## DIVISION OF MOLECULAR AND DEVELOPMENTAL BIOLOGY


| | |
|---|---|
| *Professor:* | *TAKADA, Shinji* |
| *Research Associate:* | *KOSHIDA, Sumito* |
| *Technical Staff:* | *UTSUMI, Hideko* |
| *NIBB Research Fellows:* | *KURATA, Tomoko* |
| | *AKANUMA, Takashi* |
| *Postdoctoral Fellows:* | *KAWAMURA, Akinori* |
| | *TAKADA, Ritsuko* |
| | *YAMAGUCHI, Yoshifumi* |
| | *OKUBO, Tadashi* |
| *Graduate Students:* | *ISHITANI, Shizuka* |
| | *TAKAHASHI, Jun* |
| *Technical Assistants:* | *TAKASHIRO, Kayoko* |
| | *SASAKI, Michiko* |
| | *ODA, Ritsuko* |
| | *SHIBATA, Chitose* |
| *Secretaries:* | *MATSUDA, Mitsuko* |
| | *UKAI, Sakie* |


During morphogenesis of vertebrates, the embryonic body is gradually divided into sub-regions that are specified to give rise to functional units. Most of the processes of regionalization and specification are regulated by cell signaling molecules. So far, spatial distribution of secreted signals, called morphogens, has been considered to regulate regionalization and specification during embryogenesis. However, the molecular basis to generate tightly regulated gradient formation of secreted signal proteins, including machinery to secrete these proteins, and specific activation of their target genes in particular cells, has not yet been discovered. To better understand this molecular basis, we are currently examining the biochemical characteristics of Wnt proteins and the molecular mechanism to activate specific targets.

In contrast, some regionalization processes have also been shown to be regulated by different manners. One of the typical examples is the segmentation of somites. Somites are the morphologically distinct segmental units that are transiently formed during early vertebrate development and subsequently give rise to metameric and fundamental structures such as the vertebrae of the axial skeleton, their associated muscles, and tendons. The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the ìsegmentation clockî. To gain insight into the molecular mechanism underlying this specific regionalization, we are also characterizing genes involved in somite segmentation.

### I. Molecular mechanism to secret Wnt proteins

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis, as well as in carcinogenesis. Most Wnt proteins transmit signals locally, presumably since their secretion and transport are under tight control (Figure 1). Although the molecular mechanism underlying their secretion and transport remains largely unknown, recent successes in identifying various molecules involved in these processes provide further clues.

One important step regulating the extracellular transport of various secreted signal proteins involves post-translational modification with lipid moieties. A fatty acid modification, i.e. acylation, occurs with Wnt, Hedgehog (Hh), and Spitz (Drosophila Transforming Growth Factor $\alpha$). In the case of Wnt, Nusse and co-workers reported that murine Wnt-3a is S-palmitoylated at a conserved cysteine residue at the 77th residue (Cys77). A mutant form of mouse Wnt-3a, in which the palmitoylated Cys77 is substituted with alanine (C77A), shows diminished ability to activate Wnt signaling, but is secreted normally into the culture medium. Thus, the authors proposed that palmitoylation of this cysteine residue may be required to produce an increased local concentration of Wnt on the plasma membrane. However, although their mass spectrometry analysis covered 85% of the primary amino acid sequence of Wnt-3a, there remains the possibility of additional acylation sites.

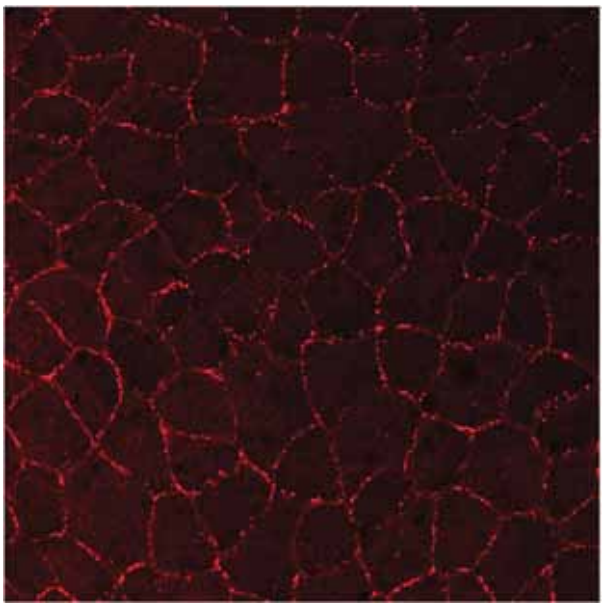

Figure 1. Secreted Wnt-3a proteins in epithelial sheet. Immunostaining of Wnt-3a proteins secreted from Xenopus epithelial cells. Wnt proteins are secreted in dot-like structure in the extra-cellular space.

There is strong evidence to suggest that acylation is involved in the processing and intracellular trafficking of Wnt prior to secretion. Genetic evidence suggests that Wnt-secreting cells require the action of specific genes, e.g., *porcupine* (*porc*) in *Drosophila* or its ortholog, *mom1,* in *C. elegans*, both of which encode proteins with structural similarities to those of a family of membrane-bound O-acyl transferases (MBOAT), which transfer acyl groups, such as a palmitoyl group, to substrates. Porc is localized at the endoplasmic reticulum (ER) and its over-expression in culture cells enhances the intracellular processing, for example, N-glycosylation, of Wingless (Wg: the Drosophila Wnt-1 ortholog). In addition, treatment with a chemical inhibitor of acyl-transferases produces defective intracellular

rafficking of Wg. Thus, *porc*-dependent acylation may regulate the processing and intracellular trafficking of Wnt, although acylation at Cys77 does not appear to be involved in these processes.

To resolve inconsistencies between studies examining the roles of Wnt acylation and to better understand the biological significance and molecular mechanism of Wnt function of acylation (Figure 2)[Ö ] We show that murine Wnt-3a is also acylated at a conserved serine residue (Ser209). Significantly, we demonstrated that this residue is modified with a mono-unsaturated fatty acid, palmitoleic acid (Figure 3). Wnt-3a defective in acylation at Ser209 is not secreted from cells in culture or in Xenopus embryos, but is retained in the endoplasmic reticulum (ER). Furthermore, Porcupine, a protein with structural similarities to membrane-bound O-acyltransferases, is required for Ser209-dependent acylation, as well as for Wnt-3a transport from the ER for secretion (Figure 2). These results strongly suggest that Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process.

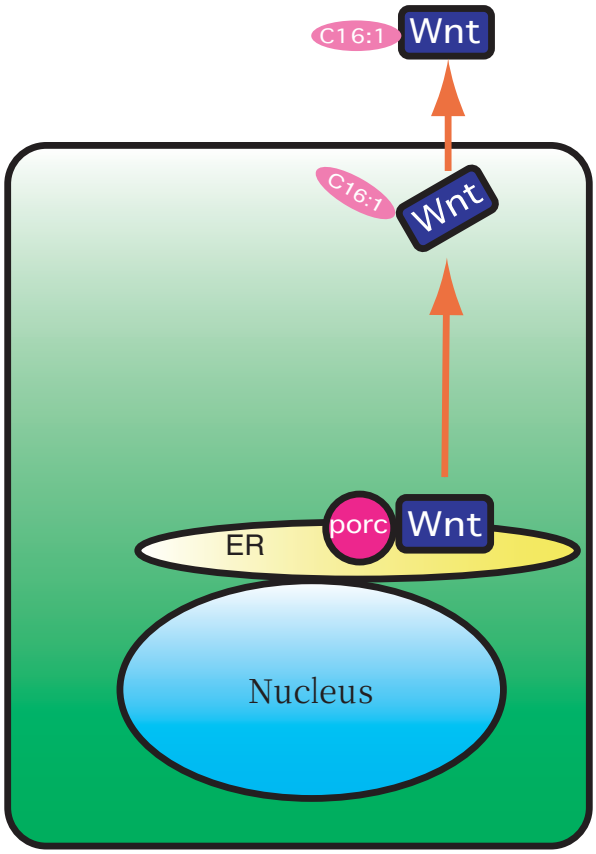


Figure 2. The function of palmitoleic lipid modification of Wnt protein. Wnt proeins are modified with palmitoleic acid (C16:1) by acytransferase, Porcupine (porc), in the ER. This modification is required for trafficking of Wnt proteins from the ER.

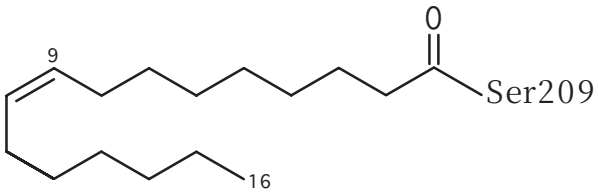


Figure 3. Structure of the lipid chain, palmitoleoyl moiety, bound to Ser209 of murine Wnt-3a protein

## II. Identification and functional characteristics of Wnt target genes during embryogenesis

To identify Wnt-target genes during mouse development, we used the induction gene-trap approach (Yamaguchi *et al.* 2005). We screened 794 trapped ES lines and recovered 2 ES cell lines that contained trapped genes responsive to Wnt-3a protein. One trapped gene, CP2L1, which encodes a transcriptional regulator, was mainly expressed in the ductal epithelium of several developing organs, including the kidney and a number of exocrine glands. The spatial and temporal expression of this gene coincided well with that of several *Wnt* genes. Furthermore, the expression of this gene was significantly decreased in cultures of embryonic tissues treated with a Wnt signal inhibitor, indicating that the in vivo expression of this gene is dependent on Wnt signaling.

We have further analyzed the developmental role of CP2L1 by generating mutant embryos defective in its function from the gene-trapped ES cell line. In *CP2L1*-deficient mice, the expression of genes directly involved in functional maturation of the ducts was specifically reduced in both the salivary gland and kidney, indicating that *CP2L1* is required for the differentiation of duct cells. Furthermore, the composition of saliva and urine was abnormal in these mice. These results indicate that *CP2L1* expression is required for normal duct development in both the salivary gland and kidney.

Interestingly, the identification of a gene essential for the maturation of the exocrine ducts and kidney should help to elucidate their underlying molecular mechanisms. Duct maturation includes both duct formation and the acquisition of physiological function. Because CP2L1 is a transcriptional regulator, genes whose expression is directly or indirectly regulated by this factor may be involved in these processes. In *CP2L1*-deficient embryos, the expression of genes involved in the physiological function of the ducts was reduced (Figure 4). In addition to the genes directly involved in physiological function, we found that the expression of *keratin* genes was also abnormal in the ducts of the mutants. Thus, CP2L1 participates in establishing the function of ducts by coordinating the expression of several genes that are involved in physiological function and generate the appropriate cellular architecture.

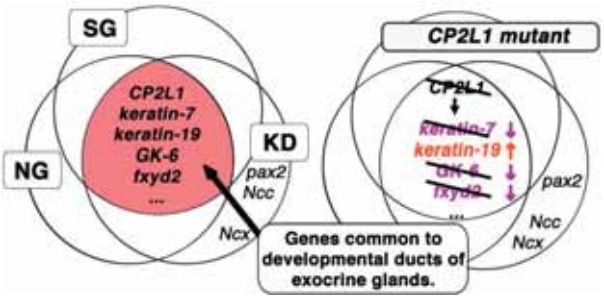


Figure 4. CP2L1 may play a common regulator in development of exocrine ducts. Several exocrine ducts express a common set of genes, and dysfunction of CP2L1 causes defects in the expression of these genes.

## III. Identification and characterization of genes required for somite development

### 3-1 Groucho-associated transcriptional repressor Ripply1 is required for proper transition from the presomitic mesoderm to somites

Prior to morphological segmentation, which is a process including inter-somitic boundary formation and mesenchymal-epithelial transition, a segmental pre-pattern, characterized by segmental gene expression, is established in the anterior PSM. The establishment of the segmental pre-pattern in the anterior PSM has been revealed to require a number of processes regulated by many transcription factors and signaling molecules. Concomitant with the transition from the anterior PSM to somites, the characteristic gene expression in the PSM is translated into the segmental structure. However, most of the events accompanying the transition from the anterior PSM to somites have remained obscure.

We show that another gene identified by our *in situ* hybridization screening, *ripply1*, encoding a nuclear protein associated with the transcriptional co-repressor Groucho, is required for this transition. Zebrafish *ripply1* is expressed in the anterior PSM and in several newly formed somites. Ripply1 represses specific gene expression in the PSM through a Groucho-interacting motif. In *ripply1*-deficient embryos, somite boundaries do not form, the characteristic gene expression in the PSM is not properly terminated, and the initially established rostrocaudal polarity in the segmental unit is not maintained, whereas paraxial mesoderm cells become differentiated. Thus, *ripply1* plays two key roles in the transition from the PSM to somites: termination of the segmentation program in the PSM and maintenance of the rostrocaudal polarity.

We then examined the role of ripply gene famity in the mouse. Its function was also analyzed in an *in vitro* system.

### 3-2 Functional analysis of other genes involved in somite development

The segmental pre-pattern established in the anterior PSM leads to morphological segmentation. Boundary formation and epithelialization are crucial processes in the morphological segmentation of vertebrate somites, but the molecular mechanisms underlying these processes are not yet clearly understood.

To gain insight into the mechanism underlying somite development, we performed an ENU mutagenesis screening of zebrafish, in addition to the *in situ* hybridization screening described above. We found that *integrinα5* and *fibronectin* were mutated in embryos showing defective boundary formation in their anterior somites. Detailed analysis with these mutantas indicated that Integrinα5-directed assembly of Fibronectin appears critical for epithelialization and boundary maintenance of somites.

These results indicate that our strategies are effective for the identification of the genes involved in the somite segmentation process. We are further searching for other genes involved in this process by both the expression screening and the mutagenesis screening methods. This systematic screening should reveal another interesting mechanism underlying somite segmentation.

## Publication List:

### Original papers

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