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**Abstract**

In most ascidians, metamorphosis of tadpole-like swimming larvae is accompanied by dynamic changes in their shape to form sessile adults. The mechanisms underlying ascidian metamorphosis have been debated for a long time. Although recent molecular studies have revealed the presence of various molecules involving in this process, the basic mechanism of the metamorphic events is still unclear. For example, it has not been solved whether all metamorphic events are organized by the same single pathway or by multiple, independent pathways. In the present study, we approached this question using the ascidian Ciona intestinalis. When the papillae and preoral lobes of the larvae were cut off, the papillae-cut larvae initiated certain trunk metamorphic events such as the formation of an ampulla, body axis rotation and adult organ growth without other metamorphic events. This observation indicates that metamorphic events can be divided into at least two groups, events initiated in the papillae-cut larva and events not initiated in this larva. In addition to this observation, we have isolated a novel mutant, tail regression failed (trf), which shows similar phenotypes to those of papillae-cut larvae. The phenotypes of trf mutants are basically different from those of swimming juvenile mutants (Sasakura, Y., Nakashima, K., Awazu, S., Matsuoka, T., Nakayama, A., Azuma, J., Satoh, N., 2005. Transposon-mediated insertional mutagenesis revealed the functions of animal cellulose synthase in the ascidian Ciona intestinalis. Proc. Natl. Acad. Sci. U. S. A. 102, 15134–15139.), which also show abnormal metamorphosis. These findings suggest a model by which ascidian metamorphic events can be classified into four groups initiated by different pathways.

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**Introduction**

Ascidians provide excellent experimental systems to uncover cellular and molecular mechanisms involved in animal metamorphosis (Cloney, 1982; Satoh, 1994). Fertilized eggs of ascidians develop quickly into tadpole larvae, which then complete metamorphosis within several days. Such quick development and metamorphosis allow easy, detailed observations of the changes during metamorphosis and make it possible to carry out various experimental treatments.

During the metamorphosis of ascidians, non-feeding swimming larvae attach to substrates and initiate a set of drastic structural changes to form sessile filter feeder adults. Although there are many structural variations in ascidian larvae, all ascidians are lecithotrophic, and organs or organ rudiments required for the post-larval phase of the life cycle do not grow or are not differentiated in the larva (Cloney, 1978). Many studies have described in detail the changes of organs and tissues during metamorphosis, such as the morphological change of papillae at the beginning of metamorphosis, contraction of the epidermis and notochord during tail regression, formation of tunic cells and immigration of mesenchymal cells (Cloney, 1978, 1982, 1990; Wright, 1981). Cloney (1982) pointed out the following ten events as the basis of ascidian metamorphosis: (1) secretion of adhesives by the papillae or epidermis of the trunk, (2) eversion and retraction of papillae, (3) regression of the tail, (4) loss of the outer cuticle layer of the larval tunic, (5) emigration of blood cells or pigmented cells, (6) expansion of visceral organs through an arc of about 90°, expansion of the branchial basket, and elongation of the oozooids of juveniles, (7) rotation, elongation, or reciprocation of ampullae, (8) release of test vesicles and expansion of the tunic, (9) phagocytosis of visceral ganglion, sensory organs, and cells of the axial complex, and (10) release of organ rudiments from an arrested state of development. Some kinds of cues derived from settlement lead to the initiation of metamorphosis, and these metamorphic events occur in coordination, because these events never occur prior to settlement and always proceed in the order enumerated above. The mechanism by which these events are coordinated is largely unknown, however.

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Several factors have been shown to act as stimuli for the initiation of metamorphosis (Grave, 1944; Hirai, 1961; Lynch, 1961; Whittaker, 1964; Cloney, 1978; Patricolo et al., 1981; Degnan et al., 1997; Bishop et al., 2001; Kimura et al., 2003; Zega et al., 2005; Roberts et al., 2007), and the papillae are known to be a critical organ for sensing stimuli. The papillae are adhesive organs consisting of three protrusions at the anterior end of the larva (Katz, 1983; Satoh, 1994). Except for its anterior tip, a single layer of flattened epidermal cells envelopes the papilla. Papillae contain epidermal neurons (Horie et al., 2008) and secrete adhesives for effective settlement at the onset of metamorphosis (Satoh, 1994). They function not only as an adhesive organ but also as a control center for the initiation of metamorphosis. Eri et al. (1999) showed that Hemps, an EGF-like secreted signaling molecule, is expressed in the papillae to induce metamorphosis. Chambon et al. (2007) observed specific activation of the MAPK signaling pathways in papillae at the onset of metamorphosis.

Significant roles of the nervous system in the initiation of metamorphosis have also been reported. Neurotransmitters have been reported to play an important role in the initiation of metamorphosis (Kimura et al., 2003; Zega et al., 2005). The presence of a neural network that connects papillae neurons to the sensory vesicle has been reported (Takamura, 1998; Hudson and Lemaire, 2001; Horie et al., 2008). These reports suggest that the papillae sense and transmit stimuli of settlement to regulate metamorphic events through the neurons. In addition to these discoveries, recent molecular studies have revealed various genes whose expression is up- or down-regulated during ascidian metamorphosis (Arnold et al., 1997; Davidson and Swalla, 2001, 2002; Nakayama et al., 2001, 2002; Woods et al., 2004, Chambon et al., 2007). Some of these genes are strongly expressed in the papillae (Nakayama et al., 2001, 2002). These studies provide good clues to understanding the regulatory mechanisms of ascidian metamorphosis by the papillae.

In contrast to the upstream mechanism, the downstream mechanism after settlement is not well understood in ascidian metamorphosis. In fact, it is not known how many pathways are present to initiate the ten above-mentioned metamorphic events. The most simplified

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**Fig. 1.** Metamorphosis of papillae-cut larvae. (A) A swimming larva, the body of which is largely divided into two parts, trunk and tail. The position of the papillae cut is shown by a red dash line. Pa, papilla; Po, preoral lobe; SV, sensory vesicle. Scale bar, 100 μm for A–D. (B) A larva just after the papillae cut. (C) A 3-dpf-old papillae-cut larva. Papillae-cut larvae showed formation of ampullae and growth of adult organs, while sensory vesicle retraction and tail absorption did not occur. The size of the sensory vesicle (SV) is larger than that of a normal juvenile shown in (D). (D) The normal juvenile has developed adult organs such as ampullae (Am), an oral siphon (OS), an endostyle (En), stigmata of the gills (St) and a digestive gland (DG). (E) A 3-dpf-old larva of Tg[MiCiTnIG]1 transgenic line. Primordia of oral siphon (OS) and atrial siphon (AS) show GFP signal. (F) A 3-dpf-old juvenile of Tg[MiCiTnIG]. Muscles of oral and atrial siphons are more developed than those in (E), and clear rows of muscle cells are seen. The longitudinal muscle is elongated from the posterior pole (arrow). (G) A 3-dpf-old papillae-cut larva of Tg[MiCiTnIG]. Oral siphon and atrial siphon muscles are more developed than those in larvae but smaller than those in normal juveniles. The longitudinal muscle is elongated from the posterior pole (arrow).
model is that papillae sense the stimuli for metamorphosis and activate a single pathway that starts all ten metamorphic events. However, this may not be the case. It has been suggested that metamorphic events are classified into several groups which are regulated by different mechanisms (Cloney, 1978; Sasakura et al., 2005). For example, it has been suggested that trunk development and tail regression are differentially controlled (Cloney, 1978). Direct evidence for this notion has come from mutant analyses (Sasakura et al., 2003a,b, 2005). Minos transposon-based transgenesis has generated an insertion mutant swimming juvenile (sj). These sj mutant larvae autonomously start certain trunk metamorphic events such as the retraction of adhesive papillae and body axis rotation without settlement, but never proceed to other metamorphic events such as tail regression, retraction of sensory vesicle and adult organ formation (Sasakura et al., 2005). This suggests that the events initiated in the sj larvae are triggered by a different pathway from the events that are not initiated in sj larvae. The gene responsible for the sj mutant is Ci-CesA, which encodes cellulose synthase (Nakashima et al., 2004; Sasakura et al., 2005), suggesting a relationship between animal cellulose and the regulatory pathways of metamorphosis.

In that case, how many pathways are present in ascidian metamorphosis? To understand the mechanisms of ascidian metamorphosis, identification of the discrete pathways initiating metamorphic events is necessary. We addressed this question by two experiments, removal of a part of larval tissues and mutant analysis, in the ascidian Ciona intestinalis. Among the ten metamorphic events, six events including papilla retraction, ampulla formation, tail regression, body axis rotation, sensory vesicle retraction and adult organ growth, were addressed in this study as the major metamorphic events. We found that these metamorphic events of Ciona can be classified into four groups initiated by different mechanisms. The present study provides a new model for the mechanisms of ascidian metamorphosis.

Materials and methods

Animals

Adults of C. intestinalis were collected from various areas in Japan. Animals were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at about 18 °C in seawater containing 50–100 μg/ml streptomycin. Larvae hatched out and swam at approximately 18 h post fertilization (hpf). Yeast extract was added at the concentration of 1 mg/ml in seawater to induce metamorphosis.

Cutting off of papillae or the tail of larvae was performed with razors. To avoid loss of the experimental larval body by entrapment at the surface of seawater, the surface of seawater was covered with polyethylene film.

Table 1

<table>
<thead>
<tr>
<th>Cut parts</th>
<th>% of animals which keep larval morphology</th>
<th>% of animals showing tail absorption</th>
<th>% of animals showing ampulla formation and body axis rotation</th>
<th>% of animals showing ampulla formation, body axis rotation and growth of adult organs without tail absorption</th>
<th>N</th>
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<tr>
<td>Papillae</td>
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<td>12.4</td>
<td>43.4</td>
<td>44.2</td>
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<td>Papillae+tail</td>
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<td>4.0</td>
<td>34.9</td>
<td>55.6</td>
<td>126</td>
</tr>
<tr>
<td>Papillae+tail tip</td>
<td>3.5</td>
<td>9.6</td>
<td>13.2</td>
<td>73.3</td>
<td>114</td>
</tr>
<tr>
<td>Tail</td>
<td>100</td>
<td>0**</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Tail tip</td>
<td>64.1</td>
<td>35.9</td>
<td>0</td>
<td>0</td>
<td>131</td>
</tr>
</tbody>
</table>

* Observation was performed at 3 dpf. ** Completion of tail absorption was judged by the formation of an epidermal chamber at the posterior end of the trunk to receive the absorbed tail.
One was that growth of the adult organs in the papillae-cut larvae was slower than in normal juveniles at the same age, and thus the trunk of the papillae-cut larvae was smaller than that of the normal juveniles. The other difference was that papillae-cut larvae did not perform sensory vesicle retraction, and thus their sensory vesicle remained as a large sphere, like that of normal larvae. Therefore, the sensory vesicle remained in the larval state in papillae-cut larvae, even after other trunk metamorphic events were initiated. These results showed that the removal of the preoral lobe and papillae leads to the initiation of ampulla formation and adult organ formation without settlement, tail regression, or sensory vesicle retraction.

The larval preoral lobe contains both an epidermis and mesenchymal cells. In order to examine which tissue’s removal is responsible for alterations in metamorphic events, we cut larvae carefully so as to retain as many mesenchymal cells as possible in the preoral lobe. Even though most of the mesenchymal cells were left, metamorphic events in the trunk occurred without settlement and thus these manipulated larvae showed ampulla and adult organ formation (data not shown). On the other hand, when cutting of the anterior epidermis was incomplete, the experimented larvae did not

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Responsiveness of larvae to yeast extract*</th>
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<tr>
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<td>% of not metamorphosed animals</td>
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<tr>
<td>Papillae-cut larvae</td>
<td>100</td>
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<tr>
<td>Tail-cut larvae</td>
<td>7.69</td>
</tr>
<tr>
<td>Normal larvae</td>
<td>20</td>
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</tbody>
</table>

* Excision was performed at 2–3 h after hatch. The larvae were kept for 2 days to observe the phenotypic changes. Unmetamorphosed animals were treated with yeast extract (1 mg/ml in seawater). After exposure to yeast extract overnight, the phenotypic change was observed. The completion of metamorphosis was judged by tail absorption and overall morphology.

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**Fig. 2.** Requirement of cell division for the metamorphic events of papillae-cut larvae. (A) Incorporation of BrdU of a 3-dpf-old juvenile. Strong signals are detected in multiple tissues such as atrial siphon (AS), oral siphon (OS), digestive gland (DG), endostyle (En) and pharyngeal gill (Gi). Bar, 100 μm. (B) Incorporation of BrdU of a 3-dpf-old papillae-cut larva. The outline of the larva is shown by a dashed line. Signals are observed in the adult organs. The signal intensity is stronger than that in larvae but weaker than that in juveniles. (C) Incorporation of BrdU of a 3-dpf-old larva. Only a few cells show incorporation of BrdU. (D) A control papillae-cut larva showing growth of adult organs and an ampulla (Am). For easy observation, the tail of the larva was cut. (E) Incorporation of BrdU of a papillae-cut control larva. Strong signals are observed. The larva in (E) is different from that in (D). (F) A papillae-cut larva treated with aphidicolin. Neither ampullae nor adult organs grew. The tail of the larva was cut for easy observation. (G) Incorporation of BrdU of a papillae-cut larva treated with aphidicolin. No signal is detected. The larva in (G) is different from that in (F).
start trunk metamorphosis. Taken together, the evidence shows that the epidermis consisting of the papillae is the critical tissue that regulates metamorphosis.

The papilla is an organ that senses stimuli of settlement, and the stimuli may be transmitted to the posterior part of the body to initiate metamorphic events. To examine whether or not the loss of papillae affects the organism’s ability to sense stimuli, we treated papillae-cut larvae with yeast extract, which is an inductive agent of metamorphosis in C. intestinalis (Nakayama et al., 2001). After the acquisition of competence, normal larvae responded to the yeast extract to start metamorphosis and became complete juveniles (Table 2). In contrast, papillae-cut larvae did not show any response to the agent. This result demonstrates that the papillae and preoral lobe are critical in receiving the stimulus for the absorption of the tail. This result also suggests that papillae-cut larvae initiate trunk metamorphic events autonomously without the stimuli of settlement, because they do not have the capacity to receive these stimuli.

Cell division is required for adult organs growth in papillae-cut larvae

Initiation of Ciona metamorphosis is accompanied by the restart of drastic cell division, which is necessary for rapid growth of adult organs (Nakayama et al., 2005; Fig. 2A). Trunks of papillae-cut larvae grew to form adult organs. However, growth of their trunks was slower than that in normal juveniles. We wondered whether this drastic change in cell division is initiated in papillae-cut larvae as in the normal metamorphosis. To examine this question, the cell division cycle in papillae-cut larvae was monitored with bromodeoxyuridine (BrdU). While many BrdU-positive cells were observed in the normal juveniles and papillae-cut larvae (Figs. 2A–C), only a few BrdU-positive cells were observed in normal larvae, suggesting that cell division is arrested at the larval stage. BrdU-positive cells increased in number in papillae-cut larvae compared with normal larvae, suggesting that cell division is activated in papillae-cut larvae. However, the BrdU-positive signals in papillae-cut larvae were much weaker than those in the normal juveniles, suggesting that activation of cell division in papillae-cut larvae is less efficient than in normal juveniles.

Next, to address the necessity of cell division for metamorphic events and adult organ growth in papillae-cut larvae, they were treated with an inhibitor of DNA replication, aphidicolin (Satoh and Ikegami, 1981). Aphidicolin-treated papillae-cut larvae initiated ampulla and body cavity formation. However, neither elongated ampullae nor adult organs developed (Figs. 2D and F). Incorporation of BrdU was not observed in aphidicolin-treated larvae (Figs. 2E and G), suggesting that the cell division cycle was blocked by this chemical. Therefore, papillae-cut larvae form ampullae and adult organs through activation of cell division. Taken together, cell division is restarted by cutting of the papillae and preoral lobe to allow for ampulla formation and adult organ growth. Activation of cell division in the papillae-cut larvae is less efficient than in normal juveniles, resulting in slower adult organ growth. This indicates the presence of an additional mechanism for initiating adult organ growth, which is not turned on by cutting of the papillae and preoral lobe.

The preoral lobe is necessary to initiate apoptosis in the tail

Tail tissues start apoptosis after acquisition of competence for metamorphosis. Previous studies have shown that apoptosis plays a critical role for tail absorption (Chambon et al., 2002, 2007). The blockage of apoptosis in the tail results in the failure of tail absorption initiation. Papillae-cut larvae did not undergo tail absorption, even when trunk metamorphic events had been initiated. There is a possibility that apoptosis in the tail tissues of the papillae-cut larvae is suppressed, which in turn results in the failure of tail absorption. To address this issue, we observed apoptosis in papillae-cut larvae by TUNEL staining. As shown in Fig. 3A, post-competent larvae had TUNEL-positive cells at the posterior part of the tail. In contrast, post-competent papillae-cut larvae showed no TUNEL-positive cells in the tail (Fig. 3B). Similarly, tails that were cut off from the trunk showed no TUNEL-positive cells even though they reached the post-competent stage (Fig. 3C). These results suggest that initiation of apoptosis in the tail tissues requires signaling from the preoral lobe of the trunk. Cutting of the preoral lobe and papillae causes the suppression of apoptosis in the tail, which probably results in the failure of tail absorption in the papillae-cut larvae.

Metamorphosis of tail-cut larvae

Tail regression is the most dramatic event during ascidian metamorphosis. Tail regression precedes most events of trunk metamorphosis, including body axis rotation, sensory vesicle retraction and the formation of ampullae and adult organs. However, the regulatory mechanism of the timing of tail absorption and trunk metamorphic events is not known. One plausible possibility is that the tail has a repressive function against these trunk metamorphic events, and that the absorption of tail releases the trunk from repression in the normal process of metamorphosis. To examine this possibility, larval tails were cut off from the trunk at 1–2 hph, and the trunk regions were cultured. These larvae were referred to as “tail-cut larvae”. Effects were observed during the first 1–2 days after the cut. The trunks of unsettled tail-cut larvae showed no structural change or metamorphic developments, and

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Fig. 3. Apoptosis of tail cells in papillae-cut larvae and cut tail. (A–B) Apoptosis of tail cells detected by TUNEL staining in larvae at approximately 24 hpf. Clear TUNEL-positive signals (green) are observed at the posterior region of a normal larva (A), while no signal is detected in the tail cells of a papillae-cut larva (B). (C) Apoptosis of cells of a tail that was cut from its trunk, at approximately 24 hpf. No TUNEL signal is detected in the tail cells.
they maintained the larval state (Fig. 4A). Therefore, the tail does not appear to have a repressive function on trunk metamorphosis. The percentage of tail-cut larvae that initiated metamorphosis was very low (Table 1). This is because tail-cut larvae were not able to swim to attach to substrates with their papillae. Some attached tail-cut larvae started metamorphosis as in the normal larvae and became juveniles with normal morphology (Fig. 4B), suggesting that tail-cut larvae have the ability to complete metamorphosis like normal larvae. Tail-cut larvae showed responsiveness to yeast extract treatment to undergo normal metamorphosis (Table 2). This suggests that the absence of a tail does not affect the reception of adhesive stimuli to induce metamorphosis.

In papillae-cut larvae, some metamorphic events of the trunk are initiated, but sensory vesicle retraction did not start and thus metamorphosis was not completed in the normal procedure. There remains a possibility that the presence of tails represses the sensory vesicle retraction. To examine this possibility, we cut both the tail and papillae from the larvae. These animals were called "papillae-tail-cut larvae." The proportion of larvae with well-grown adult organs was higher in papillae-tail-cut larvae than in papillae-cut larvae (Table 1). However, no drastic difference was observed in the morphology between well-grown papillae-cut larvae and papillae-tail-cut larvae (Fig. 4C). The events that did not occur in papillae-cut larvae also did not occur in papillae-tail-cut larvae. From these results we concluded that the Ciona larval tail is not responsible for the initiation of metamorphic events in the trunk.

The tail regression failed mutant shows defects in metamorphosis

Results from papillae-cut experiments mentioned above show that metamorphic events can be divided into at least two groups: events that are initiated in papillae-cut larvae and those not initiated in papillae-cut larvae. The former includes ampulla formation, body axis rotation and adult organ growth, and the latter includes tail regression and sensory vesicle retraction. Although the papillae-cut experiments clearly distinguish these metamorphic events, the experiment has two technical limitations. One is that we could not observe changes in the preoral lobe region because of its loss. The second is that the damage from the surgeon has to be considered in the interpretation. To overcome these technical limitations, another experiment which does not require surgery on larvae is necessary. One strong candidate is mutant analysis.

The tail regression failed (trf) mutant is a naturally maintained mutant. The mutant frequency of trf/trf in families generated by self-fertilization of trf+/trf heterozygous animals is approximately one quarter of the siblings (Table 3), suggesting that trf is a recessive mutant of a single locus. Embryos of trf/trf mutants showed normal embryogenesis and became normal larvae. Larvae of trf/trf settled normally with their adhesive papillae, but showed abnormality after settlement. Attached trf/trf larvae did not start tail regression, and

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**Table 3**

<table>
<thead>
<tr>
<th>Family ID*</th>
<th>% of normal juveniles**</th>
<th>% of trf mutants**</th>
<th>Number of larvae included in the family</th>
<th>Number of unknown animals***</th>
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<td>21.7 (17)</td>
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<tr>
<td>2</td>
<td>75.6 (124)</td>
<td>24.3 (40)</td>
<td>7</td>
<td>8</td>
<td>164</td>
</tr>
<tr>
<td>3</td>
<td>75.3 (647)</td>
<td>24.6 (212)</td>
<td>22</td>
<td>3</td>
<td>884</td>
</tr>
</tbody>
</table>

* Three independent families were counted.
** Percentage of metamorphosed animals was calculated because the phenotypes of trf mutants were visible after settlement.
*** Animals whose phenotype could not be determined due to abnormal development.
their tails continued strokes like normal larvae (Figs. 5A–C). Regression of adhesive papillae, which is one of the earliest events of ascidian metamorphosis and is observed just after settlement (Cloney, 1978), did not occur in \( trf/\) mutants in the first day after settlement (Fig. 5B). The retraction of sensory vesicles also did not occur in \( trf/\) mutants (Figs. 5C–E). To observe apoptosis in \( trf/\) mutants, TUNEL staining was performed. TUNEL-positive cells in the tail of \( trf/\) larvae were dramatically reduced compared with that of normal larvae (Figs. 5F and G), suggesting that in this mutant, initiation of apoptosis is suppressed as in the papillae-cut larvae.

In contrast to these events, the conversion of the preoral lobe into ampullae, which can be recognized by its transparency and morphology, occurred in settled \( trf/\) mutants at the normal time (Fig. 5B). After settlement, the trunk of \( trf/\) mutants started to grow. Adult organs including the endostyle, pharyngeal gill, intestine and heart developed. The movement of cilia and the beating of heart muscle were observed in \( trf/\) mutants at several days post fertilization, suggesting that their growth was completed to form functional organs. The development of the adult organs in \( trf/\) mutants was slower than that in normal juveniles, and the

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**Fig. 5.** Phenotypes of \( trf/\) mutant larvae. (A) A 3-dpf-old normal larva. Pa, papillae; Po, preoral lobe; SV, sensory vesicle. Bar, 100 μm. (B) A 3-dpf-old \( trf/\) larva. Papillae do not retract, while the preoral lobe is converted into an ampulla which can be seen by its transparency and morphology. (C) A 3-dpf-old \( trf/\) larva in which growth of adult organs has been initiated. Adult organs such as endostyle (En) and stomach (ST) can be observed. The sensory vesicle (SV) remains a large sphere, as in normal larvae. (D) A wild-type juvenile. Most of the adult organs are functional at this stage. Am, ampulla; AS, atrial siphon; St, stigmata of pharyngeal gill; OS, oral siphon. Bar, 100 μm. Higher magnification of the sensory vesicle is shown in the inset. The outline of the sensory vesicle is visualized by a broken line. (E) A \( trf/\) larva after growth of adult organs. The trunk morphology of this larva is similar to that of a normal juvenile, while the tail remains at the posterior region. Higher magnification of the sensory vesicle is shown in the inset. The outline of the sensory vesicle is visualized by a broken line. The sensory vesicle of \( trf/\) larvae remains a large sphere. (F) TUNEL staining of 2-dpf-old normal larva. Clear TUNEL-positive signals are observed in the nuclei of tail cells (arrows). Bar, 100 μm. (G) TUNEL staining of 2-dpf-old \( trf/\) mutant larvae. A TUNEL-positive signal is not observed in the tail cells. Tunic cells show intense signals in both normal and \( trf/\) mutant larvae (arrowheads).
organisms of trf/trf mutants were smaller than those of normal juveniles at the same age (Figs. 5D and E). As a result, trf is a mutant in which a part of metamorphic events, namely tail regression, papillae retraction and sensory vesicle retraction, are not initiated by the stimuli of settlement, while other metamorphic events, including ampulla and adult organs formation, are initiated. The overall phenotypes and the resultant morphology of trf/trf mutant larvae are almost identical to those of papillae-cut larvae, thus reinforcing the papillae-cut experiment.

Three metamorphic events, papillae retraction, tail absorption and sensory vesicle retraction, did not occur in trf/trf mutants at the normal time. Among them, papillae were lost from aged trf/trf mutants. Therefore, papillae retraction took place in trf mutants, but the event proceeded at an abnormal time or by different mechanisms from those of normal metamorphosis. Tails of these trf/trf mutants started degeneration during long culturing but they were not lost. Sensory vesicle retraction did not occur in trf/trf mutants even after long culturing.

It is important to specify whether trunk metamorphosis observed in trf/trf mutant larvae is caused by the stimuli of settlement or autonomously. The following observations indicate that the stimuli of settlement are required to initiate trunk metamorphosis in trf/trf mutants. First, almost all juveniles showing trf phenotypes attached to the substrates. Second, unsettled trf/trf animals kept their larval morphology, indistinguishable from wild types. Families from two trf/+ heterozygous animals contained a considerable number of non-attached larvae with normal morphology. These larvae were treated with yeast extract to simulate the stimuli of settlement. A part of them showed trf phenotypes, suggesting that these animals have a trf/trf genotype (32%, n = 37). All of these results suggest that the stimuli of settlement are required to initiate ampulla formation, adult organ formation and body axis rotation in trf mutants. This means that the initiation of these metamorphic events is normal in trf mutants.

Re-examination of the phenotypes of swimming juvenile mutants

The mutant swimming juvenile (sj) is generated by Minos-mediated insertional mutagenesis (Sasakura et al., 2003a,b 2005). The causal gene of this mutant is Ci-CesA, which encodes cellulose synthase (Nakashima et al., 2004). Larvae of sj skip tail regression and start trunk metamorphic events, such as retraction of papillae and body axis rotation. These trunk metamorphic events occur without settlement in sj mutants, which is a major point of difference from trf mutants. In sj larvae, the trunk metamorphic events eventually cease, and they do not show adult organ growth due to the arrest of cell proliferation (Sasakura et al., 2005). This is in contrast to the papillae-cut larvae and trf mutants, which show development of adult organs. If sj larvae successfully attach to substrates, they undergo the remaining metamorphic events to become normal juveniles, suggesting that sj larvae have the ability to sense the stimuli of settlement to initiate all metamorphic events.

Although the phenotypes of sj larvae have been reported in the previous study (Sasakura et al., 2005), some of the metamorphic events have not been described in detail. We re-examined in sj mutants two metamorphic events: namely, ampulla formation and sensory vesicle retraction. The sj larvae showed a juvenile-like trunk structure at 2 dph. At this period, a transparent ampulla elongated in the normal juveniles (Fig. 6B). In contrast, preoral lobes of sj larvae elongated but did not become transparent (Fig. 6A). Transparency of preoral lobes of sj larvae increased during the successive days, but a mass of cells remained in the preoral lobe (Fig. 6C, arrowhead), a phenomenon not observed in normal juveniles. In addition, the size and morphology of the preoral lobe of sj larvae did not change during the successive culturing (Figs. 6A and C), while the ampullae of the normal juvenile were variable in both size and morphology (see Fig. 5D). Therefore, the normal process of ampulla formation did not take place in sj larvae, nor did sensory vesicle retraction. From the observations of these phenotypes, we have concluded that in sj mutant larvae, adhesive papillae retraction and body axis rotation

Fig. 6. Phenotypes of sj mutant larvae. (A) An sj larva at 2 dpf. Body axis rotation and papillae retraction start at this stage. Note that the preoral lobe (Po) of sj/sj mutant larva is not transparent. (B) A normal juvenile just after tail regression. They have a transparent ampulla (Am) at this stage. (C) An sj-mutant larva after long culturing. The transparent area in the preoral lobe is expanded, but there remains a cell mass (arrowhead) that is not observed in the ampullae of the normal juveniles. (D) A 2-dpf-old sj larva whose papillae and preoral lobe were cut off at 1 dpf. Its adult organs have started to grow. The transparent ampulla is elongated in this animal. (E) An sj larva whose tail was cut off at 1 dpf. The trunk structure of this larva is the same as that of the intact sj larvae in (A).
occurs autonomously, while the remaining metamorphic events, such as ampulla formation, sensory vesicle retraction, tail regression and adult organ formation do not take place.

The formation of ampullae and adult organs occurred in papillae-cut larvae, but not in sj mutant larvae. We wondered whether these two metamorphic events of sj larvae are regulated in the same manner as other metamorphic events in normal larvae, or whether loss of Ci-CesA would affect the initiation of these events in this mutant. To address this issue, we performed a papillae-cut experiment of sj larvae. Papillae-cut sj larvae started trunk development like papillae-cut wild-type larvae (Fig. 6D and Table 4). Thus, formation of ampullae and adult organs in sj larvae is regulated by the same mechanisms as in normal larvae, and these events may be caused by independent pathways of Ci-CesA. A tail-cut experiment was also performed in sj larvae, and had no effects on trunk structure (Fig. 6E and Table 4). This result also suggests that the tail does not regulate trunk metamorphic events in C. intestinalis.

### Discussion

In the present study, we analyzed papillae-cut larvae, and trf and sj mutants of C. intestinalis. All of these larvae showed phenotypes in which some metamorphic events occurred while others did not. This indicates that metamorphic events of Ciona, although they occur coordinately in the normal metamorphosis process, can be divided into several groups, controlled by different mechanisms. Because all metamorphic events in normal metamorphosis are initiated by the stimuli of settlement, the possible grouping suggests that the pathways diverge after settlement. Based on the results in this study, we here propose a model of the classification and pathways of Ciona metamorphic events (Fig. 7). The bases of the model are discussed in the following sections.

The trf mutant and papillae-cut larvae initiated some trunk metamorphic events

The trf mutant larvae did not show normal papillae retraction, sensory vesicle retraction or tail absorption after settlement. In contrast, trf mutants showed ampulla formation, body axis rotation and adult organ growth. This indicates that ascidian metamorphic events can be divided into two groups: events that occur in trf mutants (trf-independent events) and events that do not occur in trf mutants (trf-dependent events). Settlement was required to initiate trf-independent events in the trf mutant, indicating that the initiation of these events is normal in this mutant. Therefore, trf is the mutant that shows defect in the pathway initiating trf-dependent events; we named this pathway the “trf-pathway” (Fig. 7). For instance, because apoptosis in tail cells does not occur in trf mutants, initiation of apoptosis is regulated by the trf-pathway. Among the trf-dependent events, retraction of papillae took place in the aged trf larvae. There might be a compensatory pathway to complete papillae retraction, however. Because retraction of papillae is also affected in the sj mutant, the regulation of this event may have different origins from the other two trf-dependent events.

Although the phenotypes are quite similar, the phenomena observed in papillae-cut larvae and trf mutants did differ. In papillae-cut larvae, ampulla formation, body axis rotation and adult organ growth are initiated, while sensory vesicle retraction and tail absorption are not. Apoptosis in the tail does not occur in papillae-cut larvae. These phenotypes are identical to that of trf mutant larvae.

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### Table 4

<table>
<thead>
<tr>
<th>Cut parts</th>
<th>% of tail absorbed animals</th>
<th>% of sj larvae</th>
<th>% of animals showing ampulla formation, body axis rotation and growth of adult organs without tail absorption</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillae</td>
<td>0</td>
<td>15.8</td>
<td>84.2</td>
<td>19</td>
</tr>
<tr>
<td>Tail</td>
<td>7.7**</td>
<td>92.3</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Uncut</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>

* Observation was performed at 3 dpf.
** Completion of tail absorption was judged by the formation of an epidermal chamber at the posterior end of the trunk to receive the absorbed tail.

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Fig. 7. Model of the classification and pathways of Ciona metamorphic events. Metamorphic events are classified based on the sensitivity to cellulose and Ci-CesA, and dependency on trf. The four groups of metamorphic events are shown by different colors. Cellulose-sensitive events are shown in orange, trf-dependent events in green, and cell division-required events in light blue. Retraction of papillae is categorized in both cellulose-sensitive events and trf-dependent events. Ci-CesA and/or cellulose are likely to be involved in the suppression of the cellulose-sensitive events. The causal gene of the trf mutant is involved in a metamorphic pathway and this is called the “trf-pathway”. A metamorphic pathway activates cell division in the adult organ primordia, and initiates cell division-required events.
However, the response to the stimuli of settlement is lost in papillae-cut larvae; thus, ampulla formation, body axis rotation and adult organ growth are initiated autonomously in papillae-cut larvae. Therefore, cut of papillae can be substituted for the stimuli of settlement to induce ampulla formation, body axis rotation and adult organ growth. There are two possibilities concerning the initiation of trunk metamorphic events in papillae-cut larvae. One is that the surgeon induces a continuous stimulus that is sufficient to initiate ampulla formation, body axis rotation and adult organ growth but not sufficient for the initiation of the remaining events. In this case, normal settlement provides larvae with stimuli that are sufficient to induce all metamorphic events at once. We name this hypothesis the “different threshold hypothesis”. The second possibility is that the papillae and preoral lobe have a repressive role on the metamorphic events which were observed in papillae-cut larvae. In this case, settlement may cancel this repression to induce ampulla formation, body axis rotation and adult organ growth. We name this hypothesis the “repression-canceling hypothesis”. These hypotheses should be examined in future studies.

Papillae-cut larvae do not respond to stimuli that induce metamorphosis in normal larvae. This means that papillae and the preoral lobe are necessary to respond to the stimuli. Papillae contain epidermal neurons (Horie et al., 2008), and several reports have shown that neurotransmitters induce the initiation of metamorphic events (Kimura et al., 2003; Zega et al., 2005). Taken together, the evidence suggests that the nervous system is likely involved in metamorphosis. Because the papillae-cut experiment gets rid of papillae neurons and a part of the neurons in the preoral lobe, these neurons are likely to receive and transmit stimuli for metamorphosis to the posterior part of the body.

The neural network starting from the papillae is connected to the tail via the sensory vesicle and visceral ganglion. Therefore, firing which occur in papillae neurons can be transmitted to the sensory vesicle and tail to initiate metamorphic events. It is interesting that all trf-dependent events are involved in this network of tissues; namely, papillae, sensory vesicle and tail. Therefore, it is probable that the trf-pathway utilizes this neural network, and that trf mutants have a defect in this system. Immunostaining of neurons showed no structural abnormality in the nervous system of 1-dpf-old trf larvae (Horie and Sasakura, unpublished data), although the possibility of a defect that does not affect the morphology of the nervous system remains. In addition to the nervous network, EGF- or other ligand-mediated signaling pathway is another candidate conducting trf-pathway, as recent work showed that an EGF-repeats containing protein Hems regulates initiation of metamorphosis (Eri et al., 1999). Further analyses will clarify the molecular nature of trf-pathway.

The sj mutants and cellulose-sensitive events

In sj mutant larvae, papillae retraction and body axis rotation are initiated autonomously. The term “autonomous” means that settlement is not required to initiate the two metamorphic events in sj mutant larvae. The remaining metamorphic events, namely ampulla formation, sensory vesicle retraction, tail absorption and adult organ growth, do not start in sj larvae until settlement. From the phenotypes of sj mutants, papillae retraction and body axis rotation can be distinguished from other metamorphic events, and we call them “cellulose-sensitive events” (Fig. 7) because the causal gene of sj is Ci-CesA, which encodes cellulose synthase. Because cellulose-sensitive events are caused autonomously in the mutant, Ci-CesA and/or cellulose have a role in repressing the initiation of these events. In the normal process of metamorphosis, settlement releases these events from repression. However, “repression” by Ci-CesA and/or cellulose is not necessarily their active function. For example, the loss of cellulose from the tunic would make papillae more sensitive to small particles in the seawater to induce the two metamorphic events without settlement. In this case, the “different threshold hypothesis” described above is attractive to explain the different phenotypes of sj, trf and papillae-cut larvae.

Ascidian metamorphic events can be divided into four groups

By comparing the phenotypes of sj, trf, and papillae-cut larvae, metamorphic events of C. intestinalis can be divided into the following four groups. Group 1 comprises a cellulose-sensitive and trf-independent event, namely body axis rotation. Group 2 is a cellulose-sensitive and trf-dependent event, papillae retraction. Group 3 comprises cellulose-independent and trf-dependent events, including sensory vesicle retraction and tail absorption. Group 4 comprises cellulose-independent and trf-independent events, including ampulla formation and adult organ growth. Interestingly, the events belonging to the same group have common characteristics. As mentioned above, trf-dependent events, which include group 2 and 3 events, are possibly dependent on the nervous system. The trf-dependent events have another common characteristic, in that they are events that destroy the larval body. Group 4 events require cell division and these events are directly involved in the formation of adult bodies. We called group 4 events “cell division-required events” (Fig. 7). The presence of these shared characteristics supports the hypothesis that Ciona metamorphic events in the same group are regulated by the same pathways.

Because all metamorphic events in the normal process of metamorphosis are triggered by settlement, the pathways diverge subsequent to settlement. Events of groups 1, 2 and 3 are affected in trf and/or sj mutants, and the causal genes of these events are obviously involved in their initiation. Group 4 events are not affected in either mutant, and unknown factors independent of trf and sj trigger these events. A signaling cue affecting cell division may be the causal factor, because the group 4 events are dependent on cell division. Recent studies have shown that EGF-repeat-containing proteins play critical roles in metamorphosis (Eri et al., 1999; Davidson and Swalla, 2001, 2002; Nakayama et al., 2001, 2002; Woods et al., 2004), and it is possible that EGF proteins are involved in the initiation of group 4 metamorphic events.

The pathways that initiate the metamorphic events are likely to have crosstalk with each other. A good example is one triggering the retraction of papillae, because this event is affected by both cellulose/Ci-CesA and causal gene of trf. The phenotypes of trf mutants and papillae-cut larvae suggest crosstalk between the trf pathway and the activation of cell division, because trf mutants and papillae-cut larvae showed less efficient growth of adult organs compared with normal juveniles. In trf mutants and papillae-cut larvae, the pathway to activate cell division is “on” but the trf-pathway is “off”. Taken together, the findings suggest that the trf pathway is necessary for proper growth of adult organs. There are two possibilities for the crosstalk. One is that the trf pathway directly activates cell-division-required events, and another is that the trf pathway and activation of cell division have to be initiated at the same time for the proper growth of adult organs. The initiating pathways of metamorphic events are more complicated than the model presented in this study. Further analysis to describe details of the pathways is necessary for complete comprehension of ascidian metamorphosis.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.11.026.

**References**


