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Master Thesis

探討 NudC 調控細胞自噬的分子機制

Exploring the functional roles of NudC in autophagy

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中文摘要

細胞自噬 (Autophagy) 是真核生物體內一種降解受損胞器和物質的過程，透過細胞自噬可以維持細胞恆定並且可以透過回收降解物質幫助細胞度過逆境。在細胞自噬的過程中，受損的胞器與物質會被雙層膜構造的細胞自噬小體所包裹，細胞自噬小體會再和溶酶體融合形成自噬溶小體，接著所包裹的胞器與物質會被溶酶體帶來的酸性酵素所降解。核遷移蛋白 C (NudC)已知在有絲分裂和細胞質分裂中扮演重要的角色，除此之外，已經有其他文獻發現 NudC 突變會降低細胞自噬小體和內噬體進行反向運輸時的速率，但對於 NudC 在細胞自噬中的功能仍不清楚。近來我們透過將果蠅以及哺乳類細胞中弱化 NudC，發現在飢餓引發細胞自噬的狀況下會使 Microtubule-associated proteins 1A/1B light chain 3A (LC3)的數量下降，也會妨礙細胞自噬的進行。此外也發現 NudC 是因不同刺激引發細胞自噬時的廣泛調控因子。另一方面，目前已知有一部分的 WD 重複結構域磷酸肌醇相互作用蛋白(WIPI)成員會和 NudC 有交互作用，我們發現當在細胞中弱化 NudC 時會使 WIPI2 的數量降低。我們還有在果蠅的不同組織中弱化 NudC，發現眼睛會有縮小以及翅膀會有萎縮的表徵。未來我們將會進一部探討 NudC 詳細於細胞自噬中的功能，以及了解 NudC 對於 WIPI 蛋白於細胞自噬中的影響。

關鍵字：細胞自噬、果蠅、NudC，WIPI

Abstract

Autophagy is a conserved process in eukaryotes by which the intracellular materials are degraded for recycling. Autophagy is important for maintaining cellular homeostasis and recycling cytosolic materials in response to various stress stimulation. During autophagy, the cargo is engulfed into double-membrane structures called autophagosomes, followed by fusing with lysosome to form autolysosomes. The cargo in autolysosomes then degraded by various acidic enzyme in lysosomes. Nuclear migration protein C (NudC) plays an essential role in mitosis and cytokinesis. Although NudC mutants have been implicated reducing the velocity of autophagosomes and late endosomes retrograde transport, the function of NudC in autophagy remains unclear. Recently, we observed that knockdown of NudC results in the reduction of LC3 puncta formation and impaired autophagic flux in both *Drosophila* larval fat bodies and mammalian cells under starvation conditions. Moreover, our results indicate that NudC is a general regulator of autophagy in reponse to various stresses stimulation. The WIPI members have been reported to interact with NudC. In this study, we found that the number of WIPI1 and WIPI2 decreased in NudC knockdown cells. Furthermore, we investigated the developmental function of NudC by crossing dNudC-RNAi with various drivers in *Drosophila* and found that dNudC knockdown cause small eye and aberrant wing phenotypes. In the future, we will investigate the molecular function of NudC in

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autophagy and explore the relationship between NudC and WIPI proteins in autophagy.

Keywords: Autophagy, *Drosophila*, NudC, WIPI proteins

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Content

doi:10.6342/NTU202301996

doi:10.6342/NTU202301996

Chapter 1

Introduction

1. Autophagy

1.1 Overview of Autophagy

Autophagy is a self-degradative mechanism that is important for homeostasis through balancing energy sources and response to different cellular stress (Glick et al., 2010). In mammalian cells, there exist three distinct types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Each of them is morphologically distinct, while the cargo in all three pathways is transported to the lysosome for degradation and recycling (Parzych and Klionsky, 2014). During microautophagy, the autophagic cargoes are directly captured by lysosomes and late endosomes through membrane protrusion and invagination, and the cargoes are degraded in the endolysosomal lumen (Wang et al., 2022). In chaperone-mediated autophagy (CMA), the autophagic cargoes identified by chaperone containing a specific pentapeptide motif; are then unfolded and translocated to the lysosome for degradation (Parzych and Klionsky, 2014). In macroautophagy, the autophagic cargoes are delivered to the lysosome by the double-membrane vesicle, called the autophagosome, followed by fusing with the lysosome for degradation (Yorimitsu and Klionsky, 2005). In this study, we will

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focus on macroautophagy, hereafter referred to as autophagy.

1.2 The Machinery of Autophagy

(1) Induction

Upon the induction of autophagy in mammalian cells, the formation of autophagosomes begins at multiple sites throughout the cytoplasm. Several studies implicated that endoplasmic reticulum (ER)-associated structures called the omegasome, may recognize as initiation sites (Parzych and Klionsky, 2014). The assembly of ULK protein complex composed of ULK1, Atg13, FIP200, and Atg101 at the isolation membranes during initiation of autophagy, followed by working with other autophagy-related gene (Atg) proteins to promote autophagosome formation (Wong et al., 2011). The activation of the ULK1 complex requires dissociation from the negative regulator mTOR complex 1 (mTORC1) (Hosokawa et al., 2011) during cellular stress. Besides, the ULK1 complex can also be activated by lowering cyclic adenosine monophosphate (cAMP) levels or reducing inositol 1,4,5-trisphosphate (IP_3) production (Sarkar et al., 2009). Proteins such as WIPI1, Atg5, and Atg12 are present in the isolation membranes to facilitate autophagosome formation (Wong et al., 2011).

doi:10.6342/NTU202301996

(2) Nucleation and Elongation

After initiation, the primary double-membrane structure; called the phagophore, expands by accepting lipids from various sources such as plasma membrane (Ravikumar et al., 2010), ER (Hayashi-Nishino et al., 2009), Golgi complex (Takeshige et al., 2009), and mitochondria (Hailey et al., 2010). During nucleation, the assembly of Class III phosphatidylinositol-3-kinase (PI3K) complex composed of several proteins such as Beclin1, Atg14, p150, and PI3K Vacular protein sorting 34 (Vps34), which then activates PI3K to produce PI3P (Mehrpour et al., 2010). Following the nucleation, there are two ubiquitin-like (UBL) conjugation pathways involved in the elongation step, the ATG12 system and the microtubule-associated protein 1 light chain 3 (LC3) system (Chiarini et al., 2012), contributing to the expansion of the phagophore. The ATG12-ATG5-ATG16L1 complex associates with the phagophore membrane, while dissociating following autophagosome completion (Mizushima et al., 2003). The ATG12 system mediates the conjugation of phosphatidylethanolamine (PE) to the LC3-I forming LC3-II in the LC3 system. LC3-II then translocated from the cytosol to the membrane of the premature autophagosomes (Wong et al., 2011).

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(3) Degradation

Following the elongation step, the autophagosomes are fused with lysosomes to form autolysosomes for the degradation of the autophagic cargoes. There are various types of vesicles including late endosomes and lysosomes can be fused with autophagosomes (Eskelinen, 2005). During the fusion of autophagosomes with lysosomes, the GTPase RAB7 is activated to promote autolysosome formation (Jager et al., 2004). Besides, the SNARE machinery also has been suggested that play a role in fusion (Fader et al., 2009).

2. NudC

Nuclear migration protein C (NudC) was first identified as a gene in the regulation of nuclear movement in the asexual reproductive cycle (Osmani et al., 1990) in *Aspergillus nidulans*. NudC plays various roles in cells, accumulating data have reported that NudC can act as a modulator of cell wall thickness and nuclear positioning through its association with Lis1 (also known as NudF) (Chiu et al., 1997) in *Aspergillus nidulans*. NudC has also reported that function in dynein-dynactin function during cell migration (Wynshaw-Boris and Gambello, 2001; Cappello et al., 2011; Aumais et al., 2001). According to a conserved CS domain in NudC, it is predicted to possess chaperone activity (Zheng et al., 2011).

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It is indicated that NudC stabilizes proteins, including the dynein regulator Lis1, through its chaperone activity (Zhu et al., 2010; Xiang et al., 1995).

NudC orthologs have been identified from fungi to mammals, which show high sequence and structure conservation (Fu et al., 2016). The first mammalian NudC was identified as a prolactin-inducible gene in rat T cells (Morris et al., 1997), and other paralogs of mammalian NudC, NudCL, NudCL2, and NudCD1 genes, were cloned in 2006 (Zhou et al., 2006), 2010 (Yang et al., 2010), and 2001 (Yang et al., 2001), respectively. All NudC gene products share a similar conserved CS domain (also called p23-like domain) which implied their chaperone activities.

According to the chaperone activity of NudC described before, NudC can act as co-chaperone to regulate Hsp90 resulting in substrate handover and activation (Biebl et al., 2022). Additionally, based on the quantitative chaperone interaction network, NudC is not only interacted with Hsp90, but also likely associated with structurally related but evolutionarily distinct β-propeller folds, such as WD40, Kelch, and RCC1 (Taipale et al., 2014).

On the other hand, NudC is a dynein regulator by increasing Lis1 protein stability in the axon terminal (Kawano et al., 2022). It reported that depletion of NudC in zebrafish results in autophagosomes and late endosome accumulation in the axon

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terminal and lower velocity of autophagosome and late endosome retrograde transportation. However, the function of NudC in autophagy is still unclear, so we are curious about what NudC plays a role in autophagy.

3. WIPI proteins

WD-repeat protein Interacting with PhosphoInositides (WIPI) protein family, also called for beta-propellers that bind polyphosphoinositides (PROPPINs), is an evolutionarily conserved family of proteins. The WD-repeat is a short chain of about 40 amino acids, ending with tryptophan and aspartate residues (D. Li and R. Roberts, 2001). WD-repeat-containing proteins are involved in various functions, such as cell cycle, apoptosis, and autophagy (Buddhi Prakash Jain and Shweta Pandey, 2018). All WIPI proteins, including WIPI1, WIPI2, WIPI3/WDR45B, and WIPI4/WDR45, have 7 WD-repeats and are predicted to fold into a 7-bladed ß-propeller (Catherine Hong Huan Hor and Bor Luen Tang, 2019). In addition to the WD-repeat domain, all WIPI proteins possess the F/LRRG motif that has them bind to phosphoinositides (Dove et al., 2004). This specific motif is localized in blade 6 and allows their specific interaction with phosphatidylinositol-3-phosphate (PtdIns3P) and phosphatidylinositol-3,5-biphosphate (Proikas-Cezanne et al., 2004; Polson et al., 2010; Krick et al., 2006). The WIPI protein family's membership varies across different organisms. In yeast, there are three members (Atg18, Atg21, and Hsc2),

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two in *Dictyostelium* (Atg18 and Wdr45l) (Vincent et al., 2021), and two in *Caenorhabditis elegans* (APG-18 and EPG-6) (Zhang et al., 2013). In our studies, Atg18a, Atg18b, and CG11975 are considered WIPI homologs in *Drosophila*.

WIPI proteins are PtdIns3P-binding effectors that are components of the ATG machinery in autophagy (Proikas-Cezanne et al., 2015). They function in both the biogenesis and maturation of autophagosomes. Autophagy is regulated through the interplay between AMPK and TORC1 at the induction step. Autophagy is activated by AMPK through the activation of the TORC1 inhibitor, TSC1/2 complex. It reported that WIPI3 is a regulator in TORC1 inhibition by interaction with TSC1/2 complex. In the meanwhile, WIPI3 translocates to the nascent autophagosome interacting with FIP200 and ATG proteins (Bakula et al., 2017). After induction, the lipidation of LC3 is essential for autophagosome formation. WIPI2 has been shown to be associated with PtdIns3P production which mediates the recruitment of the ATG12-ATG5/ATG16L1 complex for following LC3 lipidation at the phagophore (Dooley et al., 2014; Fracchiolla et al., 2020). Besides, WIPI2 also interacts with RAB11A at the early stages of autophagosome formation (Puri et al., 2018). Depletion of WIPI2 not only lead to a dramatic reduction of LC3 lipidation and autophagosome formation but also causes an accumulation of PtdIns3P and DFCP1 (Polson et al., 2010; Dooley et al., 2014; Bakula et al., 2017). WIPI1 also functions

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in the early stage of autophagosome formation and has been proposed to support WIPI2 in the recruitment of ATG16L1 complex for LC3 lipidation (Bakula et al., 2018). However, it was reported that the knockdown of WIPI1 in G361 cells does not block the formation of autophagosome and LC3 lipidation, suggesting WIPI1 is dispensable for autophagosome formation (Bakula et al., 2017). In addition to LC3 lipidation, phagophore elongation requires other lipid sources, such as ER. It has been reported that WIPI4 forms a complex with ATG2 at the phagophore to conduct lipid transfer (Bakula et al., 2018). The previous report showed that double knockout WIPI3 and WIPI4 results in fusion defect of autophagosome and lysosome through the recruitment of EPG5, a tethering protein to bridge autophagosome and lysosome (Ji et al., 2021). Double knockout of WIPI3 and WIPI4 shows a more acute autophagy defect than single knockout, suggesting redundancy of function in autophagy (Ji et al., 2021).

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Chapter 2

Material and Methods

Drosophila strains

All flies were raised and crossed at 25°C

Stocks	Genotype	Source
R4-GAL4-Flip-out	hsflp; r4mCherry-Atg8a; Act5c>CD2>Gal4, UAS-GFP-nls	Gift from Tom Neufeld
Cg>mCherry-Atg8a	Cg-GAL4-mCherry-Atg8a	Generated by our lab
Luciferase-RNAi^{JF}	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01355}attP2	Bloomington *Drosophila* Stock Center #31603
dNudC-RNAi^{KK}	P{KK107790}VIE-260B	Vienna *Drosophila* Resource Center #104008
dNudC-RNAi^{Nig1}		Nig-Fly #9710R-2
CG11975-RNAi^{GD}	w[1118]; P{GD12639}v26799	Vienna *Drosophila* Resource Center #26799
CG11975-RNAi^{KK}	P{KK100592}VIE-260B	Vienna *Drosophila* Resource Center #108549
Atg18a-RNAi^{KK}	P{KK100064}VIE-260B	Vienna *Drosophila* Resource Center #105366
Atg18a-RNAi^{JF}	y1 v1; P{TRiP.JF02898}attP2	Bloomington *Drosophila* Stock Center #28061
Atg18a-RNAi^{HMS}	y1 sc* v1 sev21; P{TRiP.HMS01193}attP2	Bloomington *Drosophila* Stock Center #34714
Atg18b-RNAi^{GD}	w1118; P{GD14002}v36001	Vienna *Drosophila* Resource Center #36001
Tub-GAL4	y1 w*; P{tubP-GAL4}LL7/TM3, Sb1 Ser1	Bloomington *Drosophila* Stock Center #5138
Ey-GAL4	w*; P{GAL4-ey.H}3-8	Gift from Yao’s lab
MS1096-GAL4	w1118 P{GawB}BxMS1096	Bloomington *Drosophila* Stock Center #8860

Starvation condition (*Drosophila*)

The second instar larva is removed from food and transferred to the dish containing 20% sucrose for 4 hours to induce autophagy response.

Antibody

Antibodies	Source	Reactivity	Concentration
LC3	Cell signaling	Rabbit	1:200 for IF
GAPDH	Cell signaling	Rabbit	1:8000 for WB
Tubulin	Sigma	Mouse	1:10000 for WB
WIPI1	Santa Cruz	Mouse	1:1000 for WB
WIPI2	Abcam	Mouse	1:1000 for IF 1:5000 for WB
NudC	Santa Cruz	Mouse	1:1000 for WB
GFP	Santa Cruz	Mouse	1:1000 for WB
Myc	Cell signaling	Rabbit	1:1000 for WB
HA	Sigma	Mouse	1:200 for IF 1:8000 for WB
Flag	Sigma	Mouse	1:200 for IF 1:5000 for WB
V5	Sigma	Mouse	1:5000 for WB

Plasmid list

All flies incubate and cross at 25°C.

Plasmid	Source
pCMV-NudC-Flag	SinoBiological (HG15068-CF)
pCMV-WDR45-HA	SinoBiological (HG16339-CY)
pCMV-WDR45B-HA	SinoBiological (HG15068-CY)
pMXs-IP GFP-WIPI-1	Addgene (38272)
GFP-WIPI2	Gift from Ruey-Hwa Chen
pUAST-CG11975-V5	Generate from our lab
pUAST-V5-Atg18A	Generate from our lab
pUAST-V5-Atg18B	Generate from our lab
pUAST-Myc-NudC	Generate from our lab

Immunostaining and Confocal Fluorescence Imaging

For larval fat body dissection, the second instar larva was dissected in 1X PBS, and the collected fat bodies were fixed in 4% paraformaldehyde for 25 min. Following fixation, fat bodies were washed with 1X PBS twice, followed by 5 min DAPI staining. For immunofluorescence analysis of mammalian cells, cells were cultured on coverslips (12-545-83, Fisherbrand), fixed with methanol for 10 min, and permeabilized with 10 mg/ml digitonin (D141, Sigma) for 10 min at room temperature. Then cells were blocked with 5% goat serum in 1X PBS for 1 h, followed by incubation with the indicated primary antibodies (diluted in 5% goat serum in 1X PBS) overnight at 4ºC. After washing with 1X PBS, cells were incubated with fluorescently labeled secondary antibodies for 2 h at room temperature, followed by 5 min DAPI staining. Coverslips were mounted in 50% glycerol. Images were captured by Olympus FV3000 Confocal Microscope.

Cell culture and transfection

Drosophila S2 cells were cultured in Schneider’s medium with 10% fetal bovine serum (FBS) and 1% Penicillin-Stretomycin at 25℃. For the experiment, S2 cells were transfection with LipofectamineTM 2000 (Invitrogen) transfection reagent for 48h before harvesting. HeLa and 293 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% Penicillin-Stretomycin at 25℃. Indicated plasmids were transfected with

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Polyjet (SignaGen) and cultured for 24-48 h before harvesting.

Generation of shRNA for HeLa cell and transfection

HEK293T cells were transfected with shRNA oligos using Lipofectamine RNAi MAX (13778150, Life Technologies), and the supernatant was harvested 72 h after transfection. Then HeLa cells were infected with the supernatant for 24-48 h, followed by selection with puromycin.

Immunoprecipitation and western blotting

Cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, protease inhibitor cocktail (Roche) at 4^{o}C for 30 min. After centrifugation at 12,000 g for 10 min, cell lysates were pre-cleaned with protein G-Sepharose beads (GE Healthcare) for 1 h. The supernatants were then incubated with specific antibodies at 4^{o}C overnight, followed by incubation with protein G-Sepharose beads for 1 h. The beads were washed three times with wash buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl, 10% glycerol, 0.1% Triton X-100), mixed with SDS sample buffer, and then boiled at 100^{o}C for 10 min. Samples were separated by SDS-PAGE electrophoresis and transferred onto 0.45 µm PVDF membranes (Millipore). The membranes were blocked

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with 5% nonfat milk or 5% BSA in TBST and incubated with primary antibodies at 4 ^{o}C overnight. After washing, the membranes were soaked with horseradish peroxidase-labeled secondary antibodies detected by ECL reagents and exposed.

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Chapter 3

Results

Drosophila NudC depletion impairs starvation-induced autophagy.

Our lab has recently performed a genectic screen for *Drosophila* genes that are involved in autophagy using the GS lines (GS line are insertion lines of the P{GS} element (gene search vector)) in chromosome 2 or 3. Among the candidate genes, we found that dNudC may have an effect on autophagy in the *Drosophila* second instar larval fat bodies. Then we expressed different dNudC RNAi lines in the *Drosophila* second instar larval fat bodies and found the decrease of Atg8a puncta under starvation condition (Figure 1A). The number of Atg8a puncta in dNudC knockdown cells is less than that in the control *Drosophila* larval fat body cells (Figure 1B).

The decrease of Atg8a puncta of dNudC knockdown in the *Drosophila* larval fat bodies might be due to the increased formation of autophagosomes or blocked degradation of autolysosomes. To investigate these two possibilities, we expressed the tandem tagged GFP-mCherry-Atg8a reporter to determine the autophagic flux by crossing Cg-tandem-Atg8a flies with dNudC -RNAi^{KK} and dNudC -RNAi^{Nig1} flies. The GFP fluorescent signal is quenched by the acidic lysosomal environment, whereas red fluorescent signal from the mCherry tag persists if the autophagy progresses well. Therefore, the puncta that appear to be yellow are autophagosomes, whereas the puncta that visualized by only the red signal are autolysosomes. Compared to the control, knockdown of dNudC results in increased number of autophagosomes (yellow puncta) but not autolysosomes (red puncta) under starvation condition (Figure 1C-1E). Together, these results suggest that knockdown of dNudC in *Drosophila* larval fat bodies decreases Atg8a puncta formation and blocks autophagic flux under starvation condition.

NudC depletion impairs starvation-induced autophagy.

In addition to the *Drosophila* larval fat bodies, we investigated whether knockdown mammalian NudC in HeLa cells would also play a role in autophagy. HeLa cells stably expressed scramble control shRNA or NudC shRNA were analyzed the formation of LC3 puncta under fed and starvation conditions with or without treatment of lysosomal inhibitor bafilomycin A1 (BafA1). As shown in Figure 2B and 2C, the number of LC3 decrease in knockdown of NudC HeLa cells cultured in EBSS-induced starvation. The results are similar with the data shown in Figure 1A and 1B. We also showed that the reduction of LC3 puncta in NudC knockdown cells under BafA1 treatment. These results indicate that NudC depletion may block the autophagy pathway.

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NudC is a general regulator of autophagy.

Starvation is a kind of stimulation for autophagy. However, autophagy can also be stimulated by other stress, such as ER stress, oxidative stress, and loss of mitochondria membrane potential; Hence we wonder whether NudC plays a general role in the regulation of autophagy in response to different stresses. To solve the problem, we calculated the number of LC3 puncta in shScramble and NudC knockdown HeLa cells by treating with different stress inducers. For inducing ER stress, cells were treated with 1μg/mL of tunicamycin (TN) for 24 hours. TN is commonly used in vitro to study the effects of ER stress in various cell types (Bassik and Kampmann, 2011) by inhibiting N-linked glycosylation. For inducing mitochondria damage, 1mM of carbonyl cyanide m-chlorophenylhydrazone (CCCP), an ionophore that results in disruption of mitochondria outer membrane potential, was added to cells and cultured for 1 hour. Oxidative stress was induced by treating 1mM of hydrogen peroxide in cells for 0.5 hours. Similar to the results of EBSS starvation, the number of LC3 puncta were dramatically decreased in the knockdown of NudC HeLa cells under each stress condition (Figure 3). Taken together, these results suggested that NudC is a positive regulator of autophagy at induction under various stress conditions.

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NudC interacts with WIPI1, WIPI2, and WIPI4.

Based on previous results, we found that the depletion of NudC blocks autophagic flux, so we wonder if this autophagy defect is caused by the association between NudC and autophagy-related proteins. In starvation-treated HeLa cells, NudC-Flag does not colocalize with GFP-Atg16L1 (marker of early stage of autophagosome formation), GFP-ZFYVE (marker of phagophore elongation), GFP-Atg9 (the scramblase function in autophagosome formation), and GFP-STX17 (marker of mature autophagosome), the data are shown in Figure 4A-4D. Then, we performed immunofluorescence assay to investigate the number of autophagy-related proteins in NudC knockdown cells. The results are shown in Figure 5A-5D, indicating that depletion of NudC does not have an effect on autophagy-related protein performance.

A previous study by Daniela Bakula et al., 2017 has established the WIPI protein interactome by employing mass spectrometry (MS) and found that WIPI1, WIPI2, and WIPI4 share NudC as an interaction partner by using GFP-tagged WIPI variants overexpressing in U2OS cell line analyzed by anti-GFP immunoprecipitation (Figure 6A). Due to the important role of mammalian WIPIs in autophagy, we examined whether the knockdown of dWIPIs in *Drosophila* larval fat bodies impairs autophagy. *Drosophila* Atg18a, Atg18b, and CG11975 are orthologous proteins of mammalian

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WIPIs. According to the reference from FlyBase, Atg18a and Atg18b are orthologs to WIPI1 and WIPI2 and CG11975 is an ortholog to WIPI3 and WIPI4. As shown in Figure 6B, the knockdown of Atg18a, Atg18b, and CG11975 reduces the number of Atg8a puncta indicating that *Drosophila* WIPIs also have an effect on autophagy. Then, we found that dNudC interacts with Atg18a, Atg18b, and CG11975 by co-expressing dNudC and each of *Drosophila* WIPIs respectively in S2 cell (Figure 6C). Next, we also confirmed the binding of NudC with WIPI1, WIPI2, and WIPI4 by co-expressing NudC and each of WIPI proteins respectively in HEK293T cell. As shown in Figure 6D-6E, the results are consistent with mass spectrometry results shown in Figure 6A by conducting co-immunoprecipitation assays. However, we found that NudC does not colocalize with WIPI1 and WIPI2 in starvation-treated HeLa cells (Figure 6F). It suggests that NudC is associated with WIPI1 and WIPI2 in an indirect way.

NudC depletion reduces WIPI1 and WIPI2 puncta formation.

According to the results shown before, we confirmed that NudC and WIPIs depletion impairs autophagy and NudC interacts with WIPI1, WIPI2, and WIPI4. Now, we wonder whether the autophagy defect in the knockdown of NudC in the HeLa cell line is associated with WIPI proteins. We conducted Immunofluorescence (IF) assays performed in HeLa cells that were stably expressing scramble control

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shRNA or NudC shRNA. We found that the knockdown of NudC in HeLa cells reduces the number of GFP-WIPI1 and GFP-WIPI2 after EBSS starvation for 2 hours (Figure 7A and 7B). The number of endogenous WIPI2 also decreased in the knockdown of NudC HeLa cells under EBSS starvation for 2 hours (Figure 7C). We also confirm the WIPI2 protein expression level by western blotting (Figure 7G). These results suggest that NudC is a potential regulator of WIPI1 and WIPI2 in autophagy.

Phenotypes of *Drosophila* NudC knockdown flies

The GAL4/UAS system is a powerful tool that has been widely used in *Drosophila* for ectopic expression of transgenes in a tissue-specific manner. We tried to knockdown of dNudC in different tissue to find the phenotype in *Drosophila* adult. We investigated the phenotype of dNudC knockdown flies in different tissue, the summary table is shown in Figure 8A. The whole body knockdown of dNudC leads to a lethal phenotype. As shown in Figure 8B to 8D, the atrophied wing phenotype in dNudC knockdown flies was found by crossing with MS1096-GAL4 expressed in the wing disc. Besides, we utilized eyeless (Ey)-GAL4 expressed in the eye and observed that the eyes in dNudC knockdown flies are smaller than the control (Figure 8E-8G). We further found that the bristle on the compound eye in dNudC knockdown flies is more disordered than control by imaging through cryo-scanning electron microscopy

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(cryo-SEM) (Figure 8J-8L'). These results suggested that *Drosophila* NudC is a crucial factor in not only the adult stage but also the embryonic stage. However, there were no significant eye defects in Atg7 and Atg9 knockdown flies which crossed with Ey-GAL4 (Figure 8H-8I). It indicated that the reason results in a small eye phenotype may be autophagy-independent.

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Chapter 4

Discussion

Recent studies revealed that NudC acts as a co-chaperone regulating the Hsp70 and the Hsp90 systems to release the protein from Hsp70 to Hsp90 for improving protein folding (Biebl et al., 2022). Additionally, in a previous study, it has been reported that NudC regulates dynein function by increasing Lis1 protein stability in the axon terminal. Loss of NudC contributes to the accumulation of autophagosomes and late endosomes in axon terminals and decreases the retrograde transport velocity of vesicles (Kawano et al., 2022). Based on the studies mentioned before, suggest that NudC may have a role in autophagy; however, how NudC functions in regulating autophagy remains still unclear. Hence, we are going to investigate the function of NudC in autophagy.

In the beginning, we found that the depletion of dNudC results in the reduction of Atg8a puncta in *Drosophila* larval fat bodies through genetic screening. Atg8a is a mammalian homolog of LC3, GABARAP, and GATE16 proteins, an ubiquitin-like protein that conjugates to lipids and then localizes to the inner and outer membranes of isolated membrane and autophagosome (Shpilka et al., 2011). The number of Atg8a interacting with the inner autophagosomal membrane is internalized and finally degraded within the autolysosome; however, Atg8a localized to the outer membrane is de-lipidated and released to the cytosol. Hence, Atg8a can be considered a marker at all stages of the

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autophagy pathway (Mauvezin et al., 2014). We also investigate whether the depletion of NudC results in the reduction of LC3 in HeLa cells, stable expressing NudC shRNA. As the results in Figure 1A, the knockdown of NudC contributes to the reduction of LC3 puncta. The formation of LC3-II, a commonly used indicator of autophagosomes, occurs through the attachment of cytosolic LC3-I to phosphatidylethanolamine (PE) on the membrane of nascent autophagosomes. It involves two successive ubiquitin-like reactions (Runwal et al., 2019). NudC may have an effect on these two conjugation systems to influence the LC3 expression.

Although the autophagy marker Atg8a provides a snapshot of the autophagic compartment, it is limited to measuring the autophagic flux because the markers can be expressed similarly in response to either promotion or inhibition of autophagy (Mauvezin et al., 2014). To investigate the autophagic flux, we conduct tandem tagged (GFP-mCherry)-Atg8a assay to measure the rate of autophagic activity. The results suggest that knockdown NudC blocks the autophagic flux in *Drosophila* larval fat bodies, either in the HeLa cells.

Degradation of p62 is another general autophagy marker to measure autophagic activity because p62 directly binds to LC3 and is degraded by autophagy (Pankiv et al., 2007). Generally, upon autophagy induction, LC3-I converts to LC3-II and decreases in p62. In autophagy-deficient tissue, soluble p62, and p62-positive aggregates are

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accumulated (Komatsu et al., 2007). Our results indicate the knockdown of NudC blocks the autophagic flux, so we will further measure the pattern of p62 in both protein and mRNA levels to confirm the p62 expression in NUDC KD HeLa cells.

Autophagy is induced by not only nutrient depletion but also other various stress stimulation, such as ER stress, or hypoxia (Kroemer et al., 2010). Hence, we induced autophagy through different cellular stress in NudC depletion cells to figure out if NudC plays a general regulator of autophagy. The reduction of LC3 in NudC KD cells under various stress stimulation, including hypoxia, ER stress, and mitochondria damage. This suggests that NudC is a general autophagy regulator.

Next, we wonder whether NudC interacts with autophagy-related proteins to regulate autophagy. We chose four ATG proteins to conduct the colocalization assay, including Atg16L1, ZFYVE, Atg9a, and STX17, respectively. Atg16L1 expresses in the early stage of autophagy which forms a complex with Atg5 and Atg12 on the outer surface of the phagophore (Noor Gammoh, 2020). ZFYVE is an autophagy effector recruited to sites of autophagosome formation by their FYVE domains, the PI3P-binding motif (Kawabata and Yoshimori, 2015). Atg9 is a scramblase that functions in autophagosome formation (Matoba et al., 2020). Syntaxin 17 (STX17) is recruited to the mature autophagosomes, mediating autophagosome-lysosome fusion (Ikuko Koyama-Hondat and Noboru Mizushima, 2022). As the data were shown in Figure 3A-3D, we found that

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NudC does not colocalize with ATG proteins description before. Next, we investigate if the depletion of NudC has an effect on ATG protein stability. Referring to Figure 4A-4D, it seems like the number of Atg16L1, ZFYVE, Atg9a, and STX17 in NudC KD cells has no difference from the control. Together with the results, NudC may have no significant effect on the expression of some ATG proteins during autophagy. A more specific mechanism should be identified, and we will conduct more assays for further investigation.

According to the proteomics analysis by Bakula et al., 2017, they showed the network of all four WIPI proteins associated with a distinct and non-redundant protein. Additionally, they found that WIPI1, WIPI2, and WIPI4 share NudC as a common interaction partner. WIPI proteins with 7 WD-repeats, participate in autophagy at different stages and promote phagophore expansion by the recruitment of the LC3 conjugation machinery (Fracchiolla et al., 2019). Hence, we presume the autophagy defect in NudC KD cells may be through the interplay between NudC and WIPI proteins. First of all, we confirm that *Drosophila* WIPI protein depletion indeed results in the reduction of Atg8a. Then we conduct the Co-Immunoprecipitation assay to investigate the interaction with dNudC and *Drosophila* WIPI. NudC interacted with both *Drosophila* WIPI and mammalian WIPI proteins. While the results shown in Co- Immunoprecipitation assay do not mean that NudC has direct interaction with WIPI proteins, we will further investigate

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whether NudC direct binds to WIPI proteins and confirm the interaction domain of NudC with WIPI proteins.

Then, we found that the depletion of NudC in HeLa cells contributes to decreasing the number of WIPI1 and WIPI2; however, NudC does not colocalize with WIPI1 and WIPI2, suggesting that NudC may play an indirect regulator in WIPI1 and WIPI2 during autophagy. Due to chaperone activity of NudC has been reported, we wonder whether NudC have an effect on WIPI2 stability. In the further studies, we will conduct stability assay by treating cycloheximide (CHX), the inhibitor of protein synthesis, to investigate the stability of WIPI2 in NudC KD cells under specific time point. WIPI2 has been reported to interact with Atg16L1 and is required for LC3 conjugation by recruiting the Atg12-5-16L1 complex (Dooley et al., 2014). It is possible the depletion of NudC influences the association with WIPI2 and the Atg12-5-16L1 complex to affect the autophagy pathway. On the other hand, Ji et al., 2021 found that WDR45/45B promotes autophagosome-lysosome fusion through the recruitment of EPG5 to late endosomes/lysosomes. Double knockout WDR45 and WDR45B reduce the interaction between EPG5 and Rab7, the member of the SNARE protein. It has been reported the transportation of autophagosome need Rab-interacting lysosomal protein (RILP), which binds with ATG5, LC3, Rab7, and cytoplasmic dynein to promote autophagosome transportation (Khobrekar et al., 2020). We wonder whether NudC influences Rab7

doi:10.6342/NTU202301996

through interaction with WIPI4 to play a role in autophagosome transportation. The mechanism between NudC and WIPI proteins still remains identified.

Finally, we try to figure out other phenotypes in different tissue of *Drosophila* by genetic screening. It has shown that the depletion of dNudC leads to the lethal phenotype. Depletion of dNudC results in atrophied wing and small eye phenotypes. We further employed Cryo-Scanning Electron Microscopy (cryoSEM) to examine more detail of the small eye in *Drosophila*. The eye consists of approximately 800 ommatidia, which are unit eyes organized in staggered columns. Each ommatidium comprises a group of eight photoreceptors, encompassed by distinct accessory cells (Johnson, 2021). In the dNudC KD strain, they developed irregular arrays rather than hexagonal arrays of ommatidia in the wild-type strain. Moreover, while the bristle arrangement is highly ordered on ommatidia in the wild-type strain, those in the dNudC KD strain are disordered. The changes in the ommatidial shape may be caused by a variety of reasons, such as intensive membrane tension (Hayashi et al., 2022). It seems like the small eye phenotype in dNudC knockdown strain is autophagy-independent. We have also demonstrated the phenotypes in the dNudC KD strain of other tissue, most of them express lethal phenotypes, indicating dNudC plays a role in the development stage.

Taken together, our data reveal the potential regulator, NudC, in autophagy and suggest that the association with NudC and WIPI proteins is possible directly or indirectly

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affect autophagy. In the future, we are going to conduct more studies to provide more detail on the new aspect of the role of NudC in autophagy.

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Figures

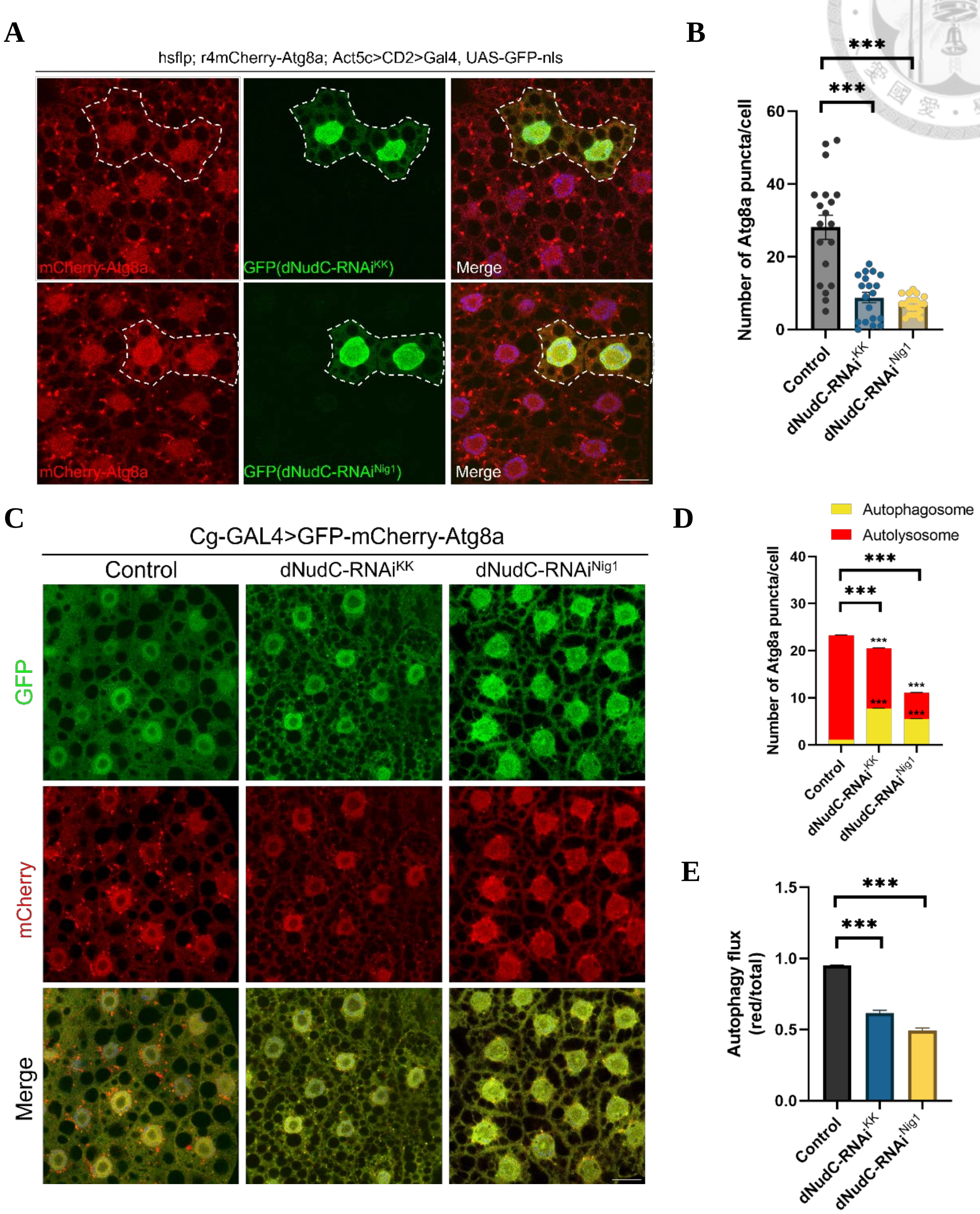

Figure 1. *Drosophila* NudC depletion impairs starvation-induced autophagy.

(A) Knockdown of NudC reduces Atg8a puncta formation under starvation conditions. GFP clone cells express NudC RNAi. NudC RNAi were crossed with R4-GAL4 to allow ectopic expression of NudC RNAi^{KK} and NudC RNAi^{Nig1} in *Drosophila* larval fat bodies. Cells outside the dotted line are control. The red dots are Atg8a puncta.

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Scale bar: 10 μm.

(B) Quantification of the number of Atg8a puncta from (A). Data are shown as mean ± SEM; n > 20 clones per genotype.

(C) Knockdown of NudC blocks autophagic flux. The early third instar of control and NudC RNAi express tandem GFP-mCherry-Atg8a driven by Cg-GAL4 were dissected under starvation conditions. Scale bar: 10 μm.

(D) Quantification of the number of total Atg8a puncta from (C). Red puncta for acidic autolysosome and yellow puncta (mCherry and GFP both exist) for autophagosome/non-acidified autolysosome.

(E) Quantification of autophagic flux from (C). Data are shown as mean ± SEM; n > 50 clones per genotype.

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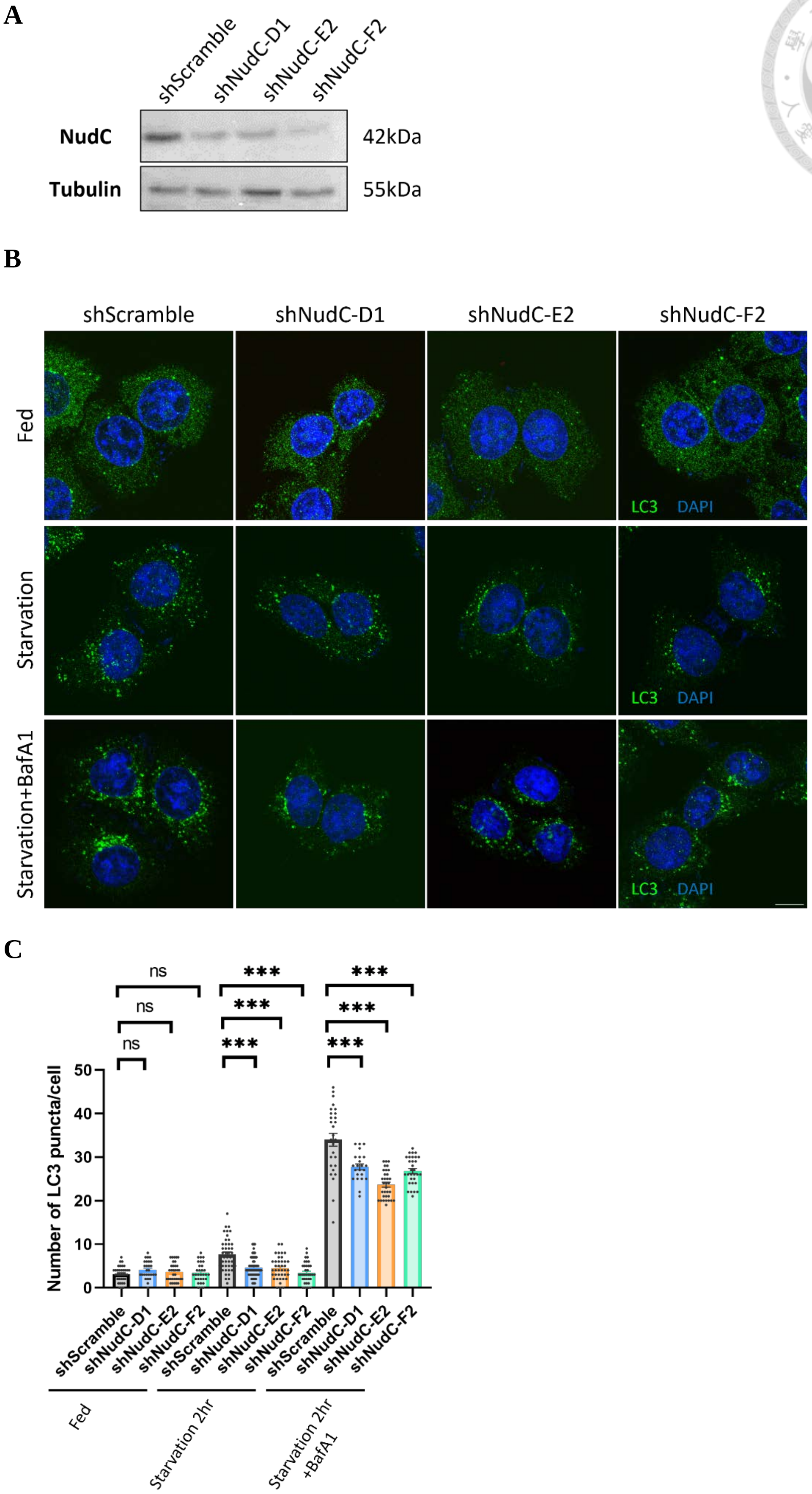

Figure 2. NudC depletion impairs starvation-induced autophagy.

(A) Western blotting analysis of NudC protein expression level in control and NudC knockdown HeLa cells.

(B) HeLa cells stable expressing scramble control shRNA or NudC shRNA were cultured in Fed condition or EBSS medium with or without BafA1 treatment. Immunostained with anti-LC3 antibody. Scale bar: 10 μm.

(C) Quantification of the number of LC3 puncta from (B). Data are shown as mean ± SEM. ***$p < 0.001$.

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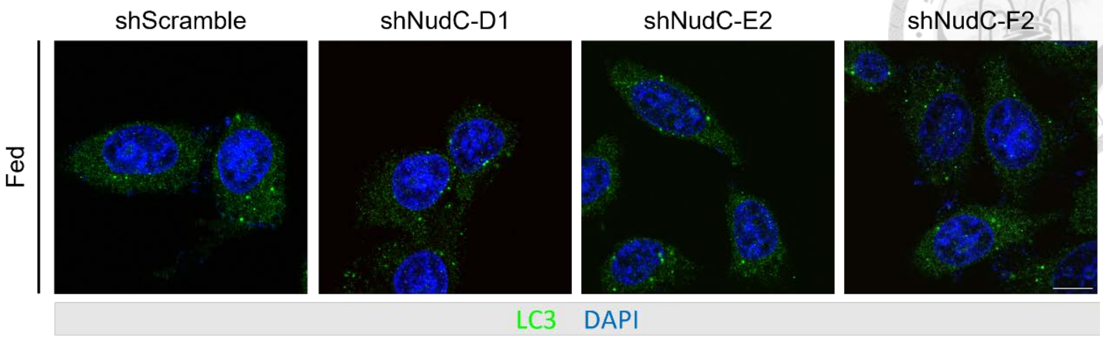
A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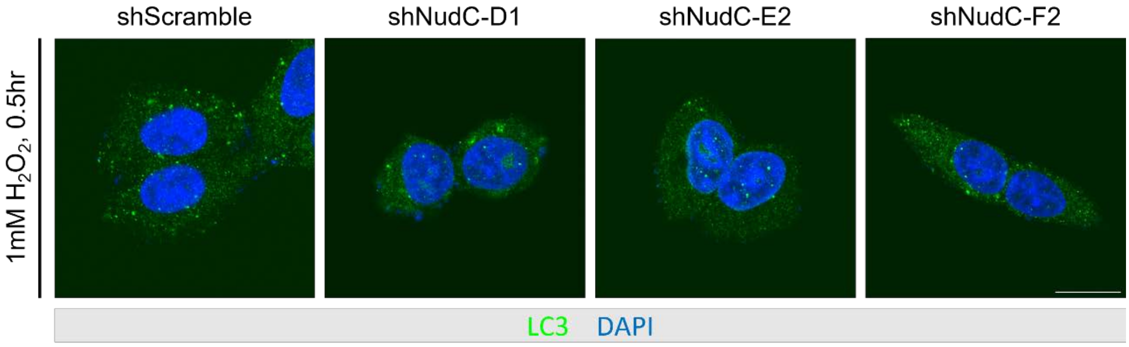
shNudC-E2
shNudC-F2
Fed
LC3
DAPI

B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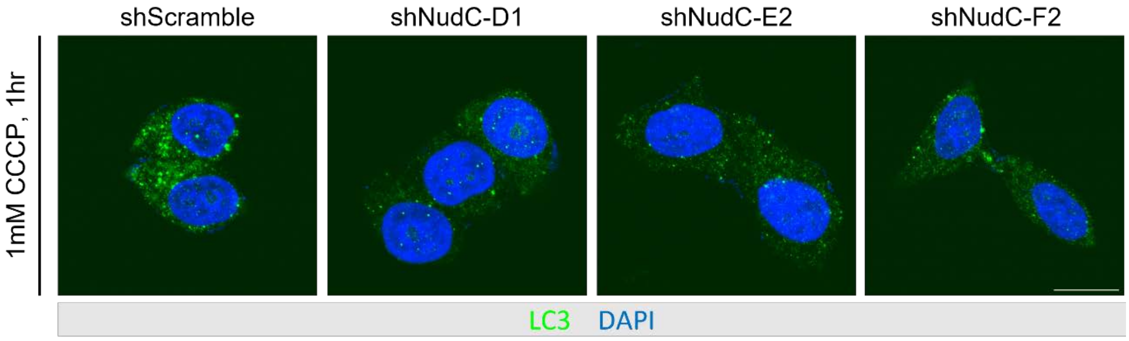
LC3
DAPI

C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shNudC-E2
shNudC-F2
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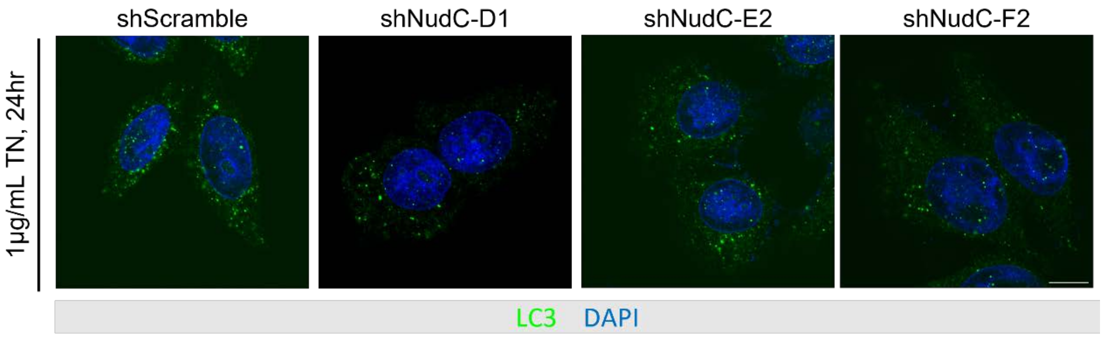
LC3
DAPI

D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shNudC-E2
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LC3
DAPI

E

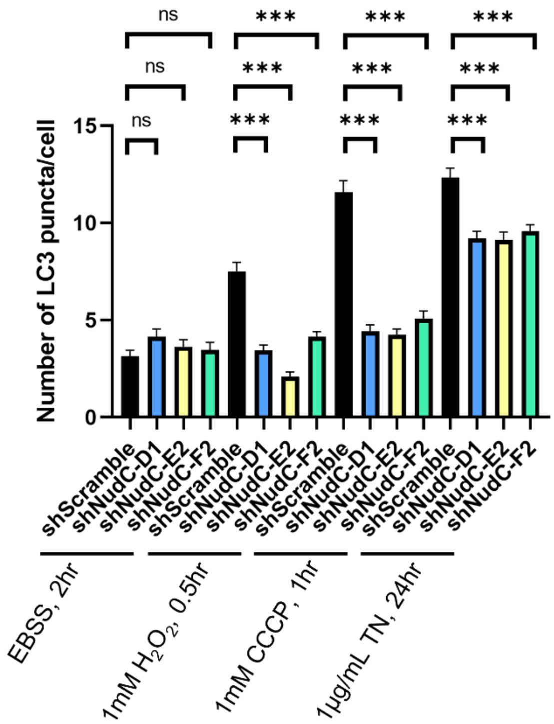

Figure 3. NudC is a general regulator of autophagy.

Autophagy-inducing treatments were applied to HeLa cells stable expressing scramble control shRNA or NudC shRNA for indicated time periods. Control or NudC knockdown HeLa cells were cultured in (A) full medium, (B) hydrogen peroxide treatment for 0.5 hours to induce oxidative stress, (C) CCCP treatment for 1 hour to induce mitochondria damage, or (D) tunicamycin treatment for 24 hours to induce ER stress. Cells were immunostained with anti-LC3 antibodies. (E) Quantification of the number of LC3 puncta under different conditions from (A-D). Data are shown as mean ± SEM. ***$p < 0.001$.

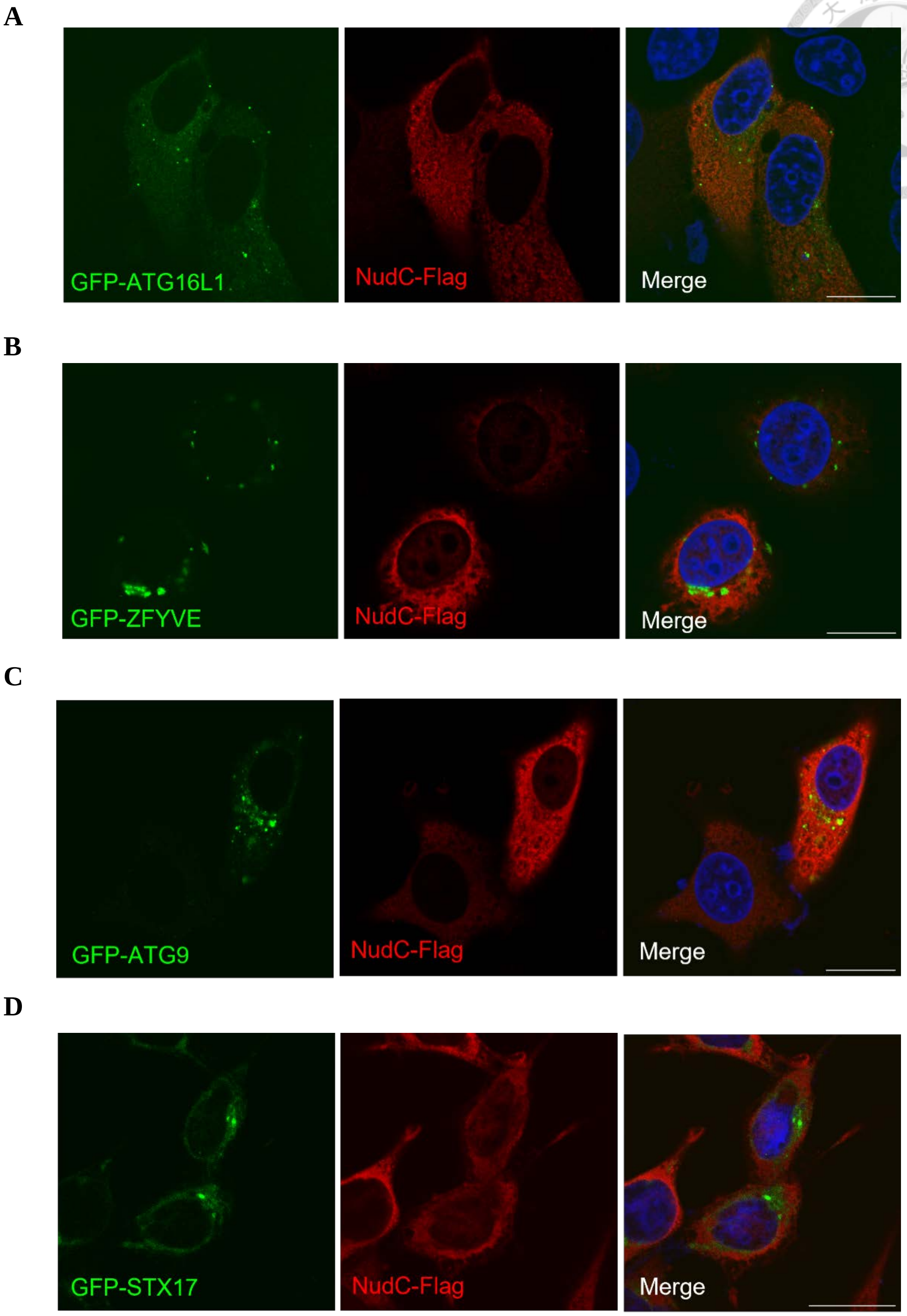

Figure 4. NudC does not colocalize with autophagy-related proteins.

NudC does not colocalize with specific autophagy-related proteins. HeLa cells transfected with NudC -Flag (red) and GFP-Atg16L1 (A), GFP-ZFYVE (B), GFP-Atg9

(C), and GFP-STX16 were cultured in EBSS medium for 2 hours and immunostained with anti-GFP antibody. Scale bar: 10 μm.

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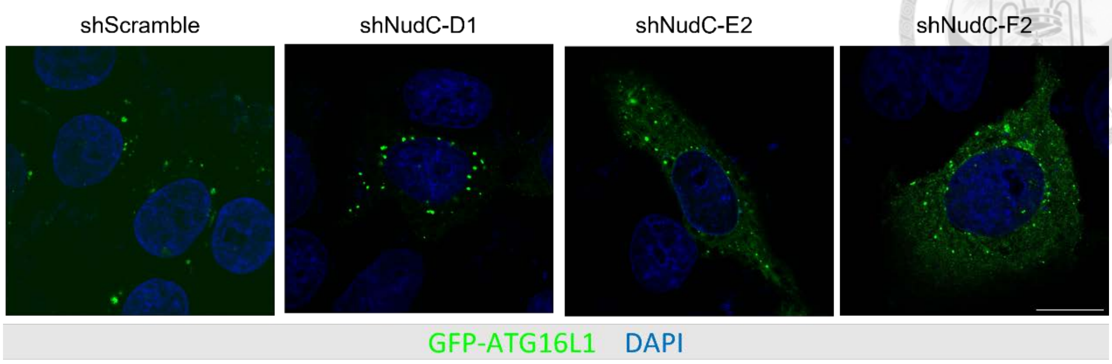
A



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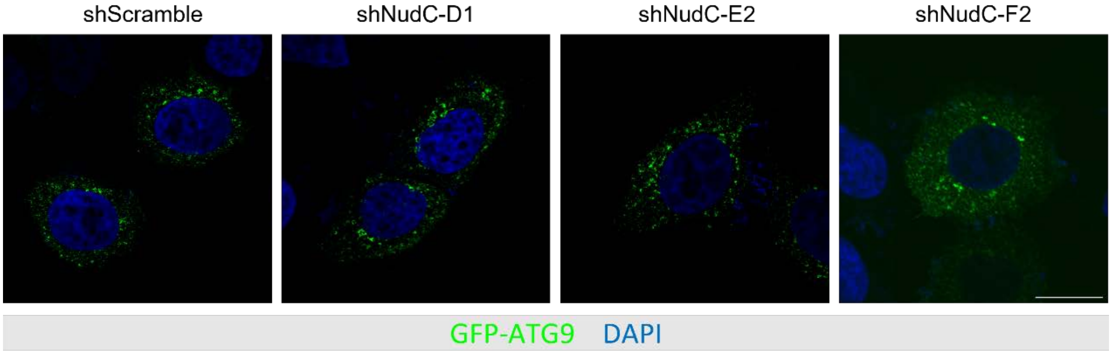

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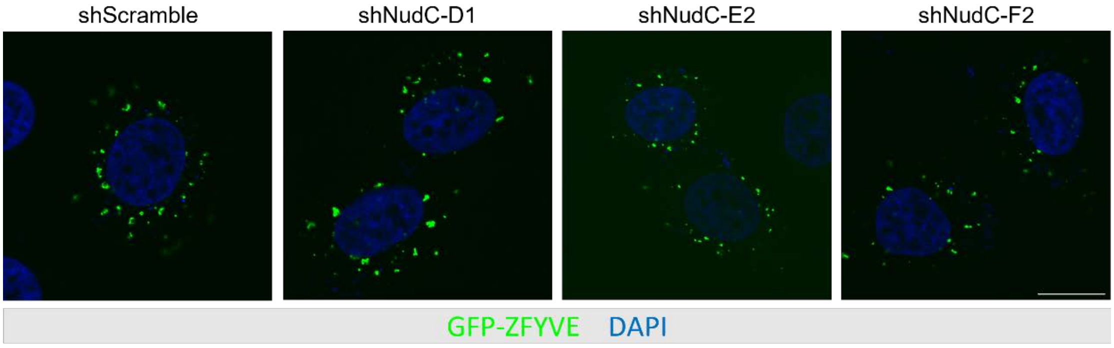

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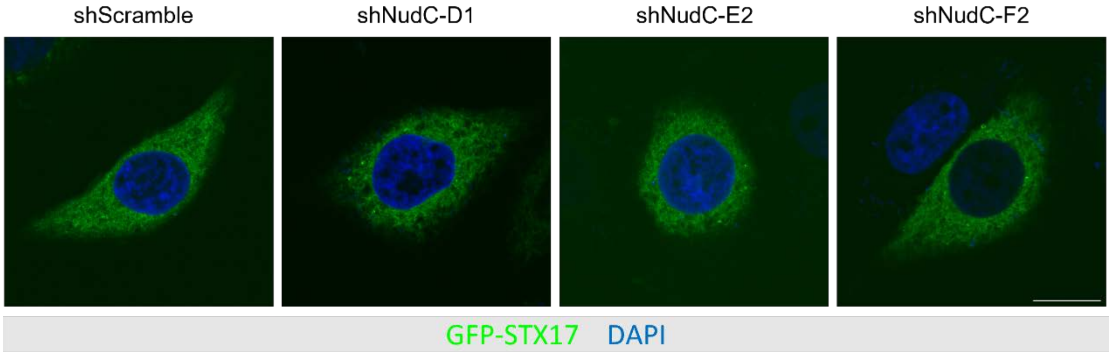

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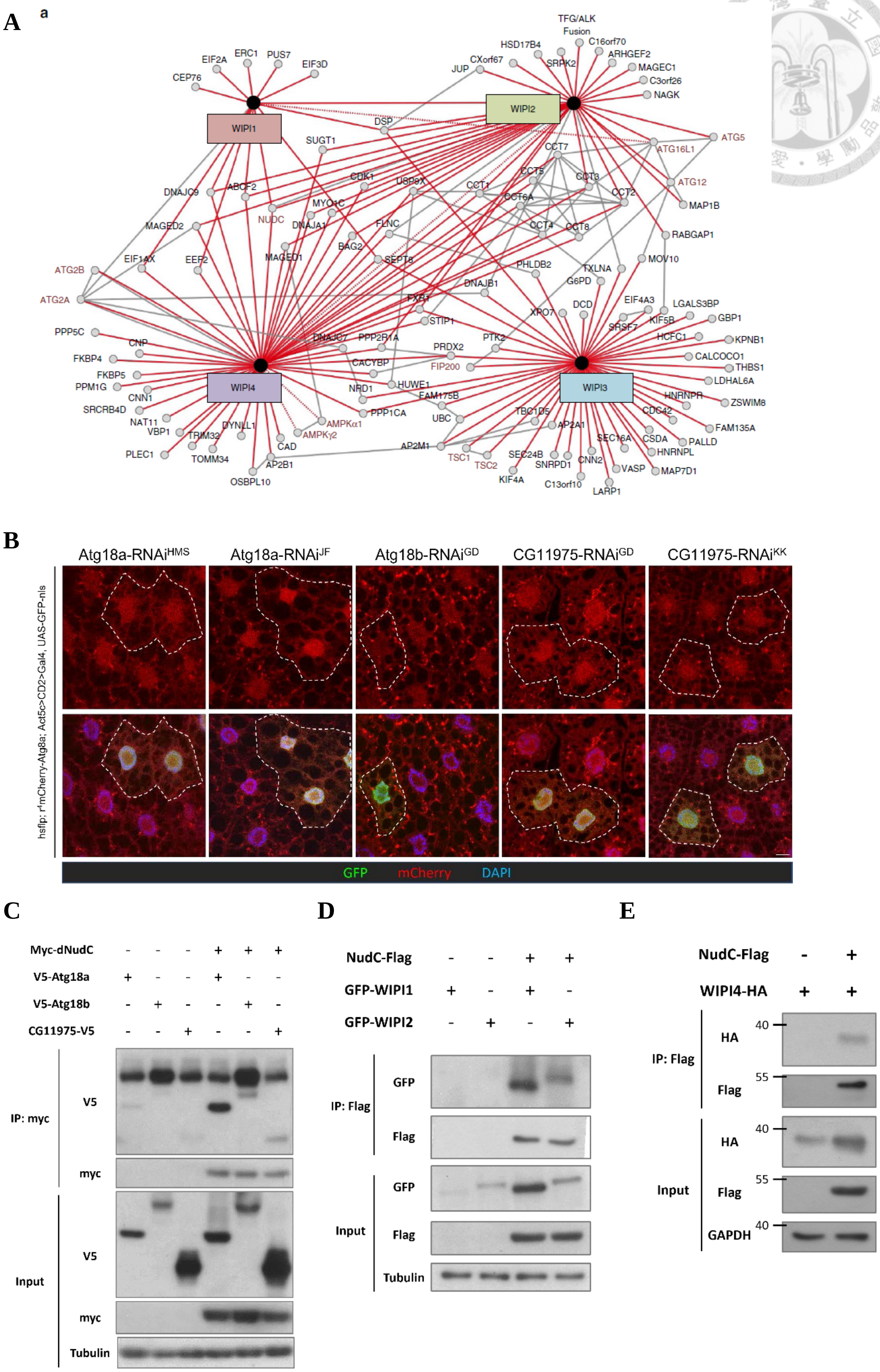
Figure 5. Depletion of NudC does not result in the reduction of autophagy-related protein number.

NudC does not lead to specific autophagy-related protein defects. HeLa cells stable expressing scramble control shRNA or NudC shRNA transfected with GFP-Atg16L1 (A), GFP-ZFYVE (B), GFP-Atg9 (C), and GFP-STX16 were cultured in EBSS medium for 2 hours and immunostained with anti-GFP antibody. Scale bar: 10 μm.

doi:10.6342/NTU202301996


A
a
WIPI1
WIPI2
WIPI3
WIPI4
CEP76
EIF2A
ERC1
PUS7
EIF3D
TFG/ALK Fusion
HSD17B4
C16orf70
CXorf67
SRPK2
ARHGEF2
JUP
MAGEC1
C3orf26
NAGK
DSP
ATG5
SUGT1
CCT7
ATG16L1
CCT5
CCT3
ATG12
CDK1
USP9X
CCT1
CCT2
DNAJC9
ABCF2
CCT6A
MAP1B
MYO1C
NUDC
DNAJA1
FLNC
CCT4
CCT8
RABGAP1
MAGED2
BAG2
MAGED1
SEPT8
MOV10
ATG2B
EIF1AX
EEF2
PHLDB2
TXLNA
G6PD
DNAJB1
ATG2A
FXR1
DCD
EIF4A3
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SRSF7
KIF5B
GBP1
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PPP2R1A
PTK2
HCFC1
KPNB1
FKBP4
PRDX2
CALCOCO1
CACYBP
FIP200
THBS1
FKBP5
PPM1G
NRD1
HUWE1
LDHAL6A
CNN1
FAM175B
HNRNPR
SRCRB4D
PPP1CA
TBC1D5
CDC42
ZSWIM8
NAT11
DYNLL1
AMPKα1
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TRIM32
AMPKγ2
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CSDA
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PLEC1
CAD
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AP2B1
TSC1
SEC24B
CNN2
VASP
MAP7D1
TSC2
SNRPD1
OSBPL10
KIF4A
C13orf10
LARP1
B
Atg18a-RNAi^HMS
Atg18a-RNAi^JF
Atg18b-RNAi^GD
CG11975-RNAi^GD
CG11975-RNAi^KK
hsflp; r^4mCherry-Atg8a; Act5c>CD2>Gal4, UAS-GFP-nls
GFP
mCherry
DAPI
C
Myc-dNudC - - - + + +
V5-Atg18a + - - + - -
V5-Atg18b - + - - + -
CG11975-V5 - - + - - +
IP: myc
V5
myc
Input
V5
myc
Tubulin
D
NudC-Flag - - + +
GFP-WIPI1 + - + -
GFP-WIPI2 - + - +
IP: Flag
GFP
Flag
Input
GFP
Flag
Tubulin
E
NudC-Flag - +
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IP: Flag
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40
55
40
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40

F

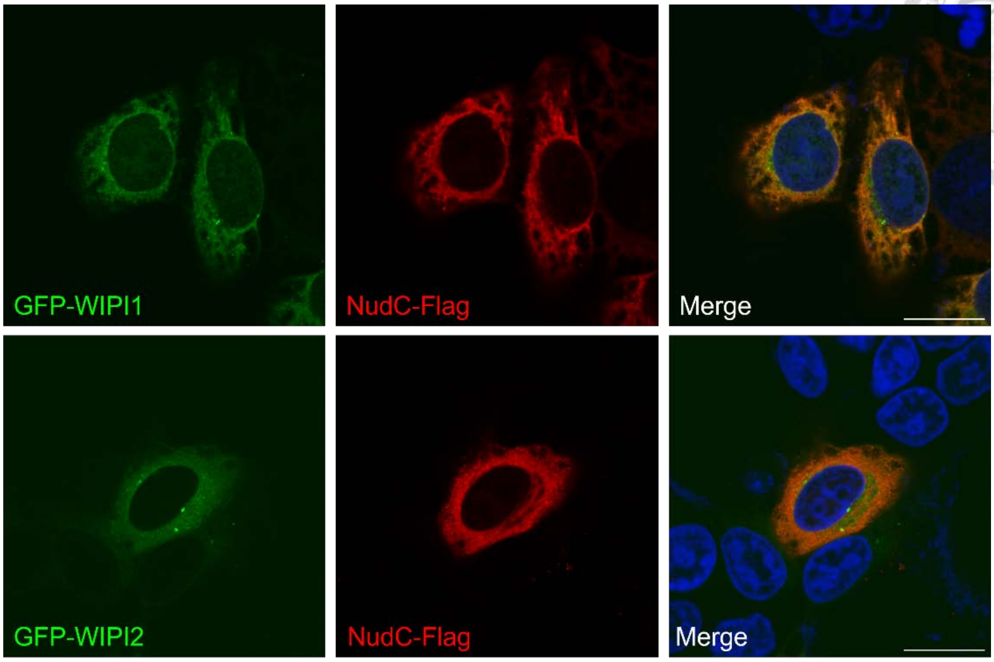

Figure 6. NudC interacts with WIPI1, WIPI2, and WIPI4.

(A) GeneMANIA was used to visualize identified WIPI protein-protein interactions.

(B) Knockdown of *Drosophila* WIPIs reduces Atg8a puncta formation under starvation condition. GFP clone cells express dWIPIs RNAi. dWIPIs RNAi were crossed with R4-GAL4 to aloe ectopic expression of dWIPIs RNAi in *Drosophila* larval fat bodies. Cells outside the dotted line are control. The red dots are Atg8a puncta. Scale bar: 10 μm.

(C) Immunoprecipitation analysis for the interaction between NudC and dWIPI.

S2 cells were transiently co-transfected myc-NudC with V5-Atg18a, V5-Atg18b, and CG11975-V5 for 48 hrs. Whole-cell lysates were immunoprecipitated with anti-myc antibody and followed by western blotting with specific antibodies.

(D) Immunoprecipitation analysis for the interaction between NudC and WIPIs.

HEK293T cells were transiently co-transfected NudC-Flag with GFP-WIPI1 and GFP-WIPI2 for 48 hrs. Whole cell lysates were immunoprecipitated with anti-Flag

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antibody and followed by western blotting with specific antibodies.

(E) Immunoprecipitation analysis for the interaction between NudC and WIPIs. HEK293T cells were transiently co-transfected NudC-Flag with WIPI4-HA for 48 hrs. Whole cell lysates were immunoprecipitated with anti-Flag antibody and followed by western blotting with specific antibodies.

(F) HeLa cells transfected with NudC-Flag (red) and GFP-WIPI1, and GFP-WIPI2 were cultured in EBSS medium for 2 hours and immunostained with anti-GFP antibody. Scale bar: 10 μm.

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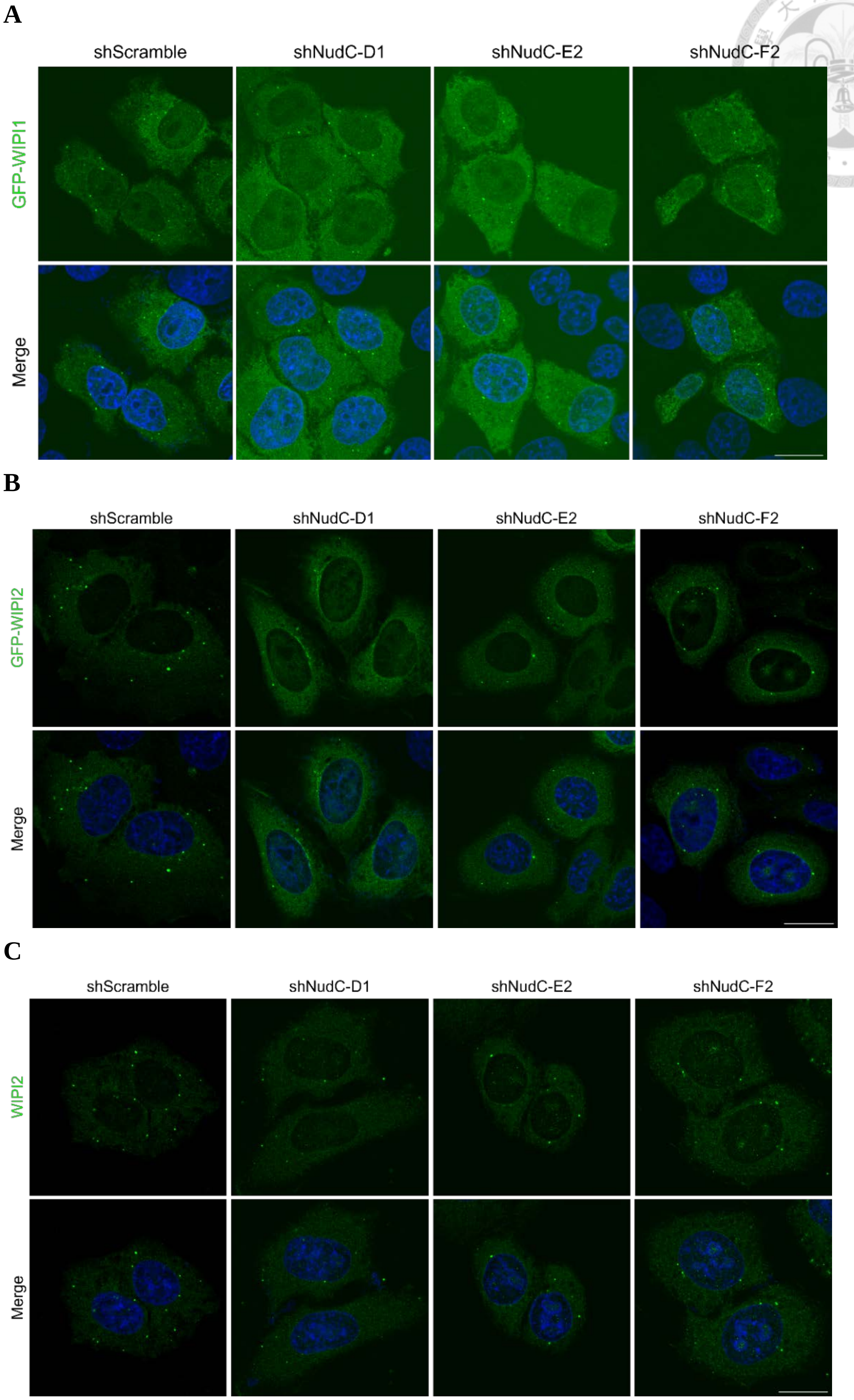
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GFP-WIPI1
Merge
B
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shNudC-E2
shNudC-F2
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C
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WIPI2
Merge

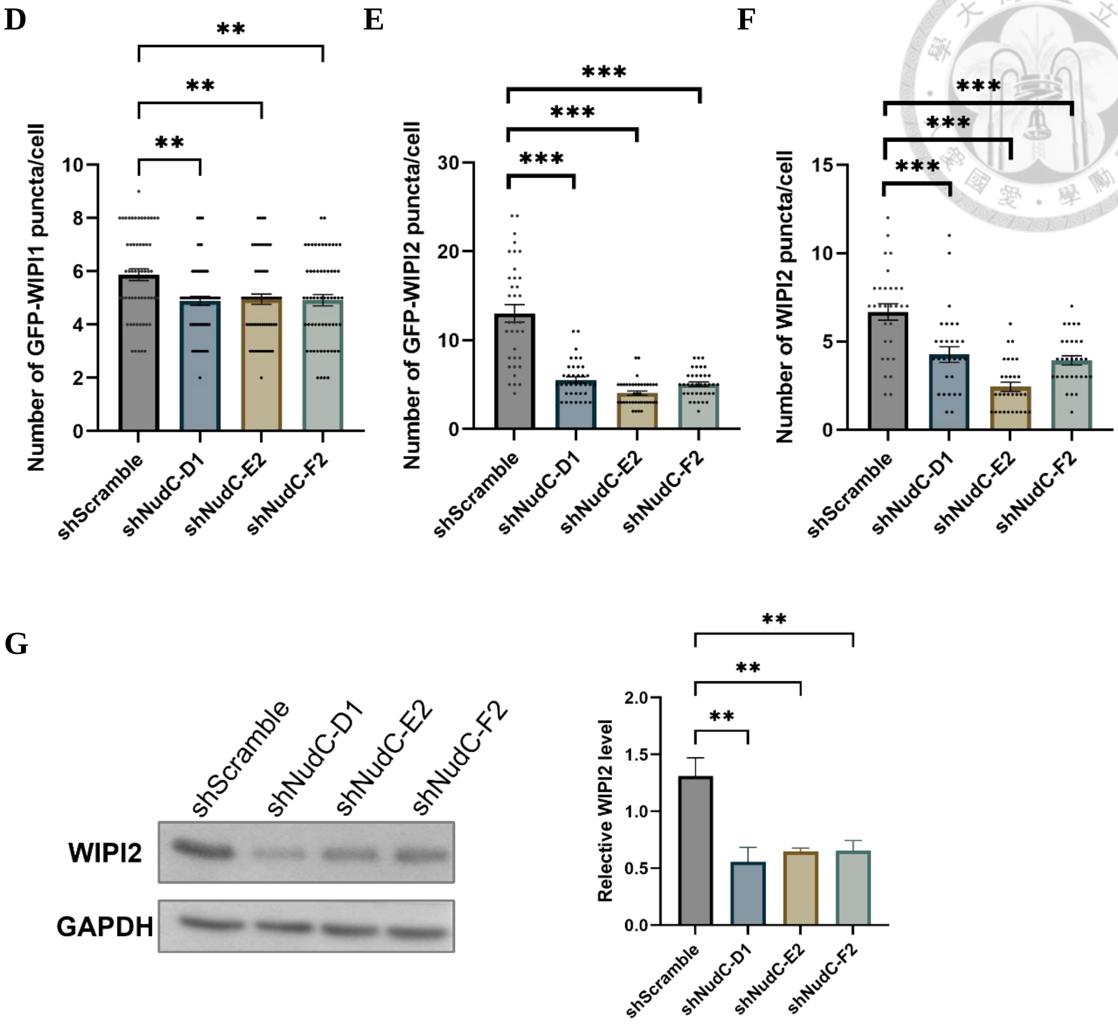

Figure 7. NudC depletion reduces WIPI1 and WIPI2 puncta formation.

(A) HeLa cells stable expressing scramble control shRNA or NudC shRNA were cultured in EBSS medium for 2 hours and immunostained with anti-GFP antibody. Scale bar: 10 μm.

(B) HeLa cells stable expressing scramble control shRNA or NudC shRNA were cultured in EBSS medium for 2 hours and immunostained with anti-GFP antibody. Scale bar: 10 μm.

(C) HeLa cells stable expressing scramble control shRNA or NudC shRNA were cultured in EBSS medium for 2 hours and immunostained with anti-WIPI2 antibody. Scale bar: 10 μm.

(D) Quantification of the number of GFP-WIPI1 puncta from (A). Data are shown as mean ± SEM. **p < 0.01.

(E) Quantification of the number of GFP-WIPI2 puncta from (A). Data are shown as mean ± SEM. ***p < 0.001.

(F) Quantification of the number of WIPI2 puncta from (B). Data are shown as mean ± SEM. ***p < 0.001.

(G) Western blotting analysis of WIPI2 protein expression level. The protein level of WIPI2 was quantified as shown on the right. Data are shown as mean ± SEM. **p < 0.01, ***p < 0.001.

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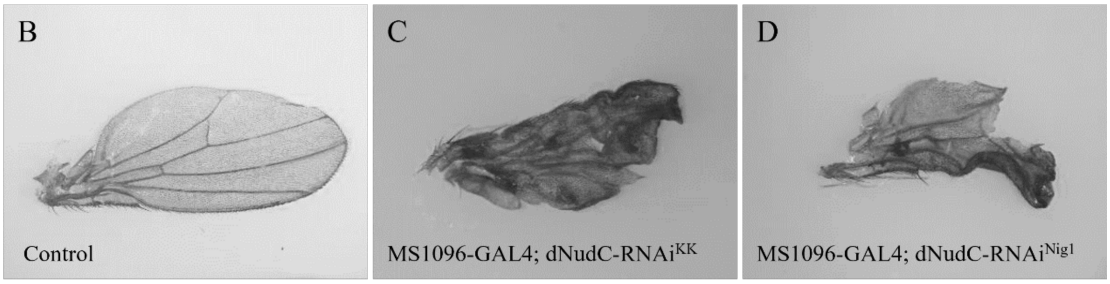
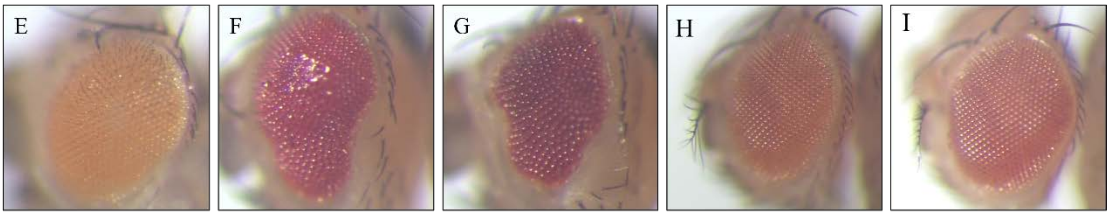
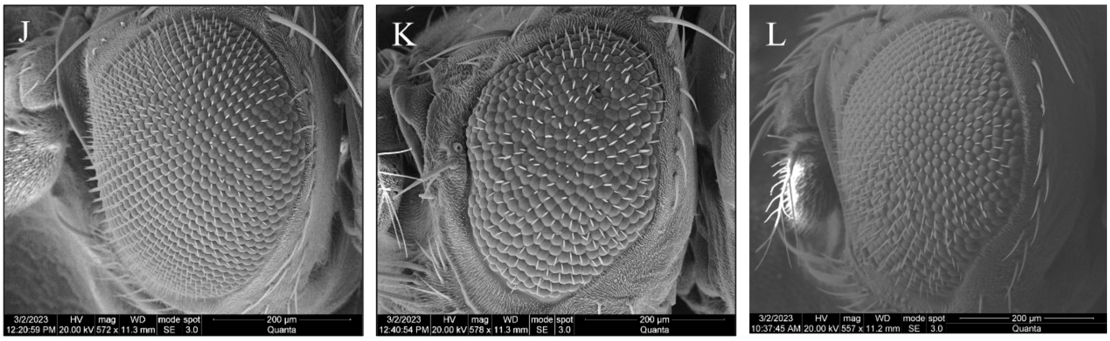
A

	Tub-GAL4	Ey-GAL4	MS1096-GAL4	En-GAL4	Repo-GAL4	24B-GAL4
dNudC-RNAi^{KK}	Larva Lethal	Small eye	Atrophied wing	Pupa lethal	Pupa lethal	Pupa lethal
dNudC-RNAi^{Nig1}	Larva Lethal	Small eye	Atrophied wing	Pupa lethal	Pupa lethal	Pupa lethal

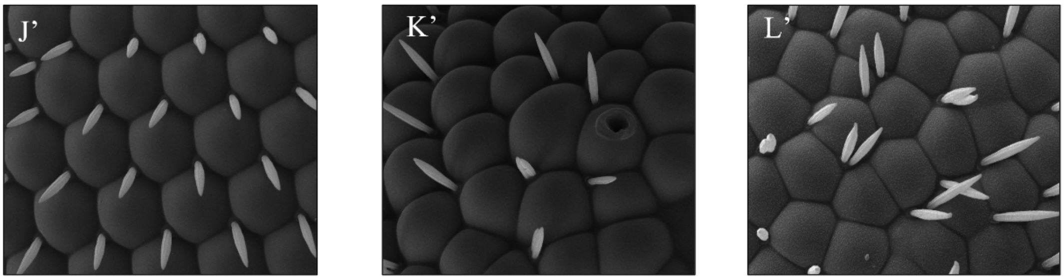




Control   Ey-GAL4; dNudC-RNAi^{KK} Ey-GAL4; dNudC-RNAi^{Nig1} Ey-GAL4; Atg9-RNAi   Ey-GAL4; Atg7-RNAi



Control   Ey-GAL4; dNudC-RNAi^{KK} Ey-GAL4; dNudC-RNAi^{Nig1}



Control   Ey-GAL4; dNudC-RNAi^{KK} Ey-GAL4; dNudC-RNAi^{Nig1}

Figure 8. Phenotypes of NudC knockdown flies.

(A) The phenotype summary of NudC knockdown flies in different specific tissue drivers.

(B-D) Knockdown of NudC results in atrophied wing phenotype. NudC RNAi were crossed with MS1096-GAL4 to allow ectopic expression of NudC-RNAi^{KK} and NudC - RNAi^{Nig1} in *Drosophila* adult wings.

(E-I) Knockdown of NudC results in a small eye phenotype that is autophagy-independent. NudC RNAi and Atg-RNAi were crossed with Ey-GAL4 to allow ectopic expression of NudC-RNAi and Atg-RNAi in *Drosophila* adult eyes.

(J-L') Cryo-scanning electron microscopy (cryo-SEM) images of NudC RNAi *Drosophila* adult eyes driven by Ey-GAL4 at different magnifications.

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