Niemann-Pick disease type C2 protein induces autophagy and inhibits growth in FM3A breast cancer cells

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Summary	Some peptides that are highly conserved between insects and mammals have anti-tumor action. Screening for inhibitors of cell growth from animal fluids may provide useful clues to anti-tumor drugs. Inducers of autophagy also have anti-tumor activity. The current authors recently studied a protein found in silkworm hemolymph, Niemann-Pick disease type C2 (NPC2). This protein, which is highly conserved among eukaryotes, was found to have anti-proliferative action on a silkworm cell line. The current study found that the silkworm NPC2 protein also inhibits the growth of FM3A murine breast cancer cells. In FM3A cells, silkworm NPC2 increased phosphorylation of AMP-activated protein kinase and decreased phosphorylation of Akt and mammalian target of rapamycin, which are regulators of autophagy. This study also found that NPC2 increased the amount of microtubule-associated protein light chain 3 (LC3)-II, an autophagosome marker, in FM3A cells. Silkworm NPC2 also induced an increase in the number of LC3-dots, a marker of pre-autophagic endosomes, in FM3A cells. When silkworm NPC2 was used to inhibit FM3A cell growth, that inhibition was attenuated by chloroquine, which inhibits autophagic activity by preventing lysosomal acidification. Murine NPC2 also inhibited growth and induced autophagy in FM3A cells. These findings suggest that NPC2 is involved in the induction and/or maintenance of autophagy and may help to elucidate the mechanisms underlying other neurodegenerative disorders such as Niemann-Pick disease.

Keywords: Niemann-Pick disease type C2, autophagy, breast cancer cell, silkworm

1. Introduction

The identification of natural products that suppress cancer cell growth provides clues to potential anti-cancer drugs. Animal fluids, including insect hemolymph, are often used as a resource with which to identify inhibitors of the growth of mammalian cancer cells (*1-5*). Some peptides are highly conserved between insects and mammals and are believed to function as tumor suppressors in humans (*6,7*). Therefore, screening for novel inhibitors of growth in animal fluids may provide clues to potential anti-cancer drugs as well as insight into endogenous anti-tumor systems.

Recent studies have revealed that autophagy is an important mechanism for suppressing tumors in animal bodies. The *BECN1* autophagy gene is monoallelically deleted in 40% to 75% of cases of human sporadic breast, ovarian, and prostate cancers (*8*). Atg genes encode canonical autophagy components and are associated with the suppression of cancer in mouse models. Mice with systemic mosaic deletion of *Atg5* and liver-specific *Atg7-/-* develop benign liver adenomas (*9*). AMP-activated protein kinase (AMPK) is an upstream kinase that positively regulates autophagy, whereas mammalian target of rapamycin (mTOR) is a negative regulator (*10*). AMPK activators and mTOR inhibitors, which are potent inducers of autophagy, have anti-tumor activity *in vitro* (*11-13*). The induction of autophagy might therefore be an effective method for controlling malignant cell growth.

The current authors recently reported that silkworm hemolymph inhibits proliferation of BmN4 cells, a silkworm-derived cell line, and that the silkworm NPC2 protein is the factor responsible (*14*). NPC2 is

highly conserved among eukaryotes, and mutations of the human *NPC2* gene are observed in 5% of patients with Niemann-Pick disease type C (*15,16*). The current authors previously reported that NPC2 activates AMPK in BmN4 cells (*14*). NPC-model mice, which carry a mutation in *Npc1*, present with a progressive loss of cerebellar Purkinje cells, hepatomegaly, and splenomegaly (*17*). Intriguingly, mice with impaired autophagy also have these features (*18-20*). These findings and the fact that AMPK is a positive regulator of autophagy led the current authors to hypothesize that NPC2 contributes to induction of the autophagy of malignant cells. This study has sought to verify whether both silkworm NPC2 and murine NPC2 induce autophagy and inhibit cell growth in the FM3A murine breast cancer cell line.

2. Materials and Methods

2.1. *Cell culture*

The FM3A murine breast cancer cell line was generously donated by Dr. Fumio Hanaoka (Gakushuin University, Japan). The FM3A cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), penicillin (100 IU), and streptomycin (100 µg/mL) at 33°C and 5% CO_2. For all cell growth assays, Western blotting, and immunofluorescent analyses, the culture medium for FM3A cells was replaced with DMEM without FBS and incubated at 33°C for 24 h in 5% CO_2. Samples containing medium were supplemented with 5% FBS instead of 10% FBS.

2.2. *Antibodies*

Anti-silkworm NPC2 antiserum was prepared as described previously (*14*). Primary rabbit antibodies to total AMPK (#2532), phospho AMPK (#2531), total Akt (#4691), phospho Akt (#9271), total mTOR (#2983), total S6K (#2708), microtubule-associated protein light chain 3 (LC3)-B (#2775), and β-actin (#4967) were obtained from Cell Signaling Technology Japan. Antibodies to phospho mTOR (#09-213) and phospho S6K (#07-018) were purchased from Millipore. Anti-rabbit IgG, HRP-Linked Whole Ab (from donkeys, #NA934) was purchased from GE Healthcare (Piscataway, NJ, USA). Anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated (from goats) (#sc-2012) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.3. *Preparation of silkworm hemolymph and recombinant proteins*

Abdominal legs of silkworm larvae (day 5 or 6 of 5th instar) were cut with scissors, and blood from the wound was collected in ice-cold tubes. Silkworm blood was incubated at 60°C for 30 min and then centrifuged. The supernatant was frozen in liquid nitrogen and stored at -80°C before use. This fraction is hereafter referred to as silkworm hemolymph. Recombinant silkworm NPC2 and murine NPC2 were prepared as described previously (*14*).

2.4. *Measurement of cell growth*

FM3A cells were suspended by pipetting and counted using a cytometer at the indicated time. To test the inhibition of growth by silkworm hemolymph, various media were prepared as follows: control (45% DMEM, 5% FBS, 50% saline, 50 IU penicillin, 50 µg/mL streptomycin); 20% silkworm hemolymph (45% DMEM, 5% FBS, 30% saline, 20% silkworm hemolymph, 50 IU penicillin, 50 µg/mL streptomycin), and 40% silkworm hemolymph (45% DMEM, 5% FBS, 10% saline, 40% silkworm hemolymph, 50 IU penicillin, 50 µg/mL streptomycin). For recombinant proteins, the sample volume was increased to 10% of the medium volume, and a vehicle (25 mM HEPES [pH 7.2], 10% glycerol, 100 mM NaCl) was used as the control..

2.5. *Western blotting and immunofluorescence analysis*

For Western blotting, the cells were harvested and lysed after treatment. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene-difluoride membrane (Millipore, Billerica, MA, USA). The membrane was immersed in 5% skim milk or 5% PhosphoBLOCKER™ (Cell Biolabs, Inc., San Diego, CA, USA) for 1 h at room temperature. After rinsing, the primary and secondary antibodies were allowed to react with Can Get Signal® reagent (TOYOBO) and detected using chemical luminescence. For immunofluorescence analysis, cells were harvested and washed with phosphate-buffered saline (PBS). Cells were suspended in ice-cold methanol and incubated at -20°C for 15 min. After washing twice in PBS, the cells were suspended in blocking solution (PBS containing 3% normal goat serum, 0.1% Triton X-100) and incubated at room temperature for 1 h. Cells were precipitated by centrifugation and suspended in blocking solution containing anti-LC3B (1:200) antibody. After incubation at room temperature for 1 h, the cells were washed three times with PBS and suspended in anti-rabbit FITC-conjugated IgG (1:200). Cells were further incubated in the dark for 1 h. Cells were then washed three times with PBS. Finally, the cells were gently mixed with ProLong® Gold Antifade with 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) and mounted on glass slides. Images were obtained on a Leica DM 4000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.6. *Statistical analysis*

For statistical analysis, experiments were performed at least twice and the data are presented as the mean ± SEM. To evaluate the growth rate, the slope of each growth curve was compared with the log-linear model. Significant differences were calculated using the Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. *Silkworm hemolymph inhibits growth of FM3A cells*

The current authors recently reported that silkworm hemolymph inhibits growth of BmN4 cells, a silkworm-derived cell line (*14*). To test whether silkworm hemolymph also inhibits the growth of mammalian cells, silkworm hemolymph was added to culture medium containing FM3A cells. The 40% silkworm hemolymph medium inhibited FM3A cell growth (Figure 1A). Boiling the hemolymph at 100°C for 5 min eliminated this inhibition of growth (Figure 1B). Since NPC2 protein in silkworm hemolymph inhibits BmN4 cell growth (*14*), the current study also evaluated whether NPC2 is capable of inhibiting FM3A cell growth. Cultures of FM3A cells with 20 μg/mL recombinant NPC2 had lower cell counts than cultures of FM3A without NPC2 (Figure 2A). The amount of NPC2 in silkworm hemolymph was determined using immunoblot analysis with anti-NPC2 antiserum. The amount of NPC2 in the hemolymph was estimated to be 240 μg/mL (*i.e.*, 20% (v/v) hemolymph contains 50 μg/mL NPC2; Figure 2B). This result suggests that NPC2 is present in a sufficient quantity in silkworm hemolymph to suppress FM3A cell growth.

3.2. *Silkworm NPC2 induces autophagy in FM3A cells*

The current authors previously found that silkworm NPC2 induces phosphorylation of AMPK, one of the major protein kinases responsible for signal transduction, in BmN4 cells (*14*). Therefore, the current authors hypothesized that NPC2 would also stimulate the phosphorylation of AMPK in FM3A cells. As expected, treatment with silkworm NPC2 increased AMPK phosphorylation in FM3A cells (Figure 2C). AMPK phosphorylation induces autophagy in mammalian cells (*10*). Therefore, the current authors hypothesized that silkworm NPC2 would induce autophagy in FM3A, resulting in apparent inhibition of cell growth. Immunoblot analysis was used to examine levels of LC3-II, an autophagosome marker, in FM3A cells treated with silkworm NPC2. Silkworm NPC2 increased the amount of LC3-II in FM3A cells (Figure 2C). mTOR, a well-known negative regulator of autophagy, was also examined. Akt and S6K, the upstream and downstream kinases of mTOR, respectively, were examined. Silkworm NPC2 decreased the phosphorylation of Akt, mTOR, and S6K in FM3A cells in a concentration-dependent manner (Figure 2C). Whether treatment with silkworm hemolymph modulated the Akt/mTOR/S6K signaling pathway in FM3A cells was then examined. Silkworm hemolymph induced both an increase in AMPK phosphorylation and a decrease in Akt, mTOR, and S6K phosphorylation in FM3A cells (Figure 2D). Silkworm hemolymph increased the amount of LC3-II in FM3A cells (Figure 2D). These findings suggest that NPC2 in silkworm hemolymph activates the AMPK and the Akt/mTOR/S6K signaling pathways, inducing autophagy in the FM3A murine cell line.

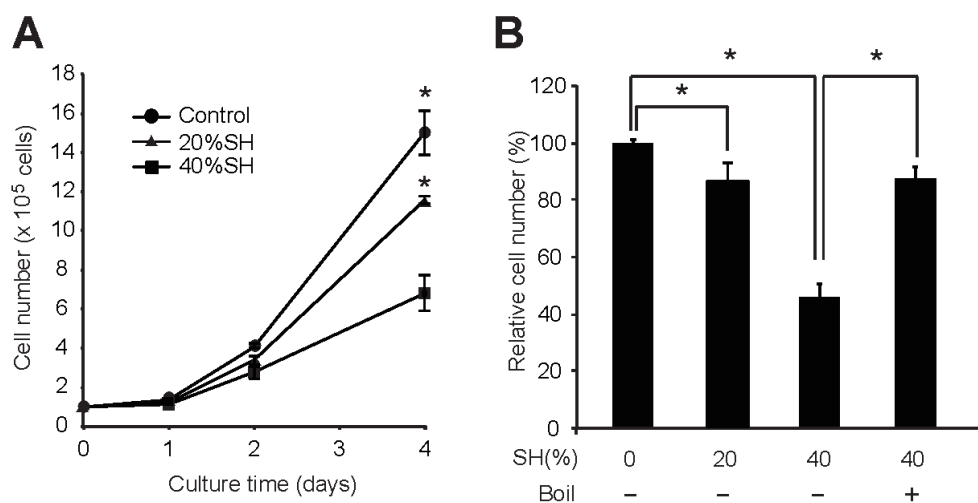Autophagosome formation is required for autophagy. Autophagosomes fuse with lysosomes to form

Figure 1. Inhibitory effects of silkworm hemolymph (SH) on FM3A murine breast cancer cell growth. (A) FM3A cells were incubated in culture medium containing 0% (circle), 20% (triangle), or 40% (square) silkworm hemolymph. Cell numbers were counted with a cytometer. Data are presented as the mean ± SEM of three experiments. Statistical significance between the slope of the control group and that of the sample groups was determined with the Student's *t*-test (*: *p* < 0.05). **(B)** Silkworm hemolymph was boiled at 100°C for 5 min before it was added to the culture medium. FM3A cells were cultured for 48 h and then counted. Data are presented as the mean ± SEM (*n* = 3). Statistical significance was determined with the Student's *t*-test (*: *p* < 0.05).
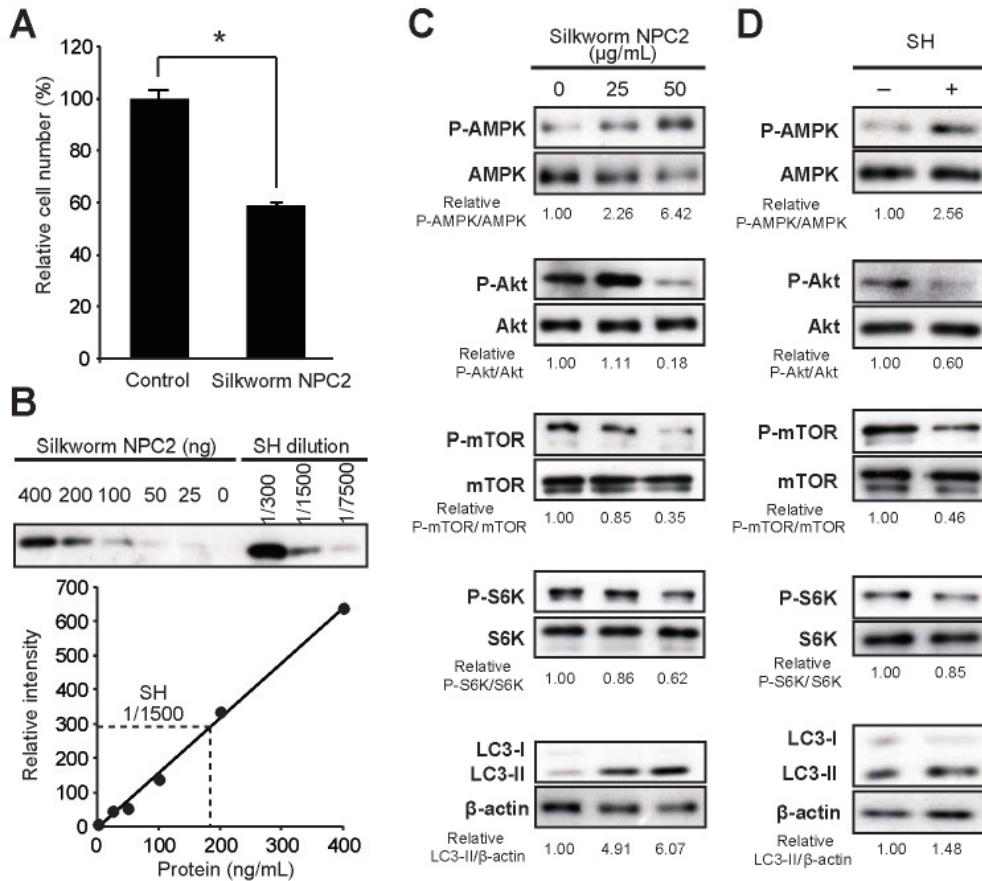
Figure 2. Silkworm NPC2 led to inhibition of growth, AMPK activation, and LC3-II accumulation in FM3A cells. (A) FM3A cells were cultured for 48 h in the presence or absence of 20 μg/mL silkworm NPC2. Cell numbers were counted with a cytometer. Data are presented as the mean ± SEM (n = 3). Statistical significance was determined with the Student's t-test (*: p < 0.05). **(B)** The amount of NPC2 protein in silkworm hemolymph was estimated using Western blotting. Indicated amounts of recombinant NPC2 protein and 10 μL of diluted silkworm hemolymph were subjected to Western blotting analysis with anti-silkworm NPC2 antiserum. Band intensities were quantified with an Image J-based densitometer. The amount of NPC2 protein in silkworm hemolymph was calculated using the linear regression curve of recombinant protein. **(C)** Images of Western blot analysis of FM3A cells treated with the indicated amount of silkworm NPC2 at 48 h are shown. **(D)** Images of Western blotting of FM3A cells treated with or without 20% silkworm hemolymph at 48 h are shown.

autolysosomes. Acidification of autophagosomes via fusion with lysosomes is required for the degradation of unnecessary organelles and bacteria captured by phagocytosis (*21*). Chloroquine prevents lysosomal acidification, resulting in the inhibition of autophagy (*22*). The current study examined the effect of chloroquine on inhibition of FM3A cell growth by silkworm NPC2. Chloroquine partially suppressed the inhibition of growth by silkworm NPC2 (10 μg/mL) in FM3A cells (Figure 3). Chloroquine's inhibition of autolysosome formation resulted in the accumulation of autophagosomes in several mammalian cell lines (*22*). The current study measured the autophagic flux affected by chloroquine and NPC2 using an accepted method (*23*). Chloroquine increased the accumulation of LC3-II in FM3A cells under experimental conditions (Supplementary Figure 1), suggesting NPC2 induced autophagic flux. These findings suggest that silkworm NPC2 induces autophagy, resulting in the inhibition of FM3A cell growth.

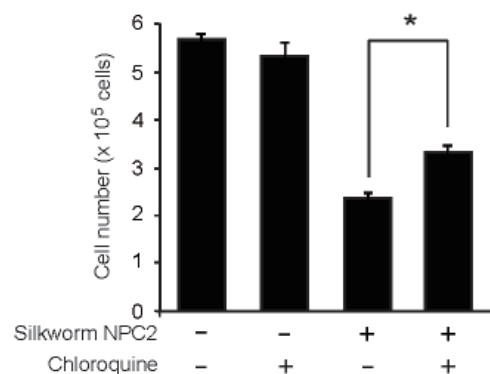### 3.3. *Murine NPC2 induced both cessation of cell growth and autophagy in FM3A cells*

Figure 3. Suppressive effect of chloroquine on inhibition of FM3A cell growth by silkworm NPC2. FM3A cells were cultured with or without 10 μg/mL silkworm NPC2 and/or 10 μM chloroquine. After culturing for 48 h, cells were counted with a cytometer. Data are presented as the mean ± SEM (n = 3). Statistical significance was determined with the Student's t-test (*: p < 0.05).

The biological activity of NPC2 in fibroblasts is interchangable among species (*14,15*). Thus, both silkworm NPC2 and murine NPC2 were predicted to
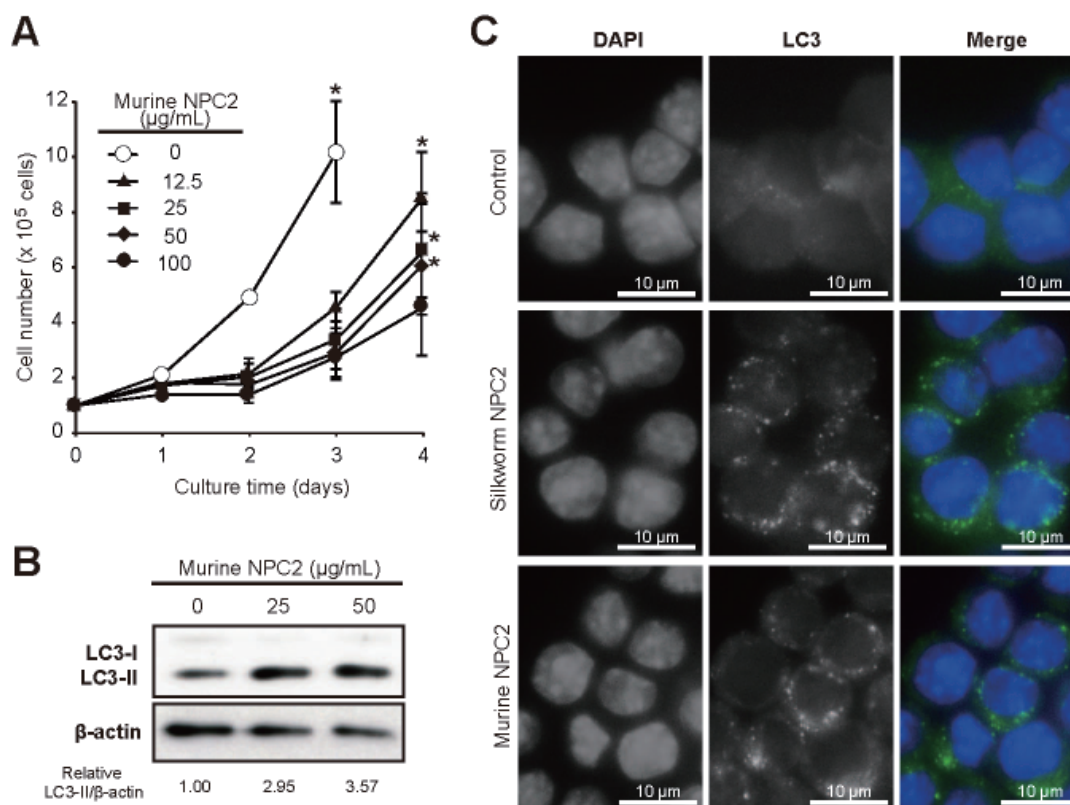
Figure 4. Inhibition of growth and induction of autophagy by murine NPC2 in FM3A cells. (A) FM3A cells were cultured with the indicated amount of murine NPC2 and cells were then counted. Data are presented as the mean ± SEM of two experiments. Statistical significance between the slope of the control group and that of the sample group was determined with the Student's *t*-test (*: $p < 0.05$). **(B)** The accumulation of LC3-II was detected after culturing for 48 h. **(C)** LC3 foci were detected with immunofluorescence analysis. FM3A cells were cultured with 20 μg/mL silkworm NPC2 or 20 μg/mL murine NPC2. Cells were fixed and permeabilized after 48 h. Anti-LC3B antibody was used as the primary antibody and anti-rabbit FITC-conjugated IgG antibody was used as the secondary antibody..

inhibit growth and induce autophagy in FM3A murine cancer cells. Murine NPC2 suppressed growth in FM3A in a dose-dependent manner (Figure 4A). Furthermore, LC3-II accumulated in FM3A cells treated with murine NPC2 (Figure 4B). Immunofluorescence analysis verified that treatment with murine or silkworm NPC2 increased autophagosome formation in FM3A cells (Figure 4C). These results suggest that both murine NPC2 and silkworm NPC2 lead to the induction of autophagy and inhibition of the growth of FM3A cells.

4. Discussion

The current findings revealed that NPC2 protein induced autophagy and inhibited cell growth in FM3A murine breast cancer cells. Both the AMPK and Akt/mTOR pathways were affected by NPC2 treatment, resulting in the induction of autophagy. To the extent known, this is the first study to report that NPC2 is a humoral factor that induces autophagy. However, non-specific inhibition caused by chloroquine cannot be ruled out as a possibility. Further analysis with more specific inhibition, such as genetic manipulation, will help to further clarify the association between the induction of autophagy and inhibition of growth by NPC2.

NPC2 is a highly conserved soluble protein in body fluids of various animal species. NPC2 is found in high concentrations in epididymal fluids (*24*). Some studies suggest that NPC2 functions as an endocrine factor (*14,25*). The current study found that both murine and silkworm NPC2 inhibited growth of FM3A cells. Thus, NPC2 appears to ubiquitously act as an anti-tumor endocrine molecule in both insects and mammals.

NPC1 is a membrane protein that is considered to function similarly to NPC2 during various physiological events because the phenotypes of NPC1-, NPC2-, and NPC1/NPC2-double knockout mice have an almost identical disease onset and progression, pathology, and neuronal storage (*26*). NPC1-deficient mice have phenotypes similar to those of mutant mice, which have phenotypes that include a defect in autophagy in terms of progressive loss of cerebellar Purkinje cells, axonal swelling, and hepatosplenomegaly. Both NPC2 and autophagy components affect hematopoiesis in hematopoietic stem cells (*20,25*) and adipogenesis in fibroblasts (*14,27,28*). The NPC1 and NPC2 proteins are thought to act together in the autophagic pathway. Recent studies suggest that NPC1 deficiency results in impaired completion of autophagy (*29,30*). The current results suggest that NPC2 is involved in the induction and/or

maintenance of autophagy. This notion may enhance the understanding of NPC and other neurodegenerative diseases.

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Supplementary Figure
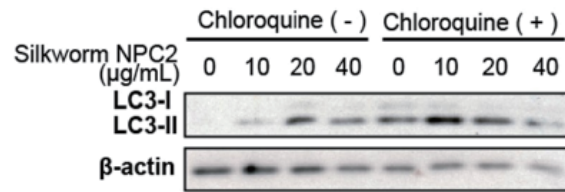
Supplementary Figure 1. Effect of chloroquine on LC3-II accumulation in FM3A cells. FM3A cells were cultured with or without the indicated amount of silkworm NPC2 and/or 10 μM chloroquine. After culturing for 48 h, the cells were subjected to Western blotting. LC3-II accumulation was detected using anti-LC3B antibody