DIVISION OF MOLECULAR AND DEVELOPMENTAL BIOLOGY

Professor TAKADA, Shinji

Assistant Professor	OKUBO, Tadashi
Technical Staff	UTSUMI, Hideko
NIBB Research Fellow	AKANUMA, Takashi
Postdoctoral Fellows	TAKADA, Ritsuko
	CHEN, Qiuhong
	YABE, Taijiro
	NAKAYAMA, Kei
Graduate Students	ISHITANI, Shizuka
	TAKAHASHI, Jun
	TAKAHASHI, Hiroyuki
	ASANO, Takuya
Technical Assistants	TAKASHIRO, Kayoko
	ISHIKAWA, Mika
	TOMIDA, Sanai
	TSUZUKI, Chisa
Secretary	UKAI, Sakie

The body and tissues of the developing embryo are repeatedly divided into sub-regions specified by characteristic gene expression and morphology. The process that gives rise to these sub-regions according to a defined pattern is called “pattern formation” or “patterning.” The most popular model to explain the patterning process is the “morphogen gradient and threshold” theory. Many genetic results indicate that secreted signal proteins such as Wnt, BMP, and Hedgehog function as morphogens in many aspects of patterning processes. In spite of the accumulation of genetic evidence, however, the biochemical characteristics of morphogens, including modification and higher order structure, remain to be elucidated. Thus, one of our major goals is to reveal the *real image* of morphogens and the molecular mechanism underlying the formation of morphogen gradients, including the secretion and extracellular transport of these morphogens.

The segmental sub-regions of the paraxial mesoderm (or somites), by contrast, appear not to be simply directed by the morphogen gradient and threshold, but by a unique mechanism proceeding periodically. Somites are sequentially generated in an anterior-to-posterior order by converting oscillatory gene expression into periodical structures. The molecular mechanism underlying this conversion and morphological segmentation, however, is not yet fully understood. Thus, another goal of our current studies is to reveal the molecular mechanism of *this other and unique mode of patterning* that underlies the periodical and sequential sub-division in somite formation.

Ⅰ. Secretion and modification of Wnt proteins

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis. Most Wnt proteins transmit their signals locally, presumably since their secretion and transport are under tight control. One important step regulating the extracellular transport of various secreted signal proteins involves post-translational modification with lipid moieties. Recently, we found that murine Wnt-3a is modified with a mono-unsaturated fatty acid, palmitoleic acid, at a conserved Ser residue (Figure 1). Wnt-3a defective in this modification 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 this Ser-dependent modification, as well as for Wnt-3a transport from the ER for secretion. These results strongly suggest that Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process. We expect that the discovery of this unexpected lipid modification might provide a clue regarding the higher-order structure of secreted Wnt proteins and their gradient formation.

To better understand the molecular mechanism of secretion and gradient formation of Wnt proteins, we are carefully examining the biochemical characteristics of Wnt proteins from cultured cells. We are also trying to reveal the role of palmotoleoylation during embryogenesis using zebrafish embryos.

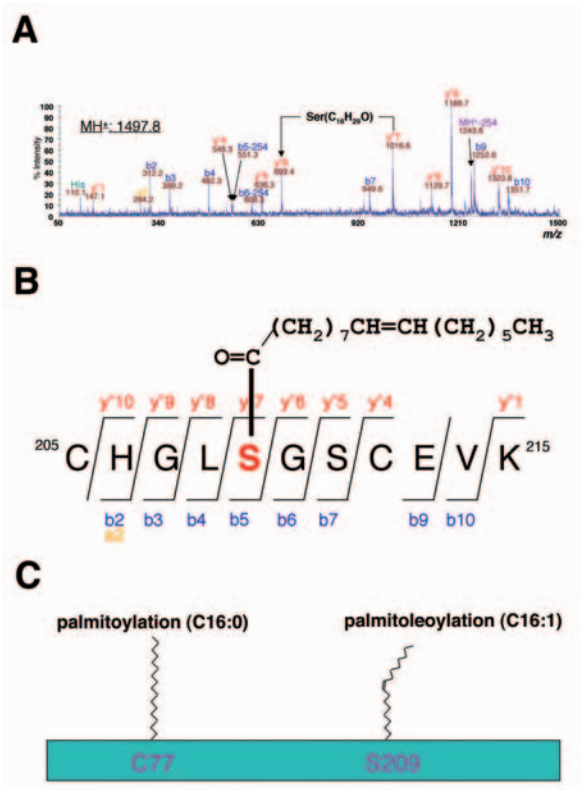

Figure 1. Lipid modifications of murine Wnt-3a protein (A, B) MASS spectrometric profile indicating modification with palmitoleic acid (C16:1) at Ser209, which is conserved among most Wnt proteins. (C) Schematic representation of lipid modification of murine Wnt-3a protein. Wnt-3a is modified with palmitolate (C16:1) at Ser209 and palmitate (C16:0) at Cys77. We found the palmitoleoylation is catalyzed by acytransferase, Porcupine (porc), in the ER. This modification is required for the trafficking of Wnt proteins from the ER.

Ⅱ. Characteristics of genes required for the development of somite or pharyngeal arches –Molecular mechanism of suppression by the Ripply family of transcriptional modulators–

The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the "segmentation clock," which has been evidenced by the cyclic expression of genes in the presomitic mesoderm (PSM). For example, *hairy/Enhancer of split* (*Espl*)-related bHLH genes, including *her1* and *her7* in zebrafish, are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction. The oscillating and anteriorly propagating wave of gene expression, which is maintained in the posterior PSM, becomes fixed to cause segmentation in the anterior PSM. 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.

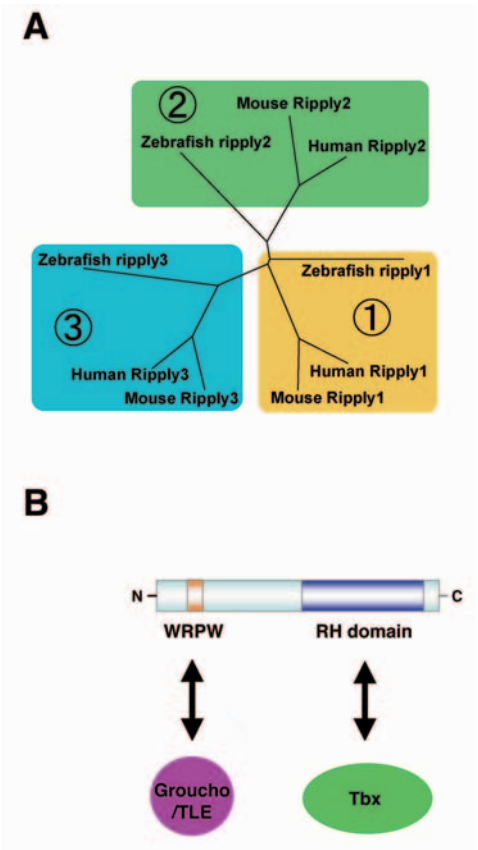

Figure 2. The Ripply family. (A) The phylogenic tree of the Ripply family. (B) Schematic structure of Ripply proteins.

We showed that a 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 (Figure 2). Ripply1 represses specific gene expression in the PSM through a Groucho-interacting motif. In *ripply*1-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.

In *ripply1*-deficient embryos, the expression of *mesp-b*, a key regulator in somite segmentation, is upregulated in a cell-autonomous manner, whereas, in embryos injected with *ripply1*mRNA, the expression of *mesp-b* is highly suppressed in the anterior PSM. These results suggest that Ripply1 regulates the proper expression of *mesp-b* in the anterior PSM.

Taking into account that the expression of *mesp-b* could be induced by T-box transcription factor (Tbx), we can speculate that Ripply1 may antagonize the function of Tbx in the transcription of *mesp* genes. The T-box family of transcription factors, defined by a conserved DNA binding domain called the T-box, regulates various aspects of embryogenesis by activating and/or repressing downstream genes. Reduced function of *T/Brachyury*, the founder member of this family, for example, causes truncated tail in the mouse. The importance of T-box transcription factors is also shown by the fact that mutations in human T-box genes cause severe congenital disorders, such as DiGeorge, Ulnar-mammary, and Holt-Oram syndromes. Furthermore, the T-box family is evolutionally conserved from C. *elegans* to insects and vertebrates, showing remarkable functional conservation across species. In spite of the biological significance of the T-box proteins, how they regulate transcription remains to be elucidated. Therefore, we carefully examined the relationship between Tbx and Ripply family proteins.

We showed that the Groucho/TLE-associated protein, Ripply, converts T-box proteins from activators to repressors (Figure 3). In cultured cells, zebrafish Ripply1, an essential component in somite segmentation, and its structural relatives, Ripply2 and Ripply3, suppress the transcriptional activation mediated by the T-box protein Tbx24, which is coexpressed with *ripply1* during segmentation. Ripply1 associates physically with Tbx24 and converts it to a repressor. Ripply1 also antagonizes the transcriptional activation of another T-box protein, No tail (Ntl), the zebrafish ortholog of Brachyury. Furthermore, injection of a high dosage of *ripply1* mRNA into zebrafish eggs causes defective development of the posterior trunk, similar to the phenotype observed in homozygous mutants of *ntl*. These results indicate that the intrinsic transcriptional property of T-box proteins is controlled by Ripply family proteins, which act as specific adaptors that recruit the global corepressor Groucho/TLE to T-box proteins.

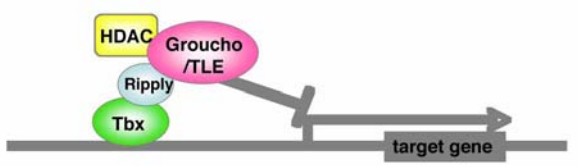

Figure 3. Transcriptional repression mediated by Ripply. Ripply converts transcriptional property of T-box transcription factor from activator to repressor by recruiting Groucho/TLE co-repressor complex.

To get further mechanistic insights into the interactions between Tbx24 and Ripply1, we next prepared several deletion or amino acid-substituted constructs of Tbx24 and Ripply1 (Figure 4A). By conducting immunoprecicipitation assays, we found that the T-domain alone, which is highly conserved among the Tbx family proteins, is sufficient for the association with Ripply1 but that another region of Tbx24 is dispensable for the interactions. On the other hand, a ~50 amino acid length sequence at the carboxyl terminus, called the Ripply homology domain, is conserved among the Ripply family proteins. Highly conserved sequences exist in this domain. Substitution of some of these sequences to an alanine stretch (Ripply1-mutFPVQ) results in a significant reduction in the transcriptional repression of the pBP-TbxX2 reporter construct (Figure 4B), indicating that this conserved sequence is required for Ripply-mediated transcriptional repression. This reduction appears to be due to decreased affinity of Ripply1-mutFPVQ for Tbx24 (Figure 4C). Consistent with this result, not only Ripply1 but also its structural relatives, Ripply2 and Ripply3, can suppress the transcriptional activation mediated by the T-box protein Tbx24 in cultured cells. Based on these results, many T-box proteins are likely to be converted from activators to repressors through specific interactions with Ripply proteins.

The identification of Ripply1 as a switching molecule for T-box genes suggests a novel transcriptional mechanism that could participate in other aspects of development, although the relationship between the Ripply family and T-box factors besides Tbx24 and Ntl remains to be elucidated. The overlapping expression of *ripply* and T-box genes, observed in various developing tissues and organs, supports the idea that the two proteins cooperate in development. Further understanding of the cooperative transcriptional regulation by Ripply and T-box proteins could help elucidate the mechanisms underlying the disproportionate activation or repression found in human genetic disorders associated with mutations in T-box genes.

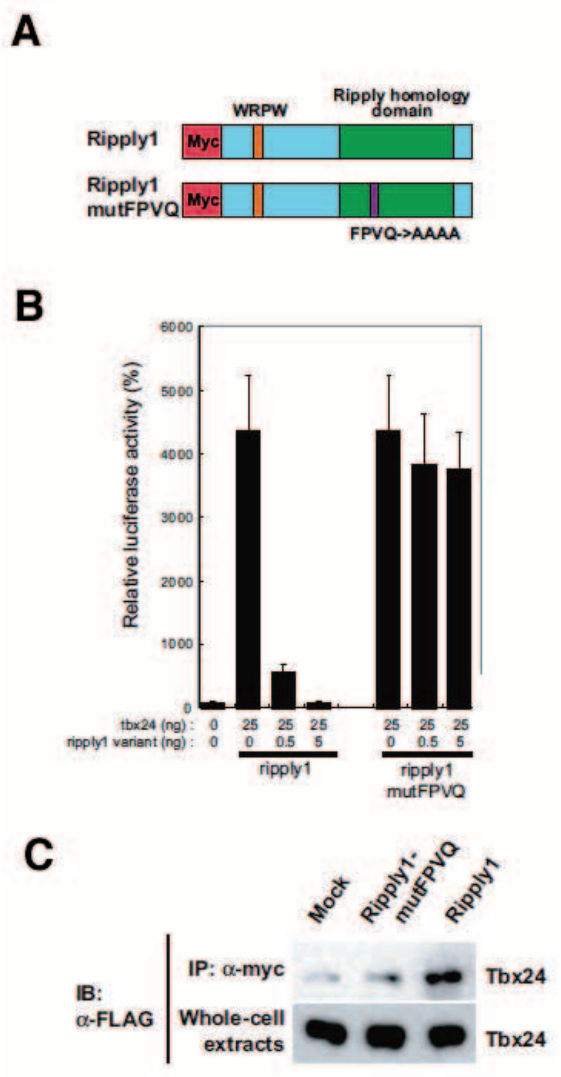

Figure 4. A conserved amino acid stretch in the Ripply homology domain required for interaction with T-box protein. (A) Schematic representation of a Ripply1 mutant used in the analysis shown in B and C. (B) *In vitro* reporter assay. (C) Immunoprecipitation to examine physical interaction between Rippply1 mutant and Tbx24.

Publication List

〔Original papers〕

- Alvarez-Medina, R., Cayuso, J., Okubo, T., Takada, S., and Marti, E. (2008). Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression Development *135*, 237-247.
- Kawamura, A., Koshida, S., and Takada, S. (2008). Activator-to-repressor conversion of T-box transcription factors by the Ripply family of Groucho/TLE-associated mediators. Mol. Cell. Biol. *28*, 3236-3244.
- Shimizu, T., Kagawa, T., Inoue, T., Nonaka, A., Takada, S., Aburatani, H., and Taga, T. (2008). Stabilized β-catenin functions through TCF/LEF proteins and the Notch/RBP-J_κ complex to promote proliferation and suppress differentiation of neural precursor cells. Mol. Cell. Biol. *28*, 7427-7441.