Cyclin-G-associated kinase GAK/Aux orchestrates glial autophagy via Atg9 phosphorylation in Parkinson's disease

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Abstract

Glia serve as double-edged swords to modulate neuropathology in Parkinson's disease (PD), but how they react opposingly to be beneficial or detrimental under pathological conditions, like promote or eliminate α -Synuclein inclusions, remain elusive. Here we present evidence that the PD risk factor Cyclin G-associated kinase (GAK)/dAuxilin (Aux) is a new component in glial autophagy. Lack of GAK/Aux promotes autophagic induction, disrupts lysosome acidification, and blocks glial clearance of substrates. Aux regulates Atg9 trafficking to autophagosomes and lysosomes for autophagosome biogenesis and autolysosome maturation, respectively, via Atg9 phosphorylation at newly identified residues T62 and T69. GAK/Aux-mediated glial autophagy is required for α -Syn degradation and contributes to a broad spectrum of parkinsonian symptoms in *Drosophila* and mice. Our findings indicate that glial autophagy is a critical component in PD progression and identify a new autophagy factor functioning in a tissue-specific manner; targeting glial autophagy is potentially a therapeutic solution for PD.

Keywords: Autophagy; Glia; Parkinson's disease; *GAK*; *dauxilin*

Introduction

63 Aberrant protein trafficking, often associated with autophagy dysfunction, is one of the
64 major mechanisms underlying neurodegenerative diseases such as Parkinson's disease
65 (PD) (Menzies et al., 2017; Nixon, 2013). Three types of autophagy: microautophagy,
66 macroautophagy, and chaperone-mediated autophagy (CMA), have been described and
67 all lead to the final delivery of cargo to the lysosome for degradation and recycling. As
68 the primary catabolic mechanism, macroautophagy (henceforth referred to as
69 autophagy) was initially characterized as a bulk and non-selective degradation pathway
70 induced by nutrient deprivation. Constitutive autophagy at the basal level, however,
71 contributes to intracellular homeostasis in non-starved cells by degrading cellular
72 materials like damaged organelles or misfolded proteins (Galluzzi et al., 2017;
73 Mizushima, 2020). Interestingly, suppression of basal autophagy in the mouse nervous
74 system results in the accumulation of ubiquitinated proteins and neurodegeneration
75 (Hara et al., 2006; Komatsu et al., 2006), whereas enhancing basal autophagy in the
76 *Drosophila* nervous system promotes longevity and oxidant resistance (Simonsen et al.,
77 2008). Furthermore, disease-associated mutations in core autophagy genes have been
78 identified, supporting the link between autophagy and PD (Deng et al., 2017; Levine
79 and Kroemer, 2019). These findings mainly focus on a role for autophagy in neurons,
80 as perturbation of such process leads to catastrophic consequences including
81 pathological protein aggregation, deleterious lesions, and cell death (Kulkarni et al.,
82 2018). Intriguingly, autophagy function in the other brain cell type, glia, is less explored.
83 Whether glial autophagy is executed via similar interplay using the same components

as in neurons or other tissues, and whether it drives or contributes to neurodegeneration in PD, remain largely unclear.

Glia serve as double-edged swords in PD via non-cell-autonomous mechanisms. For instance, microglia secrete pro- or anti-inflammatory factors to enhance or ameliorate PD progression (Gleichman and Carmichael, 2020; Ho, 2019; Subhramanyam et al., 2019). Whereas α -Synuclein (α -Syn) accumulates in neurons, evident α -Syn inclusions have been detected in microglia, astrocytes, and oligodendrocytes due to prion-like propagation from neurons to glia (Dickson, 2012; Jellinger, 2018; Peng et al., 2020; Sorrentino et al., 2019; Tanriover et al., 2020; Valdinocci et al., 2017). Neuronally secreted α -Syn also impacts on these glial cells for their reactivity and signaling to neurons (Cheng et al., 2018; Filippini et al., 2019). These findings clearly demonstrate a pivotal role for glia in PD. Nonetheless, how the α -Syn inclusions occur and persist in glia, and whether the regulatory mechanisms of its elimination depends on glial autophagy, remain elusive.

Studies from yeast and mammals provided fundamental insights into *de novo* autophagosome biogenesis mediated by the evolutionarily conserved autophagy-related (ATG) genes (Galluzzi et al., 2017; Klionsky et al., 2016; Mizushima, 2020). Upon autophagy initiation, stress induces the recruitment of ATG1/UNC-51 like kinase 1 (ULK1) complex to the phagophore assembly site or the pre-autophagosomal structure (PAS). The ATG1/ULK1 complex facilitates the assembly of class III phosphatidylinositol 3-kinase (PI3KC3) complex for PI3P production at the PAS, to

promote the nucleation of the isolated membranes (also termed phagophores) (Russell et al., 2013). Whereas phagophore expansion is achieved by two ubiquitin-like conjugation steps: the formation of ATG12-ATG5 conjugate and ATG8 lipidation, additional lipids and proteins contributing to membrane expansion are delivered by the ATG9-containing vesicles derived from the Golgi apparatus, recycling endosomes or the plasma membrane (Mari et al., 2010; Orsi et al., 2012; Puri et al., 2014; Ravikumar et al., 2010; Yamamoto et al., 2012). The expanded phagophores are sealed into a double-layered autophagosome, which eventually fuses with lysosome to form autolysosome, the milieu for degrading autophagic cargoes by acidic hydrolases. Among all steps, very little is known about how the only transmembrane ATG protein ATG9 contributes to autophagic induction and whether it plays additional roles in autophagy. It is also not clear whether ATG9 functions in glial autophagy and contributes to neurodegeneration.

Given these outstanding questions, we investigated the mechanism of glial autophagy in PD. Here we present evidence that the PD risk factor Cyclin G-associated kinase (GAK)/dAuxilin (Aux) functions in glial autophagy via Atg9 phosphorylation at two newly identified threonine residues T62 and T69. In the absence of Aux, enhanced Atg9 phosphorylation promotes its trafficking to autophagosomes and lysosomes to increase autophagosome biogenesis and block autolysosome maturation, respectively. Aux-mediated autophagic mechanism contributes to PD as lacking Aux causes a broad spectrum of parkinsonian symptoms and blocks glial clearance of pathological

substrates including α -Syn. Consistently, lack of GAK induces microglial autophagy, blocks α -Syn clearance, and leads to PD-like behaviors in mice. Taken together, our findings identify a new player in glial autophagy and demonstrate the critical requirement of glial autophagy in PD.

Results

***GAK/aux* is expressed in glia**

Identified in a behavior screen, the *Drosophila* homolog of the PD risk factor GAK, Aux, has been implicated in a broad spectrum of parkinsonian symptoms in a *Drosophila* PD model (Song et al., 2017). GAK/Aux is a serine/threonine kinase that regulates clathrin-uncoating, cell cycle progression, and hepatitis C entry (He et al., 2020; Lee et al., 2006; Shimizu et al., 2009; Zhang et al., 2005). To investigate GAK/Aux function in glia, GFP-labelled adult glia from flies expressing *UAS-mCD8.GFP* under the control of the pan-glial driver *repo-GAL4* (*repo>mCD8.GFP*) were first sorted by fluorescence-activated cell sorting (FACS) analysis. These sorted glia exhibited high *repo* (a glial-specific marker) and low *elav* (a neuronal-specific marker) expression, demonstrating their glial origin (Figures 1A and S1A). Interestingly, *aux* was expressed in these sorted glia, with an expression level similar to the non-GFP cells (Figure 1A). Consistent with the previously reported expression in microglia and astrocytes (Zhang et al., 2014; Zhang et al., 2016), mammalian *GAK* expression was also detected in the mouse immortalized microglia (IMG) cells stained positive for

ionized calcium-binding adapter molecule 1 (Iba1) or transmembrane protein 119 (TMEM119, Figures S1B, S1C, and S1G) (McCarthy et al., 2016). Treatment with either of the two siRNAs targeting *GAK* reduced *GAK* expression in IMG cells as shown by western blot (WB), RT-PCR, and immunostaining analyses (Figures S1D-S1H). *GAK* mRNA and protein expression were also reduced in mouse microglia deficient of *GAK* (*CX3CRI-Cre*^{+/-}; *GAK*^{fllox/fllox}) in the substantia nigra (SN, 36 weeks, Figures S1I-S1K). Taken together, these results indicate that *GAK/aux* is expressed in adult fly glia and mouse microglia.

Aux regulates autophagosome formation in glia

To investigate Aux function in glial autophagy, transgenic RNAi lines targeting *aux* (V16182 and BL39017) were expressed in glia using *repo-GAL4*. RNAi expression efficiently reduced *aux* expression as validated by RT-PCR, WB, and immunostaining analyses (Figures S1L-S1O). Specific markers were used to label different autophagic and endocytic organelles including Lamp1.GFP (lysosomes), mCherry.Atg8a (autophagosomes), Rab7.GFP (late endosomes), and Rab5.GFP (early endosomes) (Figure 1B). The morphology, distribution, and number of these endocytic and autophagic organelles in glia were analyzed in different brain regions including the region “Glia” enriched of glial processes, some glial nuclei, and most of the neuronal nuclei, and the region “Antenna Lobe, AL” enriched of axon neuropils in close proximity to glial processes (Figures 1C and 1D). Interestingly, downregulating *aux*

expression by *aux*-RNAi (V16182) caused a striking increase in the number of glial Atg8a-positive autophagosomes (Figures 1E and 1F). These results, confirmed by one additional *aux*-RNAi (*aux*-RNAi^{#2}, BL39017), an endogenous protein trap *pmCh.Atg8a*, anti-Atg8a antibodies, and *aux* mutant Mosaic Analysis with a Repressible Cell Marker (MARCM) clones (Figures 1G, 1K, S2A, S2C, and S2E-S2H), indicate an increase in glial autophagosome formation in the absence of glial Aux. Ultrastructural analysis by Transmission Electron Microscopy (TEM) also showed an increase in the autophagosome number upon *aux*-RNAi expression in glia (Figures 1H and 1L). Furthermore, the intensities of microtubule-associated protein 1A/1B-light chain 3 (LC3) labeling autophagosomes and LC3-Iba1 colocalization increased in mouse microglia deficient of *GAK*, indicating similar autophagosome defects (Figures 1I, 1M, and 1N). Enlarged autophagosomes were also detected in *aux* mutant MARCM clones, IMG cells treated with *GAK* siRNA, and mouse microglia deficient of *GAK* (Figures 1G, 1J, 1K, 1M, and 1O), further supporting the role of GAK/Aux in autophagosome biogenesis. Notably, expression of *aux* or *hGAK* in glia suppressed the *aux*-RNAi-induced increase in the autophagosome number (Figures 1P and 1Q). Taken together, these results indicate that Aux regulates glial autophagosome formation and the function of GAK/Aux is conserved across species.

Lack of Aux causes lysosome aggregation in glia

In addition to autophagosomes, the number, size, and AL fluorescent intensities of glial

Lamp1-positive lysosomes were increased in flies expressing *aux*-RNAi (Figures 2A-2B, S2B, and S2D). Ultrastructural TEM analysis also showed an increase in the number and size of lysosomes upon *aux*-RNAi expression in glia (Figures 2C and 2D), further supporting our immunostaining results. Interestingly, glial Lamp1 fluorescent signals exhibited dramatic increase and displayed as patches pronouncedly within the AL near the neuropils (AL in Figures 2A and S2B), suggesting lysosome aggregation. Similar lysosome aggregations were observed in IMG cells treated with the *GAK* siRNA and mouse microglia deficient of *GAK* (Figures 2E-2H). *In-vivo* time lapse analysis further demonstrated a reduction in the lysosome mobility, potentially leading to aggregation (Figure S2I). Moreover, whereas Rab7-positive late endosomes also aggregated (Figure S2J), the formation of the Rab5-positive early endosomes remained largely unaffected (Figure S2K). Taken together, these results indicate that GAK/Aux is required for glial autophagosome and lysosome formation; GAK/Aux is implicated in glial autophagy, particularly at the steps of autophagosome biogenesis and terminal lysosomal dynamics.

Lack of Aux induces glial autophagy

Based on an increase in the autophagosome number, it is conceivable that lack of glial Aux induces or blocks autophagy. Interestingly, Atg8a/LC3 protein levels of samples collected from adult fly heads or mouse IMG cells downregulating *aux* or *GAK*, respectively, revealed an increase in the Atg8a-II/LC3-II protein levels, which further

increased in the presence of Bafilomycin A1 (BafA1), an inhibitor blocking lysosome acidification and autophagosome-lysosome fusion (Mauvezin et al., 2015; Yoshimori et al., 1991) (Figures 3A-3B and S3A). The levels of phosphorylated S6K (pS6K), a downstream target of mTOR, also increased (Figures S3B and S3C). These results suggest that lack of glial Aux induces autophagy. To further assess how the initiation of glial autophagy is affected, phagophore nucleation and omegasome formation were monitored using GFP markers labeling Atg6, Atg14, and ZFYVE. The number of GFP.Atg6-, GFP.Atg14-, and GFP.ZFYVE-positive puncta were increased upon glial *aux*-RNAi expression, indicating increased formation of autophagosome precursors (Figures 3C-3F, S3D, and S3E). The size of GFP.ZFYVE-positive omegasomes were significantly smaller, suggesting that these newly formed omegasomes might be immature (Figures 3E and 3F). Taken together, these results indicate that autophagy is induced in the absence of glial Aux; Aux suppresses glial autophagy.

Glial Aux does not affect autophagosome-lysosome fusion

To further demonstrate a role for Aux in glial autophagy, autophagic flux was analyzed using a dual reporter *UAS-GFP-mCherry-Atg8a*. Whereas mCherry-positive puncta were GFP-negative and autophagy proceeds normally in the control animals, autophagic flux was impaired in the absence of glial Aux as the number of mCherry- and GFP-positive puncta increased (Figures 3G and 3J). These puncta were abnormally enlarged and exhibited non-quenched GFP signals, representing dysfunctional

autolysosomes that have already been fused but wrongly acidified. Consistently, colocalization studies and Stimulated Emission Depletion (STED) imaging of autophagosomes and lysosomes in adult fly glia expressing *aux*-RNAi revealed an increased number of enlarged and well-colocalized autolysosomes, indicating that abnormal autolysosomes are generated upon complete fusion (Figures 3H, 3I, 3K, and 3L). Ultrastructural TEM analysis also indicated an increase in the autolysosome number upon glial *aux*-RNAi expression (Figures 3M and 3N). Taken together, these results indicate that lacking glial Aux does not impair autophagosome-lysosome fusion, as increasing number of autolysosomes are detected.

To further demonstrate that fusion is not affected, RNAi lines targeting fusion factors such as *Syx17* or *vamp7* were expressed in the absence of glial Aux. Consistent with previous findings, reducing *Syx17* or *vamp7* expression caused a block in fusion (Hegedus et al., 2013), and co-expression of either RNAi reverted the *aux*-RNAi-induced increase in Lamp1-Atg8a colocalization (Figures S3F-S3H). These results suggest that autophagosomes and lysosomes fuse normally yet generate enlarged autolysosomes in the absence of glial Aux; glial Aux suppresses autophagy while autophagosome-lysosome fusion proceeds normally.

Lack of Aux blocks autophagic substrate degradation by disrupting lysosome acidification

Despite a role in autophagic induction, inhibiting *aux* expression in glia caused

accumulation of autophagic substrates such as Ref(2)P, the *Drosophila* ortholog of P62, and Ubiquitin (Ubi, Figures 4A-4D). P62 and Ubi were also accumulated in IMG cells with downregulated *GAK* expression (Figures 4E-4H). These results suggest that substrate degradation is blocked in these dysfunctional autolysosomes generated upon glial GAK/Aux depletion.

Given that the block in substrate degradation is likely due to an unfavorable environment in the enlarged and dysfunctional autolysosomes, LysoTracker dye was used to analyze the acidification of labelled organelles in adult fly brains lacking glial Aux. Interestingly, the LysoTracker intensities were significantly reduced in flies or IMG cells downregulating glial *aux* or *GAK*, respectively, indicating that these organelles are not properly acidified (Figures 4I-4J and S3I-S3J). Furthermore, the activity of the lysosomal hydrolase cathepsin B (cathB) stained by Magic Red was significantly lowered upon glial Aux depletion (Figures 4K and 4L). The processing of the other lysosomal hydrolase cathepsin D (cathD) was also affected in the absence of glial Aux, as the ratio of mature over total cathD was reduced (Figures 4M and 4N). Taken together, these findings suggest that lack of Aux induces autophagy yet impairs substrate degradation owing to insufficient lysosome acidification. Aux likely plays dual roles in glial autophagy.

Glial Aux regulates Atg9 phosphorylation

To determine the mechanism of Aux in regulating glial autophagy, transgenic flies

expressing truncated Aux lacking each domain were created and validated by WB analysis (Figures 5A and 5B). In contrast to *aux*-RNAi, overexpression of *aux* or its truncated variants caused a decrease in the glial autophagosome number, indicating a suppression effect on glial autophagy (Figures 5C and 5D). Expressing Aux lacking the kinase domain (Aux^{ΔKinase}), however, caused significantly lower suppression on the autophagosome number when comparing to Aux or other Aux variants, indicating that Aux lacking the kinase domain is a less potent regulator in glial autophagy and the kinase domain is important for Aux-mediated autophagic function.

Interestingly, phosphorylomic analysis on adult head samples collected from control and *repo>aux*-RNAi flies revealed significant differences in the phosphorylation profiles of autophagy-related proteins (Figures 5E and S4A). Particularly, Atg9 (the fly homolog of mammalian ATG9) phosphorylation is significantly upregulated at the threonine residues T62 and T69 upon glial Aux depletion (Figure 5F). Probing the relationship between Aux and Atg9, *atg9* expression in glia was first detected in sorted fly glia in a relatively high level compared to non-glial cells (Figure 5G). Moreover, Atg9 colocalized with Aux in adult fly glia whereas mouse ATG9 colocalized with GAK in IMG cells (Figures 5H and 5I). Both *atg9* mRNA and protein levels were upregulated upon *aux*-RNAi expression in glia (Figures 5J-5L and S4B). These results demonstrate that Atg9 is a downstream target of glial Aux.

Glial Aux regulates autophagic induction via Atg9 phosphorylation

Next, we determine if Aux-mediated glial autophagic induction depends on Atg9 and its phosphorylation. Whereas inhibiting *atg9* expression in glia caused a decrease in the number of omegasomes and autophagosomes (Figures 6A-6B and S4C-S4D), co-expression of *atg9*-RNAi suppressed the *aux*-RNAi-induced increase in the glial autophagosome number (Figures S4C and S4D). These results indicate that Atg9 functions downstream of Aux in regulating glial autophagosome biogenesis and autophagic induction. Similarly as *aux*-RNAi, the expression of Atg9 or phosphomimetic Atg9 mutants with the two threonine residues mutated to glutamate (Atg9^{T62E}, Atg9^{T69E}, or double mutant Atg9^{T62E-T69E}) increased the number of omegasomes and autophagosomes, while the expression of non-phosphorylatable Atg9 mutants with the two threonine residues mutated to alanine (Atg9^{T62A}, Atg9^{T69A}, or double mutant Atg9^{T62A-T69A}) inhibited omegasome and autophagosome formation (Figures 6C-6D and S4E-S4F). These results suggest that Atg9 phosphorylation at T62 and T69 is required for autophagosome biogenesis and autophagic induction in glia. Notably, co-expression of either of the three non-phosphorylatable Atg9 mutants suppressed the *aux*-RNAi-induced increase in omegasome and autophagosome formation (Figures 6E and S4G), demonstrating that glial Aux regulates autophagosome biogenesis and autophagic induction via Atg9 phosphorylation.

Glial Aux regulates Atg9 trafficking to autophagosomes in a phosphorylation-

dependent manner

To further dissect how glial Aux regulates autophagic induction via Atg9, Atg9 trafficking to autophagosomes was analyzed. Reducing *aux* expression in glia increased the colocalization of Atg9 and Atg8a-positive autophagosomes, suggesting that lack of Aux promotes Atg9 trafficking to autophagosomes (Figures 6F and 6G). Given that Atg9 phosphorylation on T62 and T629 is enhanced in the absence of glial Aux, phosphomimetic Atg9 mutants were tested for their effects on trafficking. Similarly, expressing either of the three phosphomimetic Atg9 mutants in glia increased the Atg9 colocalization with autophagosomes, indicating that Atg9 phosphorylation promotes its trafficking (Figure 6H). Co-expression of either of the three non-phosphorylatable Atg9 mutants also suppressed the *aux*-RNAi-induced increase in Atg9-Atg8a colocalization (Figure 6I), further demonstrating a role for Atg9 phosphorylation in Aux-mediated Atg9 trafficking, followed by autophagic induction. Taken together, these results indicate that Atg9 acts downstream of Aux in regulating the induction of glial autophagy; Aux promotes Atg9 trafficking in a phosphorylation-dependent manner.

Glial Aux regulates autolysosome maturation via Atg9 phosphorylation

In addition to autophagosomes, a portion of Atg9 was found to be transported to lysosomes upon glial Aux depletion, indicating that Atg9 might also play a role in Aux-mediated lysosome acidification (Figure 6J). Interestingly, inhibiting *atg9* expression in glia caused a decrease in the intensities of LysoTracker-positive puncta (Figures 6K

and 6L). In contrast to *aux*-RNAi, expression of Atg9 or either of the three non-phosphorylatable Atg9 mutants enhanced the LysoTracker intensities (Figures 6M and 6N). Co-expression of either of the three Atg9 mutants also restored the *aux*-RNAi-induced reduction in the LysoTracker intensities (Figure 6O). Altogether, these results indicate that Atg9 acts downstream of Aux to mediate lysosome acidification; Atg9 phosphorylation is critical in establishing a proper pH gradient in lysosomes for autolysosomal degradation.

Glia Aux contributes to PD-like symptoms

To investigate whether Aux-mediated glial autophagy contributes to PD, a broad spectrum of PD-like behaviors were analyzed upon glial Aux depletion. Interestingly, lacking glial Aux caused significant loss of PPM1/2 DA neurons, disrupted locomotor function, and shorten fly lifespan in an age-dependent manner (Figures 7A-7E). Some of the earlier PD-associated symptoms such as sleep defects were also detected upon glial Aux depletion (Figures S5A and S5B). Similar DA neuron loss and locomotor defects, in addition to body weight loss and impaired movement in the pole test, were also seen in mice lacking microglial *GAK* (Figures 7F-7I and S5C). Furthermore, using a genetic binary system, human α -Syn was selectively expressed in fly neurons while abolishing *aux* expression in glia. Not only that total α -Syn protein levels increased upon glial Aux depletion (Figures 7L and 7M), α -Syn proteins were also detected in sorted fly glia, despite its neuronal origin (Figure S5D). These results indicate that α -

Syn transmits from neurons to glia and accumulates in the absence of glial Aux. Whereas mouse microglia lacking *GAK* exhibited significantly shorter processes in a potentially activated state (Figures 7J and 7N), α -Syn levels were also elevated in these *GAK*-deficient microglia (Figures 7K and 7N). In addition, accumulated α -Syn exhibited increased colocalization with Lamp1 or LC3 in microglia deficient of *GAK*, suggesting that α -Syn degradation by autophagy in these microglia is blocked (Figures 5E-5I). Taken together, GAK/Aux-mediated glial autophagy is pathologically relevant and targets α -Syn for degradation during PD progression.

Discussion

Glial autophagy vs. neuronal autophagy

Neuronal autophagy has been considered as one of the major mechanisms underlying PD. More is known about how autophagosomes are formed *de novo* at the axon terminals to traffic back to soma where lysosomes are abundant, and how autolysosomes are generated to degrade unwanted organelles or proteins once efficiently fused (Cheng et al., 2015; Maday et al., 2012). This mechanism is useful under the pathological condition when target proteins start to accumulate. Dysfunctional neuronal autophagy, thus, aggravates pathological consequences. Although autophagy components and mechanisms might be universal among all tissues, it remains unclear how autophagy in glia is operated. Our findings indicate that most of the *atg* genes are expressed in glia in similar or potentially higher levels compared to

neurons (Figure 6A). Silencing their expression selectively in glia, leading to the block of glial autophagy, results in locomotor abnormalities and DA neurodegeneration, suggesting that *atg* gene expression in glia might act in similar means as in neurons and are equally important for PD-like behaviors and DA neuron survival (Figures 6B-6D). In addition, a recent study also uncovers dysfunctional glial autophagy linking to abnormal α -Syn degradation in microglial *Atg7* knockout mice (Choi et al., 2020). Findings in the present study further demonstrate the importance of glial autophagy, yet it remains unclear how neuronal and glial autophagy differ and how glial autophagy contributes to neurodegeneration in PD. It is noteworthy to mention that the overall morphology or distribution of lysosomes and autophagosomes remains largely intact upon reducing *aux* expression pan-neuronally (Figure S7). These results suggest that Aux functions separately in neurons vs. glia, implicating differential requirement of glial autophagy vs. neuronal autophagy in PD.

GAK/Aux is a new player in glial autophagy

We identify GAK/Aux as a new component in glial autophagy. Aux regulates glial autophagy via Atg9 phosphorylation, hence the trafficking of membrane sources to orchestrate autophagosome biogenesis and autophagic induction. On the other hand, Aux-mediated Atg9 activity also regulates autolysosome maturation by mediating lysosomal acidification to develop a properly acidic environment for degradation. These combined activities ultimately coordinate autophagy function in glia. In addition

to its role in autophagic induction, Atg9 has also been reported to function in intraluminal vesicle formation and compartmentalized acidification within amphisomes and autolysosomes (Bader et al., 2015). As the only integral membrane protein in the core autophagy machineries, Atg9 might regulate amphisome/autolysosome activity by providing membrane sources to form the intraluminal vesicles required for proper lysosome acidification similarly as providing membrane sources to PAS for autophagosome biogenesis. Intriguingly, our findings pinpoint a role of Atg9 phosphorylation in these processes. Whereas Aux plays a role in regulating Atg9 phosphorylation, enhanced Atg9 phosphorylation upon glial Aux depletion promotes its transport to autophagosomes or lysosomes to regulate autophagosome biogenesis or autolysosome maturation, respectively.

Atg9 phosphorylation at different residues has been reported in promoting its anterograde trafficking to initiate autophagy (Feng et al., 2016; Zhou et al., 2017). Mammalian Src and ULK1 kinases phosphorylate mATG9 N-terminus at Y8 and S14, respectively, to enhance mATG9 interaction with the adaptor protein AP1 complex, promoting mATG9 trafficking from the plasma membrane to initiate autophagy (Zhou et al., 2017). Yeast Atg1 also phosphorylates Atg9 C-terminus to recruit Atg18 to the PAS for autophagosome membrane expansion (Papinski et al., 2014). Interestingly, our results show that Atg9 phosphorylation at the N-terminus T62 or T69 promotes its colocalization with Atg8a and omegasome formation, indicating a role in autophagic induction. Once phosphorylated, similar mechanisms might be employed to enhance

Atg9 interaction with AP1/2 complex or Atg18 to promote autophagy.

Given that Atg1-mediated Atg9 phosphorylation is crucial for Atg9 trafficking and autophagic induction, we tested whether Aux interacts with Atg1. Co-immunoprecipitation analysis indicated that Aux physically interacts with Atg1, suggesting that Aux might act via Atg1 to regulate Atg9 phosphorylation at T62 and T69 (Figure S8). It has been shown that mTOR senses the nutrient-rich condition and blocks autophagic induction by phosphorylating mammalian ULK1 at S757 to prevent its activation (Kim et al., 2011). Similarly, GAK/Aux might also act as a sensor, but to sense pathological signals, to prevent Atg1 activation and autophagic induction by modulating Atg1-mediated Atg9 phosphorylation.

On the other hand, it is also possible that GAK/Aux mediates glial autophagy via its interaction with the AP1/2 adaptor complexes in clathrin-mediated trafficking. GAK/Aux might phosphorylate AP1/2 to enhance its interaction with Atg9, thereby promote Atg9 trafficking and autophagic initiation, whereas Atg9 phosphorylation might be a secondary consequence upon glial Aux depletion. Nonetheless, our findings indicate that earlier Rab5-mediated endocytic traffics are not affected by glial Aux depletion (Figure S2K). Aux lacking the clathrin-uncoating activity also does not affect glial autophagy (Figures 5C and 5D). Furthermore, general inhibition of clathrin-mediated internalization decreases autophagosome biogenesis (Puri et al., 2013; Ravikumar et al., 2010). Based on these findings, it is unlikely that Aux regulates glial autophagy via its uncoating function, as lack of Aux induces rather than suppresses

glial autophagy.

Aux-mediated glial autophagy functions in α -Syn clearance

Unlike neurons, glia are not polarized, despite the terminal processes are often in close proximity to axon neuropils. The intimate interaction between glia and axons suggest a possible function for glial autophagy in mediating degradation of substances transmitted from neurons. For instance, autolysosomes at the terminal glial processes are readily available for degrading neuronal α -Syn, whereas axonal autophagosomes have to travel back to soma to generate autolysosome for degradation. Given the relative positions, it seems likely that glial autophagy is a more energy efficient route compared to neuronal autophagy when degrading substrates under pathological conditions. Despite the benefits of inducing glial autophagy, lack of GAK/Aux also disrupts lysosome acidification and blocks substrate degradation. Owing to its dual roles, the attempt to initiate the glial clearance program ultimately failed, leading to α -Syn accumulation, a block in further transmission, and neurodegeneration in PD. Considering that prominent inclusions of α -Syn have been detected in astrocytes, microglia, and oligodendrocytes (Dickson, 2012; Tanriover et al., 2020), identified GAK SNPs might cause reduced *GAK* expression in PD, then critically controls the onset of glial autophagy to allow α -Syn either propagates from cell to cell in a prion-like fashion, locally accumulates due to a failure in autophagic substrate degradation, or to be eliminated by the induction of glial autophagy.

462

463 **Aux-mediated glial autophagy contributes to PD**

464 As most of the hypotheses that underlie PD pathology are often driven by a neuronal
 465 origin of dysfunctional machineries or cellular defects, glia have only recently been
 466 purposed as possible drivers for neurodegeneration (Gleichman and Carmichael, 2020).
 467 For instance, the Alzheimer's disease (AD) risk factor gene *Apolipoprotein E (APOE)*
 468 is predominantly expressed in astrocytes (Xu et al., 2006) and its genetic mutations are
 469 associated with an increasing risk of developing AD, supporting a role for genetic
 470 causes from glia driving neurodegeneration. Here we add to the list a PD risk factor of
 471 glial origin in modulating PD pathology. Although the relationship between GAK
 472 deficiency and PD is still uncertain, lacking glial GAK/Aux leads to a broad spectrum
 473 of parkinsonian symptoms in both *Drosophila* and mice. These include dysfunctional
 474 locomotor activity, DA neurodegeneration, and some of the earlier symptoms such as
 475 sleep defects. As an identified Aux downstream target, glial Atg9 depletion also causes
 476 similar defects in autophagy and PD-like behaviors. Considering the role of glia as a
 477 double-edged sword in PD, the seemingly contradictory roles of GAK/Aux in
 478 autophagosome biogenesis and autolysosome maturation might independently
 479 dominate at different period of time, leading to differential glial responses that
 480 aggravate or ameliorate the disease progression. These findings provide new insights
 481 on PD pathology from a glial perspective and elucidate the mechanism of glial
 482 autophagy. Future work will be required to investigate how GAK/Aux plays different

roles in glia and neurons, and how it contributes to neurodegeneration in a tissue-specific manner.

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504

505 **Author contributions**

506 S.Z, L.W, S.Y, and M.S.H conceived and designed the study. S.Z, L.W, S.Y, S.L, H.W,
507 L.S, J.O, M.Z, M.W, Y.Z, R.W, K.Y, T.L, W.Y, and M.S.H performed the experiments.
508 S.Z, L.W, S.Y, S.L, C.L, and M.S.H analyzed the data. M.S.H wrote the paper with the
509 input of S.Z, L.W, S.Y, and S.L. All authors read and approved the manuscript.

510

511 **Declaration of Interests**

512 The authors declare no competing interests.

513

514 **Figure Legends**

515 **Figure 1. Aux regulates autophagosome formation in glia** (A) Gene expression was
516 analyzed in GFP-positive adult glia sorted by FACS analysis from flies expressing
517 *UAS-mCD8.GFP* under the control of *repo-GAL4* (*repo-GAL4>UAS-mCD8.GFP*).
518 Note that GFP-sorted glia exhibit high *repo* and low *elav* expression. *aux* is expressed
519 in both neurons and glia. (B) Schematic diagram on endocytic and autophagic
520 trafficking. Markers used to label different organelles include: Rab5.GFP for early
521 endosomes, Rab7.GFP for late endosomes, Lamp1.GFP for lysosomes, and
522 mCherry.Atg8a for autophagosomes. (C and D) Illustration (C) and representative
523 images (D) of an adult fly brain with the olfactory associative center mushroom body
524 (MB), antenna lobe (AL), and the gustatory subganglion (SOG). Dashed squares in

yellow and blue indicate the regions “Glia” enriched with GFP- and Repo-positive glia (green and magenta) and “AL” enriched with axon neuropils. Neuronal nuclei are Elav-positive (red). **(E and F)** Representative images (E) and quantifications (F) of glial autophagosomes in the Whole brain, Glia, and AL. Autophagosomes (red) are labeled with *repo-GAL4* driven *UAS-mCherry.Atg8a*. Note that downregulating glial *aux* induces an increase in the autophagosome number in both regions Glia and AL. **(G and K)** Glial MARCM clones of *aux* mutants (*aux*^{L78H}, *aux*^{G257E}, and *aux*^{I670K}) expressing *UAS-mCh.Atg8a* by *repo-GAL4*. Note that the number and size of glial autophagosomes per clone increase in *aux* mutants. **(H and L)** Representative TEM images (H) and quantifications (L) of autophagosomes in the control and *repo>aux-RNAi* adult fly brains. Note an increase in the number of autophagosomes (pink asterisks) when *aux-RNAi* is expressed in glia. **(I,M,N)** Representative images (I) and quantifications (M and N) of LC3-positive autophagosomes in mouse substantia nigra (SN) microglia deficient of *GAK* (*CX3CR1-Cre*^{+/+}; *GAK*^{flox/flox}) at 36 weeks. Note the intensities and size of LC3-positive autophagosomes and the LC3-Iba1 colocalization increase upon microglial *GAK* depletion. **(J and O)** Representative images (J) and quantifications (O) of LC3-positive autophagosomes in IMG cells treated with the *GAK* siRNA. Note that LC3-positive autophagosomes are enlarged upon abolishing *GAK* expression. **(P and Q)** Co-expression of fly *aux* or human *GAK* (*hGAK*) suppresses the *aux-RNAi*-induced increase in glial autophagosome number. Scale bars and the numbers of brains quantified are indicated in the Figures. For TEM, more than 40 images from at least 5

independent fly brains of each genotype were analyzed. Data are shown as mean $\pm$ SEM. P-values of significance (indicated with asterisks, ns no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are calculated by two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test. Approximately 30 confocal Z-stack sections were taken with 0.4 μm each, and representative single layer images acquired at the similar plane of brains across all genotypes are shown. Pearson's R values for colocalization are calculated based on methods described. Quantification figures from the same image but with different parameters quantified are shown under the same label. All genotypes analyzed are included in Key Resources Table. All data in subsequent Figures are present and analyzed as described in Figure 1 unless stated otherwise.

Figure 2. Lack of glial Aux induces lysosome aggregation (A and B) Representative images (A) and quantifications (B) of glial lysosomes in the Whole brain, Glia, and AL. Lysosomes (green) are labeled with *repo-GAL4* driven *UAS-Lamp1.GFP*. Note that downregulating glial *aux* induces an increase in the lysosome number and size in Glia region and lysosome fluorescent intensities in AL. (C and D) Representative TEM images (D) and quantifications (C) of lysosomes in the control and *repo > aux-RNAi* adult fly brains. Note an increase in the number and size of lysosomes (yellow arrows) with the presence of large lysosome-like structures (red arrows) when *aux-RNAi* is expressed in glia. (E and F) Representative images (E) and quantifications (F) of

Lamp1-positive lysosomes in IMG cells treated with the *GAK* siRNA. Note that Lamp1-positive lysosome intensities increase upon abolishing *GAK* expression. (**G** and **H**) Representative images (G) and quantifications (H) of Lamp1-positive lysosomes in mouse SN microglia deficient of *GAK* (*CX3CR1-Cre^{+/-}; GAK^{flox/flox}*) at 36 weeks. Note the Lamp1-positive intensities and the Lamp1-Iba1 colocalization increase upon microglial *GAK* depletion. Scale bars and the numbers of brains quantified are indicated in the Figures. Data are shown as mean $\pm$ SEM. P-values of significance (indicated with asterisks, ns no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are calculated by two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 3. Lack of glial Aux induces autophagy but does not impair autophagosome-lysosome fusion (**A** and **B**) Representative WB images (A) and quantifications (B) of Atg8a-I and II protein levels in the control and *repo>aux-RNAi* adult fly brains. Note that lack of glial Aux increases both Atg8a-I and II protein levels, which further increases in the presence of BafA1. More than three independent replicates are analyzed. (**C** to **F**) Representative images (C and E) and quantifications (D and F) of GFP-positive puncta labeling phagophore nucleation (*repo>UAS-GFP.Atg6*) and omegasome formation (*repo>UAS-GFP.ZFYVE*) in adult fly glia with the indicated genotypes. Note that glial Aux depletion induces phagophore nucleation and omegasome formation in glia. (**G** and **J**) Autophagic flux is impaired as examined by expressing the flux reporter *UAS-GFP-mCh-Atg8a* in adult fly glia. Note that GFP-

and mCh-positive autolysosomes are enlarged and potentially dysfunctional as shown by the non-quenched GFP signals. **(H,I,K,L)** Representative confocal/STED images (H and I) and quantifications (K and L) of Lamp1- and Atg8a-positive puncta in adult fly brains with the indicated genotypes. Note that Aux depletion in glia increases the number and size of glial autolysosomes and the colocalization of autophagosomes and lysosomes. **(M and N)** Representative TEM images (M) and quantifications (N) of autolysosomes in the control and *repo>aux*-RNAi adult fly brains. Note an increase in the number of autolysosomes (blue arrows) when *aux*-RNAi is expressed in glia. Scale bars and the numbers of brains quantified are indicated in the Figures. Data are shown as mean $\pm$ SEM. P-values of significance (indicated with asterisks, ns no significance, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$) are calculated by two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 4. Lack of glial Aux blocks autophagic substrate degradation by disrupting lysosome acidification (A-D) Autophagic substrates such as Ref(2)P and Ubiquitin (Ubi) accumulate in *repo>aux*-RNAi adult fly brains as revealed by immunostaining and WB analyses. **(E-H)** WB analyses reveal an increase in P62 (E and F) and Ubi (G and H) protein levels upon GAK depletion in IMG cells. **(I-L)** The number and fluorescent intensities of LysoTracker Green- (I and J) or Magic Red-positive (K and L) puncta decrease upon glial Aux depletion. **(M and N)** The processing of the lysosomal protease cathD is analyzed by WB analysis. Note that lacking glial Aux alters cathD

processing and the ratio of mature over total cathD levels decreases. More than three independent replicates are analyzed for WB analysis. Scale bars and the numbers of brains quantified are indicated in the Figures. Data are shown as mean $\pm$ SEM. P-values of significance (indicated with asterisks, ns no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are calculated by two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 5. Atg9 is a downstream target of glial Aux (A and B) Schematic diagram of Aux protein domains and expression of different Aux protein variants by WB analysis. (C and D) Representative images (C) and quantifications (D) of glial autophagosome number in adult fly brains expressing Aux variants lacking different domains. Note that autophagosome number is reduced when expressing different variants, with Aux lacking the kinase domain exhibit a less potent effect. (E) Phosphorylomic analysis of adult fly brains from control and *repo>aux-RNAi* animals. Note that phosphorylation of various Atg proteins is greatly altered upon glial Aux depletion. (F) Atg9 phosphorylation on two threonine residues T62 and T69 is enhanced by glial Aux depletion. (G) *atg9* is expressed in GFP-positive adult fly glia sorted by FACS analysis. (H and I) Mouse ATG9 colocalizes with GAK while the fly Atg9 colocalizes with Aux in IMG cells and adult fly brains, respectively. *EGFP-Atg9* transgenic flies were used for colocalization analysis. (J-L) Both *atg9* mRNA (L) and protein levels (J and K) increase in adult fly brains upon glial Aux depletion. *atg9* mRNA levels were analyzed

in glia sorted by FACS analysis. More than three independent replicates are analyzed for WB analysis. Scale bars and the numbers of brains quantified are indicated in the Figures. Data are shown as mean $\pm$ SEM. P-values of significance (indicated with asterisks, ns no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are calculated by two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 6. Aux regulates autophagic induction and autolysosome maturation via Atg9 phosphorylation (A-E) Representative images (A and C) and quantifications (B,D,E) of glial GFP.ZFYVE-positive omegasomes in brains with the indicated genotypes. Note that the expression of *atg9*-RNAi or the non-phosphorylatable Atg9^{T62A} or Atg9^{T62A-T69A} mutants reduces while the expression of Atg9 or the phosphomimetic Atg9^{T69E} or Atg9^{T62E-T69E} increases the glial omegasome number. Co-expression of either of the three non-phosphorylatable Atg9 mutants suppresses the *aux*-RNAi-induced increase in the glial omegasome number (E). **(F-I)** Representative images (F) and quantifications (G-I) of Atg9-Atg8a colocalization in adult fly glia with the indicated genotypes. Note that the expression of *aux*-RNAi or either of the three phosphomimetic Atg9 mutants promotes Atg9 trafficking to autophagosomes. Co-expression of either of the three non-phosphorylatable Atg9 mutants suppresses the *aux*-RNAi-induced increase in Atg9-Atg8a colocalization. **(J)** The colocalization of Atg9- and Lamp1-positive lysosomes is also increased upon glial Aux depletion. **(K-O)**

Representative images (K and M) and quantifications (L,N,O) of Lysotracker intensities and puncta number in adult fly brains with the indicated genotypes. Note that the expression of *atg9*-RNAi or *aux*-RNAi decreases the intensities and number of Lysotracker-positive puncta, while the expression of either of the three non-phosphorylatable Atg9 mutants results in the opposite. Expressing either of the three non-phosphorylatable Atg9 mutants restores the *aux*-RNAi-induced reduction in the Lysotracker intensities. All transgenic flies expressing Atg9 or its variants were made with a N-terminal 3XHA tag for protein detection. Scale bars and the numbers of brains quantified are indicated in the Figures. Data are shown as mean $\pm$ SEM. P-values of significance (indicated with asterisks, ns no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are calculated by two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 7. Glial GAK/Aux contributes to PD-like symptoms (A) Illustration of an adult fly brain with different DA neuron clusters. The selected areas with PPM1/2 clusters are enclosed by the red dashes, enlarged below the illustration, and visualized in B. (B and C) Representative images (B) and quantifications (C) of DA neuron number in the adult fly brains with the indicated genotypes. Note that DA neuron number in the PPM1/2 cluster decreases in an age-dependent manner. (D and E) Fly locomotor activity and lifespan are disrupted upon glial Aux depletion. (F and G) DA neuron number in SN decreases in mice deficient of microglial *GAK* at 36 weeks. (H

and **I**) Footprint analysis and pole test of mice deficient of microglial *GAK*. Note that the locomotor activity of these mice is apparently disrupted starting at 21 weeks. (**J,K,N**) Representative images (**N**) and quantifications (**J** and **K**) of α -Syn intensities in Iba1-positive microglia deficient of *GAK* at 21 weeks. Note that *GAK*-deficient microglia exhibit significantly shorter processes, amoeboid-like structures, and increased α -Syn intensities. (**L** and **M**) Representative images (**L**) and quantifications (**M**) of α -Syn intensities (green) in adult fly brains expressing *aux*-RNAi (glia) and 6XMyC-*SNCA* (neurons). Note that α -Syn intensities significantly increases upon glial Aux depletion. (**O**) A model illustrating Aux functions in glial autophagy. Aux regulates autophagic induction, autophagosome biogenesis, and autolysosome maturation via Atg9 phosphorylation and trafficking. In the absence of glial Aux, Atg9 phosphorylation at T62 and T69 is enhanced, promoting its trafficking to autophagosomes and lysosomes, for function in the early and late stages of glial autophagy. Illustrations in blue represent conditions under glial Aux depletion. Scale bars and the numbers of brains quantified are indicated in the Figures. Data are shown as mean $\pm$ SEM. P-values of significance (indicated with asterisks, ns no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are calculated by two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

Materials and Methods

Drosophila genetics

Flies were raised under standard conditions at 25°C with 70% humidity. All fly crosses were carried out at 25°C with standard laboratory conditions unless noted otherwise. All strains were obtained from Bloomington *Drosophila* Stock Center (BDSC), the Vienna *Drosophila* RNAi Center (VDRC), Tsinghua Fly Center, or as gifts from colleagues. MARCM clonal analyses were carried out using flies with the following genotypes: *hs-FLP; UAS-mCherry.Atg8a* or *UAS-Lamp1.GFP; FRT^{82B}tub-GAL80^{ts}/FRT^{82B}aux*. Flies were heat-shocked at 37°C for 30 minutes 4-5 days after cross, then recovered at 25°C. 3- or 10-day-old adult fly brains were dissected. Detailed fly genotypes for all experiments are listed in Key Resources Table.

Mouse genetics

The B6;129S6-*Gak^{tm2Legr}*/Mmjax mice (Cat. #36793-JAX, The Jackson laboratory, Bar Harbor, ME, USA) possess loxP sites flanking exon 1 of *cyclin G associated kinase* (*GAK*) gene. *CX3CR1-Cre* transgenic mice were kindly provided by Jiawei Zhou (Institute of Neuroscience, Chinese Academy of Sciences). *CX3CR1-Cre^{+/-}* littermates were used as controls. Mice were housed in the animal facility with maximum of five mice per cage under a 12 hours light/dark cycle at 22 ± 2°C. Food and water were available *ad libitum*. The use and care of animals was approved and directed by the Animal Care and Use Committee of National Center for Protein Science Shanghai.

Cell line and transfections

IMG cell line was a gift from Chih-Hao Lee (McCarthy et al., 2016). Cells were cultured in Dulbecco's modified Eagle medium (Cat. #11965092, Gibco, New York, NY, USA) with high glucose (4.5 g/L), 10 % fetal bovine serum (Cat. #10099141, Gibco) and 100 units/mL penicillin-streptomycin (Cat. #15140-122, Gibco) at 37°C with 5% CO₂ humidified atmosphere. IMG cells were transfected using Lipo 2000 (Cat. #11668019, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, cells were seeded on a 12-well plate overnight. 40 pmol siRNA (GenePharma) and/or 2 μ l Lipo 2000 was diluted with 100 μ l Opti-MEM (Cat. #31985070, Gibco), mixed, and incubated at room temperature for 20 minutes, then added dropwise to cells cultured at 37°C with 5% CO₂. After 6 hours, cells were changed into growth medium and harvested after 72 hours.

Molecular biology

Plasmid cloning: Plasmids were constructed by polymerase chain reaction (PCR) using DNA templates from *Drosophila aux*, *atg9*, human *GAK*, and human α -Syn. These plasmids express: Aux, Aux truncated variants including Aux ^{Δ Kinase} (aa124-948), Aux ^{Δ PTEN} (aa1717-aa2121), Aux ^{Δ CBD} (aa2542-3174), Aux ^{Δ J} (aa3217-3441); Atg9, EGFP-Atg9, Atg9 mutants including Atg9^{T62A}, Atg9^{T69A}, Atg9^{T62A-T69A}, Atg9^{T62E}, Atg9^{T69E}, Atg9^{T62E-T69E}, human *GAK*; human α -Syn. The corresponding DNA sequences were subcloned into *pUAST-attB* vector containing a 3XFlag or a 3XHA epitope tag, *pJFRC19-13XLexAop2-attB* vector (Cat. #26224, Addgene, Watertown,

MA, USA) containing a 6XMyC epitope tag by Genscript (Nanjing, China). Fly microinjection was conducted by the *Drosophila* Core Facility, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

Primers used for amplification:

aux-F: CGCGGAATTCGGCGAGTTCTTTAAGTCGCTCAACCTCAAC

aux-R: CGCGCTCGAGTTACGCATTAAACATATTTTGCTGCGTGGC

GAK-F: CGGCCGCGAATTCATCGATAGATCTGATATCAACAAGTTTG

GAK-R:

GAAAGCTGGGTCGAATTCGCCCTTTCAGAAGAGGGGCCGGGAGCCCTG

SNCA-F: CCGGGCCTGGGATGTATTCATGAAAGGACTTTCAAAGG

SNCA-R: ACAAAGATCCTCTAGATTAGGCTTCAGG

qRT-PCR: Total RNAs were extracted from adult fly heads or IMG cells using

TransZol Up (Cat. #ET111-01, TransGen, Beijing, China). cDNAs were reversely

transcribed using HiScript III RT SuperMix (Cat. #R323-01, Vazyme, Nanjing, China).

qRT-PCR reactions were performed using ChamQ Universal SYBR qPCR Master Mix

(Cat. #Q711-02, Vazyme) and ABI 7500 RT-PCR system. The mRNA levels were

normalized to *rp49* (flies) or *Actin* (IMG cells), respectively. The expression levels

were analyzed by $\Delta\Delta$ CT method.

Primers used are listed below:

Flies:

atg1, *atg5*, *atg6*, *atg7*, *atg8a* as described previously (Yan et al., 2019).

757 *rp49*-F: CCACCAGTCGGATCGATATGC

758 *rp49*-R: CTCTTGAGAACGCAGGCGACC

759 *aux*-F: CAGCGGCTACCACCAATCCTTC

760 *aux*-R: TCGGTCGGCGTCCTGATAGA

761 *repo*-F: AAGTAGCAACAGCAGCCCAG

762 *repo*-R: CTTTGGCCACTGCGACACTT

763 *elav*-F: AGCATCCTGTTCTGTGACG

764 *elav*-R: CGCGATTAGCGAGCTTTTGT

765 *atg9*-F: AGCAGAAGCACGGATTCACA

766 *atg9*-R: GCAGTGCATCACAAAGGCAA

767 IMG cells:

768 *Actin*-F: CTAAGGCCAACCGTGAAAAG

769 *Actin*-R: ACCAGAGGCATACAGGGACA

770 *GAK*-F: GGTCATCCAGTCTGTGGCTAAC

771 *GAK*-R: TTGATTGCAGACTCCACACC

772

773 **Immunohistochemistry**

774 Adult fly brains: Brains were dissected and fixed in 4% formaldehyde for 40 minutes,

775 then washed with PBT (PBS + 0.1% TX-100) for 3 times and dissected further to

776 remove additional debris in PBS solution. Clean and fixed brains were blocked in PBT

777 solution with 5% Normal Donkey Serum (NDS) and subsequently stained with primary

antibodies at 4°C overnight and then secondary antibodies at room temperature for 2 hours. Primary antibodies used: mouse anti-Atg8a (1:1000, gift from Chao Tong), rabbit anti-Ref(2)P (1:500, Cat. #ab178440, Abcam, Cambridge, UK), mouse anti-Mono- and polyubiquitinated conjugates antibody (FK2, 1:500, Cat. #BML-PW8810, Enzo Life Sciences, Farmingdale, NY, USA), rabbit anti-dATG9B (1:200, Cat. #NB100-98679, Novus Biologicals, Littleton, CO, USA), mouse anti-Repo (1:100, Cat. #8D12, Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, USA), rat anti-Elav (1:500, Cat. #7E8A10, DSHB), and DDC (1:300, Jay Hirsh, primary).

IMG cells: Cells were seeded on glass coverslips in 12-well plates the day before fixation with cold methanol (Cat. #100141190, Sinopharm, Beijing, China) for 15 minutes, followed by washing with PBT (PBS + 0.1% TX-100) for 15 minutes, then blocked in PBS with 5% NDS for 1 hour. Cells were stained with primary antibodies at 4°C overnight and then secondary antibodies at room temperature for 2 hours. Primary antibodies used: rabbit anti-Iba1 (1:500, Cat. #019-19741, Wako), rabbit anti-TMEM119 (1:500, Cat. #ab185333, Abcam), rabbit anti-Lamp1 (1:400, Cat. #ab24170, Abcam), rabbit anti-LC3B (1:200, Cat. #2775, Cell Signaling Technology, CST, Danvers, MA, USA), and rabbit anti-GAK (1:50, Cat. #12147-1-AP, Proteintech, Chicago, IL, USA). Nuclei were labeled by DAPI (Cat. #C1005, Beyotime, Shanghai, China) at room temperature for 5-10 minutes.

Mouse brain slices: To prepare brain slices, mice were anesthetized with isoflurane and perfused with 0.9% saline, followed by 4% paraformaldehyde in PBS. The brains were

removed and post-fixed overnight in 4% paraformaldehyde in PBS at 4°C. After dehydration in 20% sucrose, a series of coronal sections (10 µm thick) across the Substantia Nigra were cut by a Thermo CryoStar NX50 cryostat (ThermoFisher Scientific, USA). The sections were permeabilized with 0.3% Triton X-100 in PBS containing 5% normal goat serum for 1 h at 37°C. After blocking with 5% normal goat serum in PBS, the samples were incubated with primary antibodies at 4°C overnight. Primary antibodies used: rabbit anti-TH (1:500, Cat. #AB152, Merck), rabbit anti-Iba-1 (1:500, Cat. #019-19741, Wako), goat anti-Iba-1 (1:100, Cat. #ab5076, Abcam), rabbit anti-GAK, and mouse anti- α -Synuclein (1:50, Cat. #sc-58480, Santa Cruz Biotechnology). After three washes with 1XPBS, samples were incubated for 1 hour with secondary antibodies conjugated with Alexa-fluorescein.

For all samples, secondary antibodies used were from Jackson ImmunoResearch (West Grove, PA, USA): donkey anti-mouse Cy3 (1:1000, Cat. #715-166-151), donkey anti-rabbit Cy3 (1:1000, Cat. #711-166-152), donkey anti-rat Cy3 (1:1000, Cat. #712-165-153), donkey anti-mouse Cy5 (1:500, Cat. #715-175-150), donkey anti-rabbit Cy5 (1:500, Cat. #711-175-152) and donkey anti-rat Cy5 (1:500, Cat. #712-175-150). All samples were mounted in Vectashield mounting media (Cat. #H-1500-10, Vector Laboratories, Burlingame, CA, USA).

LysoTracker staining

Live adult fly brains were dissected in PBS, and immediately incubated in LysoTracker Green DND-26 (1:1000, Cat. #L7526, Life technologies) solution with PBS (pre-warmed at 37°C) for 5 minutes at 25°C. Images were taken by Nikon A1 microscope. Live IMG cells were seeded on 8-well chamber slides overnight, and incubated in LysoTracker solution (1:10000) with anti-fluorescence quenching DMEM (Cat. #A1896701, Gibco). Images were taken by Nikon Spinning Disk at 37°C in 5% CO₂.

Cathepsin B detection with Magic Red

Adult fly brains were dissected in PBS, and stained with 1× Magic Red Cathepsin B dye for 10 min, according to the manufacturer's instruction (Magic Red® Cathepsin B assay kits, Cat. #ICT937, BioRad). The red fluorescent intensities representing cathepsin B activity in the cells were monitored under the Nikon A1 microscope.

***In-vivo* time lapse analysis**

Live adult fly brains were dissected and kept in saline solution for tracing the GFP puncta in a single focal plane using LSM 710 NLO (Zeiss, Germany) microscope with a 20X water objective. The trafficking speed was analyzed by Imaris using Brownian motion tracking mode.

Transmission electron microscopy

Adult fly brains were fixed, embedded, stained, and dehydrated according to previous protocols (Li et al., 2013). Ultrathin sections (70 nm) of each brain were cut with a Diatomediamond knife on a Leica EM UC7 ultra-microtome (Leica Microsystems, Germany) and collected on copper grids. Images were acquired with Talos L120C transmission electron microscope (Thermo Fisher Scientific, Waltham, MA, UAS) operating at an acceleration voltage of 120 kV.

Phosphorylomics

Samples for phosphorylomic analysis were prepared and analyzed as described previously (PTM Bio, Hangzhou, China (Yang et al., 2012)). 300 fly adult brains per sample were sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 mins at 56°C and alkylated with 11 mM iodoacetamide for 15 mins at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB (tetraethylammonium bromide, TEAB) to urea concentration less than 2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 for a second 4 hour digestion. After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for TMT kit/iTRAQ kit. Briefly, one unit of TMT/iTRAQ reagent were thawed and reconstituted in acetonitrile. The

peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted and dried by vacuum centrifugation. The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (15 cm length, 75 μ m i.d.). The gradient was comprised of an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 mins, 23% to 35% in 8 mins and climbing to 80% in 3 mins then holding at 80% for the last 3 mins, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system. The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent protocol that alternates between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion was conducted. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z.

Biochemistry

Western blot analysis: Fly adult heads were homogenised using a motorised pestle (Cat. #116005500, MP Biomedicals, Irvine, CA, USA) in lysis buffer (0.4% NP-40, 0.2 mM EDTA, 150 mM NaCl, 20% glycerol, 100 mM Tris-HCl pH7.5, 2% Tween 20, 0.5 mM

phosphodiesterase inhibitors, and 1 mM PMSF). Proteins were separated on SDS-PAGE gels and transferred to PVDF membranes (Cat. #IPFL00010, Millipore, Billerica, MA, USA). The membranes were blocked by 5% fat-free milk diluted in PBS with 0.1% Tween-20 for 40 minutes. Samples were incubated with the primary antibodies at 4°C overnight and HRP-conjugated secondary antibodies at room temperature for 2 hours. Primary antibodies used: rat anti-Aux (1:1000, in this study), rabbit anti-GABARAP+GABARAPL1+GABARAPL2 antibody (1:10000, Cat. #ab109364, Abcam), rabbit anti-Ref(2)P (1:500, Cat. #ab178440, Abcam), mouse anti-Mono- and polyubiquitinated conjugates antibody (FK2, 1:500, Cat. #BML-PW8810, Enzo Life Sciences), rabbit anti-dATG9B (1:200, Cat. #NB100-98679, Novus Biologicals), rabbit anti-pS6K (1:1000, Cat. #9205S, Cell Signaling), rabbit anti-S6K (1:1000, Cat. #9202S, Cell Signaling), rabbit anti-CathD (1:1000, Cat. #ab75852, Abcam), mouse anti- α -Tubulin (1:5000, Cat. #T9026, Sigma), rabbit anti-Myc (1:2000, Cat. #0912-2, HuaAn Biotechnology), mouse anti- α -Syn (1:1000, Cat. #180215, Invitrogen, Carlsbad, CA, USA), and mouse anti-Flag (1:1000, Cat. #F3165, Sigma, St. Louis, MO, USA). Bands were visualized by Clarity Western ECL Substrate (Cat. #WBKLS0500, Millipore). Results are representative of at least three biological replicates. For IMG cells, cells were treated and lysed in 2× SDS loading buffer at 95°C for 10 minutes. Primary antibodies used: rabbit anti-LC3B (1:200, Cat. #2775, CST), and rabbit anti-GAK (1:50, Cat. #12147-1-AP, Proteintech), mouse anti-P62 (1:1000, Cat. #ab56416, Abcam) and mouse anti-Ubiquitin (1:1000, Cat. #3936S, CST). For all examples, secondary

antibodies used are from Jackson ImmunoResearch: goat anti-mouse-HRP (1:5000, Cat. #115-035-003), goat anti-rabbit-HRP (1:5000, Cat. #111-036-003) and goat anti-rat-HRP (1:5000, Cat. #112-035-003).

BafilomycinA1 Treatment: BafA1 (Cat. #BML-CM110-0100, ENZO) was diluted in DMSO (Cat. #D8371-50, solarbio) in a concentration of 1 mM, then added to 0.5 ml of liquid yeast for a final concentration of 4 μ M. Yeast containing BafA1 was added to the top of the fly food in standard vials kept overnight to allow the evaporation of residual DMSO. Flies of different genotypes (25 females and 25 males) were then put in each vial and kept at 25°C for 5 days. After treatment, flies were collected for western blot analysis.

anti-Aux antibodies: anti-Aux antibodies were generated by ABclonal Biotechnology co., Ltd (Harrogate, UK). *aux* cDNA fragment encoding aa694-1157 was subcloned into the pET-28a-SUMO vector, and purified proteins were injected into rats using standard protocols to generate the antibodies.

FACS analysis

Adult fly brains were dissected in Schneider's medium (Cat. #21720024, Invitrogen) and dissociated in 0.18 units/ml fresh papain (Cat. #LK003178, Worthington, Freehold, NJ, USA) and 2.5mg/ml liberase TM (Cat. #5401119001, Roche, Basel, Switzerland) with PBS. Approximately 100 or 400 brains were dissected for qRT-PCR or WB analysis, respectively. Papain was activated at 37°C for 15 min before use. After

dissociation for 1 hour, cold Schneider's medium was added to inactivate the enzyme. Samples were then filtered through cell strainer (Cat. #352340, BD Falcon, Franklin Lakes, NJ, USA) into a flow cell tube (Cat. #352003, BD Falcon), centrifuged, and re-suspended in the Schneider's medium. The suspension was then kept on ice and sent to Flow cytometer (FACSMelody, BD). Fluorescent GFP or RFP signals were monitored to sort adult fly glia. For transfected IMG cells, cells were harvested and filtered through cell strainer as described above. Fluorescent Cy5 was used to sort IMG cells.

Behavior analysis

Mouse footprint gait analysis: To perform footprint gait analysis, mouse hindpaws and forepaws were coated with black and red nontoxic paint, and trained to walk along a 33-cm long and 8-cm wide open-top runway (with 6.35-cm high walls). A fresh sheet of white paper was placed on the floor of the runway for each run. The footprint patterns were assessed quantitatively by stride length(Wertman et al., 2019).

Mouse pole test: Mice were placed with a 55-cm metal pole of 1-cm diameter covered with adhesive tape to facilitate traction. Upon positioned head-up at the top of the pole, the time required for mice to turn (t-turn) and climb down completely was recorded.

Fly lifespan analysis: The following fly strains were crossed to *repo-GAL4* and used for lifespan analysis: isogenic *w¹¹¹⁸*, *UAS-LacZ*, *UAS-aux-RNAi* (V16182). These strains were separately back-crossed to isogenic *w¹¹¹⁸* for five generations in order to clean the genetic background before assessing longevity. Newly eclosed male or female

flies (1 to 2 days old; n=400 for each genotype) were collected and placed separately in 20 vials (each contains 20 unisex flies). Flies were transferred to new vials with standard fly food every other days, and the number of dead flies were counted. All experiments were repeated at least three times, and the percentage of flies survived (live/total, %) was analyzed. Data points were drawn in curves using Prism software.

Fly locomotion analysis: The rapid iterative negative geotaxis (RING) assay was used to analyze climbing ability as described previously (Cao et al., 2017; Gargano et al., 2005; Song et al., 2017). Briefly, flies of different ages (3, 10, and 20 days old) were collected and placed in vials with fly food (no yeast) for 1 day before transferring to the cylinders for experiments. A total of 100 flies were analyzed in ten cylinders (inner diameter: 20 mm; height: 200 mm) placed side by side, and each contains ten unisex flies. An initial mechanical shock was applied for six times so that all flies were tapped down to the bottom of the cylinder and synchronized. Climbing distances for each fly were measured and averaged by RflyDetection software, which allows automatic detection of fly position within the cylinder using video images captured every 6 s (Sony digital camera, HDR-CX220E). At least three independent experiments were performed. Data were shown as mean $\pm$ SEM.

Fly sleep analysis: The parental and F1 generation flies were raised on standard medium in an incubator set to 25°C with 12 hour light/12 hour dark (LD) cycle. The flies were collected at the age of 0-2 days old and mated for 2 days. Each mated female was then loaded into sleep tubes (PGT5x65 Pyrex Glass, Trikinetics) that contained 2% agar and

5% sucrose at one end. The other end was sealed with parafilm with a hole for air-circulation. The tubes were placed into the DAM2 *Drosophila* activity monitor system (Trikinetics, Waltham) for sleep recording. Activity was recorded for five consecutive days including entrainment for the first two days. Data from the following three days were averaged for further analysis. The original data acquired by the DAM system software were processed by DAMfileScan113. Sleep analysis was then performed using an in-house program called SCAMP2019v2(Donelson et al., 2012) in Matlab (MathWorks). Parameters of total sleep and sleep latency were calculated. Statistical analyses were performed using GraphPad 8. Significance differences were determined using Kruskal–Wallis tests for datasets that did not have a normal distribution and one-way ANOVA for datasets normally distributed, followed by multiple comparisons tests.

Confocal microscopy and statistical analysis

Images of adult fly brains and IMG cells were acquired by scanning a serial Z-stack of average 25-30 sections, each of 0.4 μm thickness, using Nikon A1, C2, or Spinning Disk confocal microscope (Tokyo, Japan) with the 40X or 60X oil objective. The whole brain was positioned so that they can be scanned anteriorly to posteriorly (top to bottom). Super-resolution images were taken by STED SP8 (Zeiss, Germany) for a single layer section on the desired regions using 100X oil objective. For statistical analyses of number and size, original and unmodified images were imported into ImageJ (National Institutes of Health), and the intensity threshold for the relevant

channel was set so that maximum number of dots were selected without miscounting two adjacent dots into one. For colocalization measurements, primary images were imported and analyzed automatically using the colocalization plugin with the intensity correlation tool, showing as Pearson's correlation coefficient (R), Manders' colocalization coefficients (tM1 or tM2), or the plot profile to show the mean radial fluorescence. All data were imported into GraphPad Prism 8 and shown in scatter plots or column bar graphs. For calculating the statistical significance, two-tailed unpaired t-test or ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used. P value less than 0.05 is considered significant. ns: no significance, $p \geq 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

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Figure 1

Figure 2

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Figure 3

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