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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 the development of somites and pharyngeal arches.

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. 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.

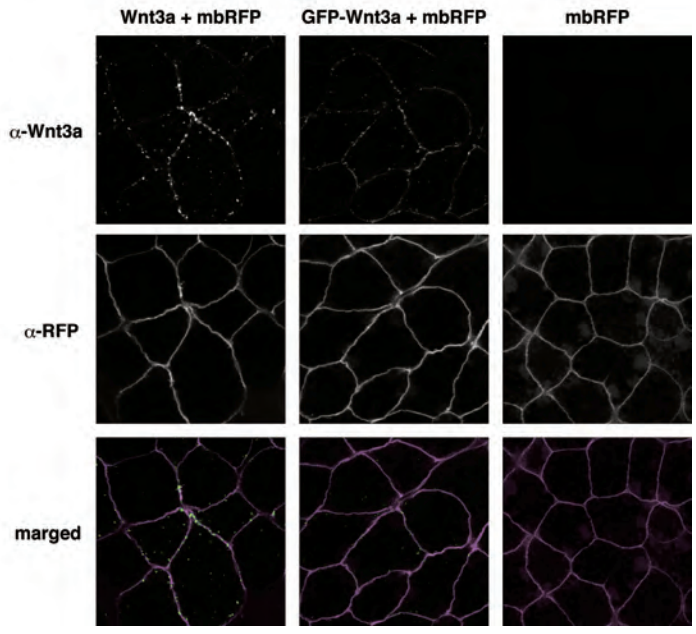

Figure 1. Visualization of Wnt-3a proteins secreted from Xenopus epidermal cells. A GFP-tagged form of Wnt-3a protein, as well as the authentic form, can be observed as punctate signals around the edge of cells. We can use the GFP tagged-Wnt proteins for visualization of extracellular trafficking of Wnt proteins.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2009. The former title is indicated by an asterisk (*).

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 palmitoleoylation and the molecular mechanism underlying the extracellular transport of Wnt proteins during embryogenesis using zebrafish and frog embryos (Figure 1).

II. Characteristics of genes required for the development of somite or pharyngeal arches -----The function of mouse Ripply1 and 2 in the rostro-caudal patterning within a somite -----

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 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.

The spatial pattern of somites is characterized not only by the periodical borders between neighboring somites, but also by the rostral and caudal compartments within a somite. These compartments are subsequently segregated and re-fused with adjacent compartments to form vertebra. In addition, the rostro-caudal pattern defines the migration of neural crest cells and motor axons. In contrast to the many patterning processes that have already been revealed, the rostro-caudal patterning of a somite is unique in that a spatial pattern is established with temporal periodicity. However, the precise molecular mechanism by which the rostro-caudal pattern is established remains unclear.

We showed that a gene identified by our *in situ* hybridization screening, *ripply1* is required for this transition. Ripply proteins suppress Tbx-mediated transcription by recruiting the Groucho/TLE co-repressor. Rippy1 and 2 are expressed in the anterior PSM and in several newly formed somites in zebrafish and mouse embryos (Figure 2). In *ripply1*-deficient zebrafish embryos somite boundaries do not form, the characteristic gene expression in the PSM is not properly terminated, and the initially established rostro-caudal patterning in the segmental unit is not maintained, whereas paraxial mesoderm cells become differentiated. Thus, *ripply1* plays a role in the maintenance of the rostro-caudal patterning.

In addition, Notch signaling and Mesp2 activity are also required for the rostro-caudal patterning of a somite. For instance, mouse embryos defective in Notch signaling, caused by knocking out *Dll1* or *Presenilin1*, show rostralized somites, whereas those lacking Mesp2 activity exhibit caudalization of their somites. Consistent with their roles in rostro-caudal patterning, the Notch and Mesp2 active domains become contracted in the caudal half of S0 (the prospective somite in the most anterior PSM) and in the rostral half of S-1 (the prospective somite posterior to S0), respectively. Interestingly, *Mesp2* is required for *Ripply2* expression, indicating that *Mesp2* suppresses its own expression by activating Ripply2 expression. Thus, rostro-caudal patterning appears to be established through molecular interactions between these molecules.

Given that Mesp2 expression is dynamically changed in the anterior PSM in association with the traveling wave of Notch activity, characterization of the dynamism of Mesp2 expression and Notch activity would be important for understanding the mechanism of rostro-caudal patterning. Furthermore, the effect of Ripply on the dynamic movement of Mesp2 expression and Notch activity would be revealed. Therefore, we examined the dynamic processes of this patterning by exhaustive examination of periodical changes in the location of the Notch active domain and the Mesp2 protein domain in wild-type and *Ripply*-deficient mouse embryos at several distinct phases of the segmentation cycle. We examined the spatial movement of Notch activity with Mesp2 protein localization in wild-type embryos and those

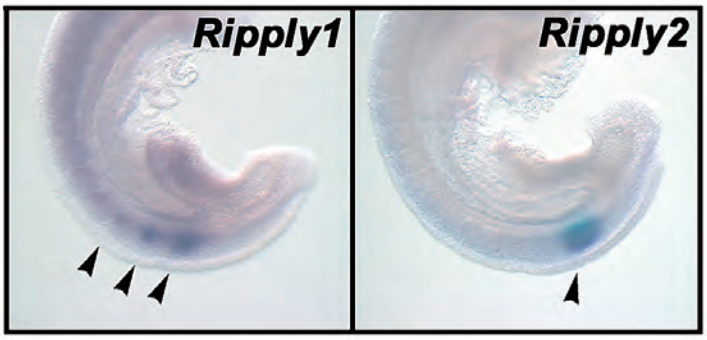

Figure 2. Expression patterns of mouse Ripply1 and 2 in somite development. Both Ripply1 and 2 are segmentally expressed in the anterior presomitic mesoderm.

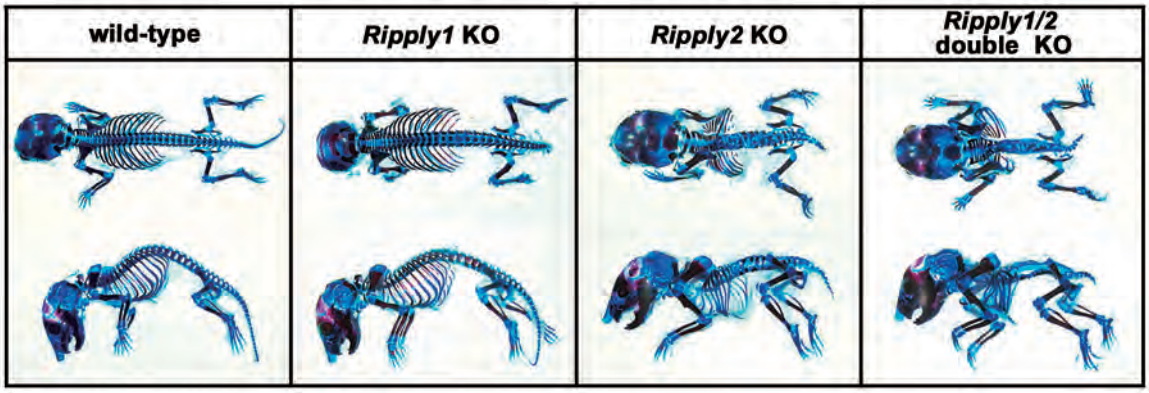

Figure 3 Segmental abnormalities observed in Ripply1 and 2 double knockout embryos. Segmental defects of somites appear in structures of vertebrae and ribs. Ripply1 deficiency enhances rostralized phenotype of Ripply2 knock-out embryos in somite development. .

defective in the two *Ripply* genes expressed in the PSM.

Mesp2 protein appears first as a thin band in the traveling Notch domain. In wild-type embryos, the Mesp2 band expands anteriorly to the expression front of Tbx6, an activator of *Mesp2* transcription. Notch activity becomes localized further anteriorly to this Mesp2 domain, but does not pass over the anterior Mesp2 domain generated in the previous segmentation cycle. In *Ripply1/2*-deficient embryos, the Mesp2 band becomes more expanded and the Notch domain is finally diminished. Interestingly, *Ripply1/2*-deficient embryos exhibit anterior expansion of Tbx6 protein domain, suggesting that Ripply1/2 regulates *Mesp2* expression through Tbx6 degradation. We propose that the rostro-caudal pattern is established by dynamic interaction of Notch activity with two Mesp2 domains, which are defined in successive segmentation cycles by Notch, Tbx6 and Ripply1/2.

In addition to these roles of Ripply1 and 2 in somite segmentation, we have also examined the role of another Ripply gene in the development of pharyngeal arches.

Publication List

〔Original paper〕

- Agalliu, D., Takada, S., Agalliu, I., McMahon, A.P., and Jessell, T.M. (2009). Motor neurons with axial muscle projections specified by Wnt4/5 signaling. Neuron *61,* 708-720.