Developmental Biology

Stimulatory and inhibitory G-protein signaling relays drive cAMP accumulation for timely metamorphosis in the chordate *Ciona*

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Abstract

Larvae of the ascidian *Ciona* initiate metamorphosis tens of minutes after adhesion to a substratum via its adhesive organ. The gap between adhesion and metamorphosis initiation is suggested to ensure the rigidity of adhesion, allowing *Ciona* to maintain settlement after losing locomotive activity through metamorphosis. The mechanism producing the gap is unknown. Here, by combining gene functional analyses, pharmacological analyses, and live imaging, we propose that the gap represents the time required for sufficient cAMP accumulation to trigger metamorphosis. Not only the Gs pathway but also the Gi and Gq pathways are involved in the initiation of metamorphosis in the downstream signaling cascade of the neurotransmitter GABA, the known initiator of *Ciona* metamorphosis. The mutual crosstalk of stimulatory and inhibitory G-proteins functions as the accelerator and brake for cAMP production, ensuring the faithful initiation of metamorphosis at an appropriate time and in the right situation.

eLife assessment

This **important** work substantially advances our understanding of the molecular mechanisms underlying the timing of the initiation of metamorphosis of the Ciona ascidian tadpole larva. Through the combination of gene knockdown experiments and fluorescent molecular reporters the authors provide **compelling** evidence about a crosstalk between different G protein mediated signalling pathways and are able to place different signalling molecules within a signalling network. The work will be of interest to molecular, developmental and marine biologists and to scientists working on animal metamorphosis.

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Introduction

Metamorphosis is a widespread feature of animal development that allows them to have different functions between larval and adult stages [1]. As larvae become adults, the shape and various characteristics of their physiology, gene expression, behavior, and lifestyle change. With these changes, animals can focus on a limited number of biological activities at each stage to increase feeding, growth, dispersal, and reproduction efficiencies. This biphasic mode of life is a widely conserved and ancient trait of animals, as evidenced by its presence in groups that maintain primitive states [2], indicating that this long-conserved feature has contributed to animals' flourishment. Therefore, to understand animal evolution, it is essential to elucidate the mechanisms underlying metamorphosis.

A key characteristic of metamorphosis is that both internal and external conditions determine its initiation. Sufficiently matured (or metamorphically competent[3]) larvae start metamorphosis only when they meet an appropriate external condition to be adults. Various organic and inorganic external stimuli suited to the lifestyle of adulthood trigger metamorphosis. Many marine invertebrates exhibit a benthic lifestyle at the adult stage[4]. Their planktonic larvae have an adhesive organ that secretes adhesives and triggers metamorphosis when they adhere to a substratum. Upon starting metamorphosis, their larvae lose locomotive organs and transition into benthic adult forms. Determining the timing of metamorphosis is important for benthic animals, particularly sessile ones, to ensure their future survival and reproduction, as they become unable to move after metamorphosis. Therefore, sessile animals are suspected to have elaborate mechanisms to start metamorphosis only when a firm adhesion is achieved at an appropriate place. Although several reports describe this kind of phenomenon[5–7], the mechanism by which the external condition is turned into an internal mechanism to trigger metamorphosis at the right timing remains elusive.

Ascidians are marine invertebrate chordates that are the closest living relatives to vertebrates[8,9]. The chordate features of ascidians are best represented by their tadpole larval shape. Like vertebrate tadpoles, ascidian larvae swim by beating their tail through neuromuscular activities[10,11]. However, ascidians lose the tadpole shape during metamorphosis, and they exhibit a sessile lifestyle at the adult stage[12,13]. Ascidian metamorphosis comprises several key events, such as tail regression, body axis rotation, and adult organ growth[14] (**Figure 1A**).

Adhesion to a substratum triggers ascidian metamorphosis[14]. Ascidian larvae have adhesive organs called adhesive papillae, usually triangular protrusions at the anterior end. Adhesive papillae secrete a lectin-type mucus substance that is suspected to aid in binding to the substratum[15]. Moreover, adhesive papillae serve as mechanical sensors[16]. The papilla is a neuronal organ that includes epidermal neurons positive for vesicular glutamate transporter (vGLUT) and GABA, which innervate axons toward the sensory vesicle (larval brain)[17–20]. The papilla neurons are responsible for initiating metamorphosis[16,21–23]. Moreover, our recent study suggests the involvement of a mechanoreceptor channel (the TRP channel) in the responsiveness to mechanical stimuli[22]. However, a simple physical adhesion is not sufficient to trigger metamorphosis. In our study using the model ascidian *Ciona intestinalis* Type A (this species has been suggested to be renamed *C. robusta* [24–26], and hereafter we call it *Ciona*), continuous adhesion for about 30 min is necessary before starting metamorphosis[27]. When larvae detach before reaching the critical period, they must adhere for 30 min again for metamorphosis. Therefore, ascidian larvae are suspected of sensing the duration of adhesion and initiating metamorphosis only when adhesion is firm enough to be maintained for the required period. *Ciona* larvae continue tail beating during adhesion to push their body to the substratum. We showed that the strength of force generated by this swimming activity influences the timing of

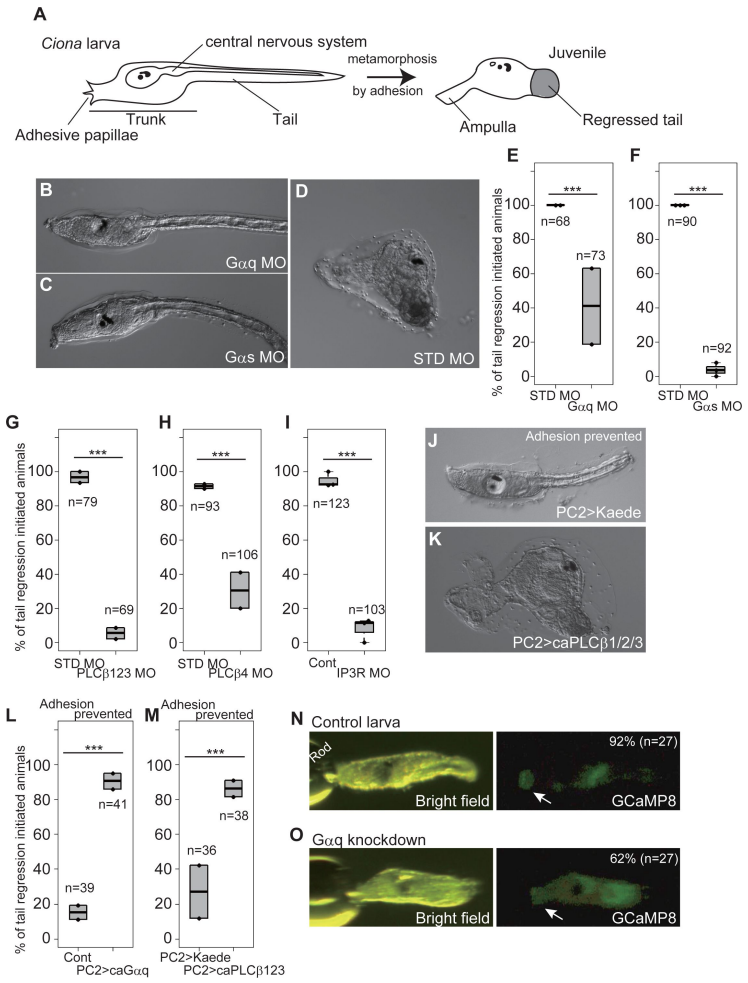

Figure 1.

Gq and Gs pathways are required for *Ciona* metamorphosis.

(A) Schematic illustration of *Ciona* metamorphosis.
(B) A larva knocked down with *Gαq* using the antisense morpholino oligonucleotide (MO). Metamorphosis did not initiate at 2 days post-fertilization (2 dpf).
(C) A *Gαs* knockdown larva.
(D) A control animal injected with the standard (STD) MO. Metamorphosis initiated, as indicated by the completion of tail regression.
(E) Effect of *Gαq* knockdown on the percentage of metamorphosis initiation (indicated by the initiation of tail regression), shown as box-and-whisker plots. Dots indicate experiment replicates. ***, p<0.001 (Fisher's exact test). n, number of examined larvae in total.
(F) Effect of *Gαs* knockdown.
(G-I) Effects of *PLCβ1/2/3*, *PLCβ4*, and *IP3R* knockdowns.
(J-M) Results of the experiments where adhesion was prevented by tail amputation and laying larvae on agar-coated plates.
(J) A control larva overexpressing the *Kaede* reporter in the entire nervous system using the *PC2 cis* element.
(K) A larva overexpressing constitutive active (ca)*PLCβ1/2/3*.
(L) Effect of ca*Gαq* overexpression.
(M) Effect of ca*PLCβ1/2/3* overexpression.
(N-O) Live imaging of Ca^{2+} transient, monitored by injecting *GCaMP8* mRNA.
(N) Snapshot of the control larva injected with *GCaMP8* mRNA. An increase in the fluorescence intensity in the adhesive papillae (arrow) was observed. Rod, the position of the glass rod used to stimulate papillae. The percentage and number exhibit the rate of animals showing Ca^{2+} transient in the papillae.
(O) A larva injected with *Gαq* MO plus *GCaMP8* mRNA. An increase in the fluorescence intensity in the papillae (arrow) was not observed even though the brightness of the whole body was raised. See also **Figure S1**.

metamorphosis initiation[22]. In this phenomenon, abolishment of swimming activity elongates the time from settlement until metamorphosis is initiated. The mechanisms for measuring the duration of adhesion and the strength of the force generated by adhesion are unknown.

To elucidate the mechanisms triggering ascidian metamorphosis, the signaling pathways must be characterized. Many studies have discovered signaling molecules as possible inducers of ascidian metamorphosis[28–35]. These molecules include neurotransmitters, suggesting that transmission of excitation in the nervous system, starting from the adhesive papillae, is crucial for metamorphosis. Recently, our group reported that the inhibitory neurotransmitter GABA plays a pivotal role in initiating *Ciona* metamorphosis[36]. Knockout and knockdown of the genes encoding GABA synthase (GAD), vesicular inhibitory amino acid transporter (VIAAT or VGAT), and metabotropic GABA receptor (GABABR) resulted in the perturbation of metamorphosis. Moreover, GABA administration induces metamorphosis without adhesion. Our previous studies demonstrated that GABA induces the secretion of gonadotropin-releasing hormone (GnRH)[36,37]; however, it remains unknown how the inhibitory neurotransmitter activates neuronal functions for initiating metamorphosis.

In this study, we addressed the characterization of the downstream cascade stimulated by GABA. Because GABABR is a G-protein-coupled receptor (GPCR)[38], we searched for trimeric G-proteins necessary for metamorphosis. These G-proteins are heterotrimers of an α β and γ subunit[39]. Upon ligand binding, GPCR exchanges GDP of the α subunit to GTP, then the GTP-bound α subunit and the βγ complex are released from the GPCR. Although both the GTP-bound α subunit and the βγ complex have activities, the α subunit mainly determines the reaction specific to the G-protein type. We found that three G-proteins are activated in the downstream GABA cascade, resulting in cyclic adenosine monophosphate (cAMP) elevation. Because the signaling includes stimulating and inhibiting cAMP synthesis, *Ciona* initiates metamorphosis only when a sufficient quantity of cAMP is accumulated due to sustained adhesion. Our results revealed the ingenious mechanism that permits *Ciona* to start metamorphosis only when achieving an appropriate adhesion firm enough to relinquish swimming ability and commence sessile adult life.

Results

Characterization of G-proteins necessary for metamorphosis initiation

We knocked down the genes encoding G alpha (Gα) proteins[40] using antisense morpholino oligonucleotides (MOs). We found that two genes corresponding to Gαq and Gαs are necessary for metamorphosis. Neither morphant showed any signature of metamorphosis even though both were allowed to adhere to the base of culture dishes, which was sufficient for control larvae to initiate metamorphosis (**Figure 1A-F**).

Gαq activates phospholipase C beta (PLCβ) to produce inositol triphosphate (IP3)[41]. IP3 is received by its receptor on the endoplasmic reticulum (ER) and releases calcium ion (Ca^{2+}). The *Ciona* genome encodes two PLCβ (PLCβ1/2/3, PLCβ4) and one IP3 receptor (IP3R) (**Figure S1** and **Table S1**). We knocked down *PLCβ1/2/3*, *PLCβ4*, and *IP3R* genes. The knockdown larvae of these three genes failed to start metamorphosis (**Figure 1G-I**), suggesting that Gαq initiates metamorphosis by the conventional Ca^{2+} pathway mediated by PLCβ and IP3/IP3R. To confirm this, we overexpressed a constitutively active form of Gαq (caGαq)[42] and of caPLCβ1/2/3[43] in the entire nervous system with the *cis* element of the gene encoding prohormone convertase 2 (PC2)[44–46]. When the microinjected animals reached the larval stage, they were cultured on agar-coated dishes after tail amputation to prevent adhesion. In this condition, the control larvae

rarely started metamorphosis because of the absence of adhesion (**Figure 1J**). In contrast, ca*Gαq*- or ca*PLCβ1/2/3*-overexpressed larvae initiated metamorphosis without adhesion (**Figure 1K-M**).

Adhesive papillae exhibit a Ca^{2+} increase soon after sensing an adhesive stimulus[16,22]. We examined whether this Ca^{2+} transient is dependent on the Gq pathway. Compared to controls, significantly fewer larvae injected with *Gαq* MO plus *GCaMP8* mRNA exhibited GCaMP8 fluorescence elevation in the adhesive papilla upon stimulation (**Figure 1N**, O). This result suggests that the Gq pathway is activated upon adhesion to cause a Ca^{2+} transient in the adhesive papilla.

Gs pathway initiates metamorphosis by activating cAMP synthesis

The involvement of Gs in metamorphosis was confirmed by the overexpression of a constitutively active form of Gαs (caGαs)[47] in the nervous system. This overexpression resulted in the initiation of metamorphosis without adhesion (**Figure 2A-C**). The Gs pathway activates adenylate cyclase (AC) to produce cAMP[48]. We previously reported that cAMP can induce metamorphosis[37]. Because externally added cAMP is not a strong inducer of metamorphosis, we attempted to confirm this hypothesis through another experiment. Theophylline increases cAMP by inhibiting the cAMP-degrading enzyme phosphodiesterase (PDE)[49]. We treated wild-type larvae with theophylline after tail amputation to prevent larvae from adhesion. Most theophylline-treated larvae completed tail regression without adhesion (**Figure 2D-F**). Theophylline has several target proteins in addition to PDE[50]. To further confirm that cAMP is responsible for the initiation of metamorphosis, we overexpressed photo-activating AC (bPAC)[51] in the nervous system. The *bPAC*-overexpressed larvae regressed their tails without adhesion, suggesting that a cAMP increase triggers metamorphosis (**Figure 2G**).

Using bPAC, we addressed whether enhanced cAMP production facilitates the initiation of metamorphosis. At 24 hours post-fertilization (hpf), only a few animals in both the *bPAC*-overexpressed and control groups initiated metamorphosis because 24 hpf is somewhat too early for *Ciona* larvae to be metamorphically competent[27] (**Figure 2H**). Even in this condition, the *bPAC*-overexpressed group exhibited a statistically higher rate of metamorphosis-initiated larvae. The proportion of animals initiating metamorphosis increased over successive time points, with the *bPAC*-overexpressed group consistently showing a statistically higher rate of metamorphosis compared to controls. These results support the idea that cAMP plays a role as a timer for *Ciona* metamorphosis; accumulation of this molecule to exceed a threshold could trigger metamorphosis. Because many of the *bPAC*-overexpressed larvae did not initiate metamorphosis at 24 hpf, similar to control larvae, cAMP accumulation does not alter the timing of acquiring metamorphic competence.

Gq-Gs pathways work in the adhesive papillae for metamorphosis

The above results showed that activation of the Gq and Gs pathways are the key events in initiating metamorphosis. Gαq is necessary to induce Ca^{2+} transients in the adhesive papillae, suggesting that the Gq pathway functions in this region. If both Gq and Gs function in the papillae, they should be expressed in the adhesive papilla. The transcriptome analysis of the larval papilla region showed that the genes encoding Gq and Gs pathway proteins are expressed in this region (**Figure S2** and **Table S1**). Therefore, Gq and Gs could function in the papilla to initiate metamorphosis.

As shown above, theophylline induced metamorphosis without settlement. However, when papillae were removed from larvae, theophylline failed to induce metamorphosis (**Figure 3A-C**). This suggests that cAMP elevation in the adhesive papillae is essential for starting metamorphosis. The overexpression of *caGαq*, *caPLCβ1/2/3*, *caGαs*, and *bPAC* by the *PC2 cis* element resulted in the

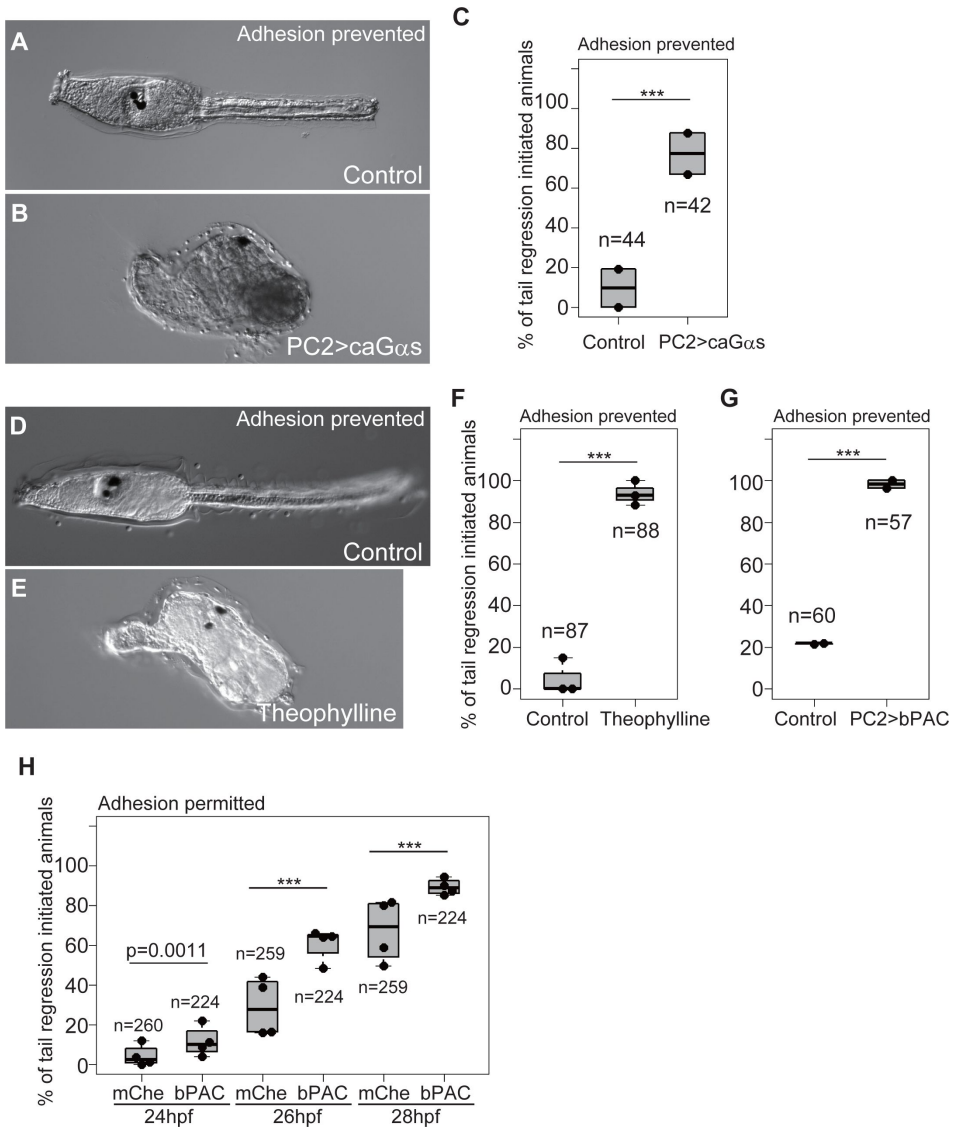

Figure 2.

cAMP initiates *Ciona* metamorphosis.

(A) An adhesion-prevented control (uninjected) larva at 2 dpf. Metamorphosis did not initiate.
(B) An animal with adhesion prevention and overexpression of ca*Gas* in the entire nervous system. Metamorphosis initiated.
(C) Effect of ca*Gas* overexpression.
(D) A control (uninjected) larva.
(E) A theophylline-treated animal. Metamorphosis initiated.
(F) Effect of theophylline treatment.
(G) Effect of *bPAC* overexpression in the nervous system.
(H) Effect of *bPAC* overexpression over time. In this experiment, adhesion was permitted. Control groups (overexpressed with mCherry) exhibited a slower initiation of metamorphosis than bPAC-overexpressed groups.

initiation of metamorphosis without adhesion. Like theophylline, amputation of the papillae inhibited them from starting metamorphosis (**Figure 3D**), confirming that activation of the Gq and Gs pathways in the adhesive papillae triggers metamorphosis.

If the Gs pathway is activated in the adhesive papillae, a cAMP increase should be observed in this region upon adhesion. We examined this possibility using a fluorescent cAMP indicator called Pink Flamindo (PF)[52]. After stimulation, the fluorescence of PF in the papillae temporarily decreased on average by 0.83-fold (n=5) from the initial intensity, followed by a gradual increase to 1.17-fold over successive time points (**Figure 3E**). Such an increase in fluorescence intensity was not observed in the adhesive papillae of the larvae that had failed to initiate metamorphosis following stimulation (n=4, **Figure S3**). Therefore, increased cAMP in the papillae serves to indicate that sufficient stimuli of adhesion have been received to induce metamorphosis. This strengthens our hypothesis that cAMP accumulation in the adhesive papillae determines the initiation of metamorphosis.

GABA in the adhesive papillae is responsible for metamorphosis

Our previous study demonstrated that GABA is the chief neurotransmitter that induces metamorphosis[36]. To gain insight into the relationships between the GABA, Gq, and Gs pathways, we addressed whether GABA functions in the adhesive papillae for initiating metamorphosis, similar to Gq and Gs.

The previous studies and our transcriptome data suggest that GAD is expressed in the adhesive papillae[53] (**Table S1**). Moreover, GABA-immunopositive signals have been detected in the papillae[19,53]. Therefore, papillae can provide GABA to stimulate themselves. Next, we examined whether the adhesive papillae can receive GABA to initiate metamorphosis. We showed that the metabotropic GABA receptor is responsible for the initiation of metamorphosis[36]. Using whole-mount *in situ* hybridization, the previous study did not detect the expression of two GABAB receptor (GABABR) genes in the papillae[53]; however, our transcriptome data detected the low-level expression of three genes encoding GABABR (**Table S1**) in the papillae, suggesting that adhesive papillae could receive GABA. GABA can induce metamorphosis without adhesion (**Figure S4A**)[36]. However, amputation of adhesive papillae eliminated this activity (**Figure S4B-C**), suggesting that GABA reception by the papillae is responsible for starting metamorphosis.

The larval brain is the major domain expressing *GABABR*[53]. Therefore, it remains possible that GABA signaling in the brain stimulates the papillae in a retrograde manner to initiate metamorphosis. The connectome analyses of the larval nervous system did not suggest a nerve that inputs into the papillae from the brain[54,55]; however, retrograde stimulation would be possible through the volume transmission of a signaling molecule. To examine this possibility, we isolated larval fragments anterior to the brain (**Figure S4D**), followed by the administration of GABA and theophylline. Through the application of these chemicals, anterior fragments exhibited increased clarity, elongation, and retraction of papillae, which are the signatures of metamorphosis (**Figure S4E-G**). Because their responses to GABA were weaker than those observed in the theophylline treatment, we further examined whether the anterior fragments can respond to GABA by monitoring the expression of GABA-responsive genes. By comparing expression levels between control and GABA-administered larvae, we made a list of the genes upregulated by GABA in the papillae (**Table S2**). Among them, we compared the expression levels of two genes (KY21.Chr5.240 and KY21.Chr8.489) between GABA-administered and control anterior fragments. GABA-treated anterior fragments expressed these genes at levels at least three times higher than controls (**Figure S4H**). We concluded that GABA is secreted from and stimulates the papillae to start metamorphosis.

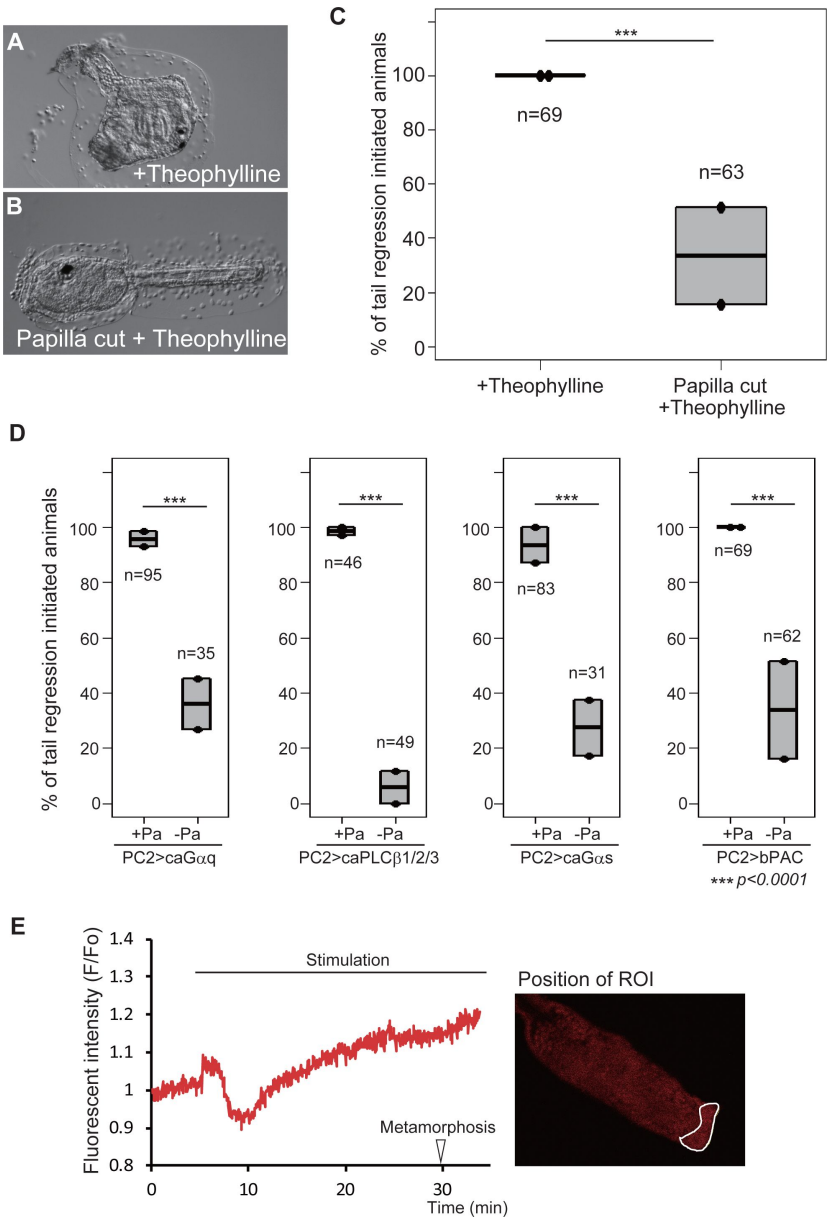

Figure 3.

Gq and Gs pathways are activated in the adhesive papillae to initiate metamorphosis.

(A) A theophylline-treated animal. Metamorphosis initiated.
(B) A papilla-amputated larva treated with theophylline. Metamorphosis did not initiate.
(C) Effect of papilla amputation on theophylline treatment.
(D) Effects of papilla amputation on ca*Gαq*, ca*PLCβ1/2/3*, ca*Gαs*, and bPAC overexpression.
(E) Measurement of cAMP concentration, as indicated by the fluorescence of Pink Flamindo. Left, the quantification of Pink Flamindo fluorescent intensity is shown for a larva that initiated metamorphosis at the time indicated by an arrowhead. The time of stimulation of the adhesive papillae with a glass rod is denoted by a bar. Right, the position of the region of interest (ROI). See also **Figure S3**.

Gs/cAMP is downstream of the Gq pathway

Our next question is: What are the upstream or downstream relationships between GABA, Gq/Ca2+, and Gs/cAMP in the adhesive papillae? We first examined the relationships between the Gq and Gs pathways. Theophylline ameliorated metamorphosis-failed phenotypes in *Gαq*, *PLCβ*, and *IP3R* knockdowns (**Figure 4A-E**). Moreover, *Gαq* morphants initiated metamorphosis when ca*Gαs* was overexpressed in the nervous system (**Figure 4F**). These results suggest that the Gq pathway is upstream of the Gs/cAMP pathway. *Gαs* knockdown larvae exhibited Ca^{2+} transients in the adhesive papillae upon stimulation (**Figure 4G**). Because this Ca^{2+} transient is Gq-dependent (**Figure 1O**), this result confirms that the Gs pathway is downstream of the Gq-dependent Ca^{2+} increase.

To gain further insight into the epistatic order of the Gq and Gs pathways, we overexpressed constitutive active forms of Gq pathway proteins in *Gαs* morphants with the *PC2 cis* element. If Gq is upstream of the Gs pathway, forced activation of the Gq pathway by caGαq or caPLCβ1/2/3 would not ameliorate the *Gαs* MO effect. Indeed, ca*PLCβ1/2/3*, which rescued *Gαq* morphants well, failed to rescue *Gαs* morphants (**Figure 4H-L**). In contrast, ca*Gαq* significantly ameliorated the metamorphosis-failed phenocopies of *Gαs* morphants (**Figure 4M**), contradicting the hypothesis that Gq is upstream of the Gs pathway. One possibility is that the Gq pathway stimulates cAMP synthesis through a massive Ca^{2+} increase and protein kinase C (PKC) activation[56,57]. Indeed, *Ciona* larvae can synthesize cAMP without Gs, as suggested by the partial rescue of *Gαs* morphants by theophylline treatment (**Figure 4N**). These data suggest that Gαs-independent AC could be a target of the Gq pathway. We concluded that the Gq pathway is upstream of the Gs pathway in the signaling cascade initiating *Ciona* metamorphosis.

Interaction between GABA and Gq pathways

We next investigated the relationships between the GABA and Gq/Gs pathways. As our previous study showed, GABA pathway knockdown by *GAD* or *GABABR1* MO disrupted the initiation of metamorphosis (**Figure 5A**)[36]. Overexpression of ca*PLCβ1/2/3* and theophylline treatment ameliorated the metamorphosis-failed phenocopies of *GAD*/*GABABR1* morphants (**Figure 5B-D**). These results suggest that the GABA pathway is upstream of the Gq and Gs pathways. We observed Ca^{2+} transients in the adhesive papillae of *GAD* knockdown larvae. *GAD* morphants rarely exhibited a Ca^{2+} increase after stimulation (**Figure 5E**). Because the Ca^{2+} transient in the papillae is Gq-dependent, this result confirms that the GABA pathway is upstream of, or at least in parallel with, the Gq pathway.

However, puzzling results were obtained when we administered *Gαq*- and *Gαs*-knockdown larvae with GABA. If GABA is upstream of the Gq and Gs pathways, this chemical does not induce metamorphosis when the Gq or Gs pathway is disrupted. However, GABA significantly ameliorated the metamorphosis-failed phenocopies of *Gαq*, *PLCβ*, and *Gαs* morphants (**Figure 5F-H**). These results could be explained by assuming enhancement of the Gq pathway by GABA through PLCβ and another GABA-mediated metamorphic pathway bypassing Gq components. These possibilities are examined in the next section.

Contribution of Gi to metamorphosis

The function of GABA as the potential upstream factor of Gq could be easily explained if GABABR is coupled with Gq. Although a few studies suggest this possibility[58], Gi is regarded as the major G-protein coupled with GABABR[59]. A previous study suggested that the *Ciona* genome encodes one typical Gαi protein[40]. The gene encoding this Gαi is strongly expressed in the entire larval nervous system including adhesive papillae[60]. The knockdown of this gene exhibited a significantly reduced rate of metamorphosis (**Figure 6A**); however, the reduction was not conspicuous, suggesting the presence of another Gαi regulating metamorphosis.

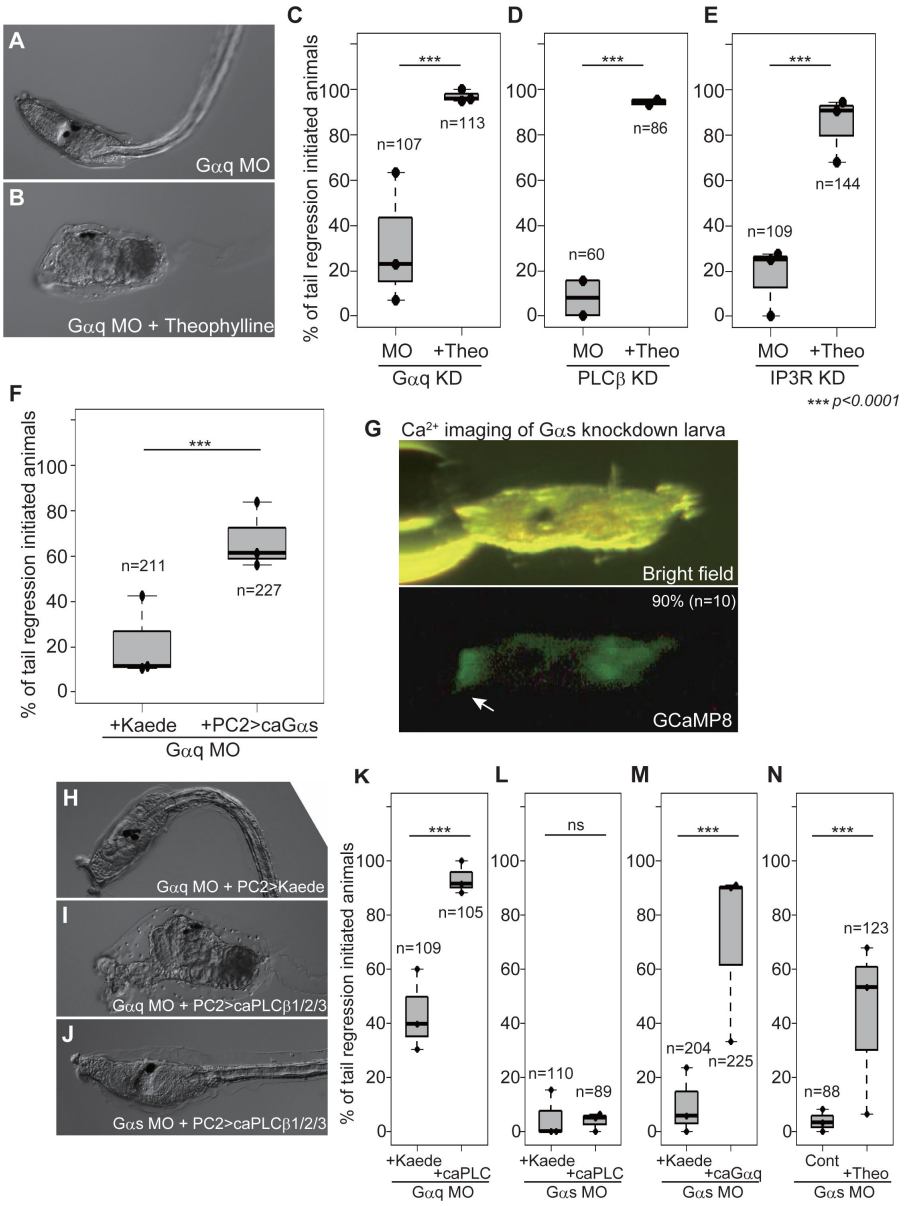

Figure 4.

Relationships between Gq and Gs pathways in metamorphosis.

(A) A *Gαq* knockdown larva.
(B) Theophylline ameliorated the effect of *Gαq* knockdown.
(C) Effect of theophylline on *Gαq* knockdown.
(D) Effect of theophylline on *PLCβ* knockdown. In this experiment, *PLCβ1/2/3* and *PLCβ4* were knocked down simultaneously.
(E) Effect of theophylline on *IP3R* knockdown.
(F) Effect of ca*Gαs* overexpression on *Gαq* knockdown.
(G) *Gαs* is not necessary for Ca^{2+} transient in the adhesive papillae (arrow) upon adhesion.
(H) A *Gαq* knockdown and *Kaede*-overexpressed larva.
(I) A *Gαq* knockdown and ca*PLCβ1/2/3*-overexpressed larva.
(J) A *Gαs* knockdown and ca*PLCβ1/2/3*-overexpressed larva.
(K) Effect of ca*PLCβ1/2/3* overexpression on *Gαq* knockdown.
(L) Effect of ca*PLCβ1/2/3* overexpression on *Gαs* knockdown.
(M) Effect of ca*Gαq* overexpression on *Gαs* knockdown.
(N) Effect of theophylline treatment on *Gαs* knockdown.

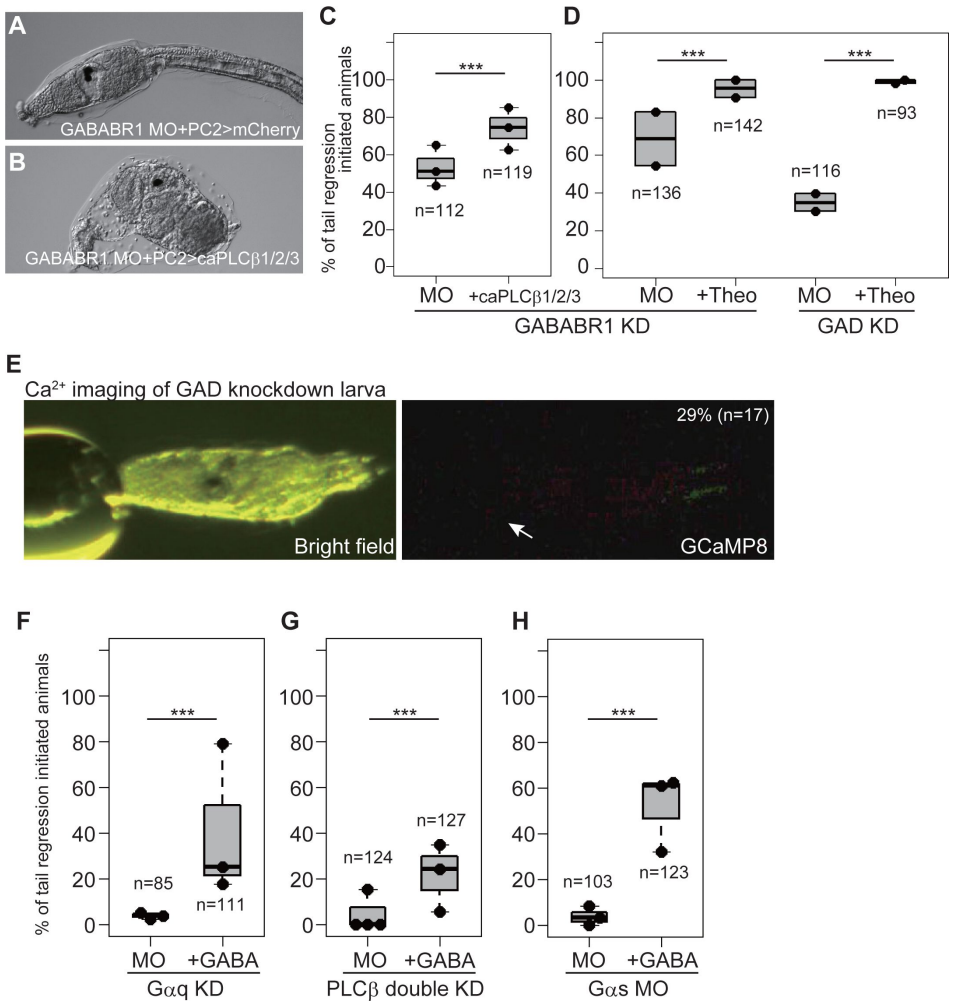

Figure 5.

GABA functions in the adhesive papillae to induce metamorphosis.

(A) A *GABABR1* knockdown and *mCherry*-overexpressed larva.
(B) A *GABABR1* knockdown and ca*PLCβ1/2/3*-overexpressed larva. Metamorphosis initiated.
(C) Effect of ca*PLCβ1/2/3* overexpression on *GABABR1* knockdown.
(D) Effect of theophylline on *GABABR1* and *GAD* knockdowns.
(E) GABA is necessary for Ca^{2+} transient in the adhesive papillae (arrow).
(F) Effect of GABA on *Gαq* knockdown.
(G) Effect of GABA on *PLCβ* knockdown.
(H) Effect of GABA on *Gαs* knockdown. See also **Figure S4**.

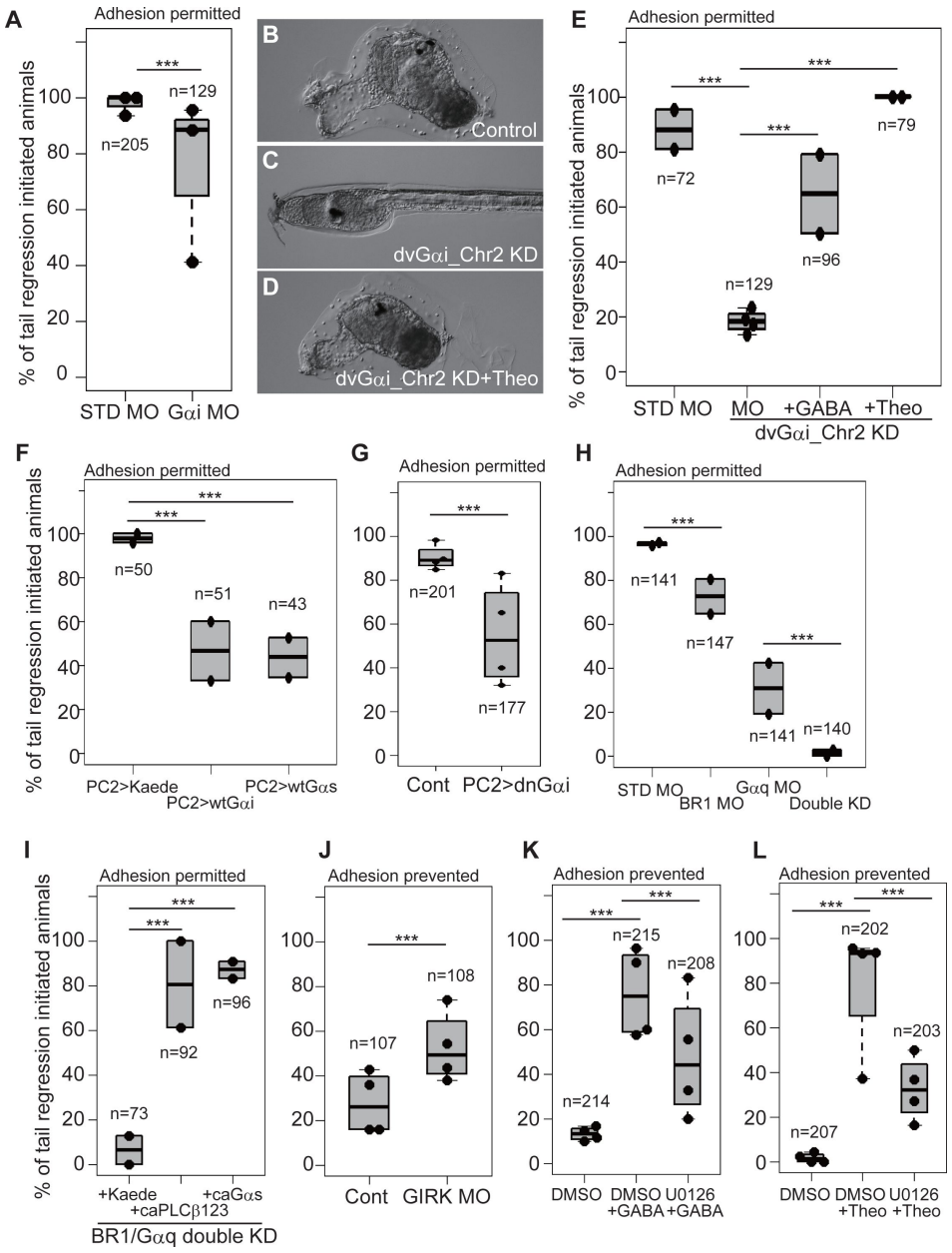

Figure 6.

Gi is required for metamorphosis initiation.

(A) Effect of *Gai* knockdown.
(B) A control animal injected with STD MO.
(C) A *dvGai_Chr2* knockdown larva.
(D) A *dvGai_Chr2* knockdown and theophylline-treated animal.
(E) Effects of GABA and theophylline on *dvGai_Chr2* knockdown.
(F) Effects of wt*Gai* and wt*Gas* overexpression.
(G) Effect of dn*Gai* overexpression.
(H) Effect of *GABABR1* and *Gaq* simultaneous knockdown.
(I) Effects of ca*PLCβ1/2/3* and ca*Gas* overexpressions on *GABABR1* and *Gaq* simultaneous knockdown.
(J) *GIRK* knockdown promoted metamorphosis initiation.
(K) Effect of U0126 on GABA treatment.
(L) Effect of U0126 on theophylline treatment. See also **Figure S5**.

A previous study showed that *Ciona* has four *Ciona*-specific Gα proteins[40]. We found three gene models (KY21.Chr2.875, KY21.Chr4.943, and KY21.Chr9.455) encoding these divergent Gα proteins from the newest genome database[61]. Our phylogenetic analyses showed that the divergent Gα proteins have a strong affinity to the Gαi/o family proteins (**Figure S5A**). Moreover, the amino acid residues at the C-terminal end of KY21.Chr2.875 are more similar to human Gαi than the other Gα proteins (**Figure S5B**). The C-terminal residues, particularly glycine at position 3, are essential for determining the G-protein partner of GPCR[62]. We tentatively name this protein dvGαi_Chr2. The transcriptome data showed that the gene encoding dvGαi_Chr2 is strongly expressed in the papillae (**Table S1**). The *dvGαi_Chr2* knockdown larvae failed to initiate metamorphosis (**Figure 6B-C**), and this phenocopy was rescued by theophylline and GABA (**Figure 6D-E**), suggesting that *dvGαi_Chr2* is necessary for metamorphosis. This strengthens the hypothesis that GABABR regulates metamorphosis through Gi. The rescue of *dvGαi_Chr2* morphants by GABA could be attributable to a redundant function of the typical Gαi protein in the papillae.

We further explored the involvement of Gi upon metamorphosis as the upstream factor of the Gq-Gs pathways. Gi is known to activate PLCβ through the Gβγi complex[63,64]. Released βγ is inactivated by overexpressing normal (usually GDP-bound) Gα subunits because GDP-Gα quenches the βγ complex[65]. Overexpressing wild-type *Gαi* and *Gαs* in the nervous system suppressed metamorphosis (**Figure 6F**), suggesting that the activated βγ complex is necessary for initiating metamorphosis. To confirm that the wild-type Gαi exerts its effect through the sequestration of the βγ complex, we overexpressed a dominant-negative form of Gαi (dnGαi)[66,67] which has reduced GDP/GTP affinity while maintaining βγ binding activity. dnGαi significantly reduced the occurrence of metamorphosis (**Figure 6G**), suggesting that the negative effect of Gαi on metamorphosis occurs through interaction with the βγ complex.

If Gβγ-mediated PLCβ activation is used in *Ciona* metamorphosis, PLCβ receives two independent inputs (Gαq and Gβγi) for its activation. These pathways could compensate for each other. We noticed that the MOs for GABA pathway genes and the *Gαq* MO did not strongly disrupt metamorphosis compared to the *Gαs* MO (**Figure 1E-F**; 5C-D). The compensatory role could explain this phenomenon. Indeed, the simultaneous knockdown of *GABABR1* and *Gαq* resulted in a strong impairment of metamorphosis (**Figure 6H**), and these morphants initiated metamorphosis by overexpression of ca*PLCβ1/2/3* or ca*Gαs* (**Figure 6I**).

If the role of the GABA/Gi pathway relies specifically on PLCβ activation in the mechanism of metamorphosis, GABA administration would not rescue *PLCβ* morphants. However, GABA weakly but significantly induced metamorphosis in *PLCβ1/2/3* plus *PLCβ4* double-knockdown larvae (**Figure 5G**). Therefore, GABA/Gi is likely to activate another pathway that bypasses PLCβ. Gβγi is known to activate group III AC[56]. Its *Ciona* counterpart (AC5/6) is expressed in the adhesive papillae (**Figure S2B**), which could explain the results of the rescue experiments. In addition, there are two other major targets of Gβγi. One is the G-protein-activated inwardly rectifying potassium (GIRK) channel[68]. Our RNA-seq data on the papillae indicated the expression of two GIRK channel genes (**Table S1**). The GIRK channel negatively regulates the excitation of neurons through hyperpolarization. If the metamorphosis of *Ciona* is induced by the excitation of papilla neurons as suggested previously[16,22], the GIRK channel is likely to regulate metamorphosis negatively. Indeed, the knockdown of one GIRK channel gene weakly enhanced the initiation of metamorphosis without settlement (**Figure 6J**). Although the negative role of the GIRK channel supports the involvement of Gβγi in the pathway of metamorphosis, this does not explain the PLCβ-independent activation of the metamorphic pathway by GABA/Gi.

The third function of Gβγi is to activate MAPK signaling through positive regulation of MEK[69]. PLCβ does not mediate this pathway. It has been suggested that MAPK signaling is required to induce metamorphosis[70,71]. To show that MEK activation is necessary for inducing

metamorphosis mediated by GABA, we treated adhesion-prevented larvae with GABA plus U0126, a potent MEK1/2 inhibitor repeatedly used in ascidians[72,73]. U0126 reduced the rate of metamorphosis induction by GABA (**Figure 6K**).

cAMP is also known to activate the pathway that involves MEK1/2[74]. We found that U0126 antagonized the effect of theophylline on metamorphosis (**Figure 6L**). Therefore, GABA and cAMP have the same target (MEK1/2) to initiate metamorphosis, suggesting their compensatory function and/or that cAMP synthesis is upregulated by GABA-Gβγi. These bypassing pathways could explain the amelioration of the phenocopies of *PLCβ* and *Gαs* morphants by GABA (**Figure 5G and H**).

The constitutive function of wild-type Gαq

We found that *Ciona* wild-type Gαq (wtGαq) has constitutive activity. In contrast to wt*Gαs* and wt*Gαi*, overexpression of wt*Gαq* did not arrest metamorphosis (**Figure S6A**). Rather, its overexpression induced metamorphosis without settlement (**Figure S6B**). The constitutive wt*Gαq* activity was confirmed by the rescue of *Gαs* morphants (**Figure S6C**). As mentioned above, caGαq also has these activities (**Figure 1L**). ca*Gαq*-overexpressed larvae had a somewhat rounder trunk shape, abnormal tail bending, and frequent tail twitching, perhaps due to a constitutive increase in the cytosolic Ca^{2+} concentration (**Figure S6D**). Overexpression of wt*Gαq* did not show such abnormalities (**Figure S6E**), suggesting that wtGαq has milder activity than, or works through a different mechanism from, caGαq. To gain further insight into the constitutive effect of wtGαq, we constructed a dominant-negative form of Gαq[66] that has reduced GDP/GTP binding activity while maintaining βγ binding activity. Overexpression of dn*Gαq* weakly inhibited metamorphosis (**Figure S6F**), suggesting that GDP/GTP exchange is important for the constitutive activity of wtGαq.

Discussion

Among chordates, the tunicate ascidian is the only group that exhibits a sessile lifestyle at the adult stage. Understanding how ascidians acquired the mechanism to metamorphose into sessile adults is important for elucidating the evolution of chordates[75,76]. Through extensive molecular, physiological, and pharmacological analyses, we identified signaling molecules responsible for the initiation of metamorphosis of the ascidian *Ciona*. Combining the results with previous knowledge, we described a schematic that best represents the cascades running in the adhesive papillae upon adhesion to initiate metamorphosis (**Figure 7**). This working hypothesis will be the basis for future research on ascidian/tunicate metamorphosis. The hypothesis explains the characteristics of *Ciona* metamorphosis, the mechanisms of which have not yet been elucidated. The genes, proteins, and signaling pathways in this schematic are essential targets for elucidating how the ancestor of ascidians acquired the metamorphosis system peculiar to this group during evolution.

Mechanisms measuring duration and strength of adhesions

One mystery of *Ciona* metamorphosis is that its initiation requires continuous adhesion with the adhesive organ for a certain period. This requirement is suggested for the faithful achievement of metamorphosis only when adhesion is firm enough to allow the transition out of the free-living larval stage. Our previous study suggested that approximately 30 min of adhesion is necessary[27]. A shorter duration of adhesion does not trigger metamorphosis. Larvae that experience short-term adhesion (such as detaching before 30 min) need to adhere again for 30 min to initiate metamorphosis, suggesting that the experience of temporal adhesion is erased. Our recent study showed that the force given to the papillae by adhesion also affects metamorphosis initiation: weaker force extends the time requirement[22]. Therefore, *Ciona* larvae likely possess a

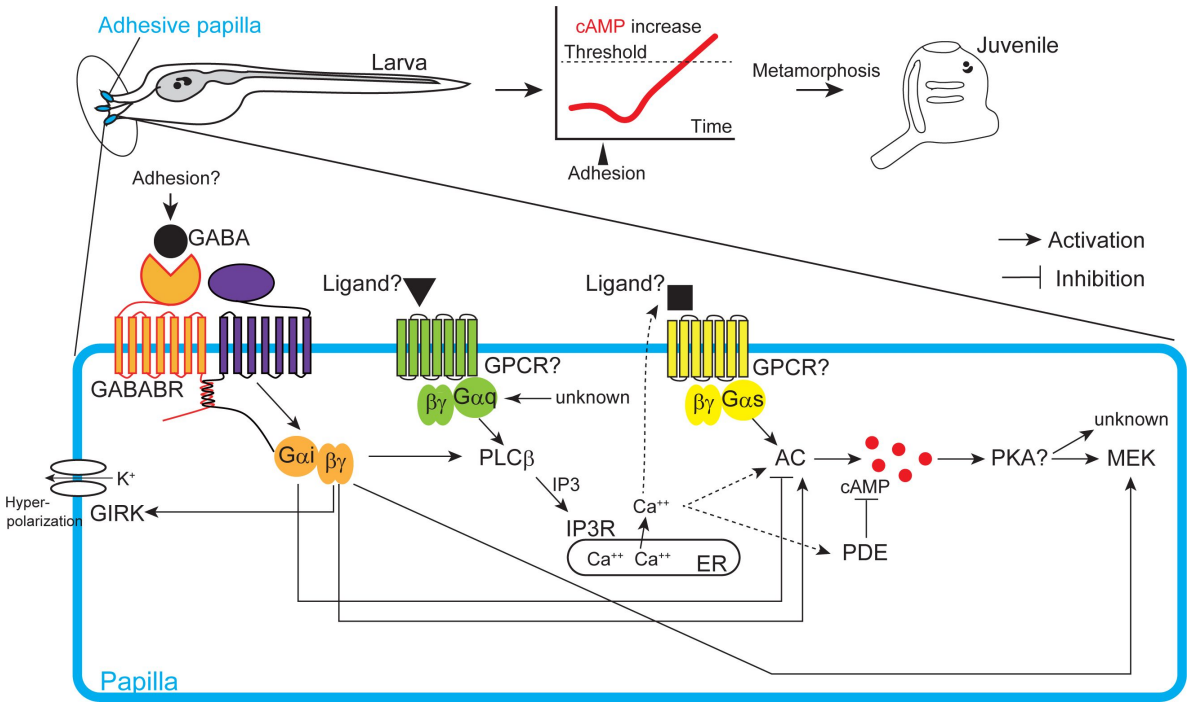

Figure 7.

Schematic illustration of the signaling cascades for initiating *Ciona* metamorphosis. AC, adenylate cyclase; ER, endoplasmic reticulum; GPCR, G-protein coupled receptor; PKA, protein kinase A.

mechanism that somehow measures the duration and strength of adhesion and the ability to cancel transient excitations of the adhesive organ. Because fewer than 300 neurons constitute the larval nervous system of *Ciona*[54,55,77], how the larva measures the strength and duration of adhesion with its simple nervous system is an important question to address the mechanisms of metamorphosis.

This study showed that cAMP is an essential second messenger molecule for triggering metamorphosis. Because PDE constitutively degrades cAMP, accumulation of this molecule requires persistent and/or strong activation of the metamorphic pathway that overcomes PDE activity. In other words, the duration and strength of adhesion could be converted into the quantity of cAMP, and when its quantity reaches a threshold, metamorphosis can be initiated. The time between the start of adhesion and metamorphosis initiation may be the period necessary to accumulate sufficient cAMP. This "cAMP timer" mechanism does not demand a complicated neuronal network system and could work in the simple nervous system of *Ciona*.

G-protein signaling relay for metamorphosis

This study showed that three major G-proteins are involved in the initiation of metamorphosis (**Figure 7**). This complicated mechanism contrasts the simple determination of metamorphosis initiation by cAMP accumulation. What factor promoted the ancestor of ascidians to acquire this complicated signaling network of metamorphosis? We suspect that incorporating multiple components increases the chance of generating the crosstalk between molecules, providing rigidity and flexibility to the system. Particularly, the involvement of Gi may have been important for achieving metamorphosis at the correct time and condition. GABA and Gi have inhibitory and excitatory functions[78,79]. As we discussed, *Ciona* larvae must ignore transient adhesion or stimulation on the papilla, which may occur frequently during swimming by colliding with an object or by strong water flows. At the same time, larvae must maintain sensing ability and start metamorphosis when they encounter an appropriate stimulus. Through the GIRK channel and Gαi, GABA can suppress the excitation and cAMP accumulation in the papilla. The GIRK channel could increase the excitation threshold of papilla neurons, allowing them to ignore weak stimuli. The transient reduction of cAMP (**Figure 3E**) after papilla stimulation guarantees the requirement of long-time adhesion irrespective of the initial quantity of cAMP. This cAMP reduction could be partly explained by the inhibitory function of Gαi on ACs. PDE1 is known to be activated by Ca^{2+}/calmodulin[80]. Because PDE1 is abundantly expressed in the papillae, the increase in Ca^{2+} upon adhesion could also trigger sudden cAMP reduction through PDE1.

Together with its inhibitory function, GABA can activate the Gq pathway, which promotes metamorphosis through Gβγi. Group III AC and MEK could also be the targets of Gβγi. When adhesion is long enough, their activations somehow overcome the inhibitory functions of GABA. The GABA pathway is an ideal player that fulfills multiple requirements in the regulation of metamorphosis through its excitatory and inhibitory functions. We suspect that papillae themselves are the source of GABA for metamorphosis initiation. Because VIAAT/VGAT is not expressed in the papillae, the secretion of GABA from the papilla neurons requires an atypical mechanism. GABA is detected in the cell body of the neurons[19]. The release of cytosolic GABA may be triggered by the reception of mechanical stimuli using the TRP channel[22]. A VIAAT-independent cytosolic GABA release is observed in pancreatic beta cells[81]. Curiously, papilla neurons express transcription factor Islet[82].

GPCRs for initiating metamorphosis and atypical Gαq activity

Future studies need to specify the mechanisms underlying Gαi, Gαq, and Gαs activation as well as the interaction between them more precisely. The activation of trimeric G-proteins relies on GPCRs, suggesting the presence of the receptors coupled with Gi, Gq, and Gs for metamorphosis initiation (**Figure 7**). Among them, Gi is likely to be coupled with GABABR; however, the expression of GABABR in the papillae is weak, and their interaction needs to be clarified in the

future. We found that wild-type Gαq exhibits constitutive activity that does not require activation through adhesion. The constitutive Gαq activity requires GTP binding. If a GPCR owns this Gαq activation, its ligand should be supplied constitutively. Wild-type *Ciona* does not initiate metamorphosis without adhesion even though Gαq is expressed in the adhesive papillae, suggesting that the quantity of Gαq is strictly regulated to prevent autonomous metamorphosis in normal conditions.

We did not identify the GPCR coupled with Gs in the metamorphic mechanism. We suspect that GnRH receptors (GnRHRs) are strong candidates for this role. Our previous studies showed that GnRHs can induce metamorphosis[36,37] as a downstream factor of GABA. Among the four genes encoding GnRHR proteins, *GnRHR1* and *GnRHR2* are expressed in the adhesive papillae[83]. These GnRHRs stimulate cAMP signaling, and the ligand GnRHs for GnRHR1 are also expressed in the papillae[83–85]. Therefore, GnRHs could be secreted from adhesive papillae as downstream molecules of GABA and stimulate the Gs pathway through GnRHR1. Future studies targeting the regulation of the secretion and reception of GnRH peptides after stimulation of papillae will improve our understanding of the signaling cascade conducting *Ciona* metamorphosis.

Understanding whether the G-protein signaling relay occurs in cell-autonomous or non-autonomous fashions is crucial for deepening our understanding of *Ciona* metamorphosis. Each papilla comprises approximately 20 cells, including four papilla neurons (PNs). We showed that activation of Gq and Gs pathways in PNs is sufficient for initiating metamorphosis. This coincides with the previous reports[22,23] that the loss of PNs by disrupting the POUIV transcription factor caused the complete arrest of metamorphosis. However, we do not think PNs are the only cells that activate G-proteins, because Ca^{2+} and cAMP imaging showed upregulation of fluorescence in the entire papillae[16,22]. A recent study also reports Ca^{2+} transient in another cell type than PNs[34]. Other papilla cells are likely responsible for activating G-proteins by directly responding to adhesion and by receiving signal input from adjacent cells, thereby enhancing the signal strength to accumulate a sufficient quantity of cAMP in the PNs to initiate metamorphosis. In this study, technical limitations prevented us from characterizing cells exhibiting increases in Ca^{2+} and cAMP; we need to address this question in future studies.

Evolutionary implications

Sessile or benthic marine invertebrates lose locomotive activity when they undergo metamorphosis triggered by adhesion to a substratum[86]. These animals are suspected of having a system to control the adhesion state, allowing them to repeat attachment and detachment before meeting the appropriate conditions for metamorphosis. For example, barnacle larvae “walk” on the substratum before adhering firmly by secreting cement[87]. Like *Ciona*, the larvae of sessile/benthic animals may have a system to initiate metamorphosis only when appropriate adhesion is provided, while erasing stimuli from transient and inappropriate adhesion. GABA serves as the metamorphosis inducer of some benthic invertebrates, including mollusks and echinoderms[88–90]. Moreover, GPCR is implicated as the mediator of settlement and metamorphosis induction in hydrozoans, mollusks, and barnacles[88,91,92]. In the abalone and barnacle, the dual use of Gq and Gs pathways in metamorphosis has been suggested[93,94]. Supported by these shared features in the metamorphic mechanisms, our working hypothesis about the initiation of *Ciona* metamorphosis (**Figure 7**) will serve as a cue to elucidate how marine benthic invertebrates regulate their metamorphosis.

Materials and Methods

Animals

Ciona intestinalis Type A wild types collected from Onagawa Bay (Miyagi, Japan) and Onahama Bay (Fukushima, Japan) were cultivated in closed colonies by the staff of the National BioResource Project, Japan. They were kept under a constant light condition to prevent gamete release. Eggs and sperm were collected surgically from gonadal ducts, and insemination was carried out in dishes. To prevent larvae from initiating metamorphosis, the tail's posterior half was manually cut with a scalpel. Removing the tail prevents larvae from swimming efficiently, and these larvae are usually unable to adhere to a substrate. These adhesion-prevented larvae do not metamorphose, since adhesion is required to initiate metamorphosis. Larvae developed from dechorionated eggs were cultured on a 2% agar-coated dish after tail amputation to prevent metamorphosis. Because larvae stick to plastics, tail amputation is insufficient to avoid their adhesion to the culture dish.

Pharmacological treatment

Larvae or larval anterior fragments isolated with a scalpel were administered overnight with 700 µM GABA (Fujifilm Wako #010-02441) or 1 mM theophylline (Sigma-Aldrich #T1633) dissolved in seawater, or 4 µM U0126 (Promega #V1121) dissolved in DMSO.

Plasmids

The open reading frame (ORF) of Kaede was removed from pSPCiPC2Kaede by inverse PCR with PrimeStar GxL DNA polymerase (Takara-bio #R050). The ORFs of *Gaq*, *Gas*, *Gai*, and *PLCβ1/2/3* were PCR amplified using full-insert cDNAs[95]. These PCR fragments were fused with the In-Fusion HD Cloning kit (Clontech #639650). The region encoding XY linker[43] was deleted from *PLCβ1/2/3* cDNA by inverse PCR to create a constitutively active form. The mutations were introduced by inverse PCR to create constitutive active or dominant-negative forms of *Gα* cDNAs. The introduced mutations are as follows: ca*Gaq*, corresponding to Q223L, abolishes GTPase activity[42]; ca*Gas*, corresponding to Q227L, abolishes GTPase activity[47]; dn*Gai*, corresponding to S47C[66,67]; and dn*Gaq*, corresponding to S54N[66]. The ORF of *bPAC*[51] was PCR amplified and inserted between the *Bam*HI and *Eco*RI restriction sites of pSP-Kaede[96] to create pSPbPAC. The PCR-amplified *PC2 cis*-element[46] was inserted into the *Bam*HI site of pSPbPAC. The plasmid DNAs were linearized by a restriction enzyme and purified with the Qiaquick Gel Extraction Kit (Qiagen #28706) before microinjection. The ORF of *Pink Flamindo*[52] was PCR amplified and inserted into the *Eco*RV restriction sites of pBS-HTB[97,98] to create pHTBPinkFlamindo. The plasmids pHTBGCaMP8[22] and pHTBPinkFlamindo were linearized using *Xho*I for subsequent *in vitro* synthesis of *GCaMP8* and *Pink Flamindo* mRNA, respectively. *GCaMP8* mRNA was synthesized with the MEGAscript T3 kit (Thermo Fisher Scientific #AM1338), the Poly (A) Tailing kit (Thermo Fisher Scientific #AM1350), and the Cap structure analog (New England Biolabs #S1404). Pink Flamindo mRNA was synthesized with the mMESSAGE mMACHINE T3 Transcription kit (Thermo Fisher Scientific #AM1348).

Microinjection

Unfertilized eggs were dechorionated in sterilized seawater containing 1% sodium thioglycolate (Fujifilm Wako #590-11762) and 0.05% actinase E (Kaken Pharmaceutical #650164) as previously described[99]. The microinjection solution included 2 mg/ml of Fast Green FCF (Fujifilm Wako #061-00031), 0.5-1.0 mM of morpholino oligonucleotide(s) (MOs)[100], 5 ng/ul of plasmids, and/or 1 mg/ml of *GCaMP8* mRNA for imaging[22]. For cAMP imaging, 1 mg/ml *Pink Flamindo* mRNA was dissolved in water without Fast Green, since this chemical emits fluorescence. Microinjected unfertilized eggs were inseminated in a gelatin-coated plastic dish. After the seawater was exchanged to remove excess sperm, the fertilized eggs were cultured at 18 °C or 20 °C overnight until the larval stage. MOs are listed below. Standard control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'; *GAD* ATG MO[36], 5'-ACCTCCAAGCCGATTGTTTCTGCAT-3'; *GABABR1* ATG MO[36], 5'-GCTTACGACTTTACATAACCTTACA-3'; *Gaq* ATG MO, 5'-

GGCATATTTGTGACTATAATGACG-3'; *Gαs* ATG MO, 5'-AAAGCAACCCATTGGCATTATCGAC-3'; *PLCβ1/2/3* splicing MO, 5'-GTGTTACTTACGCTTTCTCTA-3'; *PLCβ4* splicing MO, 5'-AACCACCAACCACCAACCTTTTG-3'; *IP3R* splicing MO, 5'-AATGATGGTTTAAAATTGCCACCTG-3'; *Gαi* ATG MO, 5'-GTGGAGACTGTGCAACCCATGATTC-3'; *dvGai_Chr2* ATG MO, 5'-CCATCTTGAGTAATCCAGGCTTTTA-3'; *GIRK channel* ATG MO, 5'-TCTGCTGGTTCAGTAATAGACATAG-3'.

Photographing and imaging

Photographs were taken with an AxioImager Z1 and AxioObserver Z1 (Carl Zeiss). The images were treated with AxioVision Rel.4.6 or Zen (Carl Zeiss) and Photoshop 2021 (Adobe) software. Imaging of the Ca^{2+} transient was carried out according to previous reports[16,22]. The tails of *GCaMP8* mRNA-injected larvae were removed by a scalpel at 15-17 hours after fertilization (hpf). At 30-40 hpf, the larvae were mounted onto a plastic dish under an M165FC stereo microscope (Leica Microsystems), and their adhesive papillae were stimulated by a glass rod using a three-axis coarse manipulator M-152 (Narishige Japan). Immediately after the tip of the rod had contact with the papillae, visible light was shut off and the fluorescence of GCaMP8 was taken with a DFC 310FX digital camera (Leica), Las version 4.12 (Leica), and the movie-capturing function of Windows 10. Images were treated with Photoshop 2021.

Imaging of cAMP was taken by confocal laser scanning microscopy (FV1000, Olympus). The movies were treated with ImageJ (National Institutes of Health). The Pink Flamindo mRNA-injected larvae were immobilized on Poly L lysine-coated glass-bottom dishes at 20-21 hpf. For stimulation of their adhesive papillae with a glass rod, an electric manipulator MM-89 (Narishige) and hydraulic micromanipulator MMO-202ND (Narishige) were used.

RNA sequencing

Larval anterior portions, including adhesive papillae and the other trunk region, were separately isolated by a scalpel around 22-25 hpf at 18°C and collected in Isogen (Nippon Gene #317-02503) soon after isolation. To identify GABA-responsive genes, half of the tail was amputated from larvae around 21-24 hpf, followed by GABA administration for 2 hours before treatment with Isogen. In each experiment, approximately 50 fragments were collected, and two biological replicates were taken. Total RNA was isolated according to the manufacturer's instructions. Ribosomal RNA was depleted from the total RNA using the NEBNext rRNA Depletion kit (New England Biolabs #E6310), followed by the conversion of the remaining RNA into an Illumina sequencing library using the NEBNext Ultra Directional RNA Library Prep kit (New England Biolabs #E7760). Following library preparation, validation was performed using the Bioanalyzer system (Agilent Technologies) to assess size distribution and concentration. Subsequently, sequencing was carried out on the NextSeq 500 platform (Illumina) employing the paired-end 36-base read option. Reads were mapped on the *Ciona intestinalis* Type A reference genome (HT model; *http://ghost.zool.kyoto-u.ac.jp/default_ht.html*) and quantified using CLC Genomic Workbench version 22.0 (Qiagen). RNA-seq data sets were deposited in the NCBI Gene Expression Omnibus (GEO) under accession numbers SAMN40712866 to SAMN40712873, which will appear online upon publication of this paper.

The read counts were normalized by calculating the number of reads per kilobase per million (RPKM) for each transcript in each sample using CLC Genomic Workbench version 22.0 (Qiagen). Eight candidate genes showing a minimum 1.5-fold increase following GABA administration, along with notably higher expression in the papillae in comparison to the trunk (an approximately 4-fold increase in a sample), were chosen for quantitative PCR analysis to assess their response to GABA.

Quantitative PCR

Total RNA was isolated from the anterior tips of larvae treated with the chemical using Isogen (Nippon Gene), following the manufacturer's instructions. After isopropanol extraction, genomic DNA removal and reverse transcription were carried out using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio #RR047). Quantitative RT-PCR (qRT-PCR) was done using a SYBR Premix Ex TaqII Dimer Eraser (Takara Bio #RR091) and a Thermal Cycler Dice Real Time System III (Takara Bio). The gene encoding elongation factor 1α (*EF1α*) was used to normalize RNA quantity according to a previous study[98]. qPCR primers are listed below. KY21.Chr5.240, 5'-TCTTCTCAAAGTTGCACATTCC-3' and 5'-CAGCAGCAACCAAACGATAAAC-3'; KY21.Chr8.489, 5'-CAATGCAACTTTGACTGCATAC-3' and 5'-TCCAAACTGCATTCCACATATC-3'; *EF1α*, 5'-CATGTCACGGACAGCGAAACG-3' and 5'-CAATGTGTGTTGAGGCATTCCAAG-3'.

Statistical analysis

Differences between conditions were evaluated by Fisher's exact test. Statistical analyses and most graph visualizations were conducted utilizing R x64.4.1.2 and RStudio software.

Phylogenetic analysis

We performed a tBlastn search[101] against the HT gene model of *Ciona*[61] using the amino acid sequences of human PDE, PLCβ, and *Ciona* Gα proteins as queries, followed by a reciprocal Blast search. We aligned the amino acid sequences from the HDc domain of PDE, the EF-hand_like, PLCXc, PLCYc, and C2 domains of PLCβ, and the whole open reading frame of Gα proteins using the M-COFFEE program[102,103]. After the removal of unnecessary residues, maximum likelihood trees were constructed using MEGA software version 11[104], employing the WAG amino acid substitution matrix[105]. The trees were assessed with 1,000 bootstrap pseudoreplicates.

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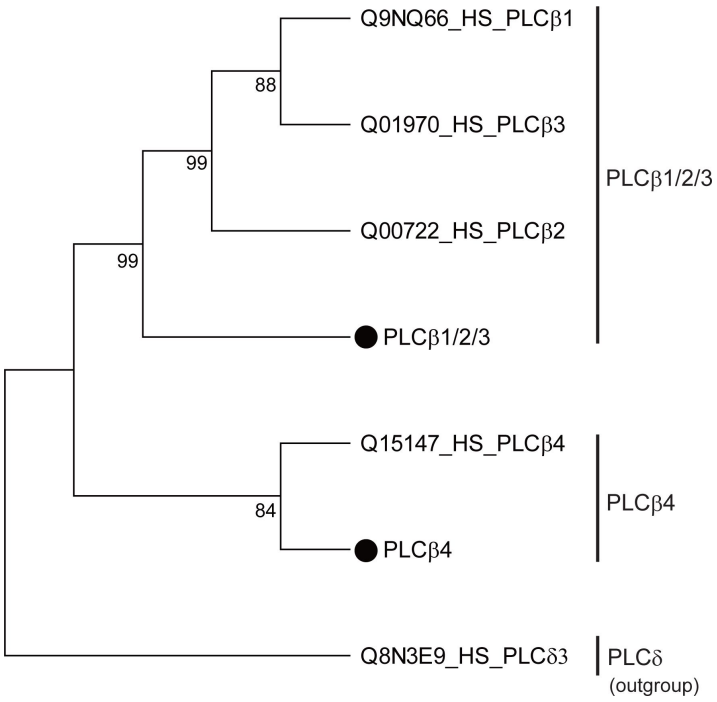
Author Contributions

A.H., N.M.T., A.O., Y.W., and Y.S. performed the experiments. Y.S. designed the experiments. T.H., M.H., and A.S. performed RNA-seq data and phylogenetic analyses. H.S., T.H., and K.H. supervised the work. Y.S. wrote the manuscript with consultations by A.H., N.M.T., and K.H. All authors contributed to the manuscript preparation.

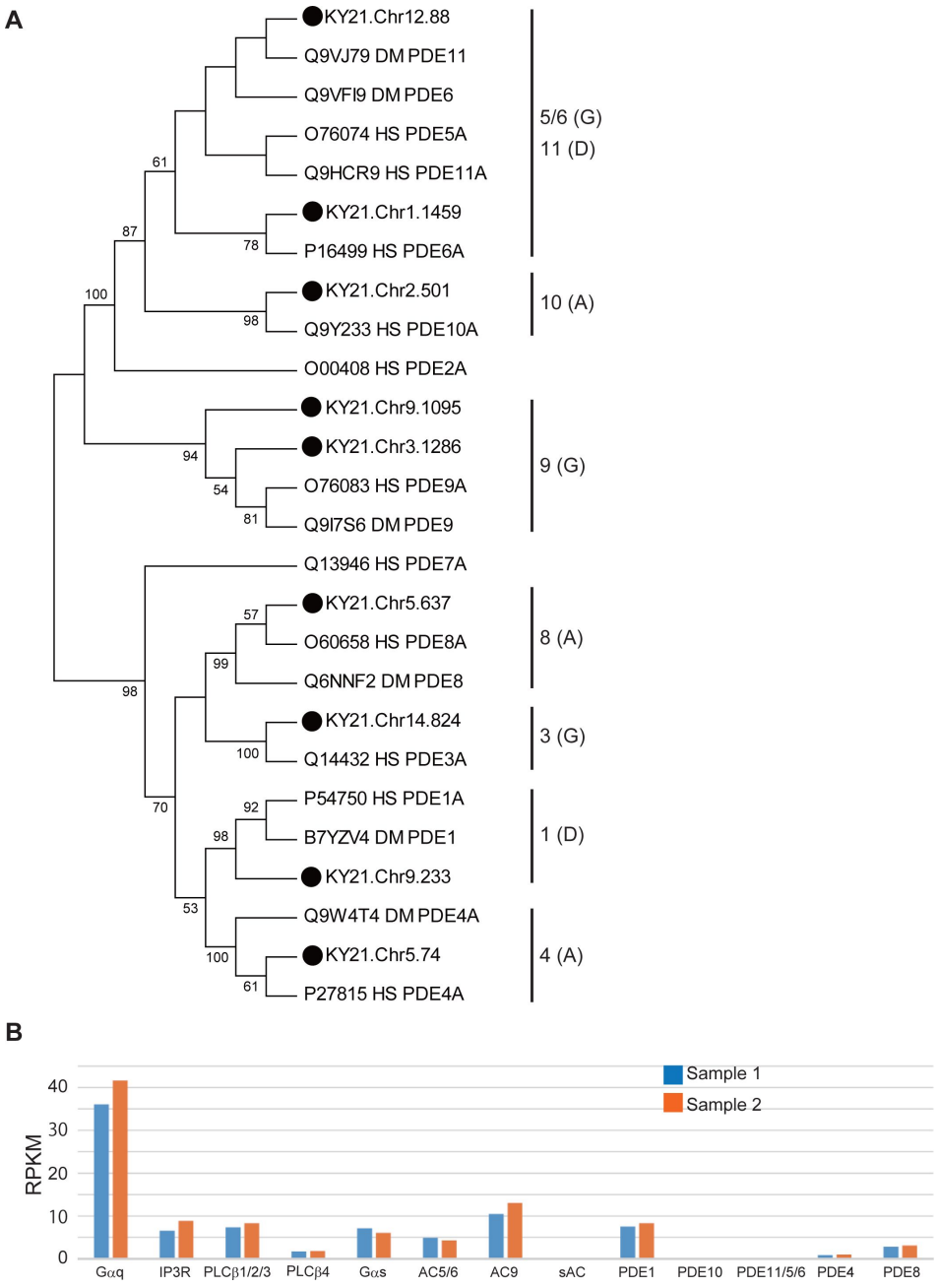
Supplementary Figures



Supplementary Figure S1.

Ciona phospholipase β proteins

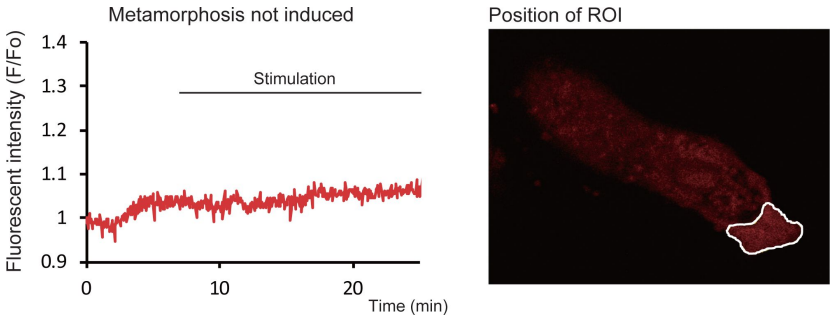
Phylogenetic relationships between PLCβ proteins, as revealed by the maximum likelihood method. The number beside each branch indicates the percentage of times that a node was supported in 1,000 bootstrap pseudoreplications. Scores equal to or exceeding 50% are shown. *Ciona* PLCβs are indicated by black circles. Human homologs are indicated by accession numbers in the Uniprot database (*https://www.uniprot.org*) and "HS" for *Homo sapiens*.



Supplementary Figure S2.

Ciona phosphodiesterase repertoire

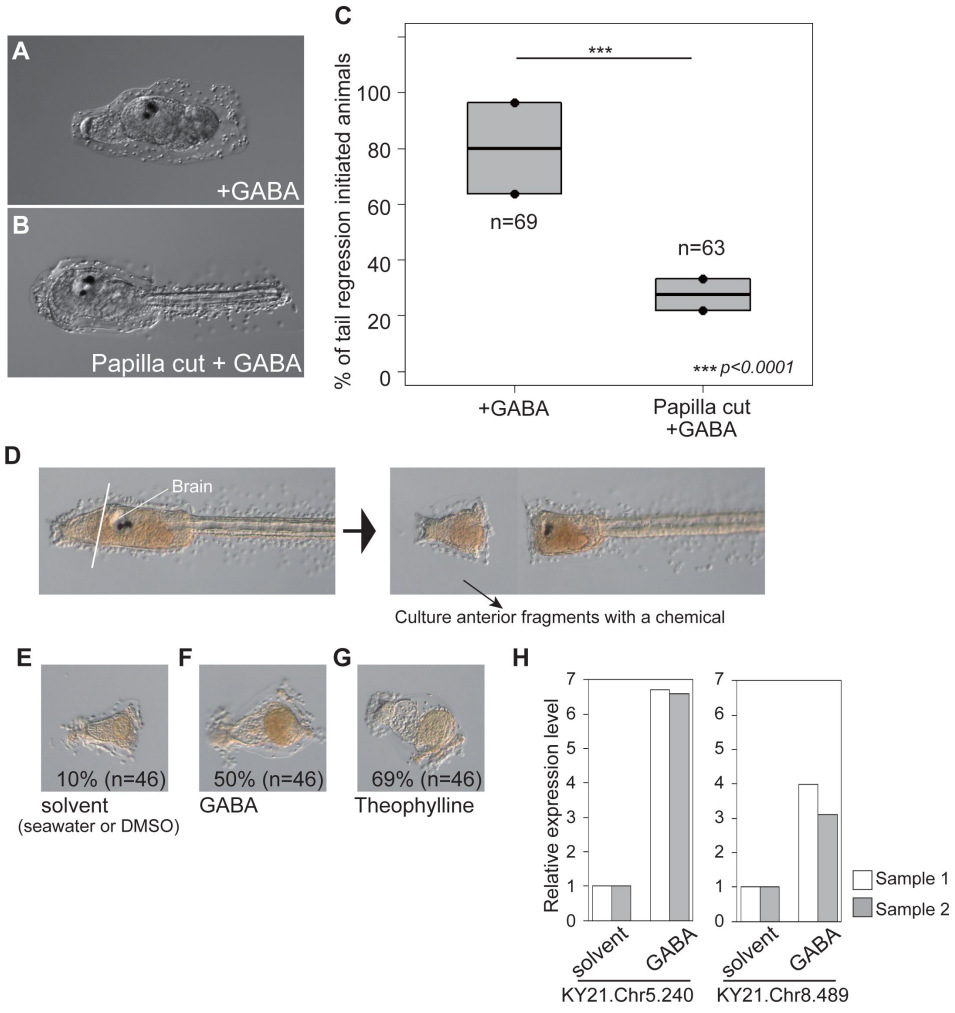
(A) Phylogenetic relationships between PDEs. Black circles and gene models indicate *Ciona* protein models in the Ghost database (*http://ghost.zool.kyoto-u.ac.jp/default_ht.html*). The numbers and letters in parentheses indicate the PDE groups and their suspected substrates. A, cAMP; G, cGMP; D, dual substrate. Human and insect proteins are shown by Uniprot (*https://www.uniprot.org*) accession numbers, as well as "HS" for *Homo sapiens* and "DM" for *Drosophila melanogaster* proteins. (B) Expression profiles of the genes related to Gq and Gs pathways, as revealed by RNA-seq of the papilla region. RPKM, reads per kilobase of exon per million mapped reads. See also **Table S1**.



Supplementary Figure S3.

cAMP increase in the papillae is coupled with metamorphosis initiation

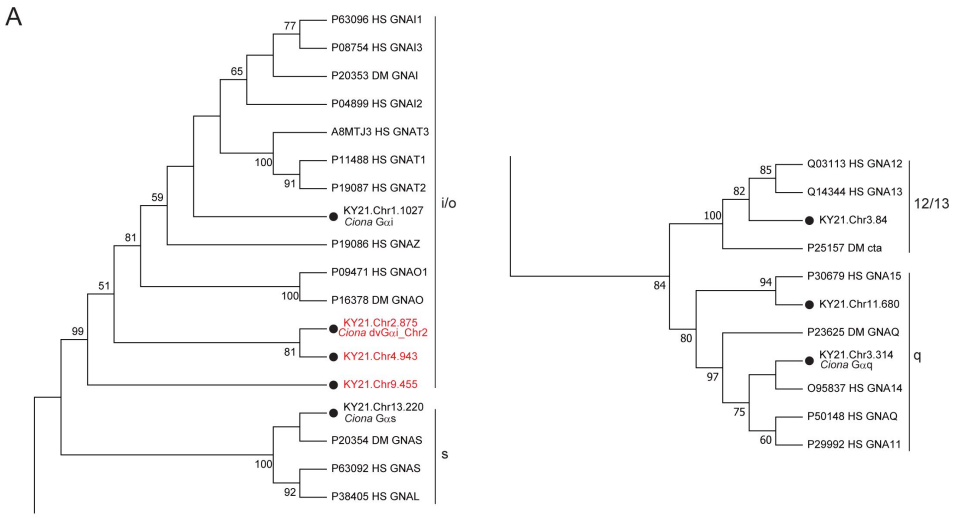
Measurement of cAMP concentration in the larva that did not initiate metamorphosis upon stimulation of the papillae, as indicated by the fluorescence of Pink Flamindo. Left, the quantification of Pink Flamindo fluorescent intensity of a larva that did not initiate metamorphosis; right, the position of the region of interest (ROI).



Supplementary Figure S4.

GABA stimulates the adhesive papillae to initiate metamorphosis.

(A) A GABA-treated animal initiated metamorphosis.
(B) A papilla-amputated larva treated with GABA. Metamorphosis was not initiated.
(C) Effect of papilla amputation on GABA treatment.
(D-H) The sensory vesicle is not required for the anterior region to respond to GABA.
(D) Schematic of the experiment.
(E) A control anterior fragment at 2 dpf.
(F) Anterior fragment treated with GABA. The percentage indicates the proportion of the fragments exhibiting the morphological signature of metamorphosis.
(G) Anterior fragment treated with theophylline.
(H) Expression levels of two GABA-responsive genes in the control- and GABA-treated anterior fragments, as revealed by quantitative RT-PCR.



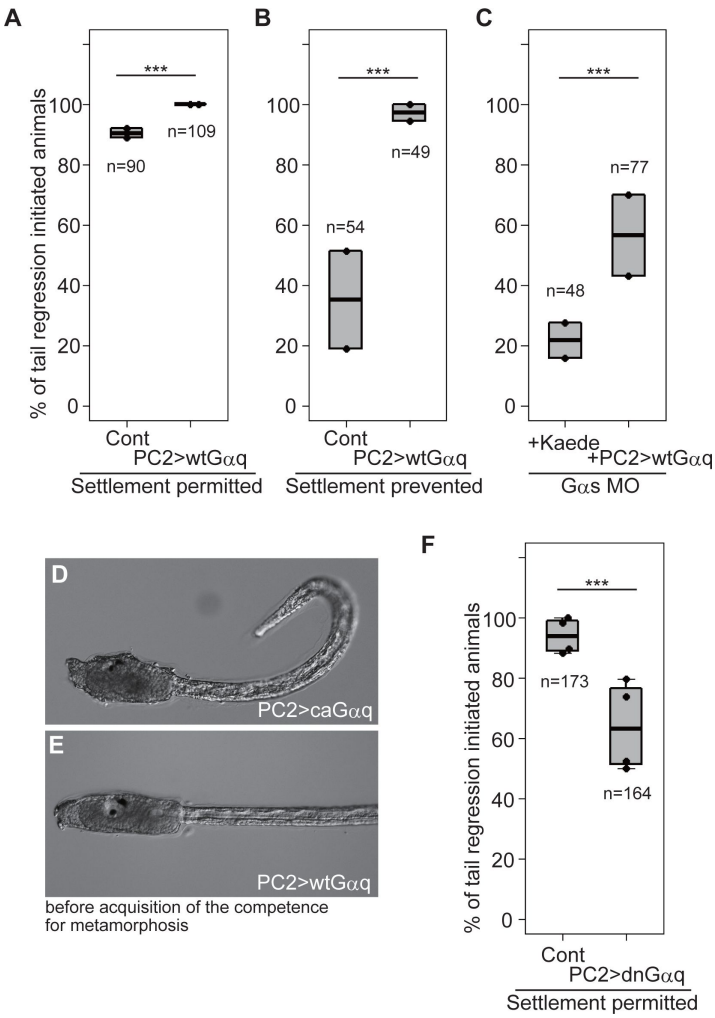
B

		Residue from the C-terminus						
		-5	-4	-3	-2	-1		
Ciona	Gαq	E	Y	N	**L**	V		
	Gαs	Q	Y	E	**L**	L		
	Gαi	**D**	**C**	**G**	**L**	**F**		
	dvGαi_Chr2	**D**	L	**G**	**L**	M	T	E
Human	Gαq	E	Y	N	**L**	V		
	Gαs	Q	Y	E	**L**	L		
	Gαi1	**D**	**C**	**G**	**L**	**F**		
	Gαi2	**D**	**C**	**G**	**L**	**F**		
	Gαi3	E	**C**	**G**	**L**	Y		
	Gαo	G	**C**	**G**	**L**	Y		
	Gα12	**D**	I	M	**L**	Q		
	Gα13	Q	L	M	**L**	Q		

Supplementary Figure S5.

Ciona has atypical Gαi proteins.

(A) Phylogenetic relationships between Gα proteins, as revealed by the maximum likelihood method. The three atypical Gα proteins are highlighted in red.
(B) Comparison of the five C-terminal amino acid sequences of Gα proteins that are shown to be essential for their functions, including two additional amino acids for dvGαi_Chr2. Residues conserved with human Gαi1 are shown in red.



Supplementary Figure S6.

Autonomous activity of wild-type Gαq.

(A) Effect of wt*Gαq* overexpression in the adhesion-permitted condition.
(B) Effect of wt*Gαq* overexpression in the adhesion-prevented condition.
(C) Effect of wt*Gαq* overexpression on *Gαs* knockdown.
(D) Morphology of ca*Gαq*-overexpressed larva at 1 dpf (before initiation of metamorphosis).
(E) Morphology of wt*Gαq*-overexpressed larva at 1 dpf.
(F) Effect of dn*Gαq* overexpression in the adhesion-permitted condition.

Signaling pathway	Protein name	Gene model	RPKM in sample 1	RPKM in sample 2
Gq	Gαq	KY21.Chr3.314	41.59881513	36.02858074
	IP3R	KY21.Chr8.925	8.870796516	6.554075453
	PLCβ1/2/3	KY21.Chr4.150	8.334938214	7.340493689
	PLCβ4	KY21.Chr13.136	1.822537572	1.713163972
Gs	Gαs	KY21.Chr13.220	6.051024382	7.109659594
	AC5/6	KY21.Chr14.273	4.323604525	4.916251967
	AC9	KY21.Chr14.609	13.05474647	10.4735061
	sAC	KY21.Chr10.472	0.066171013	0.035521714
	PDE1	KY21.Chr9.233	8.313399966	7.51787065
	PDE10	KY21.Chr2.501	0.01522946	0.030884959
	PDE11/5/6	KY21.Chr12.88	0.038744008	0.059477542
	PDE3	KY21.Chr14.824	4.688783832	4.554092009
	PDE4	KY21.Chr5.74	0.987514578	0.846482876
	PDE5/6	KY21.Chr1.1459	19.60592812	17.62571189
	PDE4/8	KY21.Chr5.637	3.101215703	2.859544055
	PDE9	KY21.Chr3.1286	0.116675399	0.307599189
Gi	Gαi	KY21.Chr1.1027	39.77613559	40.01167225
	dvGαi_Chr2	KY21.Chr2.875	125.3721017	134.2964969
	KY21.Chr4.943	KY21.Chr4.943	174.9101985	156.462334
GABA	GAD	KY21.Chr7.1221	3.514925048	2.987859536
	VIAAT/VGAT	KY21.Chr2.793	0.052692645	0.035619784
	GABABR1	KY21.Chr9.470	0.471066035	0.716482511
	GABABR2	KY21.Chr6.66	0.117407574	0.136471718
	GABABR2	KY21.Chr10.1059	1.816748932	0.751407557
	GIRK channel	KY21.Chr11.476	1.725547688	1.670994082
	GIRK channel	KY21.Chr13.97	3.392949104	2.96035066
Negative control[1]	VACHT	KY21.Chr1.783	0.005117048	0.025943113

1 The gene suggested not to be expressed in the papillae by *in situ* hybridization and immunostaining

Supplementary Table S1.

Expression levels of genes related to G-protein signaling during metamorphosis, as revealed by RNA-seq of the larval anterior tip including papillae

KY21 gene model	Human homolog	[1]GABA vs Control Sample 1	[1]GABA vs Control Sample 2	[2]Papillae vs Trunk Sample 3	[2]Papillae vs Trunk Sample 4
KY21.Chr1.1223		2.33	1.97	Not determined	Not determined
KY21.Chr2.1293		2.07	3.09	Not determined	Not determined
KY21.Chr2.219		1.78	1.8	Not determined	Not determined
KY21.Chr2.1069	ASNS: asparagine synthetase (glutamine-hydrolyzing)	6.37	11.6	22.05	49.41
KY21.Chr5.240	Serine peptidase inhibitor Kunitz type 3	1.79	2.11	12.57	14.39
KY21.Chr8.1171		1.56	1.52	11.08	10.04
KY21.Chr3.438		2.38	1.91	8.61	9.16
KY21.Chr11.358		3.57	3.39	4.69	1.2
KY21.Chr9.130	CTH: cystathionine gamma-lyase	2.16	2.75	4.68	3.26
KY21.Chr9.1057	ASS1: argininosuccinate synthase 1	3.69	6.23	4.5	3.31
KY21.Chr3.1611		1.69	1.54	4.48	7.14
KY21.Chr1.732		2.34	1.66	4.37	2.74
KY21.Chr8.489	AIFM2: apoptosis-inducing factor, mitochondria-associated 2	1.85	2.05	4.01	1.77
KY21.Chr7.224	B4GALT1: beta-1,4-galactosyltransferase 1	2.43	1.51	3.99	4.16

1 The RPKM in the GABA-treated sample was divided by the RPKM in the solvent (seawater)-treated sample to calculate the scores.
2 The RPKM in the papilla region was divided by the RPKM in the remaining trunk region to calculate the scores.

Supplementary Table S2.

List of candidate genes upregulated by GABA

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Editors

Reviewing Editor
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Heidelberg University, Heidelberg, Germany

Senior Editor
Albert Cardona
University of Cambridge, Cambridge, United Kingdom

Reviewer #1 (Public Review):

Summary:
In this manuscript, the authors use gene functional analysis, pharmacology and live imaging to develop a proposed model of diverse G protein family signalling that takes place in the papillae during the ascidian Ciona larval adhesion to regulate the timing of initiation of the morphological changes of metamorphosis. Their experiments provide solid evidence that antagonistic G protein signalling regulates cAMP levels in the papillae, which provides a threshold for triggering metamorphosis that is reflective of a larva keeping a strong and sustained level of contact with a substrate for a minimum period of approximately half an hour. The authors discuss their reasoning and address different specific aspects of their proposed timing mechanism to provide a logical flow to the manuscript. The results are nicely linked to
the ecology of Ciona larval settlement and will be of interest to developmental biologists, neurobiologists, molecular biologists, marine biologists as well as provide information relevant to antifouling and aquaculture sectors.

First, they knock down the G proteins Gaq and Gas to show that these genes are important for Ciona larval metamorphosis. They then provide evidence that the Gaq protein acts through a Ca2+ pathway mediated by phospholipase C and inositol triphosphate by showing that inositol phosphate and phospholipase C gene knockdown also inhibits metamorphosis, while overexpression of Gaq or phospholipase C allows larvae to undergo metamorphosis even in the absence of their mechanosensory cue, which is deprived by removing the posterior half of the tail and culturing the larvae on agar-coated dishes. The authors used calcium imaging which is a genetically encoded fluorescent calcium sensor to show that Gq knockdown larvae lack a Ca2+ spike in their papillae after mechanostimulation, confirming that Gaq acts through a Ca2+ pathway. Similarly the authors show that overexpression of Gas also enables larvae to metamorphose in the absence of mechanostimulation, suggesting a role for both Gaq and Gas in this process.

To confirm that Gas acts through cAMP signalling, the authors use pharmacological treatment or overexpression of a photoactivating adenylate cyclase to increase cAMP, and show that this also enables larvae to metamorphose in the absence of mechanostimulation, but only when their adhesive papillae are still present. Transcriptome data indicate that both Gs and Gq pathway genes are expressed in the adhesive papillae of the Ciona larva. One missing detail seems to be the need for evidence that cAMP is elevated in the papillae directly as a result of Gs activation. The authors use a fluorescent cAMP indicator, Pink Flamindo, to show that cAMP increases in the papillae upon adhesion to a substrate. Complementary to this, larvae that fail to undergo metamorphosis lack a cAMP increase in papillae. However, it is unclear whether the measured larvae that failed to undergo metamorphosis were wildtype or Gas knockdown larvae. If they were Gas knockdown larvae, this could provide evidence that cAMP does act downstream of the Gas activation.

The authors then provide evidence that GABA signalling within the papillae is acting downstream of the G proteins to induce metamorphosis. Transcriptome data shows that the genes for the GABA-producing enzyme, and for GABAb receptors, are both expressed in papillae. Pharmacological experiments show that GABA induces metamorphosis in the absence of mechanosensory cues, but only in larvae that retain their papillae. To show that GABA signalling within the papillae, rather than from the brain of the larva is important, the authors also demonstrate that anterior segments of larvae lacking the brain can also be stimulated to metamorphose by GABA, and show changes in gene expression caused by GABA.

The authors then use a combination of pharmacology and knockdown experiments in the presence or absence of mechanosensory cues to show that Gq/Ca2+ signalling acts upstream

of Gs/cAMP signalling. As the elevation of cAMP by pharmacology or photoactivating adenylate cyclase rescued GABA pathway mutant larvae, the Gq and Gs pathways were concluded to be downstream of GABA signaling. However, GABA treatment could still induce Gaq- and Gas-knockdown larvae to metamorphose, suggesting an alternative pathway to metamorphosis, which the authors deduce to be through a third G protein, Gai. They identify an unusual Gai protein that based on transcriptome data is strongly expressed in the papillae. Gai knockdown larvae fail to metamorphose but are rescued by GABA treatment, which can be explained by a potential additional Gai protein being still present (transcriptome evidence suggests this although it is not further confirmed experimentally, for example by hybridization, immunohistochemistry, fluorescent labelling, or knockdown). The authors then use overexpression and knockdown experiments to show that the Gai protein acts through Gβγi complex to activate phospholipase C. Their experiments also indicate a potential for a complementary or compensatory role for Gai and Gaq signalling through Gβγi. By inhibiting the potassium channel GIRK through knockdown, and the MAPK pathway gene MEK1/2 by pharmacology, the authors also establish a role for these in their proposed model of signalling, allowing GABA and cAMP to compensate or interact with each other.

Strengths:
The strength of this paper is the meticulous and extensive experiments, which are carefully designed to be able to precisely target specific genes in the putative signalling pathway to build step by step a complex model that can demonstrate how metamorphosis of the ascidian larva is timed so as to only undergo metamorphosis when strongly attached to a suitable substrate. The unique possibility of inhibiting mechanosensory-induced metamorphosis by removing some of the tail and smoothing the attachment substrate allows the authors to investigate potential effects on both activation and inhibition of metamorphosis, and to confirm that specific signalling pathways are clearly downstream of the initial
mechanosensory stimulation. The study is also clear about which aspects of the model still remain unknown, such as which ligands and receptors may be responsible for the binding and activation of Gaq and Gas. Experiments testing metamorphosis of just the anterior region of the larvae nicely demonstrates the need for signalling in the region of the papillae, as do experiments where the papillae are removed, which then block metamorphosis in treatments that would otherwise stimulate it. The final model is a nice end point and makes a clear summary of how the extensive experiments all fit together into a cohesive potential signalling network, which can be built upon in the future.

Weaknesses:
The paper has few weaknesses, however the main difficulty it poses is that due to the sheer number of precise experiments carried out and the complexity of the interwoven signalling pathways, it quickly becomes very difficult to follow exactly what is going on when and why or to keep track of the story as it develops. To improve this, an initial section in the results could be included showing a summary of the known G proteins in Ciona, their types and potential downstream signalling or upstream receptors, where known, and their expression levels in papillae. This could be in the form of a table and/or include the phylogenetic tree from the supplementary data. This would help clarify why the study first focuses on Gaq and Gas, and only later looks at Gai. This could be supplemented by a schematic workflow giving an overview of the experimental process of the study. A second minor weakness (understandable as the focus of the study is metamorphosis induced by mechanosensory stimulation) is that the study does not take into account any potential role for other types of sensory modalities (light, chemicals) that may also feed into the regulation of Ciona larval metamorphosis. This aspect would be interesting to discuss in light of the recent paper suggesting that some sensory cells in the Ciona adhesive papillae are polymodal and detect both chemicals and mechanical stimuli (Hoyer et al. 2024 Current Biology 34(6): 1168 - 1182).

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Reviewer #2 (Public Review):

Summary:
This work aims to characterize the neural signaling cascade underlying the initiation of metamorphosis in Ciona larvae. Combining gene-specific functional analyses, pharmacological experiments, and live imaging approaches, the authors identify the molecular players downstream of GABA to initiate Ciona metamorphosis. The results of this study may serve as a useful framework for future research on animal metamorphosis.

Strengths:
The authors did a great job in connecting their experiments with previous findings on Ciona metamorphosis. Taking advantage of the Ciona model system, they meticulously conducted genetic manipulation and pharmacological experiments to test the epistatic relationships among the signaling players controlling the initiation of Ciona metamorphosis.

Weaknesses:
The causal relationship between cAMP accumulation and the initiation of metamorphosis was not clearly demonstrated by the life-imaging observation with the fluorescent cAMP indicator (Pink Flamindo). It is a pity that this experiment was only conducted using normal larvae to compare those who underwent metamorphosis versus those who failed to initiate metamorphosis. This approach should be applied to some of the genetic manipulation and pharmacological experiments, to strengthen their main thesis on the "cAMP timer" mechanism.
On several occasions, the interpretation of the results seems to be imprecise and may lead to misunderstanding. This should be improved by rewriting the descriptions of those results and carefully comparing the differences in results from various treatments and experiments.

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Author response:

We would like to thank all reviewers for their valuable comments that help us to improve our manuscript. We will make the following modifications in the revised manuscript:

(1) To reduce the complexity of the experiments we carried out, we will summarize trimeric G proteins in *Ciona* in the first paragraph of the Result section and explain how we focused on Gas and Gaq in the initial phase of this study.

(2) As the reviewer 1 suggested, the polymodal roles of papilla neurons are interesting. We will add a discussion regarding this aspect. The sentences will be like the following:

“The recent study (Hoyer et al., 2024) provided several lines of evidence suggesting that papilla neurons can serve as the sensors of several chemicals in addition to the mechanical stimuli. This finding and our model seem mutually related because these chemicals could modify Ca2+ and cAMP signaling. The use of G protein signaling may allow *Ciona* to reflect various environmental stimuli to initiate metamorphosis in the appropriate situation, both mechanically and chemically.”

(3) As both reviewers suggested, imaging cAMP on the backgrounds of some G protein knockdowns and pharmacological treatments is important, and we will carry out some of these experiments.

(4) According to reviewer 2's comment, we will carefully modify the text about interpreting the results so that the descriptions suitably reflect the results.

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