Mutations in the *Drosophila* ortholog of the vertebrate Golgi pH regulator (GPHR) protein disturb endoplasmic reticulum and Golgi organization and affect systemic growth

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Summary
Sorting of secretory cargo and retrieval of components of the biosynthetic pathway occur in organelles such as the Golgi apparatus, the endoplasmic reticulum and the endosomes. In order to perform their functions in protein sorting, these organelles require a weakly acidified lumen. In vitro data have shown that Golgi luminal pH is in part regulated by an anion channel called Golgi pH Regulator (GPHR). Mammalian cells carrying a mutated GPHR version present an increased luminal pH leading to delayed protein transport, impaired glycosylation and Golgi disorganization. Using *Drosophila* as a model system, we present here the first phenotypic consequences, at the organism level, of a complete lack of GPHR function. We show that, although all individuals carrying complete loss-of-function mutations in the *dGPHR* gene can go through embryonic development, most of them die at late larval stages. The *dGPHR* mutations are, however, sublethal and can therefore generate escapers that are smaller than controls. Using cellular and molecular readouts, we demonstrate that the effects of *dGPHR* mutation on larval growth are not due to Insulin signaling pathway impairment and can be rescued by providing dGPHR in only some of the larval tissues. We reveal that, although functionally exchangeable, the invertebrate and vertebrate GPHRs display not completely overlapping sub-cellular localization. Whereas the mammalian GPHR is a Golgi-only associated protein whose inactivation disturbs the Golgi apparatus, our data suggest that dGPHR is expressed in both the ER and the Golgi and that dGPHR mutant flies have defects in both organelles that lead to a defective secretory pathway.

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Key words: GPHR, *Drosophila*, Golgi, Growth control, Endoplasmic reticulum

Introduction
The main function of the Golgi apparatus is to sort molecules that are transported through this organelle en route to the plasma membrane, the extracellular medium and the endosomal/lysosomal compartments. As for each individual organelle, the function of the Golgi apparatus depends on the establishment and stringent maintenance of a distinct pH (Casey et al., 2010). In mammalian cells, luminal acidification of the Golgi apparatus is essential for its function and to maintain cellular homeostasis (Weisz, 2003). Indeed, when the acidic luminal pH is artificially alkalinized, the trafficking, processing and glycosylation of cargo proteins and lipids are impaired. As a result, some proteins become misrouted and the morphological integrity of the Golgi is compromised (Axelsson et al., 2001; Rivinoja et al., 2006). Consequently, mutations that alter Golgi luminal pH have been shown to perturb cell metabolism leading to congenital diseases and cancer (Weisz, 2003). The acidic pH of organelles, including the Golgi, is regulated by a balance between the proton pump V-ATPase, which is the sole proton delivery source, and the counterion channel GPHR (Moriyama and Nelson, 1989; Nishi and Forgac, 2002; Schapiro and Grinstein, 2000; Wu et al., 2001). This voltage dependent anion channel dissipate the membrane potential formed by the proton influx allowing proton pump to transfer more protons into the Golgi lumen and therefore facilitating Golgi luminal acidification and hence Golgi function. Cells in which GPHR is inactive show elevated luminal pH of the Golgi but not of the endosomal/lysosomal compartments (Maeda et al., 2008). Using a keratinocyte-specific GPHR-knockout mice model, Medea and collaborators have recently shown that GPHR is essential for the homeostasis of the epidermis including the formation of lamellar bodies and for establishing its barrier function (Tarutani et al., 2012). *Drosophila* has recently been established as a good alternative model system to study the Golgi apparatus that shares many morphological and functional similarities with the mammalian one (Kondylis and Rabouille, 2009; Kondylis et al., 2007; Sisson...
et al., 2000). Drosophila is also very useful for studying the anatomical and physiological consequences associated with gene inactivation at the organism level. We present here the molecular and phenotypic characterization of null loss-of-function alleles of the Drosophila ortholog of the mammalian GPHR anion channel. We show that the complete inactivation of dGPHR is not always associated with fly lethality but dramatically impairs its developmental growth independently of the insulin signaling (IS) pathway. We also demonstrate that although dGPHR is indeed the functional ortholog of the human GPHR, its subcellular localization and function differ from the mammalian protein. Whereas mammalian GPHR is a Golgi-only protein require for pH maintenance, the Drosophila ortholog co-localized with markers of both endoplasmic reticulum and Golgi and is required for correct organization of both organelles.

Materials and Methods

Drosophila melanogaster strains and maintenance

The following strains were used in this work: dGPHR(1) (this work), dGPHR(KD6/74) (Kyoto#140780), elav-Gal4 (BL4584), c601-Gal4 (BL30844), Mel2-Gal4 (BP32790), daughterless-Gal4 (BL95460), Drosophila-Gal4: RFP (BP380907), UAS-ACT-βgal: RFP (BP17718), UAS-ymyRFP (BP71718). The UAS-dGPHR, UAS-dGPHR-AHA, UAS-huGPHR, UAS-huGPHR::HA and UAS-hams-GPHR flourescent proteins were obtained by P element mediated insertion of pCAST constructs containing full length cDNA coding for dGPHR, dGPHR-AHA, huGPHR, huGPHR::HA and hamsGPHR, respectively (molecular details available upon request).

Flies were flown at 25°C on a yeast/cornmeal medium. For 1 litre of food, 8.2 g of agar (VWR, cat. no. 20768.361), 80 g of corn meal (Flourtech, Farigel maize H1) and 80 g of yeast extract (VWR, cat. no. 24979.413) were cooked for 10 min in boiling water; 5.2 g of molybdenum sodium salt (Merek, cat. no. 106756) and 14 ml of 95% propanic acid (Carloerba, cat. no. 409553) was added when the food had cooled down.

Measurement of weight, larval size, pupariation, and adult emergence

Adult size was estimated based on the weight of 3-day-old flies. The weight of multiple replicates (minimum of three) of a pool of five females or male was calculated using a precision balance (Mettler Toledo, AG245). Larval size was estimated by collecting and freezing larvæ (n=20) twice a day (morning and evening) after an initial 3 hr period of egg deposition. Larvae were frozen and mounted in 80% glycerol in PBS. Pictures were taken on a black background using a ProgResC5 CCD camera (JenOptik) mounted on a stereomicroscope. The body surface of each larva was calculated using ImageJ. Masks covering the surface of the larva were generated using the threshold tool. Surface values were displayed in pixels. To examine the time of pupariation and adult emergence, 40 eggs were collected for 5 hr, and the number of new pupa or adults was counted every 24 hr.

Determination of wing cell size and cell number

For each genotype, eight to ten wings were photographed. Cell size and total cell numbers in the wing were estimated by counting the number of wing hairs within the constant pixel area to determine relative pixel area per cell. Relative cell number was estimated by dividing the total wing pixel area by the pixel area per cell.

Clone induction

Mitotic clones were generated by FLP-mediated mitotic recombination (Xu and Rubin, 1993). Induction of dGPHR(KD24) mutant clones in wing imaginal discs was obtained by crossing females yw;FB-FRT159 ubi-GFP FRT151 ubi-GFP FRT151 to males dGPHR(KD24) FRT151/ yw;FB-FRT159 ubi-GFP FRT151. Larvae of the progeny were heat shocked at L2 stage (48–72 hr after egg deposition, AED) and observed 24 hr later. Mutant clones were identified by absence of GFP.

Mouth hook contractions

Larvae reached mid late third instar (120 hr AED). Larvae of the progeny were heat shocked at L2 stage (48–72 hr after egg deposition, AED) and observed 24 hr later. Mutant clones were identified by absence of GFP.

Quantitative real-time PCR

Mouth hook contractions

Larvae were rinsed 30 seconds in PBS and transferred to agar plate and quantified for the frequency of mouth-hook contraction by individual larva at 22°C. Typically, the assay time was 20 min. The contraction frequencies of individual larva remained consistent throughout the assay.

Homogenate

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Results

CG8090 encodes the invertebrate ortholog of GPHR

Following an EMS mutagenesis, we isolated an allele carrying a point mutation in a gene referred as CG8090 in Flybase (http://flybase.org). Protein prediction analysis indicates that the CG8090 protein (Fig. 1B,C) corresponds to a Drosophila ortholog co-localized with markers of both endoplasmic reticulum and Golgi and is required for correct organization of both organelles.

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In addition, the onset of both pupariation and adult emergence are delayed in dGPHR mutant larvae (Fig. 2C). In contrast, the smaller body size of dGPHR mutants compared to wild-type controls (Fig. 2D). Thus, the smaller body size of dGPHR mutants is likely to be caused by slower growth rate during the late larval period rather than by precocious pupariation. Note that few dGPHR mutants can reach pupal (20% ± 7 SD of expected pupae) and adult (5% ± 2 SD of expected adults) stages.

To test whether this overall size reduction was due to a decrease in cell size, cell number or both, we quantified these parameters in adult wing blades. As shown in Fig. 2E, both cell size and cell number were reduced in dGPHR mutant when compared to controls. The similar penetrance and strength of the phenotype observed between dGPHR homozygous mutants and dGPHR<sup>k34</sup>/Df(2R)ED2426 or dGPHR<sup>LL03674</sup>/Df(2R)ED2426 allelic combinations (data not shown) demonstrate that dGPHR<sup>k34</sup> and dGPHR<sup>LL03674</sup> are complete loss-of-function alleles as far as growth control is concerned.

dGPHR is not a component of the Insulin pathway
dGPHR mutant larvae present a reduced growth rate and a developmental delay, which are hallmarks of mutants affecting the Insulin signaling (IS) pathway (Hietakangas and Cohen, 2009). To investigate whether dGPHR is implicated in IS, we analyzed the expression of two known transcriptional targets of the IS pathway in dGPHR mutants, namely 4E-BP and InR, encoding the Insulin receptor (Puig and Tjian, 2005). Both 4E-BP and InR transcription is upregulated in response to repressed Insulin signaling. We found that transcription of 4E-BP and InR mRNAs is unaffected in first instar larva mutant for dGPHR (Fig. 3A). This suggests that dGPHR does not directly act in the IS pathway. However, as expected when larval growth is reduced, dGPHR mutants displayed reduced Insulin and Ecdysone levels (measured by the transcriptional rate of E74B, a direct target of the Ecdysone receptor), signaling activities in late stages of larval development (supplementary material Fig. S1). To further confirm that dGPHR does not act via the Insulin pathway, we followed the progeny of dGPHR mutant mitotic clones in wing imaginal disc. Indeed, previous reports indicated that cells defective in IS pathway component present defective growth rate and hence give rise to smaller clones than wild-type cells (Hietakangas and Cohen, 2009). As shown in Fig. 3B, dGPHR mutant cells grew and divided as well as neighboring cells giving rise to clones of similar size than the one derived from wild-type cells. Since imaginal cells can compensate their reduced division rate by increasing cell size, we compared the size of dGPHR mutant and wild-type cells by phalloidin staining and found no major difference (Fig. 3C). It is known that larval growth is largely based on an increase in cell size, which is accomplished by endoreplication, a modified cell cycle, consisting of successive rounds of DNA synthesis without intervening mitoses. We thus investigated the requirement of dGPHR in endoreplication by analyzing the nuclei size of dGPHR mutant cells induced during embryogenesis and observing in late third instar larvae (120 hr AED). Although dGPHR mutant larvae (120 hr AED) display salivary gland cells with reduced nuclei size when compared to controls (supplementary material Fig. S2), no obvious differences in nuclei size were noticed between dGPHR mutant cells and the wild-type cells, in salivary glands with MARCM clones mutant for dGPHR (Fig. 3D). Altogether, our data indicate that dGPHR does not directly act via the Insulin signaling pathway or in the endoreplication process, which are both critical regulators of larval growth. Moreover, by using a food intake assay, such as feeding with colored yeast (data not shown), analyzing metabolic markers (supplementary material Fig. S3A) or quantifying mouth hook contractions (supplementary material Fig. S3B), we could exclude the hypothesis that the smaller sizes of dGPHR larvae reflect the inability of dGPHR larvae to feed properly.
dGPHR protein localizes to the endoplasmic reticulum and to the Golgi

The vertebrate GPHR protein has been shown to be localized and to function in the Golgi apparatus (Maeda et al., 2008). In order to test whether its invertebrate ortholog has a similar sub-cellular localization, we overexpressed an HA-tagged version of the dGPHR via the Gal4-UAS system. When expressed in a stripe of cells along the antero-posterior boundary of the wing imaginal disc using the patched-Gal4 driver (Fig. 4A), dGPHR-HA was detectable in two distinct subcellular patterns, one surrounding the nucleus and the other inside the cytoplasm and more punctiform (Fig. 4B,C). Double labeling with specific organelle markers demonstrated that the ring-like staining largely co-localized with the ER marker RFP::KDEL and the punctiform
one to a fraction of the Golgi apparatus (labeled with Galt::RFP) (Okajima et al., 2005). Similar expression patterns were observed when the human GPHR was expressed in wing disc cells (Fig. 4D,E).

The vertebrate GPHR proteins are the functional orthologs of dGPHR

The differential subcellular localization of GPHR in human and Drosophila cells prompted us to test the functional relationship between the vertebrate and invertebrate GPHRs. For that, we asked whether the vertebrate GPHR protein could functionally compensate for the lack of dGPHR function in flies. To do so, the human or the hamster GPHR proteins were expressed in dGPHR mutant flies using a ubiquitous Gal4 driver (daughterless-Gal4) and the weight of the emerging adult flies was used as a readout of body size. As shown in Fig. 5A, expression of the dGPHR protein or its two vertebrate orthologs was sufficient to fully rescue dGPHR mutant growth defects and developmental lethality (data not shown). This indicated that the vertebrate GPHRs can compensate for a lack of dGPHR and that these proteins are therefore functional orthologs. Although dGPHR is predicted to be ubiquitously expressed (Flyatlas, http://flyatlas.org), we decided to investigate in which tissue dGPHR function was required to ensure optimal growth during Drosophila development. We took advantage of the ability of UAS-dGPHR transgene to rescue the growth defect of dGPHR

Fig. 3. dGPHR mutations do not impact the Insulin pathway signaling. (A) mRNA quantification by qRT-PCR of two transcriptional targets of the IS pathway. dGPHR mutations do not affect InR and 4E-BP transcription. (B–D) Confocal microscope section of a third instar wing imaginal disc containing mitotic clones of dGPHR mutant cells (absence of green staining), and wild-type (bright green staining) sister clones. (B) dGPHR mutant clones and wild-type sister clones have similar size. (C) Size of wild-type and dGPHR mutant cells labeled with phalloidin is similar. (D) Nuclei size of salivary gland cells is not affected by a mutation in the dGPHR. Scale bars: 50 μm (B,D), 20 μm (C).
dGPHR inactivation affects both ER and Golgi organization and impairs protein secretion

We then tested whether dGPHR inactivation could have effects on ER and Golgi organization. Using Galt::RFP and RFP::KDEL as Golgi and ER markers, respectively, we analyzed salivary glands of third instar larvae at 96 hr AED (a time window that precedes the growth defect phenotype of dGPHR mutants, Fig. 2D) and demonstrate that dGPHR inactivation was associated with severe ER and Golgi disorganization phenotypes (Fig. 6A,B). Whereas RFP::KDEL forms a uniform network in wild-type salivary gland cells, it accumulates basally and asymmetrically in the center of the cell in proximity to the nucleus in mutant tissue (Fig. 6A). The global organization of the Golgi apparatus was also affected by dGPHR inactivation. The regular dot-like pattern detected in wild-type cells carrying a Galt::RFP transgene was disrupted in mutant cells although not uniformly. Whereas in some regions of the mutant cells (labeled ‘a’ in Fig. 6B), Golgi structure appears similar to control, in others (labeled ‘b’), Galt::RFP staining appears more fragmented and fainter than in controls. To insure that these effects were not an indirect consequence of the dGPHR mutation on growth retardation (i.e. on Insulin signaling reduction), we tested whether similar phenotypes could be observed in chico\(^{1}\) mutant salivary glands (at 96 hr [data not shown] and 120 hr AED). As shown in supplementary material Fig. S4, chico\(^{1}\) salivary gland cells present a homogeneous distribution of both RFP::KDEL and Galt::RFP, similar to wild-type glands. To further characterize the defaults observed in organelles associated with the secretory pathway, wild-type and mutant cells were labeled with the ER exit site marker sec16. As already observed with Golgi and ER markers, the distribution of sec16 positive structures was quite heterogeneous in mutant cells. Whereas in some compartments of mutant cells, tER sites were uniformly scattered and spaced (labeled ‘a’ in Fig. 7B) as in wild-type controls, in others (labeled ‘b’) the sec16 positive structures were more fragmented (Fig. 7B). The cell compartments in which sec16 staining was fragmented correspond to domains in which RFP::KDEL was abnormally accumulated (Fig. 7B). This disorganization of ER was confirmed using electron microscopy. Whereas TEM pictures of wild-type salivary gland cells present large vesicles of secretion surrounded with well-organized ER ribbon, the cytoplasmic organization of dGPHR mutant cells was strikingly different (supplementary material Fig. S5). The size of secretory granules was 5 times smaller in mutant than in wild-type cells. In addition, the very well-organized ER ribbon with associated ribosomes seen in wild-type cells was no longer detectable in dGPHR mutant cells. The abnormal ER organization associated with dGPHR mutation, led us to test the functionality of secretory pathway. For this purpose, we make use of a sec::GFP reporter construct that allows us to visualize the secretory process in vivo. In contrast to control cells in which the sec::GFP protein is present throughout the cytoplasm in a uniform pattern, its expression pattern was very similar to that of the ER markers in dGPHR mutant cells (supplementary material Fig. S6). Altogether, these data indicate that in the absence of functional dGPHR, both the Golgi apparatus and the ER are disorganized indicating that the dGPHR protein is required to shape the ER-Golgi apparatus and that its inactivation affect the secretory pathway functionality.

Discussion

We report here the first analysis of a Golgi pH Regulator mutant in Drosophila melanogaster. In contrast to what is expected for a loss-of-function mutation in a gene thought to be involved in fundamental cellular processes, complete removal of maternal (data not shown) and zygotic dGPHR function is not fully lethal and can generate viable flies. This is rather unexpected knowing...
**Fig. 5.** The dGPHR is the functional ortholog of the mammalian GPHR. Rescue experiment of the dGPHR mutant phenotype through ubiquitous (A) or tissue specific (B) overexpression of the Drosophila and mammalian GPHR cDNAs. The hamster and human GPHR cDNAs are as efficient as the Drosophila ortholog to rescue the growth defects observed in dGPHR mutant flies (A). This indicates that the vertebrate and invertebrate orthologs are functionally equivalent. (B) Whereas overexpression of the dGPHR cDNA in muscle is not sufficient to rescue dGPHR mutant growth defects, gut or neuronal overexpressions are. Values indicated by * are statistically significant ($p < 0.05$).

**Fig. 6.** dGPHR inactivation induces ER and Golgi disorganization. Wild-type and dGPHR mutant salivary gland cells carrying an endoplasmic reticulum marker (RFP::KDEL, A), a Golgi marker (Galt::RFP, B). (A) The RFP::KDEL marker is uniform in control cells and concentrated both basally and nearby the nuclei in mutant cells. (B) The Galt::RFP expression pattern is a regular network in control cells. In mutant cells, Galt::RFP staining is heterogeneous within the cells. In some regions, such as region ‘a’, Galt::RFP expression is similar to wild type, whereas in region ‘b’ it is more fragmented and weaker. This indicates that dGPHR inactivation is affecting organization of both ER and Golgi. Scale bars: 25 μm.
that functional redundancy is relative rare in flies and clearly less pronounced than in vertebrates. Although the complete mouse knockout phenotype has not been yet reported, a recent report indicate that mice lacking GPHR in keratinocytes exhibited hypo-pigmented skin, hair loss and scaliness, indicating that mammalian GPHR is essential for epidermis homeostasis (Tarutani et al., 2012). One possible explanation for the relative milder phenotype in Drosophila could be that pH acidification is under the regulation of multiples molecules or pathways. Another possibility would be that dGPHR is not functioning as Golgi lumen acidificator. This later hypothesis is, however, unlikely as we have shown that the vertebrate GPHR can compensate for the absence of the Drosophila ortholog, suggesting that the two proteins are performing similar functions. It should, however, be mentioned that our results indicate that the mammalian and flies GPHR do not have the same sub-cellular localization. Whereas the vertebrate GPHR is a Golgi only associated protein, its mammalian homolog structure is made of Golgi stacks linked to form a single copy organelle forming a ribbon (Rabouille et al., 1999; Ripoche et al., 1994). Further studies will be required to understand whether these differences could be explained by morphological or functional differences between flies and vertebrate secretory pathway. Although dGPHR mutant embryos can develop into adult, those are much smaller that wild-type flies indicating that Golgi function is regulating growth. In metazoans, the Insulin/Insulin-like growth factor signaling pathway controls the growth rate of tissues according to nutrient availability. Recent studies have described that communication between different larval tissues is essential to influence overall body growth and development although in many cases, the nature of the secreted factors that mediate this inter-organ signaling is still unclear. For example, Drosophila growth is mediated by Insulin-like peptides that are produced by specific neurons in the brain and released into the hemolymph to couple nutrient uptake with systemic growth (Colombani et al., 2003; Gémardin et al., 2009). Upstream of the event is the secretion by the fat body of unknown signalling molecules that circulate in the hemolymph to reach the brain in order to control Insulin secretion (Gémardin et al., 2009). In addition, animal growth relies on nutrient processing, which involves the secretion of digestive enzymes by gut cells. Hence, one putative explanation for the dwarf phenotype associated with the dGPHR mutation could correspond to the impairment of some cells that normally produce and release proteins that favour growth, to do so. For instance, one could imagine that the Insulin producing cells and/or the fat body, and/or the gut cells have reduced capacity in secreting such molecules when the function of dGPHR is impaired. The ability of UAS-dGPHR transgene to rescue the growth defect of dGPHR mutants when expressed either in neuronal cells or gut cells are in agreement with this hypothesis and suggest that restoring secretion in one tissue can compensate for defects in other tissues. Nevertheless, further work will be required to address these issues.

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Competing Interests

The authors have no competing interests to declare.

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