Vha100-3, Vha100-4, and Vha100-5 mutants by CRISPR-Cas9 mediated genome editing (Table 2). Mutations with small deletions were identified by genomic DNA PCR (Supplementary Figure S2) and used for further analysis (Figure 2A). Homozygous Vha100-3 D3 and Vha100-5 D5 mutant flies are viable and fertile with normal appearance of body structure and tissue morphology. Previous studies have shown that the expression of Vha100-3 is restricted to the testes in adult males, while the mRNA of Vha100-5 is undetectable by in situ hybridization in fly larvae (Wang et al., 2004; Allan et al., 2005). We reason that these two isoforms might perform specific physiological roles, but are dispensable for fly development. Therefore, we moved on to examine the roles of Vha100-1, Vha100-2, and Vha100-4 in fly wing development.

As Vha100-1 (Hiesinger et al., 2005), Vha100-2 D2, and Vha100-4 D4 are homologous lethal mutants, somatic mosaic clones were generated using the FLP-FRT system to examine their roles in wing development. The FLP recombinase catalyzes exchange of the homologous chromosome arms and induces the generation of homologous mutant cell clones with randomized size and location (Xu and Rubin, 1993). Importantly, the wild type cells surrounding the mutant clones serve as rigorous internal control for developmental studies (Theodosiou and Xu, 1998). Taking advantage of the FLP-FRT system, we evaluated whether Vha100-2 D2 and Vha100-4 D4 (Figure 2A) behave as loss-of-function alleles by examining their mRNA levels in mutant mosaic clones. Both Vha100-2 (Figure 2B) and Vha100-4 (Figure 2C) were ubiquitously expressed in wing

the genome (Supplementary Figures S1A–D). The -1, -2, -3, -4, and -5 isoform comprises 855, 834, 904, 844, and 814 amino acid residues, respectively. A hidden Markov model (Krogh et al., 2001; http://www.csb.dtu.dk/services/TMHMM-2.0/) predicted that these isoforms have closely similar structures with seven putative transmembrane regions, which are conserved among the five isoforms (Figure 1). High conservation is also evident for the hydrophilic amino and hydrophobic carboxy terminals of fly Vha100 isoforms (Figure 1). Detailed analysis of sequence
Vha100-2 Is Specifically Required for Wing Cuticle Formation

We first examined the overall requirement of V-ATPase activity during wing development, and the contributions of distinct Vha100 isoforms were further dissected. Two mutants of the regulatory C subunit Vha44 were tested (Supplementary Figure S3A) and both of them resulted in various developmental defects including loss of wing marginal bristles and nicking of wing margin (Supplementary Figures S3B–D). We then investigated whether Vha44 regulates Notch (N) and Wingless (Wg) signaling activity, two major signaling pathways that were affected by mutations of other V-ATPase subunits (Vaccari et al., 2010; Kobia et al., 2013; Gleixner et al., 2014; Portela et al., 2018; Ren et al., 2018). In the wild type imaginal wing disk, the N target gene cut is produced in a narrow stripe of cells along the dorsal/ventral (D/V) boundary (Supplementary Figure S3E). In Vha44<sup>K05440</sup> mutant clones, the expression of Cut is abolished when the clones are located at the D/V boundary (Supplementary Figure S3F). Clones of Vha44 mutant cells also displayed various degrees of accumulation of the ligand molecule Delta (Dl) and N protein itself, which were detected as intracellular puncta (Supplementary Figures S3G–J). During fly wing development, the Wg protein is generated at the D/V boundary (Supplementary Figure S3K) and transported throughout the wing disk to activate down-stream targets (Swarup and Verheyen, 2012). In Vha44 mutant cells, the expression level of Wg was not significantly affected, while the distribution of Wg protein in signal receiving cells was altered (Supplementary Figure S3L). As a consequence, the activity of Wg signaling was dampened in Vha44 mutant cells (Supplementary Figures S3M,N), which was monitored by a fz3-LacZ reporter (Sato et al., 1999). In addition, we found that Vha44<sup>K05440</sup> mutant cells displayed aberrant cellular cortex morphology (Supplementary Figures S3O,P) and likely underwent apoptosis (Supplementary Figures S3Q,R).

In contrast to the Vha44 mutants, fly wings harboring homozygous Vha100-2<sup>D2</sup> mutant clones displayed wrinkles in the wing surface, without disruption of the vein patterns...
and wing margin integrity (Figures 3A,B). In agreement with the lack of patterning defects in the adult wing, the expression of Cut remained unaltered in Vha100-2D2 mutant clones (Supplementary Figure S4A). The expression level and distribution of Wg, N, and DI proteins were also normal in Vha100-2D2 mutant cells (Supplementary Figures S4B–D), suggesting that Vha100-2 is not required for these two pathways. Furthermore, phalloidin staining showed that Vha100-2D2 mutant had little influence for cell morphology (Supplementary Figure S4E). Wrinkling of the wing surface is often associated with defective cuticle deposition (Moussian, 2010). To further characterize the role of Vha100-2 on wing cuticle formation, we analyzed the cuticle impermeability by FB28 and Eosin Y staining (Wang et al., 2016, 2017). Wild-type wings with an intact cuticle layer is almost resistant to Eosin Y penetration (Figure 3A). By contrast, wings harboring homozygous Vha100-2D2 mutant clones showed patches of Eosin Y staining (Figure 3B). Similarly, a polysaccharide-specific dye FB28 was able to penetrate through the cuticle of wings bearing Vha100-2D2 mutant clones (Figures 3C,D). We further confirmed the role of Vha100-2 in cuticle integrity by knock-down of Vha100-2 expression in the posterior compartment of the wing. Regional specific inhibition of Vha100-2 led to wrinkles in the RNAi expressing area as well as diffusion of the FB28 dye (Figure 3E). Consistent with the adult wing phenotype, RNAi knock-down led to attenuation of Vha100-2 mRNA level when examined in the wing disk (Figure 3F). Taken together, our results suggest that Vha100-2 might be involved in cuticle formation of the wing.

**Vha100-4 Is Required for Proper Activation of the Wg Signaling Pathway**

When Vha100-4D4 mutant clones were induced in the wing, marginal notches and bristle loss were observed (Figures 4A,B). These marginal defects are typically resulted from disruption of N or Wg signaling activity (Logan and Nusse, 2004). Interestingly, we found that neither the target Cut nor the signaling molecules (N and DI) were changed in Vha100-4D4 mutant cells (Supplementary Figure S5). On the other hand, aggregation of Wg proteins were observed in some mutant cells (Figures 4C,D), and the expression of the Wg activity reporter fz3-LacZ (Figures 4E,F), and sens-GFP (Figures 4G,H) were dampened in a subset of Vha100-4D4 mutant clones. Collectively, these findings provide evidence that Vha100-4 is likely involved in Wg signaling activation during fly wing development.

**Vha100-1 Performs Redundant Function With Vha100-4**

Although previous studies suggest that Vha100-1 functions in the neuronal system to regulate fly development (Hiesinger et al., 2005; Wang et al., 2014), we noticed that ectopic veins were formed in wings bearing Vha100-1I mutant clones (Figures 5A,B). However, N and Wg signaling were not affected in Vha100-1I mutant cells (Supplementary Figures S6A,B). In addition, we noticed that Vha100-1I mutant cells did not undergo apoptosis (Supplementary Figure S6C) and displayed normal cell morphology (Supplementary Figure S6D). The overall patterning and wing margin integrity was not affected by Vha100-1I mutant clones, suggesting that Vha100-1 might function in specific processes during vein cell differentiation.

Mutations of Vha100-1, Vha100-2, and Vha100-4 were unable to fully recapture the wing defects caused by Vha44 mutant, despite that they are all essential genes required for normal development. These results indicate that these isoforms may function redundantly and the loss of single isoform can be compensated by the remaining family members in certain developmental contexts. To test this hypothesis, we crossed Vha100-1I, Vha100-2D2, and Vha100-4D4 flies with each other and examined the wing phenotypes in the trans-heterozygous progenies. For each mutant allele, the heterozygotes were indistinguishable with wild type controls (Figures 5C–E). The wings of Vha100-1I/Vha100-4D4 flies were severely malformed (Figure 5F), indicating a strong genetic interaction and functional redundancy between Vha100-1 and Vha100-4. Combination of Vha100-2D2 with either Vha100-1I (Figure 5G) or Vha100-4D4 (Figure 5H)
shows no effect on fly development and wing morphology, suggesting that Vha100-2 likely functions independently of the other two isoforms.

DISCUSSION

The V-ATPases are highly conserved multi-subunit pumps that transport hydrogen ions in exchange for ATP. Present in the endo-membranes of all eukaryotic cells, they are well known regulators for acidification of various intracellular compartments (Forgac, 2007). Studies in the larval midgut of the tobacco hornworm Manduca sexta are among the first reports to reveal that V-ATPases also pump protons across the plasma membranes of many specialized animal cells (Wieczorek et al., 2009). The great diversity of functions that the V-ATPases serve in eukaryotic organisms is recognized as a remarkable feature (Forgac, 2007; Wieczorek et al., 2009). Several subunits of the V-ATPases are encoded by multiple isoforms, and tissue specific expression of different isoforms has been demonstrated as a common strategy to fulfill the diverse requirement for V-ATPases (Toei et al., 2010). However, whether different isoforms are used in the same tissue to execute distinct functions is still an open question.

Here we report that isoforms of the V-ATPase α subunit are differentially required for Drosophila wing development. We provide genetic evidence that three of the five Vha100 isoforms (Vha100-1, Vha100-2, and Vha100-4) are involved in fly wing development. Somatic clonal analysis show that Vha100-1 regulates vein formation, while Vha100-2 functions in cuticle deposition and Vha100-4 participates in Wg signaling transduction. It is likely that Vha100-1 regulates vein formation through pathways transduced by other signaling molecules, such as bone morphogenetic protein (BMP), and epidermal growth factor (EGF) during fly wing development (Blair, 2007). Our results strongly support a model that V-ATPases might exist as multiple subtypes composed by diverse subunit isoforms in the same cells to meet the diversified demands. The accumulation of signaling molecules and alteration of signaling activities are not always fully penetrant in Vha100 mutant cells, which is also noticed in previous studies on other V-ATPase subunits (Yan et al., 2009; Vaccari et al., 2010; Ren et al., 2018). During tissue development, the V-ATPase regulates acidification of cellular organelles which are necessary for protein sorting, trafficking, and turnover (Forgac, 2007). It is conceivable that disrupting V-ATPase activity results in aberrant trafficking and degradation of signaling molecules, a highly dynamic process that might be only partially captured by conventional genetic analysis. In addition, our compensation tests suggest that beyond their specialized roles, Vha100-1 and Vha100-4 likely function redundantly for wing patterning and growth. This redundancy may also help to explain the minor defects observed when Vha100-1 and Vha100-4 single mutant clones were induced in the wing. Our observations suggest that the V-ATPases subtypes are able to constitute a coordinated network in cells. Further studies are required to dissect the exact mechanisms underlying the mode of action for V-ATPases.

Previous studies using RNAi knock-down experiments indicate that Vha100-2 is involved in the development of multiple fly tissues (Wang et al., 2014; Overend et al., 2016; Mondragon et al., 2019; Nagata et al., 2019). We find that Vha100-2 is the sole isoform required for wing cuticle integrity. This is the first report regarding a role of V-ATPases in insect cuticle formation. The cuticle is a multifunctional tissue that covers the whole body of insects, which provides significant protections from life threatening dangers such as dehydration, predators and pathogen infection (Moussian, 2010). It is possible that V-ATPases might regulate membrane trafficking and secretion of the cuticle components in wing epithelia cells in a similar fashion as reported in Caenorhabditis elegans (Liégeois et al., 2007). However, V-ATPases may as well function in other steps during cuticle formation, which needs to be further explored.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

DM performed the mosaic screen and identified the Vha44 mutants. DM, YC, and NJ performed the immunostaining analyses. IZ performed the mRNA in situ hybridization. DM, YC, NJ, and IZ performed the statistical analyses for wing phenotypes. DM prepared the original draft. JS and IZ reviewed and edited the manuscript. JS and IZ supervised the project. All authors approved the final manuscript submitted for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00723/full#supplementary-material
REFERENCES

Allan, A. K., Juan, D., Davies, S. A., and Julian, A. T. D. (2005). Genome-wide survey of V-ATPase genes in Drosophila reveals a conserved renal phenotype for lethal alleles. *Physiol. Genom.* 22, 128–138. doi: 10.1152/physiogenomics.00233.2004

Bassett, A. R., Tibbit, C., Ponting, C. P., and Liu, J. L. (2013). Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. *Cell Rep.* 4, 220–228. doi: 10.1016/j.celrep.2013.06.020

Blair, S. S. (2007). Wing vein patterning in *Drosophila* and the analysis of intercellular signaling. *Annu. Rev. Cell Dev. Biol.* 23, 293–319. doi: 10.1146/annurev.cellbio.23.090506.123606

Buchting, T., Bartscherer, K., Ohkawara, B., Chaudhary, V., Spirohn, K., Niehrs, C., et al. (2010). Wnt/Flr3 signaling requires DPFR, the Drosophila homolog of the prorien receptor. *Curr. Biol.* 20, 1263–1268. doi: 10.1016/j.cub.2010.05.028

Cipriano, D. I., Wang, Y., Bond, S., Hinton, A., Jefferies, K. C., Qi, J., et al. (2008). Structure and regulation of the vacuolar ATPases. *Biochim. Biophys. Acta* 1777, 599–604.

Forgac, M. (2007). Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat. Rev. Mol. Cell Biol.* 8, 917–929. doi: 10.1038/nrm2272

Gleixner, E. M., Canaud, G., Hermle, T., Guida, M. C., Kretz, O., Huber, T. B., et al. (2014). V-ATPase/mTOR signaling regulates megalin-mediated apical endocytosis. *Cell Rep.* 8, 10–19. doi: 10.1016/j.celrep.2014.05.035

Gratz, S. J., Ukken, F. P., Rubinstein, C. D., Thiede, G., Donohue, L. K., Cummings, A. M., et al. (2014). Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics* 196, 961–971. doi: 10.1534/genetics.113.160713

Hermle, T., Saltukoglu, D., Grucenwald, J., Walz, G., Simons, M., Gru, J., et al. (2010). Regulation of Flr3-dependent planar polarity signaling by a V-ATPase subunit. *Curr. Biol.* 20, 1269–1276. doi: 10.1016/j.cub.2010.05.057

Hiesinger, P. R., Fayyazuddin, A., Mehta, S. Q., Rosenmund, T., Schulze, K. L., Zhai, R. G., et al. (2005). The V-ATPase V0 subunit a1 is required for a late step in synaptic vesicle exocytosis in *Drosophila*. *Cell* 121, 667–670. doi: 10.1016/j.cell.2005.03.012

Hinton, A., Bond, S., and Forgac, M. (2009). V-ATPase functions in normal and disease processes. *Pflugers Arch.* 457, 589–598. doi: 10.1007/s00424-007-0382-4

Inoue, T., and Forgac, M. (2005). Cysteine-mediated cross-linking indicates that subunit C of the V-ATPase is in close proximity to subunits E and G of the V1 domain and subunit a of the V0 domain. *J. Biol. Chem.* 280, 27969–27993. doi: 10.1074/jbc.m504980200

Inoue, T., Wang, Y., Jefferies, K., Qi, J., Hinton, A., and Forgac, M. (2005). Structure and regulation of the V-ATPases. *Bioenerg. Biomembr.* 37, 393–398.

Jiang, H., Samir, B., and John, L. R. (2015). Electron cryomicroscopy observation of rotational states in a eukaryotic V-ATPase. *Nature* 521, 241–245. doi: 10.1038/nature14365

Julian, A. T. D. (1999). The multifunctional *Drosophila melanogaster* V-ATPase is encoded by a multigene family. *J. Bioenerg. Biomembr.* 31, 75–83.

Kobia, F., Duchi, S., Deflorian, G., and Vaccari, T. (2013). Pharmacologic inhibition of vacuolar HC ATPase reduces physiologic and oncogenic Notch signaling. *Mol. Oncol.* 8, 207–220. doi: 10.1016/j.molonc.2013.11.002

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 587–580. doi: 10.1006/jmbi.2000.4315

Leng, X. H., Manolios, M., and Forgac, M. (1998). Function of the COOH-terminal domain of Vph1p in activity and assembly of the yeast V-ATPase. *J. Biol. Chem.* 273, 6717–6723. doi: 10.1074/jbc.273.12.6717

Leng, X. H., Manolios, M., Liu, Q., and Forgac, M. (1996). Site-directed mutagenesis of the 100-kDa subunit (Vph1p) of the yeast vacuolar (H+)-ATPase. *J. Biol. Chem.* 271, 22487–22493. doi: 10.1074/jbc.271.37.22487

Légeois, S., Benedetto, A., Michaux, G., Béliard, G., and Labousse, M. (2007). Genes required for osmoregulation and apical secretion in *Caenorhabditis elegans*. *Genetics* 175, 709–724. doi: 10.1534/genetics.106.060335

Logan, C. Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* 20, 781–810.
Wang, D., Epstein, D., Khalaf, O., Srinivasan, S., Williamson, W. R., Fayyazuddin, A., et al. (2014). Ca2+-Calmodulin regulates SNARE assembly and spontaneous neurotransmitter release via v-ATPase subunit V0 a1. *J. Cell Biol.* 205, 21–31. doi: 10.1083/jcb.201312109

Wang, J., Kean, L., Yang, J., Allan, A. K., Davies, S. A., Herzyk, P., et al. (2004). Function-informed transcriptome analysis of *Drosophila* renal tubule. *Genome Biol.* 5:R69.

Wang, Y., Carballo, R. G., and Moussian, B. (2017). Double cuticle barrier in two global pests, the whitefly *Trialeurodes vaporariorum* and the bedbug *Cimex lectularius*. *J. Exp. Biol.* 220, 1396–1399. doi: 10.1242/jeb.156679

Wang, Y., Yu, Z., Zhang, J., and Moussian, B. (2016). Regionalization of surface lipids in insects. *Proc. Biol. Sci.* 283:20152994. doi: 10.1098/rsbp.2015.2994

Wieczorek, H., Beyenbach, K. W., Huss, M., and Vitavska, O. (2009). Vacuolar-type proton pumps in insect epithelia. *J. Exp. Biol.* 212, 1611–1619. doi: 10.1242/jeb.030007

Wilkins, S., Inoue, T., and Forgac, M. (2004). Three-dimensional structure of the vacuolar ATPase: localization of subunit H by difference imaging and chemical cross-linking. *J. Biol. Chem.* 279, 41942–41949. doi: 10.1074/jbc.m407821200

Williamson, W. R., Wang, D., Haberman, A. S., and Hiesinger, P. R. (2010a). A dual function of V0-ATPase a1 provides an endolysosomal degradation mechanism in *Drosophila melanogaster* photoreceptors. *J. Cell Biol.* 189, 885–899. doi: 10.1083/jcb.201003062

Williamson, W. R., Yang, T., Terman, J. R., and Hiesinger, P. R. (2010b). Guidance receptor degradation is required for neuronal connectivity in the *Drosophila* nervous system. *PLoS Biol.* 8:e1000553. doi: 10.1371/journal.pone.01000553

Wissel, S., Harzer, H., Bonnay, F., Burkard, T. R., Neumüller, R. A., and Knoblich, J. A. (2018). Time-resolved transcriptomics in neural stem cells identifies a v-ATPase/Notch regulatory loop. *J. Cell Biol.* 217, 3285–3300. doi: 10.1083/jcb.201711167

Xu, T., and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 117, 1223–1237.

Yan, Y., Denef, N., and Schucpbach, T. (2009). The vacuolar proton pump, V-ATPase, is required for notch signaling and endosomal trafficking in *Drosophila*. *Dev. Cell.* 17, 387–402. doi: 10.1016/j.devcel.2009.07.001

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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