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TITLE

Sphingolipid depletion impairs endocytic traffic and inhibits Wingless signaling

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ABSTRACT

Sphingolipids are an important part of the plasma membrane and implicated in a multitude of cellular processes. However, little is known about the role of sphingolipids in an epithelial context and their potential influence on the activity of signaling pathways. To shed light on these aspects we analyzed the consequences of changing ceramide levels *in vivo* in the *Drosophila* wing disc: an epithelial tissue in which the most fundamental signaling pathways, including the Wnt/Wg signaling pathway, are well characterized.

We found that downregulation of *Drosophila*'s only ceramide synthase gene *schlank* led to defects in the endosomal trafficking of proteins. One of the affected proteins is the Wnt ligand Wingless (Wg) that accumulated. Unexpectedly, although Wg protein levels were raised, signaling activity of the Wg pathway was impaired. Recent work has spotlighted the central role of the endocytic trafficking in the transduction of the Wnt signal. Our results underscore this and support the view that sphingolipid levels are crucial in orchestrating epithelial endocytic trafficking *in vivo*. They further demonstrate that ceramide/sphingolipid levels can affect Wnt signaling.

KEY WORDS

Wingless; sphingolipid; ceramide; endocytic trafficking

1. INTRODUCTION

Sphingolipids influence a variety of developmental processes including cell growth, differentiation and apoptosis. They are synthesized in the ER and Golgi apparatus and are subsequently transported to the plasma membrane, where they reside in the external membrane leaflet (Futerman and Riezman, 2005). Sphingolipids were proposed to form the controversial detergent resistant microdomains, the so-called "lipid rafts", together with cholesterol. These microdomains within the plasma membrane form, as sphingolipids tend to cluster due to lateral interactions; additionally cholesterol intercalates between their mostly saturated fatty acids, giving rise to a more rigid or "liquid-ordered" patch of membrane. Due to the differential membrane composition some lipid-modified and transmembrane proteins might preferentially associate with these lipid microdomains, whereas others would avoid them (Lingwood and Simons, 2010; Simons and Ikonen, 1997). This led to the notion that sphingolipids are involved in protein sorting and trafficking (Le Roy and Wrana, 2005). It also suggested a potential role in regulating signaling activity by providing platforms for signaling complexes (Simons and Toomre, 2000). However, the effect of changing sphingolipid levels in an epithelial context, for example the consequences for signaling pathway activities, is not well understood. Ceramide, the precursor of all complex sphingolipids, is known to be an important second messenger, as well as a modulator of membrane properties. Ceramide production is induced during various stress situations and is thought to be involved not only in apoptotic signaling (Kolesnick and Krönke, 1998), but also in cell growth and differentiation. Due to its physicochemical properties ceramide tends to lateral clustering which, combined with its inverted conical shape, results in negative membrane curvature and inwards budding of membrane (Holopainen et al., 2000). It is still debated if ceramide functions directly as a second messenger or indirectly via its ability to induce changes in the membrane structure (Kolesnick et al., 2000; Van Blitterswijk et al., 2003). This influence on membrane structure further suggests a role of ceramide in endocytosis and intracellular trafficking: when fibroblasts and macrophages are treated with an exogenous sphingomyelinase in the absence of ATP the formation of endocytic vesicles was observed (Zha et al., 1998).

There is growing evidence that endocytosis and endocytic trafficking of the ligand-receptor-complexes are essential for Wnt/Wg signal transduction both in mammalian cells and in *Drosophila* (Blitzer and Nusse, 2006; Seto and Bellen, 2006). This highly

conserved signaling pathway plays a crucial role in a variety of developmental processes and deregulated Wnt signaling is implicated in various diseases, including colon cancer and degenerative diseases (Clevers and Nusse, 2012). In the canonical branch of Wnt/Wg signaling Wnt forms a ternary complex with its receptors Frizzled (Fz) and LRP5/6 (Arrow in *Drosophila*), which are activated by a series of phosphorylation events. This leads to relocalization of Dishevelled (Dvl) to the plasma membrane and the formation of receptor complexes on oligomerized Dvl clusters, giving rise to signaling platforms called "signalosomes" (Bilic et al., 2007). The binding of the scaffold protein Axin leads to the recruitment of APC and GSK3ß, which are also components of the ß-catenin destruction complex. As a consequence ß-catenin can no longer be degraded, accumulates and translocates to the nucleus, where it initiates the transcriptional activation of target genes. Several observations highlight the importance of endocytic trafficking in Wnt signaling: first, the Fz receptor stimulates its own endocytosis via Gαo and the early-endosomal GTPase Rab5, and localization in the respective endosomal compartment is reported to influence the balance between canonical and non-canonical signaling branch (Purvanov et al., 2010). Second, Niehrs and colleagues showed that acidification of an intracellular compartment is required for LRP6 receptor phosphorylation and thus Wnt signaling induction (Buechling et al., 2010; Cruciat et al., 2010). Third, it was shown in an elegant study that GSK3ß is sequestered into multivesicular bodies (MVBs) upon Wnt stimulation, so that the enzyme is secluded from its cytosolic substrate ß-catenin and by this the Wnt signal can be transduced (Taelman et al., 2010).

Given the dearth of knowledge about the role of sphingolipids in an epithelial context and their potential influence on the activity of signaling pathways we set out to analyze the consequences of changed ceramide levels, using the *Drosophila* wing disc as a suitable model for an epithelium. Since most fundamental signaling pathways, including the Wnt/Wg signaling pathway, are well characterized in the wing imaginal disc we hoped to gain insights into the effects of ceramide depletion on these pathways. To this end, we used RNAi-mediated gene knockdown against components of the *de novo* ceramide synthesis pathway (Acharya and Acharya, 2005). Our study focuses on the effects of depleting ceramide levels by downregulation of *schlank*, the only ceramide synthase gene in *Drosophila*. We found that downregulation of *schlank* does not obviously influence endocytosis and formation of the early-endosomal

compartment. It rather leads to disruption of recycling and degradative endocytic trafficking routes and consequently the accumulation of several secreted and transmembrane proteins in the cells, amongst them Wg. In spite of the higher Wg levels, the activity of the Wg signaling pathway is reduced. This reduction of Wg signaling activity is possibly connected to an early-to-late endosomal trafficking defect and highlights the importance of endocytic trafficking for Wg signal transduction. Importantly, the results reveal a hitherto unknown function for sphingolipids in Wnt signaling.

2. RESULTS

2.1 Depletion of schlank leads to growth defects, accumulation of Wg protein and reduced Wg signaling activity

To determine the effects of sphingolipid depletion in an epithelial context, we examined the effect of reducing *schlank* function (CG3576, Bauer et al 2009). This choice was based on the facts that its product is *Drosophila's* only ceramide synthase and that its downregulation results in reduced ceramide levels, and on the availability of suitable alleles. *Schlank* was previously identified in a genome-wide RNAi screen conducted in our lab to uncover growth regulators (G. Reim and K. Basler, unpublished). Consistent with this, s*chlank* depletion by two independent RNAi lines (Fig. 1A) in the posterior compartment resulted in reduced cell size, as judged by the closer arrangement of wing trichomes (Fig. 1B''), and increased levels of apoptosis (Fig. S1A). The wing margins were frequently found to be lost (Fig. 1B and Fig. S1C), a phenotype characteristic of reduced Wg signaling. Furthermore, the proximo-distal orientation of the trichomes was disturbed (Fig. 1B''), reminiscent of defects in the non-canonical Wnt or planar cell polarity (PCP) pathway. To begin to explain these observations, we monitored Wg distribution and analyzed the expression of Wg target genes. Wg protein was massively accumulating in dot-like structures upon *schlank* depletion (Fig. 1C). Whereas expression of the low-threshold target gene Distalless (Dll) was not changed (data not shown), the expression of the high-threshold target gene Senseless (Sens) was reduced (Fig. 1D, Fig. S1B, D).

These data indicate that besides the growth phenotypes, reduction of ceramide levels also leads to accumulation of Wg protein in vesicle-like structures. Unexpectedly although there is more Wg ligand, high-level Wg signaling activity seems to be impaired upon *schlank* depletion.

2.2 Wg accumulation is due to impaired ceramide synthase activity and independent of apoptosis

To confirm that the observed phenotypes are a consequence of downregulation of *schlank* and to exclude potential RNAi mediated off-target effects, we tested the hypomorphic P-element-based allele $schlank^{P365}$, in which the P-element is inserted downstream of the first *schlank* exon. We observed the same Wg accumulation phenotype as in *schlank* RNAi expressing wing discs (data not shown). Additionally, we generated a presumptive null allele, $schlank^{331}$, via imprecise excision of this P-

element, deleting the downstream half of the first exon including a predicted transmembrane domain (Fig. 1A). Again, we observed the cell-autonomous accumulation of Wg protein in *schlank*^{331} clones (Fig. 2A). Due to the impaired growth upon *schlank* depletion, we mostly worked in a Minute background to equip the *schlank* mutant cells with a growth advantage. These experiments show that the observed defects are caused by the depletion of *schlank* and are not an RNAi artifact.

We next performed rescue experiments using an *actin-Gal4* driven *UAS-schlank* transgene (either native or HA-tagged) and a genomic construct comprising the entire *schlank* locus. All of these constructs were able to fully rescue either *schlank*^{P365} or *schlank*^{331} mutant flies. In a wild-type background they did not cause any phenotype. Importantly, we also tested the allele H215D, in which the enzymatic activity of *Schlank* is abrogated (Bauer et al., 2009), and found it unable to rescue our *schlank* alleles (data not shown). RNAi induced Wg accumulation was diminished by expression of Schlank but not the Schlank mutant H215D (Fig. S2A). We further induced *schlank*^{P365} mutant clones and expressed the HA-tagged Schlank protein in the posterior compartment: again the *schlank*^{P365} induced Wg accumulation was reverted (Fig. 2B). The rescue capability of Schlank and the inability of the H215D mutant strongly suggest that the Wg accumulation is due to reduced ceramide synthase activity.

To test if the Wg accumulation is merely a consequence of elevated levels of apoptosis, we provoked apoptosis by expressing the pro-apoptotic protein Hid in the posterior compartment. In this case, the apoptosis induction (Fig. S2B) did not result in Wg accumulation (Fig. 2C). Next, we prevented apoptosis in *schlank* depleted cells by expressing Diap1 protein in the posterior compartment (Fig. S2C) and found Wg protein still accumulated (Fig. 2D). Hence this phenotype is not a consequence of the elevated levels of apoptosis upon *schlank* reduction.

Taken together these experiments indicate that the Wg accumulation phenotype is caused by reduced ceramide synthase activity and is independent of apoptosis.

2.3 Several secreted and transmembrane proteins accumulate, but their respective signaling pathway activities are not changed

To test if the reduction of ceramide levels affects wing imaginal disc cell morphology, we assessed the overall integrity of the wing disc epithelium by electron microscope analysis of wing discs expressing *schlank*^{RNAi1} in the posterior compartment. For

visualization of these cells we additionally expressed a membrane-bound Horseradish Peroxidase that could be detected via DAB staining. We were able to identify *schlank* mutant tissue using this method and found that the apical-basal cell morphology and the tissue integrity of *schlank* depleted cells was not disrupted (Fig. S2D).

Next we asked if Wg was the only protein accumulating as a consequence of *schlank* loss/reduction of function. We tested several other secreted and transmembrane proteins for accumulation upon *schlank* depletion. Antibody stainings against the Wg receptor Fz2 and its co-receptor Arrow, as well as Hedgehog (Hh), its receptor Patched, and the surface receptor Notch all revealed the same dot-like accumulation phenotype (Fig. 3, data not shown). The cell adhesion protein E-cadherin also accumulated but to a much lower extent (Fig. S3A). As there are no suitable antibodies for Dpp available, we expressed a GFP-tagged Dpp protein in the *dpp* expression domain. Interestingly, this did not show accumulation (Fig. S3B). The different behavior could be due to different endocytic trafficking routes of Dpp or reduced protein turnover in the case of E-cadherin.

We further examined if the signaling activity of the Hh, Notch and Dpp pathways was changed. To address this we made use of various readouts for the respective pathways including antibody stainings, stainings for *lacZ*-reporters of target genes and rtPCR analysis (Fig. S3C,D, data not shown). In contrast to the effect on Wg signaling, *schlank* depletion did not detectably change the activity of the Hh, Notch and Dpp signaling pathways.

In summary, we found that *schlank* depletion does not grossly affect the overall integrity of the wing disc epithelium. However it does cause the accumulation of a subset of secreted and transmembrane proteins. The extent of the accumulation varied, but with the exception of Wg there appeared to be no detectable changes in the activity of the signaling pathways. Therefore we decided to concentrate our attention on the Wg signaling pathway.

2.4 Wg accumulation is not due to more production and secretion

Two possible scenarios could account for the observed Wg accumulation: first, the accumulating Wg could be due to more production and secretion of the protein. Second, there could be less degradation of Wg protein. The cell autonomy of the observed phenotype already hinted to less degradation as a secretion defect would be expected to be non-autonomous. To test this in more detail, we monitored *wg*

transcription using a *lacZ*-reporter and performed extracellular Wg stainings, which constitute a very sensitive readout for Wg secretion. Neither the transcription of *wg* (Fig. 4A) nor the extracellular level of the Wg protein was changed upon *schlank* depletion (Fig. 4B,C), demonstrating that the observed accumulation is not caused by more production and secretion of the Wg protein. Furthermore we did not detect changes in the uptake of fluorescently labeled Dextran in *schlank* depleted cells, suggesting that the initial step of endocytosis is not disturbed (data not shown). Thus the Wg accumulation is probably due to impaired degradation after the protein was taken up.

Consistent with the hypothesis that upon *schlank* depletion there is a defect in endosomal trafficking we observed that the typical punctate localization of Wntless in the Golgi apparatus in Wg-producing cells was disrupted (Fig. 4D). Wg secretion is dependent on the Wntless/Evi (Wls) protein, which promotes Wg release and is afterwards recycled to the Golgi apparatus in a retromer-dependent manner (Port et al., 2008). Wls was apparently not as efficiently recycled back to the Golgi apparatus upon *schlank* depletion, whereas Wg secretion seemed unchanged. Therefore, we speculate that the amount of Wls in the Golgi apparatus is still sufficient to ensure proper Wg secretion, but cannot rule out secondary effects as, e.g., a change in the potency of the secreted ligand to induce Wg signaling (Franch-Marro et al., 2008).

2.5 Schlank depletion leads to enlarged Rab4/7/11 positive endosomal structures

To see where the endosomal trafficking is defective, we made use of various Rab GTPases as endosomal markers. Rab5 GTPase marks early endosomal structures, whereas Rab4 and Rab11 GTPases label the fast and slow recycling endosomes, respectively. Rab7 is used to label the late endosomal trafficking route leading to lysosomal degradation. Constructs consisting of the *tubulin* promoter driven Rab GTPase fused with an YFP-tag were employed and *schlank* clones were induced in this background. Whilst Rab5 positive endosomal compartments were not detectably altered by *schlank* depletion (Fig. 5A), Rab4/7/11 positive endosomes were massively enlarged (Fig. 5B,C,D). To check if other intracellular compartments were affected, we analyzed the status of Golgi apparatus and Endoplasmic Reticulum (ER) by testing the Golgi component ß-Cop and ER-resident KDEL-carrying proteins. We could not detect any differences in the antibody stainings against these proteins (Fig. S4A,B).

These experiments show that the processes of endocytosis or early endosome formation are not so sensitive to alteration in sphingolipid levels, in contrast to the more downstream intracellular trafficking steps.

2.6 All endosomal compartments exhibit increased Wg concentration

Given that we see accumulation of ligand (Wg) and receptor (Arr, Fz2) we were surprised to find that Wg signaling was reduced. Recent work on the Wg signaling transduction mechanism offers two possible explanations for this observation in the context of endocytic trafficking: first, it was shown that Wg has to enter the Rab5 positive early endosome for full strength signaling (Purvanov et al., 2010). It is possible that, due to defects in endocytic sorting and traveling, there is in absolute terms less Wg in Rab5 positive compartments, which is masked by Wg accumulation in other endosomal compartments. Alternatively, the underlying defect could be occurring in the more downstream steps of the degradative route such as impaired lysosomal acidification, which is essential for Wg signal transduction (Buechling T, 2010; Cruciat et al., 2010), or due to impaired formation of multivesicular bodies, which are reportedly necessary for GSK3ß sequestration in vertebrate systems (Taelman et al., 2010).

To test if less Wg in Rab5 early endosomes could account for the Wg signaling defect, we quantified the Wg protein in Rab5 early endosomes, as well as in Rab4 recycling and in Rab7 late endosomes. As Wg endocytosis is believed to be receptor-mediated (Dubois et al., 2001) and by staining for Frizzled receptor we would not be able to distinguish between ligand-bound endocytosed receptor and “empty” recycling receptor, we used Wg staining to detect endocytosed ligand-receptor complex. As markers of the endosomal compartments, we again used the YFP-Rab constructs, now in combination with *schlank* RNAi driven in the posterior compartment. In this setup, we first defined the endosome volumes in control and *schlank* depleted compartments in the same disc to test how much the different Rab-positive compartments are enlarged upon *schlank* depletion. Next, we quantified the amount of Wg in the mutant and the neighboring control compartment. This yielded quantitative information about how much Wg is accumulating overall in the *schlank* depleted situation. Third, we measured the amount of Wg that could be found inside a particular type of endosome. To do so, we analyzed the ratio of Wg signal in mutant compared to control endosomes and normalized for the endosomal volume.

Confirming our microscopic analysis of the respective Rab-positive endosomal compartments, we did not see an enlargement of the Rab5-marked endosomal compartments. In contrast, we observed a twofold enlargement of Rab4-positive endosomal structures and an even more pronounced enlargement of Rab7 late endosomal structures (Fig. 6A). Overall we found a twofold accumulation of Wg protein upon *schlank* depletion. Rab4- and Rab5-positive compartments showed a twofold increase in the concentration of Wg. In Rab7-positive endosomes, more than fourfold more Wg was measured upon *schlank* reduction (Fig. 6B).

These results indicate that Wg protein levels in Rab5-positive endosomes are increased, rather than decreased, upon *schlank* depletion (also when measured in absolute amounts and not corrected for the slightly decreased Rab5 endosomal volume), ruling out the notion that less Wg protein in Rab5 early endosomes is the cause for the reduced signaling activity.

Based on current models of Wnt/Wg signaling, a defect more downstream in the early-to-late endosomal pathway could result in reduced Wg signal transduction. The ligand-receptor complexes are thought to be sorted in a Hrs-dependent manner from Rab5-positive early endosome into multivesicular endosomes (Taelman et al., 2010). Therefore we analyzed the localization of Hrs and Vps16, both markers of multivesicular bodies, in our *schlank* reduced background. The localization of neither Hrs nor Vps16 was changed upon *schlank* depletion (Fig. 6C, S4C). In contrast, Lamp1-GFP and LysoTracker, markers of the lysosomal compartment were enlarged in the *schlank* depleted situation (Fig. 6D, S4D).

Taken together, the observed protein accumulation and the defects in the formation of Rab7 endosomes and lysosomes suggest that ceramide depletion impedes Wg signaling by disrupting the early-to-late endosomal route.

3. DISCUSSION

3.1 Sphingolipids and endosomal trafficking

In this study, we reduced sphingolipid levels in the *Drosophila* wing imaginal discs by genetic means to study the effects of changed ceramide availability, with a focus on the activity of signaling pathways in an epithelial context.

Not unexpected given the coordination of energy metabolism and growth with lipid metabolism, we observed reduced cell size and cell number due to apoptosis upon depletion of the ceramide synthase gene *schlank*. When we examined the *Drosophila* wing imaginal disc we found that overall tissue integrity was not affected. However, we did observe an accumulation of various secreted and transmembrane proteins. Analysis of the endocytic compartments revealed that the slow and fast recycling endocytic routes, as well as the late endosomal route, were disrupted. In contrast, exocytosis (secretion of Wg and Dpp), the initial steps of endocytosis and the formation of Rab5-positive early endosomal compartments were not affected.

Ceramide is able to induce membrane curvature in artificial membranes (Holopainen et al., 2000). Additionally, changes in membrane morphology and fusion were reported in lipids extracted from mice defective in long chain ceramide synthesis (Silva et al., 2012). Thus, via physical changes in membrane budding/fusion sphingolipid composition could influence endocytic trafficking.

Consistent with a problem in the early-to-late endosomal route leading to lysosomal protein degradation we observed the accumulation of various proteins upon ceramide synthase depletion. These included Hedgehog (Hh), its receptor Patched and the surface receptor Notch (Fig. 3). We saw no effect on the Hh or Notch signaling pathways, whereas earlier studies in *Drosophila* implicated glycosphingolipids in various steps in development and different signal pathways (Kraut, 2011; Singh et al., 2011). For example, in an impressive study Hamel and colleagues show that glycosphingolipids modulate the signaling activity of Notch ligands (Hamel et al., 2010). The fact that we do not find reduced signaling activity apart from Wg signaling suggests that sufficient glycosphingolipids are still produced, either by the remaining *de novo* synthesized ceramide or via salvage pathways. Taking this into account, our results show that the Wnt/Wg signaling pathway is particularly sensitive to the reduction in ceramide levels.

3.2 Sphingolipids and Wingless signaling

One of the many proteins that accumulated was the morphogen Wg. Although there was more Wg ligand in the system, the pathway activity was reduced upon *schlank* depletion. We showed that this effect is dependent on ceramide synthase activity and is not a secondary effect of apoptosis. Furthermore, we demonstrated that the accumulation of Wg is not due to increased expression or secretion and happens in the late endosomal route and to a lesser extent in the recycling endosomes.

The Wg signaling defect that we see could result from impaired protein sorting. Sphingolipids are, beside their implied role in membrane morphology, thought to be involved in protein sorting (Le Roy and Wrana, 2005; Lippincott-Schwartz and Phair, 2010; Simons and Ikonen, 1997). The partitioning or differential sorting of proteins into lipid microdomains is implicated in promoting the assembly of signaling complexes. In this regard, it is interesting that the Arrow homolog LRP6 is associated with membrane domains composed of cholesterol and sphingolipids (Yamamoto et al., 2006). A simple scenario is that this association is important for signaling by promoting receptor clustering. Interfering with or abolishing this clustering could be envisaged to result in reduced signaling efficiency. However, given the growing body of evidence that connects endosomal trafficking and Wnt/Wg signaling, a parsimonious explanation is that a disruption of endosomal routes is responsible for the observed effects on the Wg pathway.

The first link between Wg signaling activity and internalization was made in 2006 (Blitzer and Nusse, 2006; Seto and Bellen, 2006). Later in an elegant study by the Niehrs lab it was shown that in the mammalian system Wnts induce clustering of receptors and Dvl in endocytosed complexes called signalosomes, which facilitates pathway induction (Bilic et al., 2007). More recently a series of studies has highlighted various aspects of the relationship between Wnt signaling and endosomal trafficking: it could be shown that localization of Wg in Rab5-positive endosomal compartments is required for full-strength signal induction (Purvanov et al., 2010). Furthermore, acidification occurring along the early-to-late endosomal route was demonstrated to be important for Wnt signaling (Cruciat et al., 2010). Finally, it was reported that the GSK3ß kinase has to be sequestered into multivesicular endosomes for proper Wnt signaling induction (Taelman et al., 2010).

Given that reduced *schlank* expression leads to defects in endosomal trafficking and Wnt/Wg signaling is critically linked to endosomal transport it is tempting to suggest that reduced Wg signaling we observed upon *schlank* depletion is caused by the

impairment of endocytic trafficking. To further clarify the connection of Wnt signaling and ceramide levels, a cell-biological system has to be developed that provides the necessary resolution to dissect endocytic pathways during Wnt signaling upon ceramide depletion.

In summary, we present evidence that reducing sphingolipid levels in an epithelial context leads to the impairment of distinct endocytic routes. Furthermore, we show that the Wg signaling pathway is particularly sensitive to changes in cellular ceramide levels, and we believe this to be a consequence of defects at or below the level of early-to-late trafficking. This is the first demonstration of a connection between ceramide/sphingolipid levels and Wg signaling and highlights the importance of these lipids in regulation of signaling processes.

4. EXPERIMENTAL PROCEDURES

4.1 Drosophila strains

schlank alleles: *schlank* RNAi[1]: VDRC 33896 (III), *schlank* RNAi[2]: VDRC 109418 (II), *schlank*^{P365}: Bloomington 11985, *schlank*^{331}: imprecise excision of *schlank*^{P365}

Transgenes: *schlankHA* / *schlankH215DHA* / genomic rescue: all constructs were inserted in ZH-86Fb landing site via the ϕC31 integrase system.

Other stocks: *y w f; Sp/CyO; hhGal4/Tm6b, y w f;; act>CD2>Gal4 UAS-GFP, y w arm-lacZ FRT19 hsp-flp (/FM7a), y w f; Sp/CyO; UAS-hid/Tm6b, y w f; Sp/CyO; UAS-diap1*

In general, we analyzed male larvae for dissection in RNAi experiments and female larvae for *schlank*$^{P365/331}$ clonal analyses, as schlank is located on the X chromosome. *Schlank* RNAi clones were generated by crossing the respective *schlank* RNAi line to *ywf;; act>CD2>Gal4 UAS-GFP*. Larvae were heat shocked 48h after egg laying (AE) for 30 minutes at 37°C. *Schlank*^{331} (and analogous *schlank*^{P365}) Minute Rps5a clones were induced by heat shocking *yw schlank*^{331} *FRT19/yw ubi-GFPnls Min FRT19* female larvae 48h AE for 30 minutes at 37°C. For the *schlank*^{P365} clonal rescue experiment flies with the genotype *yw schlank*^{P365} *FRT19/ yw ubi-GFPnls Min FRT19;; schlankHA hhGal4/+* were used to induce clones in the above mentioned Minute background. Rescue of *schlank* RNAi was done in *ywf; Sp or CyO/+; schlankHA hhGal4/schlank RNAi*^{1} flies. For combining *schlank*^{P365} clones with various Rab endosomal markers, the following flies were heat shocked as described above: *yw schlank*^{P365} *FRT19/ yw arm-lacZ FRT19 hsp-flp; tubα-YFP-Rab4/5/7/11/+*. To test Dpp accumulation, *yw schlank*^{P365} *FRT19/ yw arm-lacZ FRT19 hsp-flp;; dppGal4 Gal80*^{ts} *GFPdpp/+* flies were heat shocked 48h AE (30 min, 37°C) and GFP-Dpp expression was induced by temperature shift to 29°C 96h AE. Apoptosis induction was done in *ywf; Sp/enGal4 Gal80*^{ts} *UAS-CD8-GFP; UAS-hid/MKRS* flies that were shifted to 29°C 24h AE. Rescue of apoptosis in *schlank*^{P365} clones was performed in the following genetic setup: *yw schlank*^{P365} *FRT19/ yw arm-lacZ FRT19 hsp-flp; enGal4 Gal80*^{ts} *UAS-CD8-GFP/+; UAS-diap1/+*. *Schlank*^{P365} clones were induced 48h AE and *diap1* expression by shift to 29°C 72h AE.

4.2 Cloning and transgene production

The Lag1 open-reading frame was based on amplification of the cDNA clone LD18904 (BDGP) and cloned into a pUASattB vector for C-terminal 3xHA tagging (Bischof et al 2012, under revision). For the ceramide synthase dead version H215D (Bauer et al., 2009) we performed site-directed mutagenesis (QuikChangeTM site-directed mutagenesis kit, Agilent Technologies) on a EcoRI/BamHI-subcloned fragment of the *schlankHA* construct using the primers:

fwd CTGGCAGATGTTCATCgATCACATGGTCACCCTGCTCCTAATG

rev CATTAGGAGCAGGGTGACCATGTGATCGATGAACATCTGCCAG

The mutated fragment was then swapped back in the pattB-*UASschlankHA* vector again via EcoRI/BamHI.

The genomic rescue fragment was based on BAC CH322-128M11 from the P[acman] library BACPAC Resources Center.

4.3 Generation of presumptive null allele schlank331

For the *schlank*^{331} allele, the Bloomington *schlank*^{P365} fly stock was used to generate an imprecise excision. This deleted the last 63 base pairs of the first *schlank* exon (including a predicted transmembrane domain) and fused the intronic region downstream of the P-element P^{365} insertion site to the remaining first exon.

4.4 Electron microscopy

For analysis, male larvae with the genotype *ywf; UAS-CD2-HRP/Sp or CyO; schlank RNAi*^{1}*/hhGal4* were dissected and immediately fixed with 2.5% glutaraldehyde in PBS for 15 minutes. After washing in PBS they were stained for 2 minutes using the DAB Peroxidase Substrate Kit (Vector Laboratories). Following 4 further washing steps the discs were postfixed for 1h with 1% OsO4 in PBS and block contrasted with 2% uranyl acetate in H2O. After dehydration in an alcohol series (50, 70, 96, 2x100% 15 min each) the discs were embedded in Epon and polymerized for 24h at 60°C. Thin sections were cut with a Reichert Ultracut E microtome and imaged with a Gatan Orius 1000 CCD camera in a Tecnai G2 spirit transmission electron microscope (FEI, Eindhoven, Netherlands).

4.5 Immunohistochemistry

Immunostaining of *Drosophila* wing imaginal discs and embryos was performed according to standard protocols. Primary antibodies used in this study were: mouse anti-Wg (4D4, DSHB, 1:1000), guinea pig anti-Sens (GP55, gift from H. Bellen, Baylor College of Medicine, Houston, 1:800), rabbit anti-cleaved Casp3 (#9961, Cell Signaling, 1:200), chicken anti-ßGal (ICLlab, 1:400), mouse anti-ßGal (Promega, 1:2000), mouse anti-Fz2 (12A7, DSHB, 1:20), rabbit anti-Arrow (gift from S. DiNardo, 1:15000), rabbit anti-Hh (1:500), mouse anti-Ptc (DSHB, 1:100), rabbit anti-pMad (gift from Ed Laufer, Columbia University, New York, 1:1000), rabbit anti-Wls (Port et al., 2008), (1:800), guinea pig anti-Hrs (gift from H. Bellen, Baylor College of Medicine, Houston, 1:100), rabbit anti-Vps16 (gift from H. Kramer, UT Southwestern Medical School, Dallas, 1:100), rabbit anti-GFP (1:200), rabbit anti-Ecadherin (1:100).

Pictures were taken with a Zeiss LSM710 confocal microscope and the Zen software. Images were processed using ImageJ and Photoshop Elements.

4.6 Quantification of endosomal compartments and Wg protein content

ywf; tubα-YFP-Rab4/5/7/11/Sp or CyO; schlank RNAi^{1}*/hhGal4* larvae were dissected and stained with Wg antibody. Z-stacks were taken with the confocal microscope. The images were further processed using the IMARIS software. A cubic volume was defined in control and mutant compartments and the overall Wg fluorescence was measured. To mark the endosomal compartments the Surface tool was used with automatic creation and the following settings: local Background: 0.6, Threshold: 20, enable split objects, Quality: above 10. This allowed assessment of the ratio of endosomal volumes in mutant versus control compartment of each disc analyzed (N=7 for each genotype). To quantify the amount of Wg in the respective endosomes, the fluorescence intensity of Wg signal in the previously defined endosomal compartments was measured and again set into relation of mutant to control compartment in each disc per endosome volume.

4.7 realtime PCR

Flies carrying the *schlank RNAi*^{1}, and as control *yw* flies, were crossed to the *C765Gal4* driver at 29°C. Male larvae were dissected in three independent experiments and RNA was extracted using the Nucelospin RNA II kit (Machery-

Nagel). Following an additional DNA digestion (DNA-free™ kit, Ambion) we used the Transcriptor High Fidelity cDNA synthesis kit (Roche Applied Science) for cDNA synthesis. *Tubulin*, *actin* and *TBP* expression was used for normalization of each experiment; otherwise the experiments were not normalized to show the variability between the replicates.

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AUTHOR CONTRIBUTIONS

J.P. and K.B. designed and carried out the experiments; A.K. and U.L. performed the EM analysis; G.R. conducted the genome-wide screen in which *schlank* was found as growth regulator. J.P, G.H. and K.B wrote the paper.

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FIGURE LEGENDS

Figure 1. Phenotypes upon *schlank* depletion. Overview of the *schlank* gene region including the alleles used and generated in this study (A). Adult wings showed notches and disturbed wing hair orientation upon expression of two independent RNAi lines under the control of *hh*-Gal4 in the posterior compartment (B). In third instar larval wing imaginal discs the RNAi expression with *hh*-Gal4 in the posterior compartment, marked by the expression of UAS-GFP, showed Wg protein accumulation as judged by Wg antibody staining (C) and reduced Wg signaling activity, detected by Sens antibody staining (D). Scale bars represent 50 µm.

Figure 2. Wg accumulation is indeed caused by *schlank* reduction and independent of apoptosis. (A) $schlank^{331}$ mutant clones, marked by the absence of GFP, were induced by mitotic recombination in a Minute background and also displayed accumulating Wg protein. (B) Providing back HA-tagged Schlank protein via *hh*-Gal4 in analogous generated $schlank^{P365}$ clones rescued the Wg accumulation specifically in the posterior compartment (compare arrows). (C) *En*-Gal4 driven expression of UAS-*Hid* in the GFP-positive P-compartment was induced 24h after egg-laying by temperature-shift to provoke apoptosis. Staining for Wg did not show protein accumulation. (D) Rescue of apoptosis by *en*-Gal4 mediated Diap1 expression 72h after egg-laying did not diminish Wg protein accumulation in $schlank^{P365}$ clones, which were marked by absence of ß-Gal staining. Scale bars represent 50 µm.

Figure 3. Several proteins accumulate upon reduced *schlank* expression. GFP-negative $schlank^{P365}$ clones were induced by mitotic recombination in a Minute background and stained with antibodies against Fz2 (A), Arrow (B), Hh (C) and Ptc (D). In all of these cases the proteins showed accumulation in the clones. Scale bars represent 50 µm.

Figure 4. Wg accumulation is due to less degradation instead of more production. Staining against ß-Gal of a *wg-lacZ* third instar wing imaginal disc expressing *schlank* RNAi with *hh*-Gal4 in the posterior compartment indicated unchanged *wg* transcription (A). (B) Detection of secreted Wg via antibody staining specifically for extracellular protein did not show differences upon posterior *schlank* RNAi expression. (C) Consistently, extracellular Wg staining was also not changed in

schlank^{P365} clones induced in Minute background. (D) In the same genetic setup, staining with an Wntless antibody revealed diminished Golgi localization of Wntless/Evi (Wls) upon *schlank* depletion. Scale bars represent 50 µm.

Figure 5. *Schlank* depletion results in enlarged Rab4/7/11-positive endosomal structures *Tubulin* promoter driven, YFP-tagged Rab5- (A), Rab4- (B), Rab7- (C) and Rab11-constructs (D) were combined with *schlank*^{P365} mitotic clones to monitor the individual endosomal compartments. *Schlank*^{P365} clones were marked by the absence of ß-Gal staining. Whereas Rab5-positive endosomes seemed to be unchanged, Rab4/7/11-positive endosomal structures were enlarged in *schlank*^{P365} clones. Scale bars represent 50 µm.

Figure 6. Wg accumulates preferentially in early-to-late endosomal route upon *schlank* reduction. (A) Wing imaginal discs carrying the above-mentioned YFP-Rab constructs and expressing *hh*-Gal4 driven *schlank* RNAi in the posterior compartment were employed to quantify the volumes of Rab 4/5/7-positive endosomal compartments. Using the automated Surface Creation tool on a defined cubicle volume in control and mutant compartment the size of the respective Rab endosomes was measured as ratio between mutant to control volumes in every disc. N=7 for each genotype, P-values paired t-Test: Rab4: 0.0003, Rab5: 0.01, Rab7: 0.0002. (B) To quantify the overall amount of Wg accumulation independent of its endosomal localization, we assessed the fluorescence intensity ratio of a Wg antibody staining in the cubicles in mutant versus control compartment (N=21). Following, we defined the concentration of Wg in the distinct endosomal compartments by applying the same method to the previously defined endosomal volumes and normalizing for the endosomal volume. N=7 for each genotype, P-values paired t-Test: Rab4: 0.01, Rab5: 0.00002, Rab7: 0.0001. (C) To test more components of the early-to-late endosomal route we performed antibody stainings for Hrs in discs harboring *schlank*^{P365} clones, which showed no difference. (D) In the same genetic setup, we analyzed lysosomes using the LysoTracker® reagent and found the signal to be enlarged in *schlank*^{P365} clones. Scale bars represent 50 µm.

SUPPLEMENTARY MATERIAL

Legends for Supplemental Figures

Figure S1. Phenotypes upon reduced *schlank* expression. (A) *Schlank* RNAi was expressed via the *hh*-Gal4 driver in the GFP-positive posterior compartment and stained for cleaved Caspase3. Apoptosis was elevated in the *schlank* mutant compartment compared to control. (B) *Actin*-Gal4 driven *schlank* RNAi clones (*schlank* depleted clones were in this case marked by the presence of GFP) were induced and wing imaginal discs were stained using the Sens antibody. Sens signal was reduced upon *schlank* depletion and this genetic setup was used for quantification in panel D. (C) Assessment of adult wing phenotypes upon *schlank* depletion using *hh*-Gal4 driven expression of two *schlank* RNAi lines; control *yw* crossed to *hh*-Gal4. N=45 for *yw* and *schlank* RNAi1, N=23 for *schlank* RNAi2 female escapers. (D) For quantification of Sens fluorescence intensity the clone area in the Sens domain was marked by hand using the ImageJ software and the ratio to the same area in control tissue next to the clone was calculated after background subtraction (mean from three areas outside the Sens expressing domain). N=13.

Figure S2. Wg accumulation is dependent on ceramide synthase activity and independent of apoptosis. Wg accumulation upon expression of schlank RNAi using *hh*-Gal4 (A) can be rescued by co-expressing an HA-tagged *schlank* cDNA (A') but not by co-expressing a enzymatic mutant *schlank* version (A''). As control for the induction of apoptosis UAS-*Hid* expression by *en*-Gal4 was induced 24h after egg-laying by temperature-shift and the discs were stained for cleaved Caspase3 (B). In the reverse experiment, the rescue of apoptosis in ß-Gal-negative $schlank^{P365}$ clones by *en*-Gal4 driven UAS-*diap1* expression 72h after egg-laying was confirmed by diminished cleaved Caspase3 staining in clones of the posterior compartment compared with clones in the anterior compartment (C). (D) Electron microscopic analysis was performed on discs expressing *schlank* RNAi under the control of *hh*-Gal4. To define the mutant compartment we used co-expression of a membrane-tagged UAS-*HRP*, which could be detected via DAB staining as dark membrane staining. By this, we were able to detect the compartment boundary (D) and to assess the tissue integrity in control (D') and *schlank* depleted cells (D'').

Figure S3. Not all proteins accumulate upon schlank depletion and Hh, Dpp and Notch signaling activity is not changed. (A) Staining wing imaginal discs carrying *schlank*^{P365} GFP-negative clones for Ecadherin did not show Ecadherin protein accumulation. (B) *Schlank*^{P365} clones (marked by the absence of ß-Gal) were combined with a *dpp*-Gal4 UAS-*GFP-dpp* overexpression. Staining for GFP revealed no accumulation of the GFP-tagged Dpp. (C) To monitor the Dpp signaling pathway activity we performed phosphoMad antibody stainings in discs harboring *schlank*^{P365} clones in Minute background. (D) Further, we tested Hh and Notch signaling pathway activity by realtime PCR. Discs from *C765*-Gal4 driven *schlank* RNAi animals at 29°C were dissected in three independent biological replicates and tested for transcription of *schlank* itself, *dpp*, *ptc* and *m8*.

Figure S4. Golgi apparatus and ER marker are not changed upon schlank depletion, whereas lysosomes are enlarged. *Schlank*^{P365} GFP-negative clones in a Minute background were stained for the Golgi marker ßCop (A) and the ER marker KDEL (B), both showing no change upon schlank depletion. (C) Also Vps16 as a marker for MVBs was not changed in *schlank* RNAi clones driven by *actin*-Gal4. (D) To test lysosomes we combined *hh*-Gal4 mediated *schlank* RNAi with a *tubulin* promoter driven Lamp1-GFP fusion and found this lysosomal marker enlarged in the *schlank* mutant posterior compartment.