

## Death by over-eating: The Gaucher disease associated gene *GBA1*, identified in a screen for mediators of autophagic cell death, is necessary for developmental cell death in *Drosophila* midgut

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### ABSTRACT

Autophagy is critical for homeostasis and cell survival during stress, but can also lead to cell death, a little understood process that has been shown to contribute to developmental cell death in lower model organisms, and to human cancer cell death. We recently reported<sup>1</sup> on our thorough molecular and morphologic characterization of an autophagic cell death system involving resveratrol treatment of lung carcinoma cells. To gain mechanistic insight into this death program, we performed a signalome-wide RNAi screen for genes whose functions are necessary for resveratrol-induced death. The screen identified *GBA1*, the gene encoding the lysosomal enzyme glucocerebrosidase, as an important mediator of autophagic cell death. Here we further show the physiological relevance of *GBA1* to developmental cell death in midgut regression during *Drosophila* metamorphosis. We observed a delay in midgut cell death in two independent *Gba1a* RNAi lines, indicating the critical importance of *Gba1a* for midgut development. Interestingly, loss-of-function *GBA1* mutations lead to Gaucher Disease and are a significant risk factor for Parkinson Disease, which have been associated with defective autophagy. Thus *GBA1* is a conserved element critical for maintaining proper levels of autophagy, with high levels leading to autophagic cell death.

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### Introduction

Autophagy is a catabolic process in which double-membrane vesicles, called autophagosomes, are formed *de novo* to engulf cytoplasm and organelles, eventually fusing with the lysosome to form the autolysosome, within which the internal contents are degraded.<sup>2,3</sup> Induction of autophagy is tightly regulated by the mTOR and Ulk1/2 kinases and their various modulators, which control the activation of the Vps34 phosphatidylinositol 3-kinase (PI3K). The PI3K complex, which includes Vps34, Beclin 1 and Atg14, produces PI3P at the sites of membrane nucleation, thereby recruiting the remaining Atg proteins. These proteins drive sequential ubiquitin-like conjugation reactions that ultimately produce membrane bound, lipidated MAP1B-LC3 (or LC3, orthologue of yeast *Atg8*). All of these steps are regulated at multiple levels, including post-transcriptional modifications and transcriptional mechanisms.<sup>4</sup> There are also negative feedback loops that regulate autophagosome turnover, so that autophagy flux levels are finely tuned to match the cellular need. In the normal cell, autophagy occurs at low levels in a constitutive manner to enable degradation and recycling of long-lived proteins, and removal of damaged

organelles and misfolded proteins that can form in the course of normal cell functioning.<sup>5</sup> As such, it is essential for maintaining homeostasis and preventing disease.

During cell stress, nutrient deprivation or growth factor withdrawal, autophagy is induced above basal levels by inactivation of mTOR and subsequent activation of Ulk1 and its targets within the PI3K Vps34/Beclin 1 complex.<sup>3,6</sup> The resulting enhanced autophagy flux is critical for the cell to mitigate the damage and overcome the stress until more tenable conditions are restored. This can be achieved, for example, by autophagy-mediated removal of accumulated unfolded proteins during ER stress, or by the recycling of macromolecules as a source of nutrients and molecular building blocks during starvation (for example, see ref. 7). In addition, autophagy eliminates damaged mitochondria by mitophagy, thereby 1. limiting radical oxygen species (ROS) formation and consequently, DNA damage, and also 2. directly preventing mitochondrial outer membrane permeabilization (MOMP) and the release of apoptogenic factors.<sup>8–10</sup> In these scenarios, autophagy ensures cell survival, and often blocks apoptotic cell death. When homeostasis is restored, autolysosomes are recycled, lysosomes are reformed,

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and autophagy flux readjusts to basal levels, by reactivation of mTOR and reduction in several Atg protein levels (e.g. Refs. 11,12,13). If these shut-down mechanisms fail to initiate, or if the induction steps are overly activated, autophagy flux levels can potentially reach a point where they are no longer beneficial, but instead indiscriminately consume cellular organelles and proteins until the cell can no longer function properly. Under such circumstances, autophagy would directly lead to cell death.

### Autophagic cell death

While the importance of maintaining the appropriate levels of autophagy for each cell's circumstance is obvious, it is less clear whether autophagic cell death is a physiologically relevant process. In fact, for a long time the existence of autophagic cell death was considered controversial.<sup>14,15</sup> Firstly, many studies, especially earlier ones, claimed to have observed autophagic cell death when they were in fact observing cell death that was accompanied by, but not caused by, autophagy.<sup>16</sup> In these cases, autophagy was likely induced to counter the death stimulus and block apoptosis, thereby representing a failed attempt to rescue the cell. The premature and often erroneous conclusions of these correlative studies paradoxically led many researchers to question the very premise of autophagic cell death.

As the field progressed, knock-out (KO) or knock-down (KD)-based targeted disruption of autophagy-specific genes was achieved, so that the nature of cell death could be determined not only through morphological observations, but also via analysis of the effects of blocking autophagy on cell death. However, a second complicating factor emerged, as studies showed scenarios in which autophagy was necessary for cell death, but that it occurred in conjunction with other cell death pathways, such as apoptosis or necroptosis. Moreover, autophagic processes have been shown to facilitate apoptosis or necroptosis, for example, by providing a scaffold for caspase activation or necrosome assembly,<sup>17-23</sup> or by targeting anti-apoptotic or survival proteins for selective degradation.<sup>22,24-26</sup> In these cases, autophagy was necessary for cell death but was only an indirect cause, leading researchers to doubt that autophagy can kill directly.

In light of the above studies and the questions they raised, in order to substantiate authentic autophagic cell death, researchers must not only conclusively prove that the stimulus triggers an increase in autophagic flux in the absence of alternative death pathways, but also prove that inhibition of various autophagic proteins that drive different steps in autophagy protects from cell death.<sup>15,27</sup> Scenarios wherein these criteria are best met are during developmental cell death in simpler model organisms such as the slime mold *Dictyostelium discoideum* and fruitfly *Drosophila melanogaster*. Under starvation conditions, and in response to cAMP, unicellular *Dictyostelium* aggregate into a multi-cellular organism that undergoes autophagy-dependent morphogenesis to form a spore-producing fruiting body. While not directly causing cell death, autophagy primes the cells so that when they are exposed to a second signal- the stalk Differentiation-Inducing Factor DIF-1- autophagic cell death results.<sup>28,29</sup> Similarly, removal of the larval midgut and salivary glands during *Drosophila* metamorphosis occurs by an

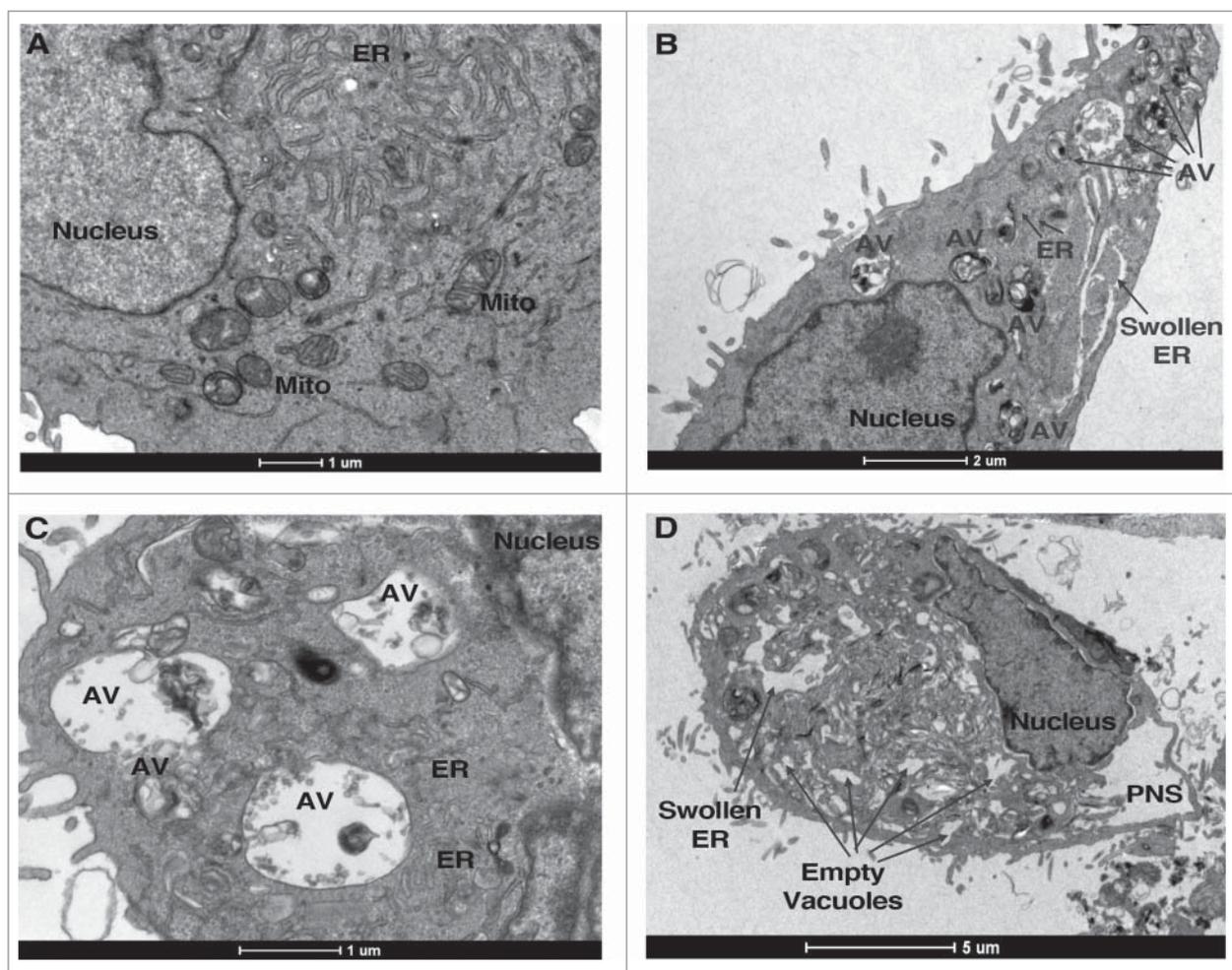
apoptosis-independent cell death that requires autophagy genes, with specific components of the autophagy pathway differentially required for each process.<sup>30-32</sup> (see also ref. 33 for review) However, complete removal of the salivary glands requires the concerted actions of both autophagy and apoptosis.<sup>30</sup>

Studies in higher mammals are more limited, since it is technically unfeasible to assess the effects of KO of multiple *ATG* genes on the death phenotype, due to early lethality and/or pathologies that develop even in tissue-specific *ATG* KOs, such as in the brain.<sup>34</sup> Yet, through the use of limited genetic manipulations and drugs that affect the pathway, autophagic cell death has been implicated in adult and neonatal animal models of cerebral hypoxia/ischemic injury,<sup>35-39</sup> and reperfusion injury of the heart.<sup>40</sup> Autophagic cell death has also been shown in cell culture models, which are more amenable to genetic manipulations. Cancer cells seem to be particularly susceptible to apoptosis-independent autophagic cell death, which was observed in response to hypoxia and oxidative stress, and to various anti-cancer drugs such as resveratrol, BH3 mimetics and betulinic acid (reviewed in ref. 41). Likewise, excessive autophagic consumption of damaged mitochondria, or mitophagy, led to melanoma cell death upon activation of the orphan nuclear receptor TR3.<sup>42</sup>

Thus, while autophagic cell death has been clearly demonstrated, the field has still not advanced due to a lack of detailed morphological analysis and limited molecular characterization of the process. We still do not understand what switches the tightly regulated autophagy pathway from functioning as a survival pathway, to becoming a lethal one. In an initial attempt to address this issue, an inhibitor screen was performed to identify modulators of a specific form of autophagic cell death, termed autosis, which was induced by a cell-permeable peptide activating Beclin 1.<sup>43</sup> Significantly, this death pathway was confirmed in pathophysiological settings, such as starvation in cell culture, hypoxia-ischemia in rat brain, and anoxia-nervosa in human patients.<sup>43,44</sup> The study showed that autosis was independent of apoptosis or necroptosis, and while it required *ATG* genes and autophagosome formation, autophagy flux was actually stalled at the degradative stage. Autosis was blocked by an inhibitor of the Na<sup>+</sup>/K<sup>+</sup>-ATPase ion channel, suggesting that cell death was a secondary response to stalled autophagy flux by a mechanism involving changes in ion transport and/or cellular osmolarity.<sup>43</sup> This study was an encouraging step in the direction of understanding an autophagy-dependent cell death, albeit an alternative form that did not involve autophagy flux.

### Defining a model system of autophagic cell death induced by resveratrol

We recently attempted to define a system of true autophagic cell death that fulfilled the strict criteria stated above in order to characterize the phenomenon at the morphological level, and to identify molecular regulators of the process.<sup>1</sup> After detailed analysis of the death phenotype, we chose resveratrol (RSV) treatment of A549 lung carcinoma cells as the model system for further study. In these cells, RSV induced a time- and concentration-dependent cell death. None of the characteristic features of apoptosis or necroptosis were observed, and neither apoptosis nor necroptosis inhibitors rescued cell



**Figure 1.** Resveratrol induces excessive autophagy in A549 cells. A. Transmission EM of control A549 cells. B-D. Transmission EM of A549 cells treated with 200  $\mu$ M RSV for 48 h. AV, autophagic vacuoles, mito, mitochondria, PNS, perinuclear space.

viability. In contrast, RSV induced abundant autophagosome accumulation with time, which we showed to represent active autophagy flux. Autophagic flux continued to increase for as long as 48 h, when cell death was most prominent. This feature clearly distinguishes the RSV-induced process from survival autophagy, such as that induced by starvation, which is transient: autophagy decreases to basal rates as the cell recovers. During RSV treatment, in contrast, the number of autophagosomes and autolysosomes continued to increase in time, to the point where they comprised most of the cytoplasmic area, and the cell was conspicuously devoid of organelles, with pronounced alterations in intracellular membranes (e.g. Fig. 1). Thus autophagy was hyperactivated in this system. Most significantly, knock-down of several different *ATG* genes attenuated both autophagy and cell death, indicating that RSV-induced death requires autophagy, i.e., it is a *bona fide* form of autophagic cell death.

#### Identifying *GBA1* as a positive mediator of autophagic cell death

Using this system, we then conducted a signalome-wide shRNA screen for genes whose KD conferred a survival advantage to RSV-induced autophagic cell death.<sup>1</sup> The screen utilized

Collecta's Lentiviral shRNA Library Module 1, which targets more than 5000 signaling-related genes with multiple shRNAs/gene. RNA-seq was used to identify shRNAs that were enriched in surviving clones after prolonged exposure to RSV. Positive hits were genes that showed a statistically significant enrichment of greater than 50% for at least 3 independent shRNAs. These were further prioritized based on a sum of ranked standardization scores algorithm. The resulting set of genes was then validated by a siRNA-based secondary viability screen, finally yielding 8 candidate genes whose KD enhanced cell viability during RSV treatment by more than 50%.

One of the top hits on this short list was *GBA1*, the gene mutated in Gaucher Disease (GD). *GBA1* encodes the lysosomal enzyme glucocerebrosidase, or acid  $\beta$ -glucosidase (GCase), which metabolizes glucosylceramide (GlcCer) to ceramide and glucose, comprising an important salvage pathway for ceramide generation. In fact, our data indicated that GCase protein and activity levels were increased upon RSV treatment in A549 cells at 48 h, concomitantly with enhanced autophagy.<sup>1</sup> This was accompanied by increased production of long-acyl chain ceramide species, and the sphingolipid derivatives sphingosine and sphingosine-1-phosphate. The increases in GCase levels correlated with the decreased viability that was observed in RSV-treated A549

cells. *GBA1* KD attenuated GCCase activity and ceramide production, and consistent with the screen results, rescued autophagic cell death. The number of cytoplasmic autophagic vesicles normally observed at late time-points of RSV treatment was reduced upon GCCase KD, and at the ultrastructural level, *GBA1* KD improved overall cell morphology, including that of the mitochondria, ER and Golgi. Interestingly, GCCase was not elevated during starvation-induced autophagy in A549 cells, suggesting that it is not a general regulator of autophagy, but specifically plays a role in scenarios wherein autophagy leads to cell death.

Altogether, our data clearly indicated a role for GCCase and ceramide production in autophagic cell death in our model system. Notably, the connection between ceramide and autophagic cell death was also suggested by a recent study in which treatment of a cell culture model of myeloid-derived suppressor cells with an inhibitor of acid ceramidase led to enhanced C16-ceramide production, autophagosome accumulation, and ultimately, apoptosis- and necroptosis-independent cell death.<sup>45</sup> Intriguingly, enhanced *GBA1* expression levels and enzymatic activity have been reported in placenta from pregnancies complicated by preeclampsia, a disorder involving hypoxia, oxidative stress, and trophoblast cell death, which jeopardizes both mother and fetus.<sup>46</sup> Moreover, levels of sphingolipids and various ceramide species were observed to be elevated in preeclampsia placenta and proven to induce autophagy in trophoblasts.<sup>47</sup> This latter study attributed ceramide production to *de novo* synthesis and reduced lysosomal hydrolase activity, but did not consider the contribution of *GBA1*. While both apoptosis (e.g. ref. 48) and necroptosis<sup>49</sup> have been observed in preeclampsia placenta, there have also been reports of increased LC3-II and autophagosome formation in both placenta from preeclampsia patients, and in cultured hypoxic trophoblasts.<sup>50</sup> More comprehensive studies are needed to determine if this represents enhanced active autophagy flux, and whether its function is to promote cell survival or death. Considering our data, it would be very interesting to determine if autophagic cell death occurs during preeclampsia, and whether it is *GBA1*-dependent.

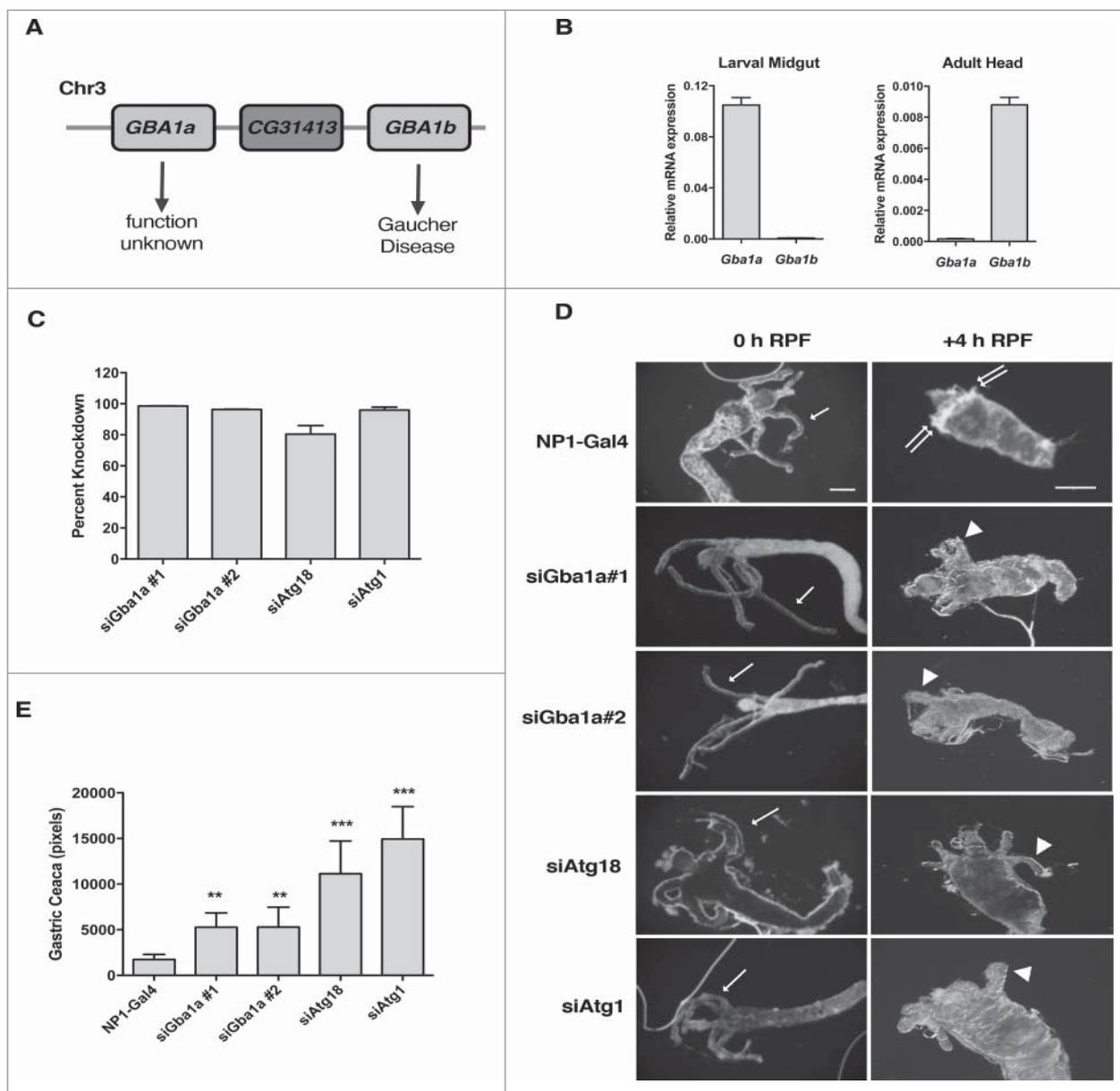
### Proving the physiological relevance of *GBA1* to developmental cell death in midgut regression of *Drosophila* larva

We wished to conclusively establish *GBA1*'s function in a validated physiological scenario of autophagy and autophagic cell death. To this end, we examined the contribution of *GBA1* to larval midgut cell death during *Drosophila* developmental metamorphosis, a critical period during which most of the larval tissues that were produced during embryogenesis are destroyed, and adult body structures are formed. While most of the cell death associated with *Drosophila* metamorphosis is apoptotic, destruction of the larval midgut requires autophagy and is caspase-independent.<sup>31</sup> The human *GBA1* gene has two orthologues in *Drosophila melanogaster*, referred to as *Gba1a* (CG31148) and *Gba1b* (CG31414), respectively. The two *Gba1* orthologues reside on chromosome 3, interposed by the CG31413 gene (Fig. 2A). Expression data from the online

resources modENCODE<sup>51</sup> and FlyAtlas<sup>52</sup> showed that *Gba1a* is almost exclusively expressed in larval and adult midguts, whereas *Gba1b* is highly expressed throughout the developmental and adult stages in a wide variety of tissues and organs. *Gba1b* has been associated with fly models of GD.<sup>53,54</sup> We confirmed the expression data by quantitative real-time PCR analysis of the *Gba1* orthologues: *Gba1a* was expressed in larval midgut at high levels in comparison to *Gba1b*, while conversely, *Gba1b* showed high expression in adult head, where *Gba1a* was barely detectable (Fig. 2B). Thus *Gba1a* is likely to be the relevant orthologue for studying autophagic cell death of the larval midgut. RNA interference (RNAi) was used to knock-down expression of *Gba1a*, and as positive controls, the autophagy genes *Atg1* and *Atg18*, specifically in the midgut, using the midgut driver NP1-Gal4 (Myo31DF-Gal4)<sup>55</sup> (see Materials and Methods for further detail). Nearly full KD efficiencies of the RNAi lines were demonstrated by quantitative real-time PCR analysis (Fig. 2C). Larval midgut cell death is initiated at the end of the third instar larval stage and the beginning of white pupa formation, referred to as 0 h relative puparium formation (RPF). The midgut undergoes a drastic regression from 0 h RPF to +4 h RPF, which can be closely followed by observing the elongated gastric caeca present at the junction of the foregut and midgut, just below the bud-like proventriculus (Fig. 2D, arrows in left-hand panels). The gastric caeca present at 0 h RPF begin to regress and are removed by +4 h RPF (Fig. 2D, see top panel, right), whereas the remaining midgut tissues gradually regress over the next 12–24 h (not shown). As previously reported,<sup>31</sup> midgut-specific KD of the autophagy genes *Atg1* and *Atg18* resulted in a pronounced delay in midgut regression, evidenced by persistent gastric caeca at +4 h RPF, when compared to the control NP1-Gal4 line (Fig. 2D, E). Importantly, morphological analysis of midguts at +4 h RPF in two independent *Gba1a* RNAi lines revealed a significant delay in midgut cell death, with only partial regression of the gastric caeca (Fig. 2D, E). These data indicate that similar to autophagy gene KD, *Gba1a* KD had a significant inhibitory effect on midgut regression during the larval to early pupal transition, albeit not as strong as that of the central regulators of the process. This data imply a physiological role for *Gba1a* in developmental autophagic cell death.

### *GBA1* dysfunction in human disease

In humans, homozygous loss-of-function *GBA1* mutations lead to GD, a lysosomal storage disease in which GlcCer accumulates as a result of the deficiency in GCCase and the subsequent inability of the lysosome to hydrolyze GlcCer to ceramide.<sup>56</sup> GD is currently treated by a combination of enzyme replacement therapy with recombinant GCCase and substrate reduction therapy. More recently, *GBA1* mutations have also been found to be an increased risk factor for Parkinson Disease (PD), and have been associated with accumulation of  $\alpha$ -synuclein aggregates as a result of defective autophagy-lysosomal function; the level of functional GCCase produced from the mutated gene correlates with severity of disease/disease risk.<sup>57</sup> Significantly, KO of *GBA1* (i.e. *Gba1b*) in *Drosophila* brains led to GlcCer accumulation and

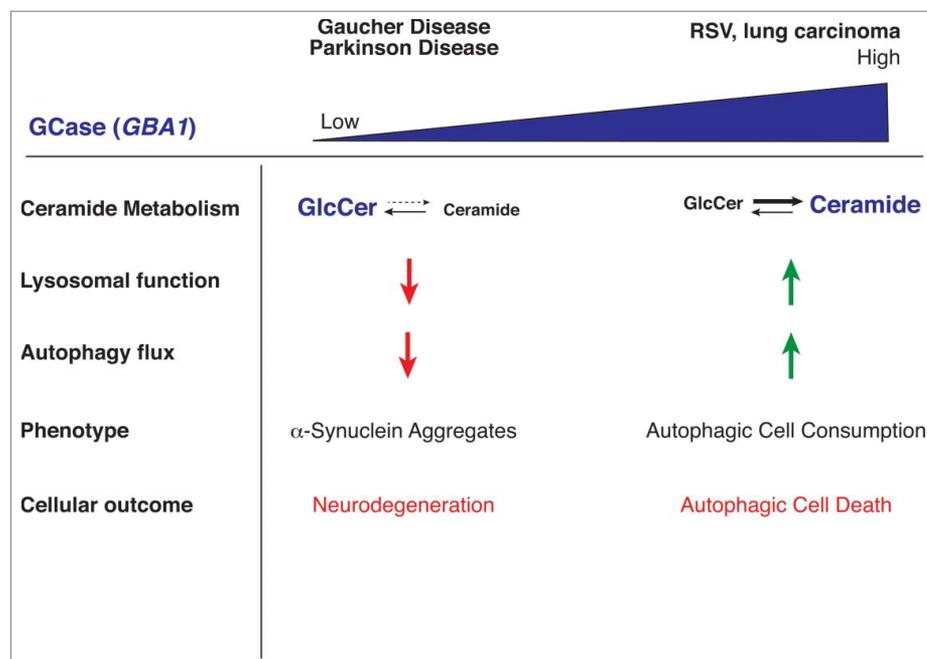


**Figure 2.** *Gba1a* is necessary for midgut regression in *Drosophila* larva. (A) Schematic representation of the *Gba1a* and *Gba1b* gene loci on *Drosophila* chromosome 3. (B) Expression levels of *Gba1a* and *Gba1b* in larval midgut and adult head were determined by quantitative real-time PCR. Assays were performed in triplicate and normalized to *Rpl32/rp49* as an internal control. (C) Percent knockdown of *Gba1a*, *Atg1* and *Atg18* in the late third instar larvae (−4 h to −1 h RPF). The indicated siRNA constructs were expressed in larvae via the midgut driver NP1-Gal4. Transcript levels were measured by quantitative RT-PCR, normalized to the internal control *Rpl32/rp49*, and are presented as relative KD of expression in comparison to expression in the NP1-Gal4 line. (D) Morphology of midguts at 0 h RPF and +4 h RPF comparing control (NP1-Gal4) pupae with pupae from NP1-Gal4>UAS-*Gba1a* RNAi #1 (siGba1a#1), NP1-Gal4>UAS-*Gba1a* RNAi #2 (siGba1a#2), NP1-Gal4>UAS-*Atg1* RNAi (siAtg1) and NP1-Gal4>UAS-*Atg18* RNAi (siAtg18) crosses. Arrows indicate gastric caeca at 0 h; double arrows, normal regression of gastric caeca at 4 h; arrowheads, persistent gastric caeca in KD larvae. Scale bar represents 200  $\mu$ m. (E) Quantitation of gastric caeca size at +4 h RPF. Data represents mean  $\pm$  SD of more than 10 midguts per genotype; statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

lysosomal pathology similar to GD, and neurodegeneration and synaptic loss, similar to PD.<sup>53,54</sup> Furthermore, an increase in autophagosome number and a block in autophagy flux were observed, consistent with defects at the autolysosome stage. These observations demonstrate a strong conservation of *Gba1* functional roles in the fly. Autophagy defects and accumulation of  $\alpha$ -synuclein inclusions were also observed in other models of inhibition of GCase by drugs, and KD or KO of *GBA1* in mice, MEFs and cell cultures<sup>58–61</sup> (also reviewed in ref. 62). Thus, GCase is critical for functional autophagy and prevention of neurodegeneration.

### Conclusion and perspective

The studies described herein demonstrate the critical role that *GBA1* plays in the regulation of autophagy, and that both loss-of-function and gain-of-function are detrimental to the balance of autophagy within the cell (Fig. 3). Too little GCase activity leads to the inability of the cellular autophagy pathway to deal with aggregating  $\alpha$ -synuclein, with dire consequences that lead to neuropathy. Too much GCase converts a beneficial pathway into a lethal one, whereby the cell literally consumes itself to death. The question still remains how changes in sphingolipid metabolism and/or



**Figure 3.** Model showing effects of different expression levels of *GBA1*. Loss of *GBA1* expression, such as that which results from mutations associated with Gaucher Disease or Parkinson Disease, leads to GlcCer accumulation, lysosomal dysfunction, defects in autophagy, and eventual accumulation of  $\alpha$ -synuclein inclusions, resulting in neurodegeneration. Enhanced *GBA1* expression, such as that observed following RSV treatment, leads to elevations in ceramide and its sphingolipid metabolites, induction of autophagy and autophagic cell death.

increased ceramide production directly cause autophagic cell death. It may have to do with differences in membrane properties and curvature that support enhanced autophagosome formation and maturation, or lysosomal recycling. Future studies that address these issues will not only further enhance our understanding of autophagic cell death, but may also be applicable to *GBA1*-related pathologies such as GD and PD.

## Materials and methods

### *Drosophila* genetics

All fly lines were maintained on cornmeal, molasses and yeast medium at 25°C. RNAi-based knockdown of specific genes was achieved by crossing midgut driver (NP1-Gal4) flies (*Drosophila* Genetic Resource Center, Kyoto (DGRC #112001)) to flies bearing UAS-RNAi lines (obtained either from the Vienna *Drosophila* Resource Center (VDRC) or the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University). UAS-RNAi lines used in the study include *Gba1a*#1 (BDSC #39064), *Gba1a*#2 (VDRC #GD14697), *Atg18* (BDSC #28061) and *Atg1* (BDSC #26731). All flies were maintained and crossed at 25°C. Larva were fed with 0.05% bromophenol blue containing food and staged by observing the midgut retention or clearing of bromophenol blue dye. Wandering clear gut larva were transferred onto a damp Whatmann paper placed in a petri dish and dissected as previously described.<sup>63</sup>

### Gene expression analysis

RNA was extracted from 30 whole heads from 5 day old adult flies and 20–30 midguts from late third instar larvae of the indicated genotypes, using TRIzol (Life

Technologies). cDNA was generated using High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time PCR based quantitation of *Gba1a*, *Gba1b*, *Atg1*, *Atg18a* and *RpL32/rp49* mRNA expression was performed using primers published elsewhere.<sup>32,54</sup> Quantitative real-time PCR was performed using FAST SYBR Green Master Mix (Applied Biosystems). Assays were performed in triplicates, normalized to *RpL32/rp49*, and analyzed according to the comparative Ct method.<sup>64</sup>

### Midgut morphology and quantitation

Animals were staged and collected at –4 h RPF (crawling 3<sup>rd</sup> instar larvae with everted spiracles), 0 h RPF (white prepupae) or +4 h RPF (white prepupae aged for 4 h). Midguts were dissected in PBS, fixed in 4% paraformaldehyde in PBS, mounted onto glass slides using IMMU-MOUNT (Thermo Scientific) and imaged using a stereozoom microscope (Olympus). Digital images were obtained using a DP50 CCD camera with Studio-Lite software (Olympus). Measurements of gastric caeca size were performed using ImageJ software for more than 10 midguts per genotype. Gastric caeca edges were traced and pixel densities were determined using the histogram tool.

### Statistical analysis

The statistical significance of differences between means was assessed by one-way ANOVA followed by Tukey's post hoc test. Values with  $P < 0.05$  were considered significant.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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