4th Progress report

June/25/2007 Nobutaka Ohgami
(Current lab: Dept. of Biomedical Sciences, Chubu University, JAPAN
: Former lab: TY Chang's laboratory)

Title: Demonstration of NPC1 as a lipid binding protein *in Vitro*

This present study aims at testing the possibility that NPC1 may directly bind cholesterol and/or other lipid.

SPECIFIC AIMS:

1. **Does azocholestanol (AC) bind to the NPC1 within the sterol-sensing domain (SSD) of NPC1?**
2. **Does NPC1, NPC2, and/or caveolin-1 form a protein complex *in vitro*?**
3. **Does NPC1 protein also bind fatty acids, and/or sphingolipids? Is the *in vitro* cross-linking between NPC1 and the azocholestanol affected by the presence of other ligands such as fatty acids or glycosphingolipids?**

(For the specific aim 1)

In order to identify a binding site within the SSD of NPC1 protein directly binding to the photoactivatable cholesterol analog (azocholestanol: AC), I will perform a proteolytic enzyme digestion with *Achromobacter lyticus* protease I (Lys-C) or trypsin to obtain the AC-labeled fragment(s). For this purpose, one of the keys to get a successful result would be to isolate the AC-labeled fragment(s) with an efficient isolation system. So far, in order to isolate a protein fragment labeled with a photoactivatable ligand, some literatures have been employing the gel system described by Schagger and von Jagow (1) (4% stacking gel, 10% spacer gel, and 16.5% separating gel), or antibodies against specific peptides (see the example (2)). However, these attempts basically require lots of pilot experiments to establish tools, such as preparing several anti-specific antibodies, if there is information about an expected binding site beforehand. Meanwhile, Kawahara et al., previously reported an interesting attempt that, after photoaffinity labeling of albumin with Ca channel antagonist followed by the proteolytic digestion, an anti-antagonist antibody which they prepared allowed them to effectively isolate the labeled fragments, resulting the identification of these labeled fragments with matrix assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS) eventually (3). Besides, in the case of the cholesterol-protein interaction as well, Ihara et al has reported that biotinylated θ-toxin (BCθ) bound with avidin beads, which has been known to be the cholesterol binding toxin, enabled them to isolate the vesicles containing the known cholesterol-binding protein, caveolin-1, the cellular cholesterol-dependently (4). Based on these reports, I came up with a possibility to efficiently isolate the AC-labeled fragments with the BCθ-avidin beads, after the proteolytic enzyme digestion of the photolabeled NPC1 protein with AC (Fig 1). For this purpose, I have performed several pilot experiments as follows: (i) BCθ-avidin beads

actually worked to isolate the vesicles containing caveolin-1 (Fig. 2, lane 3), whereas the pre-treatment with methyl beta cyclodextrin (MβCD) did not isolate it (Fig. 2, lane 4), suggesting this isolation system worked the cellular cholesterol-dependently as previously reported (4). (ii) Mammalian cells endogenously contain lots of lipids including cholesterol. Therefore, the key for this attempt would be to get rid of the cellular cholesterol as well as "free AC" completely, except for "bound AC" forming the covalent bond to the cellular proteins, otherwise BC-theta avidin beads might give me a lot of pseudo AC-labeled fragments. For this purpose, I tried to perform the cellular lipid extraction with chlroform:methanol (2:1, v:v) after the intact cell photolabeling with [^{3}H]AC. The intact cell photolabeling with [^{3}H]AC, followed by the lipid extraction clearly showed the labeled bands except for "free [^{3}H]AC" observed in the bottom of the radioluminography (Fig. 3, lane 2). In sharp contrast, the lipid extraction without the photolabeling with [^{3}H]AC did not show the labeled bands at all (Fig. 3, lane 3), suggesting the lipid extraction system did get rid of "unbound [^{3}H]AC" as well as "free [^{3}H]AC". (iii) Next, using this isolation system, I tried to isolate the AC-labeled proteins. The preliminary result showed that, after the intact cell labeling with AC, the lipid extraction followed by the isolation with the BC-theta-avidin beads showed several bands (Fig. 4, lane 2). In contrast, the isolation with the BC-theta-avidin beads without photolabeling did not exhibit these bands except for the band at 38 kDa that seems to be BC-theta, the molecular weight basis (Fig. 4, lane 3), suggesting that BC-theta-avidin beads could isolate the AC-labeled proteins. Once I make sure the reproducibility of this result, I will try to identify them with the mass spectrometer (MS). Hopefully, these bands contain unknown or known cholesterol-binding proteins including NPC1. Also, I will isolate the AC-labeled fragments in the presence of a detergent, such as Nonidet P-40 at less than 0.5% with the BCθ-avidin beads after the proteolytic enzyme digestion of the AC-labeled NPC1 protein. And then, I will try to identify the AC-labeled fragments with the MS eventually.

So far, I have been using commercial mouse anti-GFP monoclonal antibodies to isolate the AC-labeled NPC1-6xHis -CFP protein. For this project, however, since I plan to perform a large-scale purification of AC-labeled NPC1-6xHis-CFP protein after the photolabeling with AC, I tried to use other isolation system other than using the monoclonal antibodies. As a pilot experiment, I tired to use Ni-NTA resin (Novagen) to isolate the NPC1-6xHis-CFP protein after intact cell photolabeling with AC. Against my expectation, the labeling with AC followed by the isolation of NPC1-6xHis-CFP protein with the Ni-NTA resin did not work (Fig. 5, lane 2), in sharp contrast to the isolation without the labeling (Fig. 5, lane 3), presumably because of its conformational change due to photo-crosslinking to AC. This result suggests that it would be much better for this purpose to perform photolabeling with AC **after** the isolation of the NPC1-6xHis-CFP protein with the Ni-NTA resin.

(For the specific aim 2)

To find NPC1-associated protein(s), I will simply purify the NPC1 protein from the CT43-NPC1-CFP stable clones with the Ni-NTA chromatography. Once I find protein(s) besides NPC1 on SDS-PAGE,

followed by the Coomasie-blue staining, I will identify that protein by MALDI-TOF after SDS-PAGE.

(**For the specific aim 3**)
To analyze indirectly whether NPC1 protein also binds fatty acids, and/or sphingolipids, I will perform the protection assay against *in vitro* cross-linking of NPC1 with azocholestanol by other lipids such as fatty acids or glycosphingolipids.

(Figure legend)
Fig. 1. Strategy to isolate azocholestanol (AC)-labeled fragment(s) with BC-theta-avidin beads.

Fig. 2. Pilot experiment: isolation of caveolin1 with BC-theta-avidin beads. The isolation procedure with BC-theta-avidin beads basically followed the previous report (4) except for using 25RA cells, followed by Western blotting with rabbit anti-caveolin1 polyclonal antibodies (Santa Cruz).

Fig. 3. Pilot experiment: lipid extraction from 25RA cells after photolabeling with [^{3}H]AC.
Except for lane 3, 25RA cells were photolabeled using [^{3}H]AC/cyclodextrin complex (0.32 μg, 0.5 μCi). After photolysis, the [^{3}H]AC-labeled cells were washed with Hanks' buffer containing 25 mM DTT. Except for lane 1, the cellular lipids were extracted using 3 ml chlroform:methanol (2:1, v:v) at 4˚C. After removing the organic solvent by centrifugation, the cells were lysed with 10% SDS. These lysates were subjected to SDS-PAGE, followed by radioluminography.

Fig. 4. Isolation of cholesterol-binding protein using BC-theta-avidin-beads. 25RA cells were photolabeled using MβCD:AC complex (molar ratio: 64:1) **(lanes 1 and 2). For lanes 2 and 3,** after washing the cells, the cellular lipids were extracted using 3 ml chlroform:methanol (2:1, v:v) at 4˚C. After removing the organic solvent by centrifugation, the cells were lysed using 1% TX-100, 0.05% SDS. The lysates were incubated with BCθ (final concentration: 10 μg/ml) in PBS containing 0.1% BSA for 1 h at 4˚C. After incubation, the reaction mixtures were further incubated with Dynabeads M-280 Streptavidin for 1 h at 4˚C. After washing the beads, the isolated proteins were collected with 10% SDS, followed by SDS-PAGE. The isolated proteins were detected by the silver staining.

Fig. 5. Isolation of NPC1-6xHis-YFP protein with Ni-NTA resin after photolabeling with AC.
After photolabeing CT60-NPC1-6xHis-YFP stable clone with (**lane 2**) or without AC (**lane 3**), the isolation of NPC1-6xHis-YFP protein with NiNTA resin (Novagen) was carried out based on the manufacturer's manual. And then, the isolated proteins were detected with Western blotting with rabbit anti-GFP polyclonal antiserum (Abcam$_{290}$).

(Reference)
1. Schagger, H., and von Jagow, G. (1987) Anal. Biochem., 166, 368–379

2. Kuniyasu A, Itagaki K, Shibano T, Iino M, Kraft G, Schwartz A, Nakayama H. (1998) J Biol Chem., 273, 4635-4641.
3. Kawahara K, Kuniyasu A, Masuda K, Ishiguro M, Nakayama H. (2002) Biochem J., 363, 223-232
4. Wada S, Morishima-Kawashima M, Qi Y, Misono H, Shimada Y, Ohno-Iwashita Y, Ihara Y (2003) Biochemistry. 42, 13977-13986.

Attempt to isolate azocholestanol (AC)-labeled fragment(s) with BC-theta-avidin beads

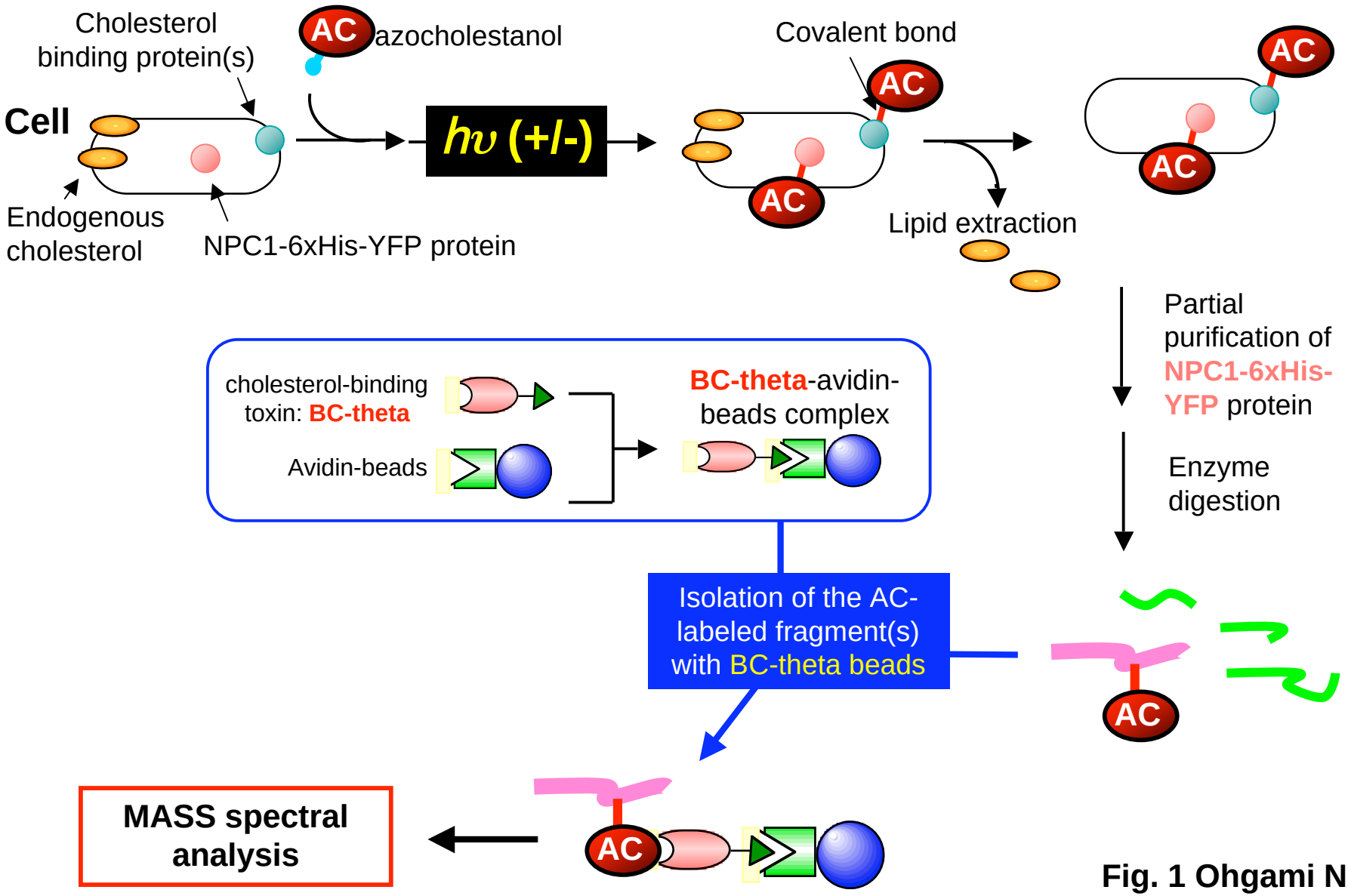


Fig. 1 Ohgami N.

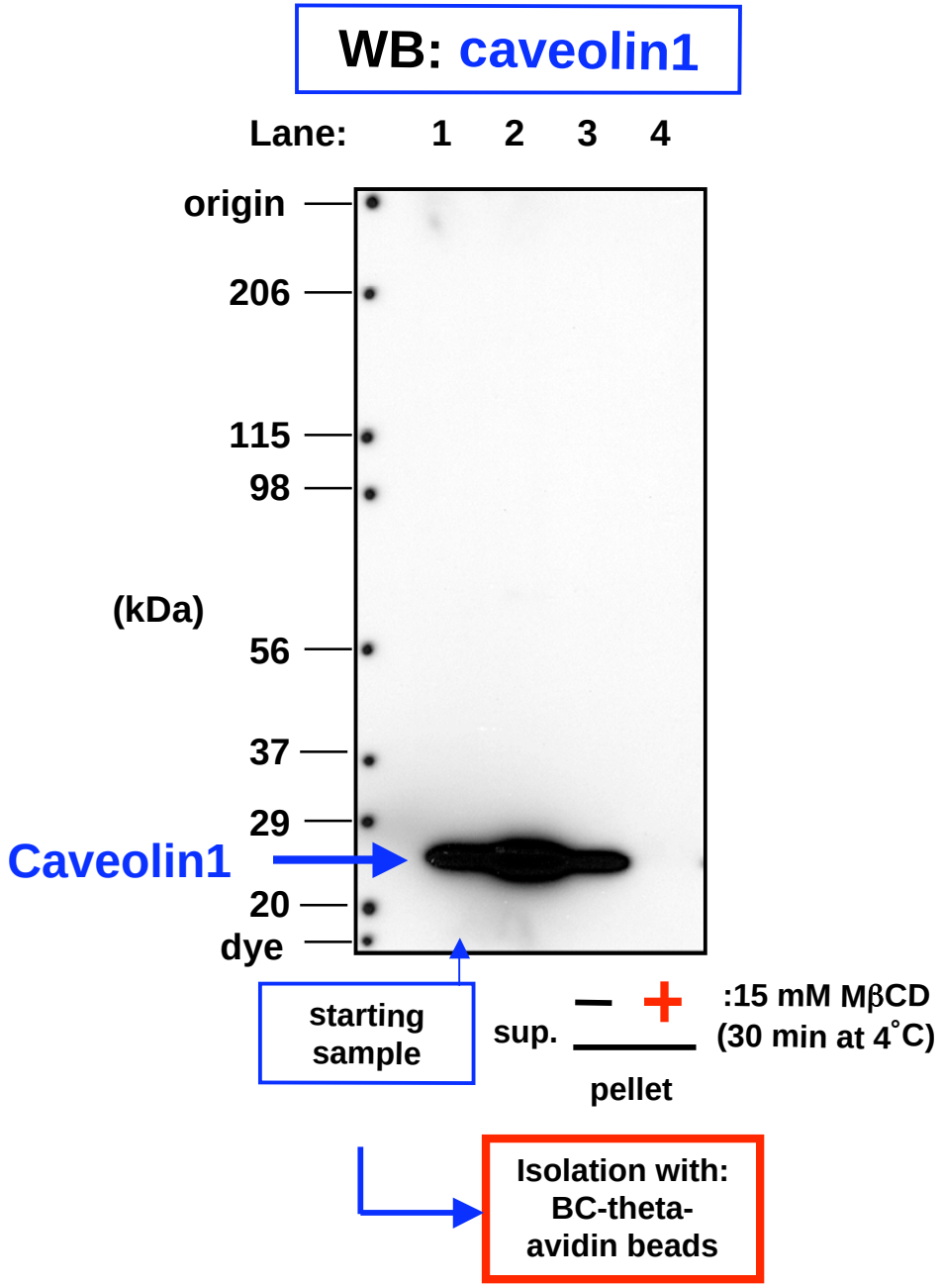


Fig. 2 Ohgami N.

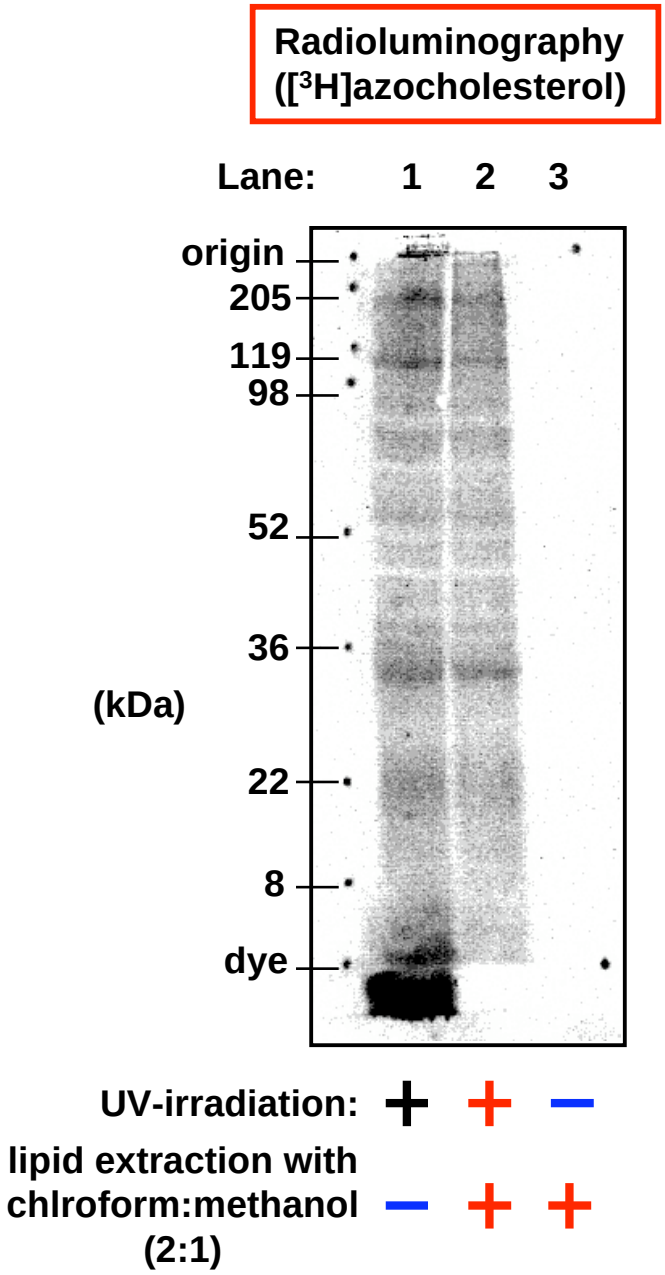
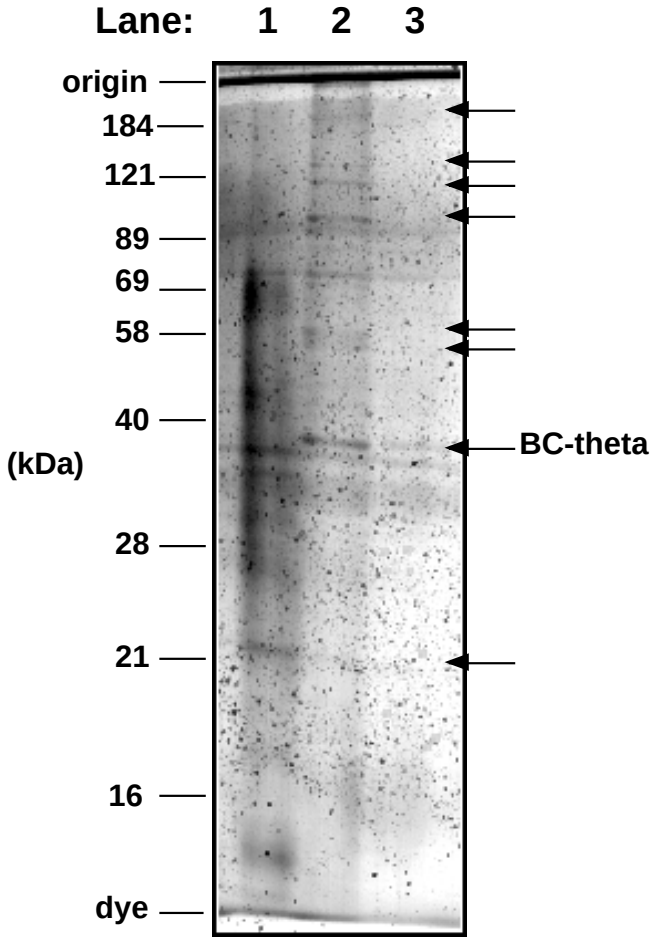


Fig. 3 Ohgami N.

Silver staining



Photolabeling with AC : + + -

lipid extraction

Isolation with BCθ : - + +

Fig. 4 Ohgami N.

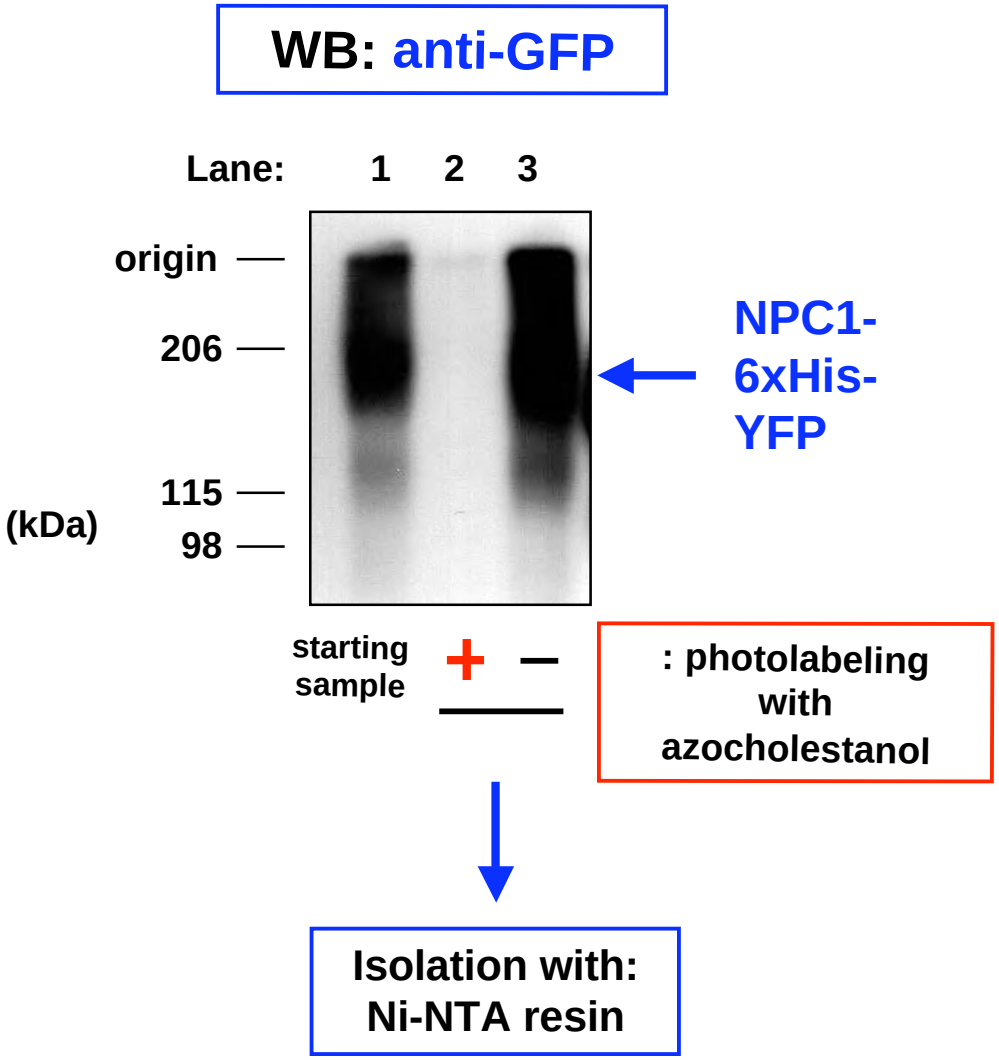


Fig. 5 Ohgami N.