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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 along some defined pattern is called "pattern formation" or "patterning." The most popular model to explain the patterning process is the "morphogen gradient and threshold" theory. Actually, many genetic results indicate that secreted signal proteins such as Wnt, BMP, and Hedgehog function as morphogens in many aspects of patterning processes. However, in spite of the accumulation of genetic evidence, the biochemical characteristics, including modification and higher order structure, of morphogens 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.

In contrast, the segmental sub-regions of the paraxial mesoderm, or somites, 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, has not yet been fully understood. Thus, another goal of our current studies is to reveal the molecular mechanism of *this other and unique modes of patterning* that underlies the periodical and sequential sub-division in somite formation.

I. Molecular mechanism for secretion 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. In the case of Wnt, Nusse and co-workers reported that murine Wnt-3a is S-palmitoylated at a conserved cysteine residue, and proposed that palmitoylation of this cysteine residue may be required to produce an increased local concentration of Wnt on the plasma membrane. In contrast, there is strong evidence to suggest that lipid modification is involved in the processing and intracellular trafficking of Wnt prior to secretion.

To resolve inconsistencies between the previous studies and to better understand the biological significance and molecular mechanism of lipid modification of Wnt, we carefully examined its modification. Unexpectedly, we found that murine Wnt-3a is modified with a mono-unsaturated fatty acid, palmitoleic acid, at a conserved Ser residue. 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 (Figure 1). 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.

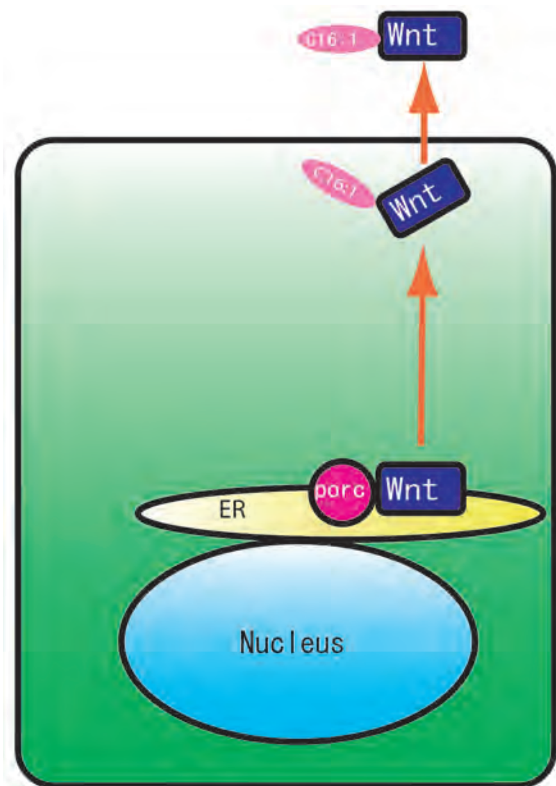

Figure 1. The function of palmitoleic lipid modification of Wnt protein. Wnt proteins are modified with palmitoleic acid (C16:1) by acytransferase, Porcupine (porc), in the ER. This modification is required for trafficking of Wnt proteins from the ER.

Ⅱ. Identification and characterization of genes required for somite development

2-1 Paf1 complex homologs are required for Notch-regulated transcription during somite segmentation

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 instance, *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. Interestingly, many genes that exhibit cyclic expression are regulated by Notch signaling in the PSM. Notch signaling is also involved in other aspects of segmentation, synchronization of the oscillating phase between cells in the PSM, and establishment of the rostral-caudal compartment within a somite.

In previous studies, we identified numerous ethylnitrosourea (ENU)-mutagenized zebrafish mutants with altered somite morphogenesis. For instance, we found that *integrin*α*5* and *fibronectin* were mutated in embryos showing defective boundary formation in their anterior somites. Detailed analysis of these mutantas indicated that Integrina5-directed assembly of Fibronectin appears critical for the epithelialization and boundary maintenance of somites. This result indicates that our strategies are effective for the identification of the genes involved in the somite segmentation process.

We are also searching for other genes involved in this process by both the expression screening and the mutagenesis screening methods. This year, we characterized one mutant obtained in our screens, *kt641*, which exhibited reduced but still striped expression of the Notch target genes in the PSM (Figure 2). In contrast to previously identified Notch signaling mutants, in which the striped expression of Notch target genes is perturbed to form salt-and-pepper patterns, *kt641* exhibited reduced but still striped expression of the Notch target genes in the PSM. We found that the gene responsible for this phenotype is a zebrafish homolog of yeast *rtf1*.

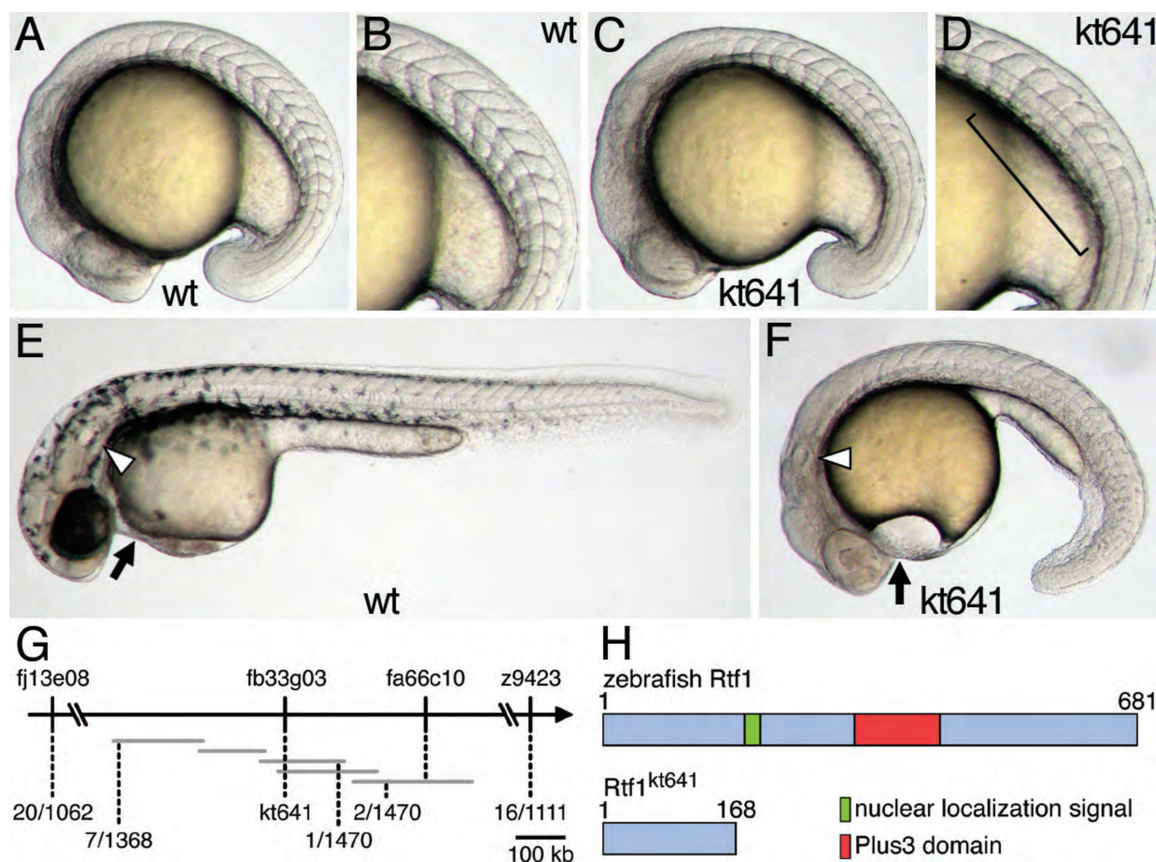

Figure 2. Phenotype of kt641 mutant and the structure of Rtf1. (A-F) Somite boundaries are disrupted in the posterior trunk of the *kt641* mutant (bracket in D). At later stages, the *kt641* mutation causes reduced pigmentation, limited tail growth, and abnormal heart (arrow) and ear (arrowhead) development (F). (G) Meiotic and physical mapping of *kt641* mutation. Horizontal gray bars represent contigs deposited in LG 13. (H) Schematic diagrams of zebrafish Rtf1 proteins encoded by wild-type and *kt641* alleles.

Rtf1 is a member of the Paf1 complex, which has been implicated in various processes, such as transcription initiation and elongation, histone modification, phosphorylation of pol II, and RNA processing and export. Members of Paf1 complex, which is composed of at least five components (Paf1, Rtf1, Cdc73, Leo1, and Ctr9), are conserved from yeast to human. Although these proteins have been implicated in RNA polymerase II-mediated transcription, their roles in vertebrate development have not been elucidated. We showed that a zebrafish mutant exhibiting a somite segmentation defect is deficient in *rtf1*. In addition, embryos deficient in *rtf1* or *ctr9* display abnormal development of the heart, ears, and neural crest cells. *rtf1* is required for proper RNA levels of the Notch-regulated genes *her1*, *her7*, and *deltaC* as well as for Notch-induced *her1* expression in the presomitic mesoderm. Furthermore, the phenotype observed in *rtf1*-deficient mutants is enhanced by an additional deficiency in mind bomb, which encodes an effector of Notch signaling. Thus, zebrafish homologs of the yeast Paf1 complex appear to preferentially affect a subset of genes, including Notch-regulated genes, during embryogenesis (Figure 3).

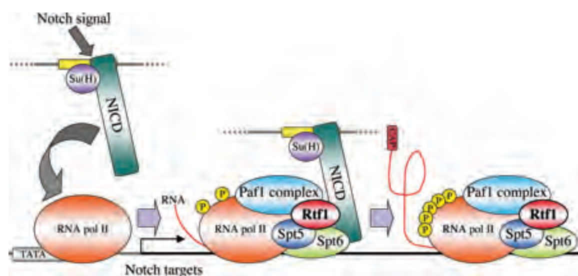

Figure 3. A model to explain Rtf1 function in the transcription of a Notch target gene. Our results suggest that the Paf1 complex may regulate the transcription of Notch targets in association with Spt5 and Spt6.

2-2 Transcriptional repression by Groucho-associated transcriptional mediator Ripply1 in somite segmentation

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 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. 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.

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 *ripply*1mRNA, 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. Therefore, we examined precisely the relationship between Tbx24 and Ripply1 in the transcription of *mesp-b*. We showed that Ripply1 is coprecipitated with Tbx24 and converts it from an activator to a repressor in culture cells. The other members of the Ripply family, Ripply2 and Ripply3, can also antagonize the transcriptional activation mediated by Tbx24. On the other hand, Ripply1 also antagonizes the transcriptional activation of another T-box protein, No tail (Ntl), both *in vitro* and *in vivo*. 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.

Publication List

〔Original papers〕

- Akanuma, T., Koshida, S., Kawamura, A., Kishimoto, Y., and Takada, S. (2007). Paf1 complex homologes are required for Notch-regulated transcription during somite segmentation. EMBO Rep. *8*, 858-863.
- Ishioka, T., Katayama, R., Kikuchi, R., Nishimoto, M., Takada, S., Takada, R., Matsuzaka, S., Reed, J.C., Tsuruo, T., and Naito, M. (2007). Impairment of the ubiquitin-proteasome system by cellular FLIP. Genes Cells *12*, 735-744.
- Takada, I., Mihara, M., Suzawa, M., Ohtake, F., Kobayashi, S., Igarashi, M., Youn, M.-Y., Takeyama, K., Nakamura, T., Mezaki, Y., Takezawa, S., Yogiashi, Y., Kitagawa, H., Yamada, G., Takada, S., Minami, Y., Shibuya, H., Matsumoto, K., and Kato, S. (2007). A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nat. Cell Biol. *9*, 1273 - 1285.