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Trans-Golgi protein p230/golgin-245 is involved in phagophore formation

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ABSTRACT

p230/golgin-245 is a *trans*-Golgi coiled-coil protein that is known to participate in regulatory transport from the *trans*-Golgi network (TGN) to the cell surface. We investigated the role of p230 and its interacting protein, microtubule actin crosslinking protein 1 (MACF1), in amino acid starvation-induced membrane transport. p230 or MACF1 knock-down (KD) cells failed to increase the autophagic flow rate and the number of microtubule-associated protein 1 light chain 3 (LC3)-positive puncta under starvation conditions. Loss of p230 or MACF1 impaired mAtg9 recruitment to peripheral phagophores from the TGN, which was observed in the early step of autophagosome formation. Overexpression of the p230-binding domain of MACF1 resulted in the inhibition of mAtg9 trafficking in starvation conditions as in p230-KD or MACF1-KD cells. These results indicate that p230 and MACF1 cooperatively play an important role in the formation of phagophore through starvation-induced transport of mAtg9-containing membranes from the TGN. In addition, p230 itself was detected in autophagosomes/autolysosome with p62 or LC3 during autophagosome biogenesis. Thus, p230 is an important molecule in phagophore formation, although it remains unclear whether p230 has any role in late steps of autophagy.

1. Introduction

Macroautophagy (simply referred to as autophagy) is a ubiquitous eukaryotic system for the turnover of cytoplasmic materials in a lysosome-dependent manner, and is carried out by evolutionarily conserved Atg (autophagy related) factors. The autophagy process is initiated by the formation of the isolation membrane (phagophore). Then, the isolation membrane expands and closes to become a double membrane vesicle called the autophagosome [1]. It has been proposed that the autophagosome membrane originates from a number of sources, including the endoplasmic reticulum (ER), the Golgi apparatus, mitochondria, and the plasma membrane (PM) [2].

It is well accepted that the Golgi apparatus is one of the membrane sources of the phagophore, and that the existence of the vesicle fusion process is essential at the early step of autophagosome formation [3,4]. In fact, pre- and post-Golgi vesicle trafficking machineries, such as SNAREs, Rabs, and tethering factors, are required for the expansion of the phagophore [3]. The multi-spanning transmembrane protein Atg9 has been supposed to play an important role for the phagophore membrane biogenesis [4]. In yeast, during starvation, Atg9 vesicles derived from the Golgi are assembled into a preautophagosomal structure, and then membranes derived from Atg9 vesicles is incorporated into the phagophores [5]. Mammalian Atg9, known as mAtg9 or Atg9a, is localized at the *trans*-Golgi network (TGN) and at endosomes in feeding cells, and cycles among the endosomes, the TGN and phagophores/autophagosomes during starvation conditions [4]. Although the regulatory mechanism of mAtg9 trafficking has been studied [6–8], the precise role and transport mechanism of membranes with mAtg9 from the Golgi for autophagosome biogenesis is not sufficiently understood. Recently, it was demonstrated that

Abbreviations: AP2, adaptor-protein 2; EBSS, Earle's balanced salt solution; ER, endoplasmic reticulum; KD, knock-down; MACF1, microtubule actin crosslinking protein 1; LC3, microtubule-associated protein 1 light chain 3; PM, plasma membrane; TGN, *trans*-Golgi network.

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AP2/clathrin-dependent endocytosis plays an important role in the transport of mAtg9 from the PM to the phagophores, suggesting that mAtg9 trafficking from the TGN to the PM is important in autophagosome biogenesis [9].

The TGN coiled-coil protein p230 (also known as golgin-245 or t-golgin-1) is a GRIP domain-containing protein (GRIP-golgin), and is peripherally associated with the cytoplasmic face of the TGN membrane [10]. p230 is required for the secretion of TNF induced with LPS in mouse macrophages [11] and is involved in HLA class I surface expression induced by INF-γ [12]. Kakinuma et al. [13] reported that p230 interacts with a protein that crosslinks microtubules to the actin cytoskeleton microfilament1 (MACF1), and that the disruption of this interaction inhibits the transport of the p230-specific cargo, GPI-anchored protein from the TGN to PM. These results suggest that p230 plays a role in the regulative transport of specific proteins from the TGN to the PM.

In this study, we investigated the role of p230 and its interacting protein, microtubule actin crosslinking protein 1 (MACF1), on amino acid starvation-induced membrane transport. p230 participation in autophagy was examined using p230 knock-down (KD) cells, and revealed that p230 and its interacting protein MACF1 cooperate in the early step of autophagy involving the transport of mAtg9. In addition, p230 itself is recruited to peripheral structures and the PM with mAtg9 in response to starvation, and is detected in the phagophores/autophagosomes with p62 or microtubule-associated protein 1 light chain 3 (LC3) during autophagosome biogenesis.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies used were as follows: mouse monoclonal anti-golgin-97 (Life technologies Inc., Rockville, MD), anti-p62 and anti-p230 (BD Bioscience, San Diego, CA), anti-LC3 (M152-3 and M186-3; MBL, Nagoya, Japan), and anti-β-actin (clone AC-15; Sigma–Aldrich, St. Louis, MO); rabbit monoclonal anti-mAtg9 (Abcam, Cambridge, UK); rabbit polyclonal anti-ACF7/MACF1 (Merck Millipore, Darmstadt Germany), anti-AP2B1 (Proteintech Group Inc., Chicago, IL), anti-LC3 (PD036), anti-p62 (PM045) (MBL), and anti-Arl1 (a gift from Dr. Kazuhisa Nakayama); and AlexaFluor^{488}-conjugated goat anti-rabbit, or anti-mouse IgG, and AlexaFluor^{546}-conjugated goat anti-rabbit or goat anti-mouse IgG (Life Technologies Inc.). E64d and pepstatin A were purchased from the Peptide Institute Inc. (Osaka, Japan), and dynasore was from Sigma–Aldrich.

2.2. Plasmid construction and transfection

cDNA fragments of MACF1 were obtained by PCR of HeLa cell oligo dT-primed cDNA as the template using the DNA primer 5′AGAATTCTTAGCACTGGAGCGGCAAAGGAAACTG3′ and 5′AGTCGACTCATCGATACGCATCCTTGTTGGGATG3′ for the p230-binding domain (amino acid number 5044–5156) or 5′AGAATTCGATACCAGCAATAGTTCTTCCCCG3′ and 5′ACTCGAGTTATCGCTTGGGACCTGGAGTCC3′ for the non-specific domain (amino acid number 5328–5430). DNA fragments were subcloned into the pSG5 expression vector, followed by attachment with an HA-tag sequence. Cells were transfected using Fugene 6 transfection reagent (Promega Corp., Madison, WI) and incubated for 24 h.

2.3. Cell culture and siRNA treatment

HeLa cells were maintained in Eagle's MEM with nonessential amino acids and 10% FCS. RNA interference was performed in HeLa cells using Lipofectamine RNAiMAX (Life Technologies Inc.) with ON-TARGETplus small interfering RNA (siRNA) (Thermo Fisher Scientific, Waltham, MA) for p230 and Stealth siRNA for MACF1 (Life Technologies Inc., HSS146438). For p230-KD experiments, one day after treatment, cells were re-treated with siRNA oligos for an additional day. p230 was tagged with the sequence GUAGAUGACUGGUCCAAUA and the nonspecific control duplex was used as a control. For MACF1-KD experiments, one day after treatment, cells were re-treated with siRNA oligos for an additional day.

2.4. Cell lysate preparation and immunoprecipitation

Cells were harvested and lysed in lysis buffer as described previously [14]. For immunoprecipitation, cells were lysed in 1% TX-100 lysis buffer. The resultant lysate was subjected to immunoprecipitation using protein A-Sepharose as described previously [14].

2.5. SDS–PAGE and immunoblotting

Cell lysates and immunoprecipitates were analyzed by SDS–PAGE (4%, 7% or 15% gels) and immunoblotting with the indicated antibodies. The immunoreactive proteins were visualized using an ECL-Prime kit or ECL-Select kit (GE Healthcare, Piscataway, NJ) by a LAS4000 imaging system (Fujifilm, Tokyo, Japan).

2.6. Immunofluorescence staining and fluorescence microscopy

After the desired incubation times, cells were fixed and immunostained as previously described [14]. Stained cells were observed with an LSM PASCAL confocal microscope with a ×63 objective (Carl Zeiss, Jena, Germany). Confocal images are presented as sections of 0.4 μm in thickness.

3. Results and discussion

3.1. The depletion of p230 or its binding partner MACF1 inhibits autophagy

To explore roles of p230 and MACF1 on autophagy, p230-KD or MACF1-KD HeLa cells were investigated. The immunoblotting analysis showed that the each protein was knocked down efficiently, whereas the amounts of MACF1 and p230 in p230-KD and MACF1-KD cells, respectively, were almost the same as those in control siRNA-treated cells (Fig. 1A). We monitored the autophagic flux in p230-KD and MACF-KD cells. After a 2 h incubation in Earle's balanced salt solution (EBSS), with or without lysosomal protease inhibitors (E64d and pepstatin A, 10 μM each), cells were subjected to immunoblotting for LC3 to monitor the conversion from LC3-I to LC3-II [15]. LC3-II was significantly accumulated in control cells with protease inhibitors in starvation conditions as compared with that in nutrient-rich conditions. In contrast, the accumulation of LC3-II was not observed in either feeding or starvation conditions in p230-KD and MACF1-KD cells (Supplementary Fig. S1). About a 1.9-fold increase of autophagic flux was observed in control cells under starvation compared with under feeding, although a lower level of autophagic flux was found in both in p230-KD and MACF1-KD cells (Fig. 1B). To ensure the effect of depletion of p230 and MACF1 on autophagy, we performed fluorescence microscopy assays of LC3-positive puncta. Upon starvation, many LC3-positive puncta were observed in control cells, whereas fewer ones were found in p230 or MACF1 siRNA-treated cells (Supplementary Fig. S2). Upon starvation, the number of LC3 puncta per cell increased only 1–1.5-fold in KD cells, in contrast to the 8-fold increase in control cells (Fig. 1C). This suggests that

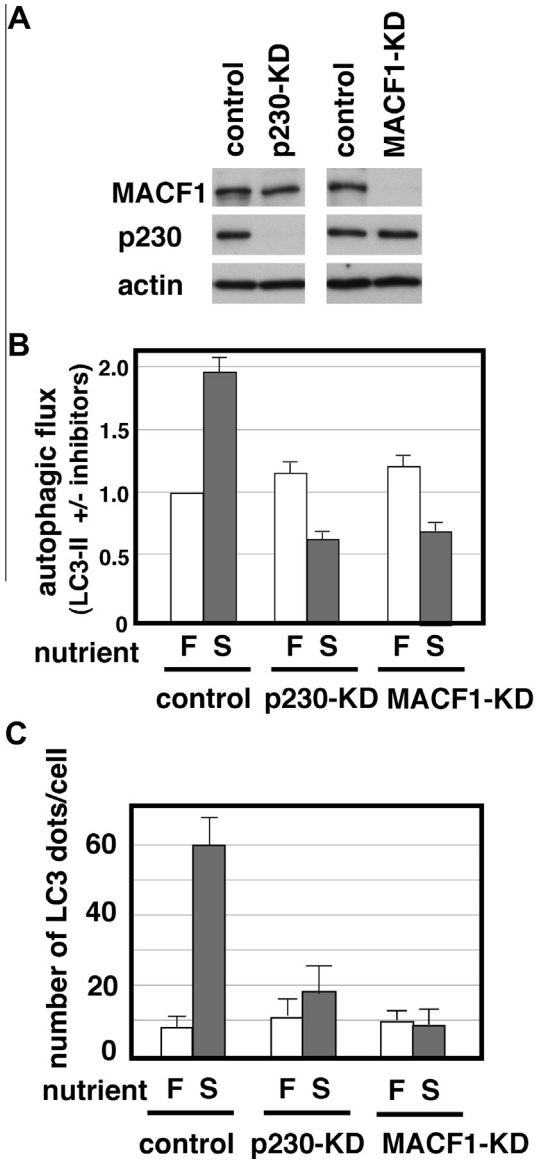

Fig. 1. The loss of p230 or MACF1 inhibits the autophagic flux. (A) Cell lysates prepared from control, p230, or MACF1-KD cells were analyzed by western blotting for the indicated protein. (B) Effect of p230- or MACF1-depletion on the autophagic flux. Immunoblotting analyses shown in Supplementary Fig. S1 were scanned for quantification of LC3-II. F: feeding, S: starvation. Autophagic flux was calculated as the ratio of LC3-II levels between samples with or without protease inhibitors, and is shown as mock-transfected cells under feeding conditions set to 1 (means ± S.D., $n = 3$). (C) Formation of LC3-positive dots was impeded in nutrient-starved p230- or MACF1-KD cells. Quantification of the formation of LC3 dots in immunofluorescence microscopy observations as shown in Supplementary Fig. S2 (100 cells each, means ± S.D., $n = 3$).

p230 has an essential role in the formation of LC3-positive structures induced by amino acid depletion.

3.2. p230 and MACF1 are involved in mAtg9 relocation under starvation conditions

Atg9 is a key regulator of autophagy induction to provide membranes for the formation of phagophores in yeast and mammalian cells during starvation [5,16]. mAtg9 is a transmembrane protein and cycles among the endocytic compartments, TGN, and phagophore membranes [4]. When amino acid starvation is induced, mAtg9 redistributes from the TGN and transiently attaches to LC3-positive structures. Given that mAtg9-depleted cells fail in the biogenesis of LC3-positive structures, mAtg9 is a candidate Atg for the delivery of membrane or membrane-associated factors to the forming autophagosomes via the TGN or endosomes [17]. To explore how p230 or MACF1 depletion affects the distribution of mAtg9, cells treated with control, p230 or MACF1 siRNA were incubated in the complete medium or EBSS, fixed, and stained with antibodies for mAtg9 and golgin-97 (a TGN marker) [18] (Fig. 2A). In control cells, mAtg9 was found both on the juxtanuclear TGN membrane and peripheral dot structures under feeding conditions, and nutrient deprivation caused the redistribution of mAtg9 away from the TGN, as previously reported [7]. In p230- or MACF1-depleted cells, mAtg9 was found in the juxtanuclear TGN region and on peripheral dots under feeding conditions, the same as in control cells. The TGN distribution of mAtg9, however, remained unchanged under starvation conditions in p230- and MACF1-KD cells. These results suggest that p230 and MACF1 play an important role in the starvation-induced transport of mAtg9-containing membranes from the TGN to the target site.

To ensure the involvement of the p230 and MACF1 interaction in mAtg9 transport, we examined the relocation of mAtg9 in starved cells by overexpressing the p230-binding domain of MACF1 (binding domain) [13]. Cells were transfected with an expression vector containing the HA-tagged binding domain (amino acids 5044–5156) or a non-specific region of MACF1 (non-specific domain: amino acids 5328–5430) [13,19], and incubated for 24 h. The transfected cells were incubated additionally in complete medium or EBSS, fixed, and immunostained for HA and mAtg9. In feeding cells, the juxtanuclear TGN membrane and peripheral dot staining of mAtg9 were observed in both cells overexpressing the binding domain and the non-specific domain. Under starvation conditions, mAtg9 relocation to peripheral structures from the TGN was impaired in p230-binding domain-overexpressing cells, in contrast to non-specific domain overexpressing cells (Fig. 2B). Next we tested the effect of p230-binding domain overexpression on LC3 puncta formation under starvation conditions. As expected, under starvation conditions, many LC3 dots were found in the non-specific domain-overexpressing cells as observed in the non-transfected cells, whereas a small number of LC3 dots were detected in the binding domain-overexpressing cells. When the numbers of LC3 dots per cell were compared between feeding and starvation conditions, we found that the number of LC3 dots per cell increased only 1.8-fold in starved cells with binding domain overexpression, in contrast to a 4- or 5-fold increase in starved cells with non-specific domain overexpression or no-transfected (Supplementary Fig. S3). The results strongly support the finding obtained from the p230-KD and MACF1-KD experiment, suggesting that the interaction of p230 and MACF1 is involved in mAtg9 relocation from the TGN to peripheral structures, which arise from amino acids starvation.

3.3. p230 remains in autophagosomes during the maturation process

The dynamics of p230 localization in starved cells were investigated by further characterization of p230 in autophagy. Cells were incubated in EBSS for 2 h with lysosomal protease inhibitors, fixed, and immunostained for p230 and p62 [20] or LC3 [21]. As shown in Fig. 3A, p230 was co-localized with both marker proteins of the autophagosome, suggesting that p230 can be incorporated into the pre-autophagosome and remains in the autophagosome, similar to p62 and LC3. The co-localization of p230 with p62 was confirmed by the findings that p230 and p62 are co-precipitated with anti-p230 antibodies in a 1% TX100 extract of HeLa cells and that the precipitated p62 is increased upon starvation (Fig. 3B). A single band of immunoprecipitated p230 was found in the cell lysate, indicating that the interaction of p230 and p62 is unlikely due to

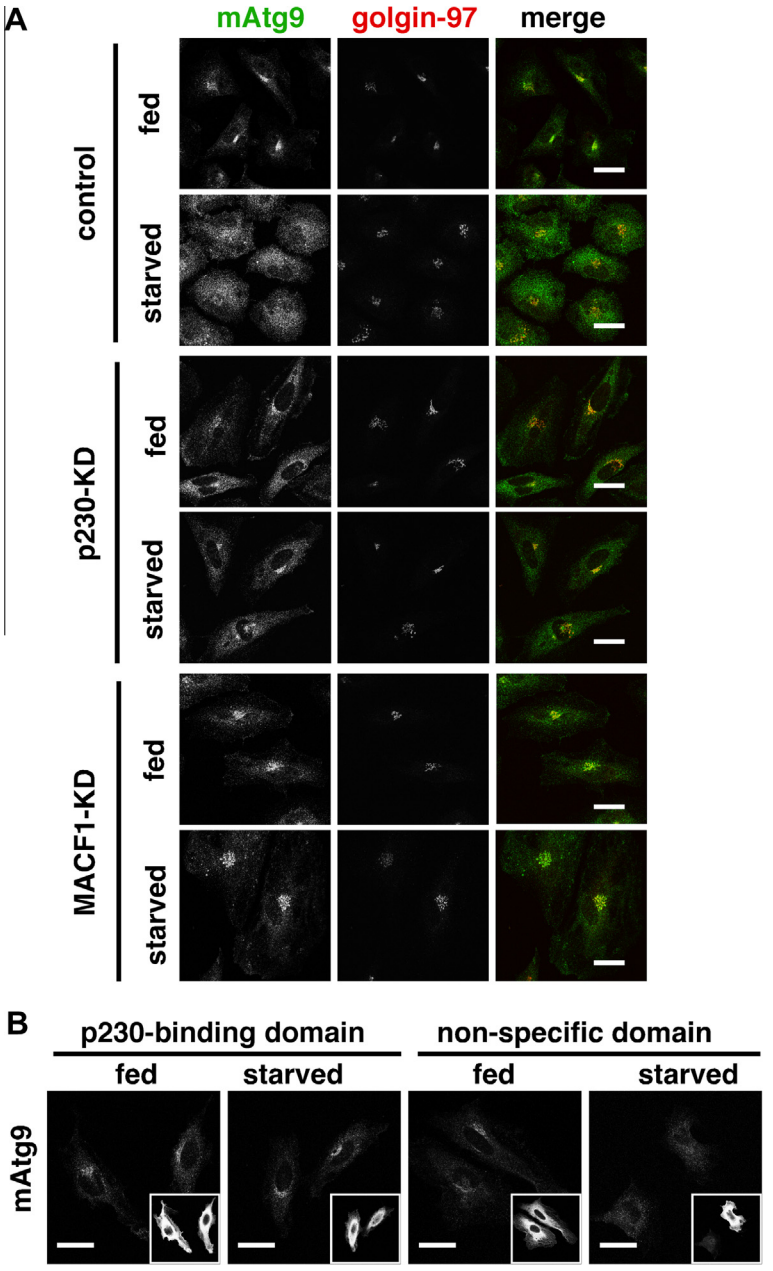

Fig. 2. p230 and MACF1 are involved in mAtg9 relocation under starvation conditions. (A) Starvation-induced relocalization of mAtg9 was inhibited in p230-KD or MACF1-KD cells. Cells were treated with control, p230, and MACF1 siRNA as in Fig. 1. Cells were incubated in complete medium (fed) or EBSS (starved), then fixed and immunostained for mAtg9 and golgin-97. Bars, 20 μm. (B) Overexpression of the p230-interacting domain of MACF1 affects the relocation of mAtg9 in autophagy-induced cells. Cells were transfected with the HA-tagged p230-binding domain of MACF1 or a non-specific domain of MACF1. After a 24-h incubation, cells were further incubated in complete medium (fed) or EBSS (starved) for 30 min, fixed, and immunostained with antibodies for mAtg9 and HA. HA-staining of each panel is shown in the inserts. Bars, 20 μm.

the aggregation of polyubiquitinated p230 with p62 [22]. As expected from its co-localization with autophagosomes, the amount of p230 was significantly decreased in amino acid starved cells (Fig. 3C). p230 is recruited onto the TGN membrane by association with Arl1-GTP through its GRIP domain [10]. However, the Arl1 content was not significantly changed between feeding and starving cells (Fig. 3C). Under starvation conditions, Arl1 was localized on peripheral structures, some of which were co-localized with LC3, in addition to the juxtanuclear structure that corresponds to the TGN (Fig. 3D). It is likely that p230 and Arl1 in the TGN-derived membrane are transported to and incorporated into the LC3-positive structure. This suggests that a portion of p230 remains in the autophagosomes and is finally digested in the autolysosomes, whereas Arl1 might be separated from mature autophagosomes like Atg16L [23].

3.4. p230 and MACF1 are involved in the autophagosome formation

Co-localization of p230 with pre-autophagosomes and mature autophagosomes raise a question whether p230 is transferred to pre-autophagosomes from the PM or endosomes. Adaptor-protein 2 (AP2) and clathrin-mediated endocytosis are required for mAtg9 sorting to phagophores [9], and membrane supply from the PM is essential for phagophore expansion [24]. Although p230 has been shown to regulate anterograde trafficking event [11–13,25], the movement of p230 itself from the TGN to the PM has not been examined. To determine the traffic route of p230 from the TGN to phagophores, we examined p230 localization in cells treated with the dynamin 2 inhibitor dynasore [26]. As shown in Fig. 4, few p230 dots that co-localized with the AP2 complex were found in mock-treated cells. A significant increase of p230 that

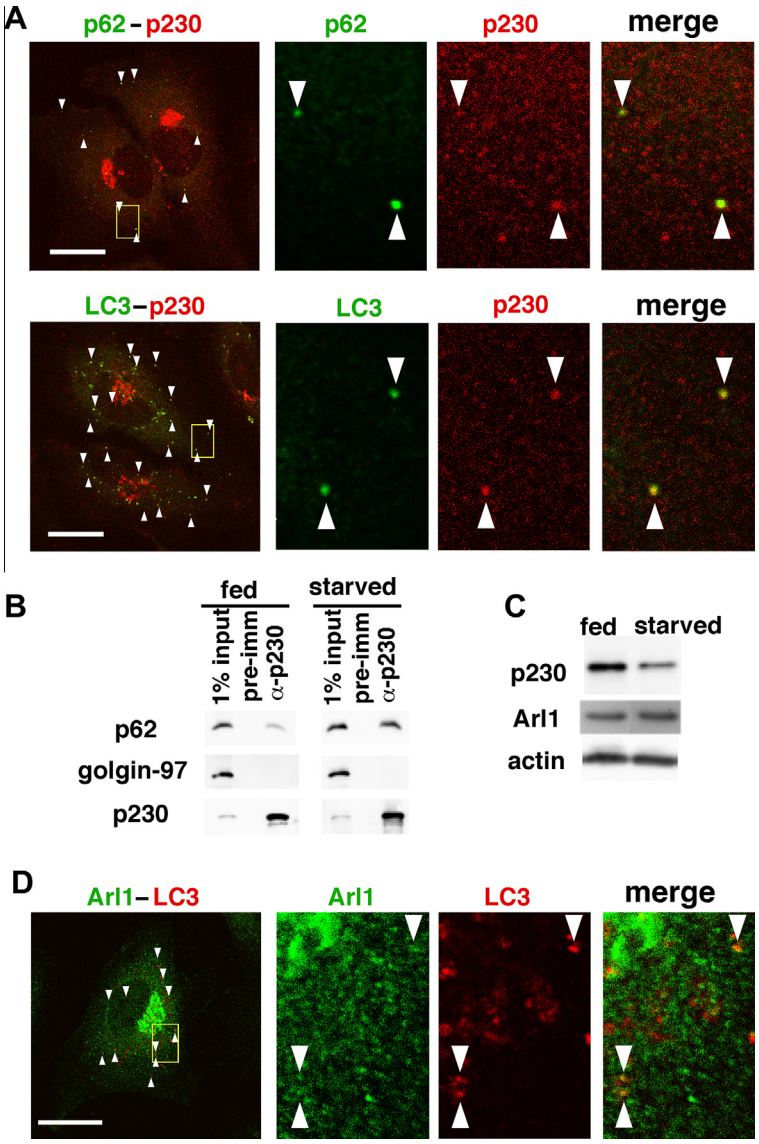

Fig. 3. p230 is localized with pre-autophagosomal structures during the maturation process. (A) Co-localized of p230 with p62 and LC3 in starved cells. Cells were incubated in EBSS for 60 min, and fixed and stained with antibodies as indicated. Enlarged profiles of boxed areas are shown. HeLa cells were treated as in Fig. 2 and stained with antibodies for LC3 and p230. Arrowheads indicate colocalized signals of p230 with p62 or LC3. (B) Co-immunoprecipitation of p230 with p62. Cells were incubated under feeding and starvation conditions as indicated in Fig. 1B, then lysed, and subjected to immunoprecipitation with antibodies for p230, followed by immunoblotting analysis for the indicated proteins. (C) p230 was decreased in nutrient-starved cells. Cells were cultured under feeding and starvation conditions, and processed for immunoblotting for the indicated proteins. (D) Arl1 localized in LC3-positive structures in nutrient-starved cells. Cells were incubated for 2 h in EBSS, fixed and stained with antibodies for Arl1 and LC3. Arrowheads indicate colocalized signals of Arl1 and LC3.

co-localized with AP2 in dynasore treated cells was observed both under feeding and starvation conditions. These results suggest that p230 is transported from the TGN to the PM and then moves to the phagophores by dynamin-mediated pinch-off of transfer vesicles, as observed for the mAtg9 carrier membranes [9].

It was reported that the interaction between p230 and MACF1 is involved in the v-SNARE VAMP7 transport from the TGN to the PM [27]. VAMP7 is thought to play an important role in homotypic fusion of the precursor of phagophores [23]. Thus, it is likely that the interaction of p230 and MACF1 is involved in transport of machineries not only for phagophore fusion, but also for membrane supply. The finding that p230-KD and MACF1-KD cells failed to form LC3-positive dots confirms the significance of these molecules in the phagophore formation, and strongly support the idea that the interaction between p230 and MACF1 is involved in a very early stage of autophagy. Taken together, previous reports and our results suggest that p230, through its interaction with MACF1, provides a molecular link between specific vesicles with mAtg9 along the microfilament railway and/or microtubules from the TGN to target structures, such as the PM. Beta-actin is known to have a crucial role in the early stage of autophagosome formation and is linked to the PtdIns3P-generation step [28]. Activation of myosin II promotes starvation-induced autophagy and mAtg9 trafficking from the TGN to peripheral structures along microfilaments [29]. It might be that the Golgi membrane decorated with p230 interacts with actin filaments through MAFC1, and that ULK1-activated myosin II drives p230-labeled transport carriers containing mAtg9 from the TGN to the phagophore. Furthermore, p230 is recruited in phagophores/autophagosome, and finally degraded in starvation dependent manner. The detailed mechanism of p230 in anterograde transport of membrane from the TGN to forming phagosomes remains elusive, and it is unclear whether or not p230 has

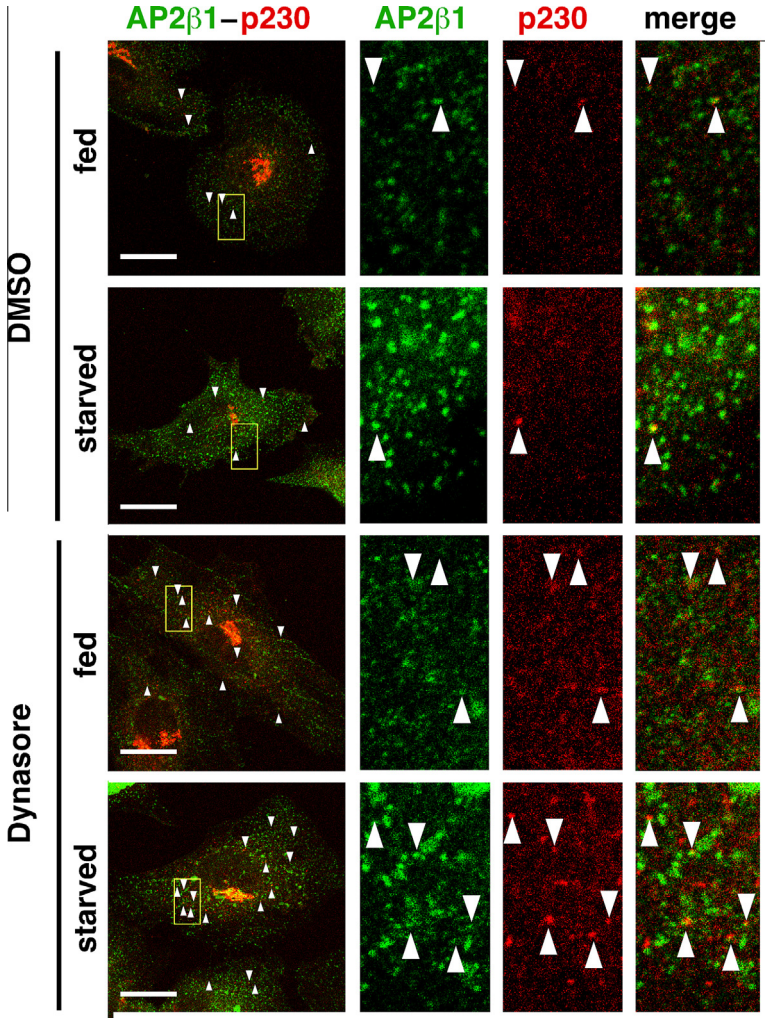

Fig. 4. p230 was transported to phagophores through the PM. p230 was localized in the PM and transported to phagophores via dynamin 2-dependent internalization. Cells were treated with DMSO or 100 μM dynasore for 15 min and then incubated in complete medium (fed) or EBSS (starved) for 30 min. At the end of incubation, cells were fixed and stained with indicated antibodies. Arrowheads indicate colocalized signals of AP2β1 and p230. Bars, 20 μm. Enlarged profiles of the boxed areas are shown.

any roles in the forming autophagosome. Further analysis of the role of p230 in autophagy is needed to understand the mechanism of autophagy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.071.

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