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Analysis of Ciliary Membrane Protein Dynamics Using SNAP Technology

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Abstract

The sensory functions of the primary cilium rely on receptors and other membrane proteins that are specifically sorted to the ciliary compartment, which is a subdomain of the plasma membrane. Defects in this process underlie a large number of human diseases yet it is poorly understood. Thus, it is of great interest to understand the mechanisms by which the cell sorts and traffics proteins to the ciliary membrane. Here we provide an overview of our method to study the sorting and trafficking of ciliary membrane proteins using SNAP technology. This technology enables pulse-chase analysis of the movement of proteins through the endomembrane system and onto the cilium.

1. Introduction

The primary cilium is a sensory organelle that cells use to monitor the extracellular environment (Pazour and Witman, 2003). Defects in this organelle cause a number of human syndromes collectively known as the ciliopathies (Cardenas-Rodriguez and Badano, 2009). As the primary cilium plays ubiquitous and extremely diverse roles in mammalian development and tissue homeostasis, the range of disease caused by ciliary dysfunction is very broad. The ciliary diseases include blindness and anosmia, cystic diseases of the kidney, liver and pancreas, diverse structural birth defects of the heart, lung, and brain along with craniofacial and other skeletal defects (Satir and Christensen, 2007).

The sensory functions of primary cilia rely on receptors localized specifically to the ciliary membrane (Pazour and Bloodgood, 2008). The ciliary membrane is continuous with the plasma membrane of the cell but is a separate compartment to which cells have the ability to enrich specific receptors. It is likely that many ciliary diseases are caused by a failure to properly localize receptors to the ciliary membrane. Consequently significant effort is being expended to understand the machinery and processes by which specific receptors are sorted to the ciliary compartment. Up to now, most studies have examined how a mutation or knockdown of a gene product affects the steady state level of a membrane protein in the cilium and inferred the function of the protein from this data. This approach has been powerful in identifying proteins that are important for ciliary assembly, but more sophisticated approaches are needed to understand the molecular functions of these proteins. Studies on trafficking to other membrane compartments have been advanced by kinetic analysis where the effects of a perturbation (mutation or knockdown) on the movement of a protein through the biosynthetic pathway are measured (Hirschberg et al., 1998). This type of analysis allows one to determine which step along the biosynthetic pathway is altered and is causing the change in the steady state level of the protein in the organelle of interest. Traditionally many of these approaches relied on following the movement of a GFP-tagged

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protein after synthesis or release from a temperature block. For a number of reasons, these approaches do not work to study trafficking to the ciliary compartment and so we are developing pulse-chase technology based on the SNAP tag to measure the rates of trafficking to the cilium. This technology was also used by Milenkovic and colleagues to study the pathway for delivery of smoothened to the cilium (Milenkovic et al., 2009).

The SNAP tag is 20 kD peptide derived from a DNA repair protein and forms irreversible covalent bonds with benzylguanine (BG) derivatives (Keppler et al., 2003). The SNAP-tag can be fused to any protein of interest allowing the fusion protein to be labeled with a BG derivative. A variety of BG derivatives are commercially available from New England Biolabs. Because these compounds can be made cell permeant and come in both fluorescent and non-fluorescent forms these BG derivatives are ideal for pulse-chase experiments. To perform a pulse-chase experiment with a SNAP tag, a cell line is first developed that expresses a SNAP-tagged protein. The pulse-chase experiment is initiated by treating the cells with a non-fluorescent BG to block all existing SNAP sites. The non-fluorescent BG is then washed out and newly synthesized protein is free to be labeled by a fluorescent BG derivative. This allows one to measure the time it takes a protein to go from synthesis to delivery to a particular compartment such as the cilium.

2. Generation of SNAP-tagged ciliary targeted proteins

The first step in the development of this method is to construct a tagged protein that is robustly and specifically targeted to the cilium. A number of mammalian integral membrane proteins have been tagged with GFP or other peptide motifs and show high specific accumulation in the cilium. These include the SSTR3 isoform of the somatostatin receptor (Berbari *et al.*, 2008; Pazour and Bloodgood, 2008), the 5HT 6 serotonin receptor (Berbari *et al.*, 2008), polycystin-1 (Xu *et al.*, 2007), polycystin-2 (Geng *et al.*, 2006), fibrocystin (Follit *et al.*, 2010) and smoothened (Corbit *et al.*, 2005). In our experience the addition of a SNAP tag next to the GFP tag does not adversely affect trafficking to the cilium. Thus, the construction of a SNAP tagged fusion protein is relatively straightforward if a GFP-tagged form has been previously analyzed.

We have focused on the trafficking mechanisms of fibrocystin, which is the product the human autosomal recessive polycystic kidney disease gene. Trafficking to the cilium is mediated by an 18 residue ciliary targeting sequence located in the C-terminal tail (Follit *et al.*, 2010). This protein is a type 1 membrane protein that contains a nearly 4000 amino acid N-terminal extracellular domain, a single transmembrane span and a short cytoplasmic-localized C-terminal tail (Ward *et al.*, 2002). To study the movement of this protein through the endomembrane system, we developed constructs where the large extracellular domain is replaced by a similar, but much smaller, extracellular domain of CD8 (Xia *et al.*, 2001). The CD8 fragment serves to ensure the correct membrane topology of the fusion protein and is also an epitope tag that can be detected by a monoclonal antibody. Two versions of the fibrocystin fusion protein were constructed where either a SNAP tag alone or a SNAP tag and a GFP tag were placed on the C-terminal end (Fig. 1). Both constructs trafficked well to cilia but most work was done with the non-GFP version (Fig. 2, 3).

3. Pulse-Chase Analysis of the trafficking of Fibrocystin-SNAP to the Cilium

To measure the kinetics of biosynthesis and transport of a membrane protein to the cilium, one first starts with a cell line that expresses the SNAP-tagged protein of interest. The existing SNAP sites are blocked with a cell permeant non-fluorescent BG. The non-fluorescent BG is then washed out and new protein synthesis occurs. It is usually helpful to stop protein synthesis with cycloheximide after a defined period of time as this reduces the label in the endoplasmic reticulum and allows for easier detection of faint label in the

cilium. Cilia-specific sorting events are likely to occur at the Golgi complex or in post-Golgi pathways. To specifically look at these steps of sorting, we developed a version of the classic technique in membrane biology where cells are held at low temperatures to block the movement of proteins at specific points in the endomembrane system (Fig. 2). Incubating cells at 16°C typically causes membrane proteins to be retained in the endoplasmic reticulum while holding the cells at 20°C causes the proteins to exit the endoplasmic reticulum and be retained at the Golgi complex (Saraste et al., 1986). In our cells, we found that 19°C was very effective at holding proteins in the Golgi complex and that most protein exited the endoplasmic reticulum and accumulated in the Golgi complex within two hours of the addition of cycloheximide and reduction in temperature. After two hours the cells are rapidly shifted to 37°C by replacing the 19°C medium with pre warmed medium and coverslips are fixed at regular intervals for several hours. The level of the signal in the cilium is measured by fluorescence microscopy and plotted as a function of time. We also monitor the steady state level of the ciliary protein by measuring either the GFP or CD8 (by immunofluorescence) signal associated with the SNAP-tagged protein. In a normal IMCD3 cell, the newly synthesized fibrocystin-SNAP construct can be first detected in the cilium about 30 minutes after shift to 37°C and reaches steady state in 2 to 4 hours.

4. Example Protocol

This protocol was designed to investigate post-Golgi trafficking in IMCD3 cells. The incubation times may vary based on cell type, your protein of interest and other factors. Cell lines with stable and uniform expression of the SNAP-tagged protein of interest are best. We use a combination of transfection (electroporation) followed by drug selection and subsequent dilution cloning to isolate a clonal line. *Prior to the trafficking protocol cells may be treated with RNAi (typically 48 hours before the trafficking assay).*

1. Grow desired number of cells in preferred vessel and media. We use 35 mM dishes with 5 coverslips to allow analysis of multiple time points. To induce ciliation, reduce the serum concentration to 0.25% for 24 to 48 hours prior to the trafficking experiment.
2. To begin the experiment, fix a coverslip and store in PBS for subsequent staining (Fig. 3A, No Block). We fix with 2% paraformaldehyde (EM Sciences Cat# 15710) in PHEM (0.05 M Pipes pH 6.9, 0.05 M HEPES, pH 7.4, 0.01 M EGTA, 0.01 M MgCl₂) for 15 minutes at room temperature, followed by a 2 minutes permeabilization with fixative plus 0.1% Triton X100.
3. *Block the SNAP epitope.* Add BG-Block (*NEB Cat# S9106*) to the cells at a final concentration of 0.04 μ M and incubate the cells at 37°C / 5% CO₂ for 20 minutes to block all existing SNAP epitopes. It is important to keep the SNAP reagents stored at -20°C and use fresh stocks for each experiment. After 30 minutes, fix a coverslip and store in PBS. At this time all existing SNAP epitopes should be blocked and no protein should label with SNAP substrates (Fig. 3B, Block).
4. *Remove the BG-Block and allow the cells to synthesize new SNAP-tagged protein.* Wash the cells 3 times with reduced serum growth media (0.25% serum) and incubate the cells at 37°C / 5% CO₂ for 30 minutes. After 30 minutes change the media once again to ensure all non-fluorescent BG-Block has been removed from the media. Incubate the cells at 37°C / 5% CO₂ for an additional hour. At this time, 1.5 hours after the BG-Block was removed, fix another coverslip and store in PBS. Removal of the BG-Block should allow the newly synthesized SNAP-tagged protein to be labeled with fluorescent substrate and should appear in the endoplasmic reticulum (Fig. 3C, ER). The amount of time after removal of the

block will vary based on expression level and size of the SNAP-tagged protein of interest and may need to be adjusted.

5. *Block Golgi exit and turn off protein synthesis.* Add cycloheximide to cells at a final concentration of 150 µg/ml to prevent protein synthesis. This allows us to follow just the newly synthesized SNAP-tagged protein of interest as it is trafficked throughout the cell. Next, buffer the media by adding Hepes pH 7.4 to 20 mM and shift the cells to 19°C to block protein exit from the Golgi complex and accumulate the newly synthesized SNAP-tagged protein at the Golgi. We float the dishes in a water bath set to 19°C located in a cold room. Incubate the cells at 19°C for 2 hours. (*During this time prepare reduced serum media with 150 µg/ml cycloheximide and pre warm in the 37°C incubator.*) After two hours at 19°C, fix a coverslip and store in PBS. The SNAP-tagged protein should have exited the endoplasmic reticulum and accumulated in the Golgi complex (Fig. 3D, Golgi block / t=0).
6. *Release protein from the Golgi and measure ciliary protein trafficking.* Add the pre-warmed media containing 150 µg/ml cycloheximide to the cells and incubate at 37°C / 5% CO₂. The SNAP-tagged protein will now exit the Golgi complex and begin trafficking throughout the cell. Fix a coverslip at desired time points. To start, fix a coverslip every 30–60 minutes for 4–6 hours and store in PBS. Shortly after the release of Golgi block the SNAP-tagged protein can be seen accumulating in the primary cilium (Fig. 3E–F, t=1 – t=4).
7. Once all coverslips have been fixed, proceed with standard immunofluorescence protocols. To label the SNAP-tagged protein incubate cells with 0.3 µM fluorescent SNAP substrate (we use TMR-Star, *NEB Cat# S9105* but many other SNAP reagents exist – choose one that suits your needs). Prepare fresh fluorescent SNAP reagent and label the cells for 1 hour at room temperature. This staining may be combined with additional primary antibodies as needed.

This protocol was designed to study the post-Golgi trafficking of the fibrocystin ciliary targeting sequence in IMCD3 cells (Follit, Jonassen and Pazour, in preparation). Incubation times may need to be adjusted depending on your protein of interest and expression levels.

5. Future Prospects

We have focused our attention on the movement of proteins from the Golgi complex to the cilium, but similar protocols can be used to measure any other step along the pathway. The technology also has great potential for live cell imaging of transport processes as cell permeant probes are available and they appear to be relatively non-toxic to cells. In addition, the availability of biotinylated BG derivatives expands the technology to biochemical approaches where the proteins are isolated using biotin-avidin technology.

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Figure 1. Schematic of SNAP-tagged ciliary constructs

A. Schematic of the fusion proteins made from JAF271 and JAF270. **B.** Mouse IMCD3 cell transfected with JAF271 and labeled with CD8 antibody (green), fluorescent BG (TMR-Star, red) and DAPI (blue). Scale bar is 5 microns. Bottom row are 2X enlargements of the cilium shown in merge, green and red channels.

Figure 2. CD8-Pkhd1-SNAP moves through the endo-membrane system

Following a non-fluorescent SNAP epitope block, newly synthesized CD8-Pkhd1-SNAP (red) is detected in the endoplasmic reticulum (column **A**) where it co-localizes with the ER marker BIP (green). Subsequent incubation at 19° C causes accumulation of CD8-Pkhd1-SNAP (red) in the Golgi complex (columns **B–D**) where it co-localizes with IFT20 (column **B**, green), golgin97 (column **C**, green) and giantin (column **D**, green). Two hours after the Golgi block is released, CD8-Pkhd1-SNAP (red) is detectable in the cilium (columns **E–F**, arrows), the Golgi complex (columns **E–F**, co-localization with IFT20 and golgin97) and a small fraction of the CD8-Pkhd1-SNAP (red) localizes with recycling endosomes (columns **G–H**, transferrin receptor TfR, Rab11, green). Top row is the merge of all three channels, middle and bottom rows are red and green channels respectively. Scale bar is 5 microns.

Figure 3. CD8-Pkhd1-SNAP traffics to the primary cilium

Mouse kidney cells stably expressing CD8-Pkhd1-SNAP show CD8 (green), SNAP (red) concentrated in the primary cilium where they co-localize with IFT27 (blue) in normal growth conditions (column **A**). Incubating the cells with a non-fluorescent SNAP substrate blocks all SNAP epitopes (lack of red staining in column **B**) although the CD8-epitope (green) is still ciliary localized and co-localizes with IFT27 (blue). Following removal of the blocking SNAP substrate the cells synthesize new CD8-Pkhd1-SNAP in the endoplasmic reticulum (red, column **C**). Incubation at 19° C causes the newly synthesized CD8-Pkhd1-SNAP to accumulate at the Golgi complex (red, column **D**) but does not affect the primary cilium (blue) or total CD8-Pkhd1-SNAP levels (green). Upon release of the temperature induced Golgi block, newly synthesized CD8-Pkhd1-SNAP traffics to the primary cilium (red, columns **E–G**). Note that prior to Golgi release, newly synthesized CD8-Pkhd1-SNAP is excluded from the zone around the base of the cilium that is labeled by IFT27 (blue). After the temperature block is released, the newly synthesized protein moves into this zone (magenta staining marked by arrowheads, panels **E–G**) presumably on its way to the cilium. Insets are 3X enlargements of the cilium. Scale bar is 5 microns.