論文の内容の要旨

論文題目　Molecular Genetic Study of Kinesin Superfamily Protein KIF12
（キネシンスーパーファミリー蛋白質 KIF12 の分子遺伝学的研究）

氏名　楊文星

Cells synthesize various distinct kinds of proteins for developing and maintaining cell morphology and functions. To distribute these proteins to their proper destinations, many motor proteins are recruited to the microtubule cytoskeleton, whose plus ends generally point to the cell periphery or axon tips of the neurons. One of the most important and well-studied motor families is kinesin superfamily proteins (KIFs). Kinesin was discovered decades ago. So far, 15 mammalian kinesin families (kinesin 1-13, 14A and 14B) have been identified, which consist of totally 45 mammalian KIF genes. All the members of KIFs have so-called “motor domain” that generally moves along the microtubules, driving through the energy from ATP hydrolysis. Although these motor domains are conserved, regions outside the motor domains can be quite divergent and unique to individual motors, enabling various cargoes to be bound. Up to now, members of KIFs have been shown to transport synaptic vesicle precursors, mitochondria, membrane organelles, cilia components, and mRNAs. In addition to function as motor proteins, a member of KIFs (KIF26A) has been also found to be a signaling regulator.

KIF12 belongs to the kinesin-12 family and contains 642 amino acid residues. It consists of an N-terminal head domain, a stalk domain and a C-terminal tail domain. Kinesin motor (22-288 aa) is located to the head domain. Coiled-coil region (376-465 aa) forms the stalk domain. And in the C-terminal, a tail domain has been identified, including a proline rich domain (469-554 aa). KIF12 was firstly identified by degenerate PCR. According to a quantitative trait loci (QTL) gene discovery system established by the Complex Trait Consortium, it has been proposed as a *cpk* (congenital polycystic kidney) modifier gene. *Kif12* transcription is regulated by HNF1α/β, which expresses in kidney and pancreatic islets. However, the molecular function and the physiological relevance of KIF12 have been still very elusive.

The peroxisome is a metabolic organelle found in all eukaryotes except the Archaezoa. Peroxisomes contain catalase, essential for turnover of H_2O_2 to reduce the oxidative stress. It also contains several β-oxidation enzymes essentially metabolizing very long chain fatty acids (VLCFA). Deficiency in peroxisome assembly or functions was reported to result in inherited disorders such as Zellweger syndrome, X-linked adrenoleukodystrophy (X-ALD) and acatalasemia, and to increase the risk to develop type 2 diabetes. Because peroxisomes do not contain genome or protein synthesis machinery, most peroxisomal enzymes are synthesized on ribosomes free in the cytosol, and targeted to peroxisomes according to the peroxisome targeting signals (PTSs) on the proteins. So far two kinds of PTSs have been

identified, called PTS1 and PTS2. The PTS1 signal is generally a SKL (Ser-Lys-Leu) tripeptide or its variants and located to the extreme carboxyl terminal of the protein. Catalase targets to the peroxisomes by its PTS1 signal. On the other hand, the PTS2 signal is a nonapeptide sequence located near the N terminus or at internal locations of proteins, sharing the consensus sequence (R/K) (L/V/I) $(X)_5$ (H/Q) (L/A) (X = any amino acid). Peroxisomal enzyme acetyl-CoA acyltransferase 1 (ACAA1) targets to the peroxisomes by its PTS2 signal.

Peroxisome matrix proteins are targeted to peroxisomes properly and efficiently to maintain peroxisome morphology and functions. How proteins import into the peroxisomes and how this targeting is regulated are two key problems about the peroxisome matrix protein targeting. Now, most studies have been focusing on the mechanism by which the peroxisome matrix protein import was accomplished. Only a few works were about the regulation the peroxisome targeting.

The purpose of the present study is to establish a good system for revealing the molecular function and the physiological relevance of KIF12, by which I found a new function of KIF12 in peroxisome matrix protein targeting though its cytoplasmic binding partner.

In this study, I firstly generated *Kif12* knockout (KO) mice. Using homologous recombination, the genomic DNA fragment covers from 2nd to 11th exons is replaced by a selection cassette, which consists of a promoter trapping β Geo and a PGK promoter-driven puro, called "βGeo/puro". This results in a translational frame shift at the downstream of the first exon of *Kif12* gene, and thus KIF12 protein is eliminated from the KO mouse. Results from Southern blotting, genotyping PCR and immunoblotting strongly suggest that *Kif12*-deficient mouse were successfully generated in this study.

Two synthesized peptides were designed for generating rabbit anti-mouse KIF12 antibodies. Peptide P12PR is located to the PRD domain of KIF12, which generates antibody 12PR. And peptide P12T is located to the C-terminal of KIF12 protein, which generates antibody 12T.

KIF12 tissue distribution assay was performed by using 12T antibody. Results showed that strong expression of KIF12 in mouse pancreatic islet and kidney. However, the lysates of mouse heart, brain, lung, spleen, fat, muscle and liver did not show any specific signals. Then I have further characterized an insulinoma-derived pancreatic beta cell line MIN6, and detected that MIN6 cells had the highest expression level of KIF12 among the analyzed samples. These results suggest that KIF12 plays some roles in maintaining appropriate function of pancreatic beta cells and kidneys. As pancreatic beta cells have the highest expression of KIF12, I used pancreatic beta cells as the experimental system for this study.

In order to assess the possible changes in organelle biogenesis and distribution, I observed the organelles and microtubules in primary cultured pancreatic islet cells. I labeled the Golgi complexes by anti-GM-130 antibody, the insulin granules by anti-insulin antibody, the lysosomes by LysoTracker, the mitochondria by MitoTracker, microtubules by

anti-α-tubulin antibody. Results showed that all above mentioned organelles or microtubules are not different in WT and KO islets cells. These results suggested that KIF12 is dispensable for biogenesis of major organelles and microtubules.

However, the down-regulation of the signal strength of a peroxisomal enzyme was specifically distinguished in the KO cells. Peroxisomal enzyme acetyl-CoA acyltransferase 1 (ACAA1) targets peroxisomes by its PTS2 signal, and catalyzes the first step of peroxisomal beta-oxidation. For peroxisome visualization, I labeled ACAA1 by its antibody to reveal its dotty signals in the cytoplasm. Although the distribution of the peroxisomes was not apparently changed, the signal strength of ACAA1 was significantly decreased in the KO cells. In order to test whether this decrease was truly due to the loss of KIF12 protein, I performed a rescue experiment using a full-length *Kif12* cDNA adenoviral vector, pKIF12FL-mCitrine. As a result, the overexpression of pKIF12FL-mCitrine in KO islet cells rescued the mean ACAA1 level to normality. These results implied that peroxisome targeting of ACAA1 was impaired in *Kif12* KO cells.

KIF12 binding partner was reported to be a regulator of peroxisome matrix protein targeting. I further performed immunofluorescence microscopy of primary cultured pancreatic islet cells with its antibody, which revealed its significant decrease in *Kif12* KO cells. And this decrease could be rescued by KIF12 overexpression.

To further confirm the impaired peroxisome targeting and its connection with KIF12 binding protein, I performed biochemical studies. As pancreatic islets are rather small compared with the brain, the intrinsic tissue is difficult to be handled as a starting material. Therefore I used mouse insulinoma-derived MIN6 cells for the biochemical studies. Results showed that *Kif12* knockdown decreased the expression levels of KIF12 binding partner and ACAA1, and this decreasing could be rescued by overexpression of KIF12 protein.

Above data suggested that KIF12 controls peroxisome matrix protein targeting through regulating the intracellular level of its binding protein. Many explanations could account for its down-regulation in KIF12 KO or knockdown cells. One is that KIF12 functions as a transcription regulator of these proteins. Another one is that KIF12 binds to these proteins, and keep it away from protein degradation machinery. To verify the first possibility, I performed quantitative real-time RT-PCR, to compare the transcription of this protein in WT and KIF12 KD MIN6 cells, using β-actin as control. Results suggested that its down-regulation is independent of KIF12. Therefore, I assessed if KIF12 could bind to it and stabilize it. To confirm this, intrinsic IP experiments and proximity ligation assay were performed. Results strongly suggested their binding in vivo. This finding suggests that KIF12 mediated-protein downregulation may be due to its selective stabilization on this protein in the cytoplasm. Future studies will reveal the precise mechanistic links between KIF12 and stabilization of KIF12 binding protein.

In the present study, I established *Kif12*-deficient models. To investigate the physiological relevance of KIF12 in development and metabolism, the newly established *Kif12* KO mice will be quite feasible, as it apparently gives no significant abnormality in development, viability and reproduction. In addition, I have generated a specific

knockdown system in MIN6 beta cells with miRNA vector and its rescue system using an RNAi-immune expression vector. This miRNA vector specifically knockdown the KIF12 protein in MIN6 beta cells to 90% of the control, which could be rescued to the intrinsic level of the protein by RNAi-immune KIF12 vector infection. This knockdown system would be useful for biochemical studies, as pancreatic islets are too small to be handled as a starting material. These model systems will allow us to study the physiological relevance and molecular pathways of KIF12 throughout different levels, from molecules to individuals.

To search for the possible cellular abnormality in *Kif12*-deficient islet cells, I performed immunofluorescence staining to visualize major subcellular organelles and cytoskeleton, such as Golgi complex, insulin granules, lysosomes, mitochondria, microtubules and peroxisomes. Majority of the subcellular structures were not apparently changed in *Kif12*-deficient pancreatic islet cells, except for the peroxisome. The most significant finding in this study was a specific decrease in the amount of proteins targeted to peroxisomes, but the distribution was apparently unaltered.

This study will be the first one which shows the functional relevance of KIF12 in peroxisome matrix protein targeting. The specificity and feasibility of these finding has been supported by the following evidences: [1] abnormality was specifically found in peroxisomes; [2] impaired peroxisome targeting was morphologically and biochemically described; [3] it was found in two different systems of cell culture, islet primary culture and the MIN6 cell line; [4] it had been confirmed by two different methods, knockout and knockdown; and [5] it could be rescued in above cases.

Specific mutations in Pex5 could impair the PTS1 protein targeting in *S. cerevisiae.* Similar phenotype was also found in plants, Chinese hamster, and human. Thus, Pex5 is essential for importing peroxisome matrix proteins carrying PTS1 or PTS2 signals. In the present study, I found that knockdown of KIF12 in MIN6 cells reduced the Pex5 protein level and the impaired peroxisomal targeting of ACAA1 protein. It is likely that this down-regulation of Pex5 protein in *Kif12*-deficient cells could be a possible cause of impaired peroxisome matrix protein targeting.

In the present study, I clarified KIF12 binding protein, and found that it was down-regulated in *Kif12*-deficient beta cells, whose mRNA levels were unchanged. These data suggested that KIF12 controls down-regulation of this protein through a post-transcriptional mechanism, such as excluding the binding of E3 ligase to this protein. These findings will collectively suggest that KIF12 acts as a new cochaperone of this protein to prevent degradation of Pex5 and maintains the appropriate levels of their expression. Because the peroxisome is an essential organelle for metabolic pathways, this cochaperone kinesin will serve for a new prevention mechanism from metabolic diseases.

In summary, I discovered a new function of KIF12 in peroxisome matrix protein targeting in pancreatic islet cells and MIN6 beta cells through regulating its binding partner. This proposes a new regulatory pathway of peroxisome targeting involving KIF12 protein.