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Collective cell migration of *Dictyostelium* without cAMP oscillations at multicellular stages

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In *Dictyostelium discoideum*, a model organism for the study of collective cell migration, extracellular cyclic adenosine 3',5'-monophosphate (cAMP) acts as a diffusible chemical guidance cue for cell aggregation, which has been thought to be important in multicellular morphogenesis. Here we revealed that the dynamics of cAMP-mediated signaling showed a transition from propagating waves to steady state during cell development. Live-cell imaging of cytosolic cAMP levels revealed that their oscillation and propagation in cell populations were obvious for cell aggregation and mound formation stages, but they gradually disappeared when multicellular slugs started to migrate. A similar transition of signaling dynamics occurred with phosphatidylinositol 3,4,5-trisphosphate signaling, which is upstream of the cAMP signal pathway. This transition was programmed with concomitant developmental progression. We propose a new model in which cAMP oscillation and propagation between cells, which are important at the unicellular stage, are unessential for collective cell migration at the multicellular stage.
Collective cell migration is ubiquitous in multicellular organisms and contributes to many organismal phenomena, including morphogenesis, wound healing, and cancer invasion. It is organized by integrated physical and chemical guidance cues between cells, such as cell–cell adhesion and diffusible factor-mediated signaling, which are integrated and act in parallel. The slime mold Dictyostelium discoideum is a model organism for the study of collective cell migration because of its morphogenesis and simple cell–cell interactions via diffusible chemical signals. Dictyostelium cells grow as unicellular organisms at the vegetative stage, but undergo transitions from a unicellular to multicellular organism by aggregation upon starvation. During aggregation, starved cells typically move towards the aggregation center to form one multicellular aggregate. This coordinated migration is achieved by the self-organization of cAMP gradients and by chemotaxis to extracellular cAMP. When Dictyostelium cells sense extracellular cAMP signals, cAMP receptors activate PI3-kinases through G proteins to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3) transiently on the plasma membrane of the cell front, leading to the transient localization of cytosolic regulator of adenylyl cyclase (CRAC) to the membrane via the Pleckstin Homolog (PH) domain that binds to PIP3, activating adenylyl cyclase. The Dictyostelium cell has three subtypes of adenylyl cyclase (ACA, ACB, and ACG), but only ACA is activated by extracellular cAMP signals. cAMP is synthesized by ACA in response to external cAMP signals and secreted to induce neighboring cells to similarly produce cAMP. Simultaneously, the transient accumulation of PIP3 at the cell front in response to external cAMP also induces actin polymerization and pseudopod formation, resulting in chemotactic migration. These reactions finally cause the propagation of cAMP signals as travelling waves called cAMP relay, resulting in chemotactic migration toward the aggregation center. That is, the correllative migrations of multiple cells are mediated by a single diffusible chemical factor, extracellular cAMP.

It has been argued that cAMP relay is also essential for the organization of collective cell migration during developmental events following the aggregation. Upon aggregation, cells form a stream which flows into a loose mound. Loose mounds become tightly packed (tight mounds) by both secretion of the extracellular matrix and the strengthening of cell–cell contacts. In tight mounds, cells differentiate into prestalk or prespore cells. Prestalk cells are sorted at the top of the mound to form the tip, which elongates and forms the front of a multicellular body (slug) to migrate as a whole. In conventional microscopic observations, optical densities of cell populations during chemotactic aggregation describe synchronous changes in cell shapes and act as an index of cAMP relay. These optical density waves have been detected in streams, mounds, and slugs, giving evidence of cAMP relay at these stages. Cell sorting to the tip of the mound also can be explained by cAMP relay. There is a difference in the response of chemotaxis toward cAMP between prespore and prestalk cells in mounds, resulting in cAMP relay guiding the sorting of prestalk cells to the tip of the mound. Cells dissociated from slugs produce cAMP upon extracellular cAMP stimulation and show chemotactic movement toward cAMP, indicating that slug cells have the ability of cAMP relay and chemotaxis toward cAMP. Furthermore, cAMP microinjection in slugs causes chemotactic attraction of some cells in the population and perturbation of the optical density wave propagation. These observations suggest that cAMP signals control cell movement in slugs. Thus, cAMP relay is regarded as an essential mechanism for organized collective cell migration, such as cell sorting and multicellular movement, in Dictyostelium cells.

In spite of these traditional views of cAMP relay for the coordination of collective cell migration in Dictyostelium, some observations suggest that the role of cAMP relay in slugs is controversial. acaA-null cells, which lack the ability of cAMP relay, normally cannot aggregate and form multicellular bodies, but the phenotypes of the mutant are rescued by constitutive activation of PKA, which is downstream of the cAMP signaling pathway, implying that Dictyostelium cells have developmental ability without cAMP oscillation. Furthermore, cAMP signals in mounds and slugs have not been investigated, whereas the cAMP relay during cell aggregation has been directly verified by live imaging of cAMP signals using sophisticated cAMP-sensitive fluorescent probes, which has revealed that intracellular and extracellular cAMP levels show synchronous oscillations in cell populations and that propagation of the oscillations changes between cells. Therefore, no clear evidence exists for cAMP relay organizing collective cell migration at multicellular stages. In this study, we investigated the dynamics of cAMP signals through the development of Dictyostelium cells by visualizing the changes in cytosolic cAMP levels ([cAMP]i), which reflect the response to cAMP relay. Our live-imaging approaches demonstrated the role of cAMP relay during aggregation and mound stages. Surprisingly, we found that [cAMP] oscillation and its propagation, which is an index of cAMP relay, gradually decreased and disappeared after slug formation. This result indicates a dramatic transition of cAMP signaling dynamics during the development of Dictyostelium cells and the possibility that oscillatory cAMP signaling is not essential for collective cell migration in slugs, which challenges the traditional view about the role of cAMP relay in the organization of collective cell migration.

Results

Flamindo2 is an indicator of cytosolic cAMP levels in Dictyostelium cells. To investigate cAMP relay in collective cell migration during the development of Dictyostelium cells, we monitored [cAMP], by using a cAMP indicator, Flamindo2. The binding of cAMP to the probe causes a decrease in the fluorescence intensity of the sensor. It has been reported that Flamindo2 can detect [cAMP], changes in aggregating Dictyostelium cell populations. We confirmed that Flamindo2 was stably expressed in Dictyostelium cells with no obvious defects in the developmental progression. The fluorescence intensity of the sensor in the cytosol of chemotactic-competent cells showed transient changes with two peaks after external cAMP stimulation; the first peak occurred 15 s after the stimulation, and the second peak gradually appeared 120 s after (Supplementary Fig. 2a, first panel). In acaA-null cells, the first peak of the response was weaker than in wild-type cells, and the second peak had completely disappeared (Supplementary Fig. 2a, second panel). When wild-type cells were treated with 4 mM caffeine, which inhibits adenylyl cyclase activities, the second peak of the fluorescence intensity after the cAMP stimulation had again disappeared (Supplementary Fig. 2a, third panel). It has been reported that cytosolic cAMP and cGMP levels show different response times to cAMP stimulation and that the first response of [cGMP], elevation occurs within 10 s of the stimulation, but the [cAMP], elevation occurs later (second peak). It has been shown using biochemical assays and FRET-based imaging analyses that the peak of ACA activity occurs 60–120 s after external cAMP stimulation. Considering Flamindo2 binds not only to cAMP but also to cGMP but with lower affinity, our results suggest that the first peak is an effect of [cGMP], elevation and that the second peak was due to only the increase in [cAMP]. This conclusion is also supported by gc- cells, which lack guanylyl cyclases (gca and gcbA) and have no cGMP production ability, showing Flamindo2 signal responses with only one peak at 90 s after the stimulation (Supplementary Fig. 2a, fourth panel). When...
cells expressing only Citrine instead of Flamindo2 in the cytosol were stimulated with cAMP, the fluorescence intensity showed no response (Supplementary Fig. 2b), indicating that changes in the Flamindo2 signal were not caused by cell deformation or other signals. Finally, the response was dose-dependent to the concentration of the applied extracellular cAMP (EC₅₀, 0.72 nM; Supplementary Fig. 2c), which is in good agreement with a previous report.

To see whether Flamindo2 is applicable to the monitoring of cAMP during the development of Dictyostelium cells, we examined cell populations in the early aggregation stage, which is the event achieved by cAMP relay and chemotaxis. Flameido2 signals showed obvious synchronous oscillations during early aggregation, and the pharmacological inhibition of ACA by caffeine treatment caused severe defects in the oscillations (Supplementary Fig. 2d). Such synchronous oscillations of Flamindo2 signals also could be detected in aggregating oscillations (Supplementary Fig. 2d). Such synchronous oscillations of cAMP signaling during aggregation could be translated into the early aggregation and tight mound stages (Table 1). The oscillations continued until mound formation. At these points, the periods became shorter in loose mounds (2.47 ± 0.28 min), but showed partial recovery in tight mounds (4.70 ± 0.56 min) (Fig. 2b, c and Table 1). Such oscillations continued until mound formation. At these points, the periods became shorter in loose mounds (2.47 ± 0.28 min), but showed partial recovery in tight mounds (4.70 ± 0.56 min) (Fig. 2b, c and Table 1). Simultaneous monitoring of Flamindo2 signals and cell movements revealed that the cell velocity oscillated with the same period as [cAMP], (Table 1) and that the two oscillations had tight correlation with each other (Fig. 3a-d). [cAMP] oscillations in the cell populations had the same intervals and were synchronized at the individual cell level, while the oscillations of the cell velocity showed some variation in the populations (Supplementary Fig. 4, 5). Cross-correlation analysis indicated that there was a phase difference of a half period between the oscillation of [cAMP], and cell velocity at the loose mound stage, but the two oscillations had the same phase at the early aggregation and tight mound stages (Table 1). These findings indicate that oscillatory cAMP signaling organizes collective cell migration until the mound formation.

**Fig. 1** Typical cAMP signaling dynamics at each developmental stage of Dictyostelium cells visualized by Flamindo2. a Spiral pattern of a [cAMP], wave in cell populations at early aggregation. b Wave propagation in an aggregating stream. c Rotational propagation in a loose mound. d Wave propagation from the top of a tight mound (right side of images) to the bottom. e A slug with a stream elongating toward the top of the images. Images were subtracted at 3-6 frame intervals to emphasize changes in fluorescence intensity. Solid and broken arrows show the positions of the first and second waves in each sequential image, respectively. Scale bars, a 1 mm, b, e 100 μm, c, d 50 μm.
We next examined the [cAMP]i dynamics during slug formation and migration. When slugs were formed through mound elongation and tip formation, [cAMP]i oscillations became weaker and finally disappeared (Fig. 2b, c). To see whether the apparent disappearance of the oscillations in multicellular bodies resulted from a desynchronization of oscillations between cells or a synchronous disappearance of the oscillations, [cAMP]i dynamics during the damping of oscillations in an elongating mound was monitored at the single cell level (Fig. 2d). When the oscillation of Flamindo2 signals in the

Fig. 2 Disappearance of [cAMP]i oscillations during development. a Fluorescent images of Dictyostelium cells expressing Flamindo2 in each developmental stage. Maximum intensity projections of Z-stack images are shown. Scale bars, 100 μm. b Time course plot of inverse Flamindo2 signals during development from the onset of aggregation to slug formation. Data were obtained 3.5–10.75 h after starvation. The mean intensity of Flamindo2 in a 30 μm² region in the cell population shown in a was measured. c Autocorrelation of Flamindo2 signals at each development stage are shown by the gray bars in b. d A fluorescence image of Flamindo2 and Histone2B-RFP in an elongating mound. The maximum intensity projection of Z-stacks is shown. Scale bar, 50 μm. e Time course plot of inverse Flamindo2 signals at the tissue (first) or individual cell level (second and third) in the mound shown in d. First, average signals in the entire region of the mound. Second, signals in 5 cells indicated by the white boxes in d. In the second graph, individual cells were tracked, and Flamindo2 intensities within each cell were measured. Third, average of the signals in the second graph.

We next examined the [cAMP]i dynamics during slug formation and migration. When slugs were formed through mound elongation and tip formation, [cAMP]i oscillations became weaker and finally disappeared (Fig. 2b, c). To see whether the apparent disappearance of the oscillations in
entire mound had almost disappeared (Fig. 2e, left: ~25 min), oscillation in single cells vanished almost at the same time (Fig. 2e, middle and right). Thus, the disappearance of the [cAMP]i oscillations in the entire mound was caused by a synchronous disappearance of oscillations in individual cells. The Flamindo2 signals of 24 migrating slugs for 20 min were observed, but no slugs showed [cAMP]i oscillations. We also measured [cAMP]i and cell velocity of the cells in slugs. Prestalk cells made up ~20% of slugs (anterior) and moved rotationally, while prespore cells made up ~80% of slugs (posterior) and moved straight (Supplementary Fig. 6a, b). Cell movements in the anterior and posterior (respectively the prestalk and prespore regions) showed oscillations with periods of 7.75 and 8.25 min. However, no obvious oscillations in [cAMP]i, associated with cell velocity were observed (Fig. 3e, Supplementary Fig. 6c, d). These results suggest that the dynamics of cAMP relay changes after slug formation and that the collective cell migration in slugs does not depend on oscillatory cAMP signaling.

### Verification Flamindo2 functions as a cAMP indicator in slugs.

To see whether the absence of [cAMP]i oscillations was due to the loss of Flamindo2 function in slugs, we stimulated cells dissociated from the slugs with external cAMP and monitored [cAMP]i. Prestalk and prespore cells showed similar [cAMP]i responses to external cAMP stimulation (Fig. 4a, first), which is consistent with the results obtained from previous biochemical assays. The responses were completely suppressed when the cells were treated with caffeine (Fig. 4a, second). The dose-dependency of the response showed an EC50 of 250 ± 136 nM and 58 ± 22 nM in the prestalk and prespore cells, respectively (Fig. 4b), values that are ~100-fold higher than in the unicellular phase (Supplementary Fig. 2c). The difference of EC50 between prestalk and prespore cells was not significant. These findings verified Flamindo2 was functional in slug cells. We next examined the response of intact slugs by using a micropipette containing cAMP solution. The response of slugs to cAMP stimulation has been investigated by the direct injection of cAMP solution into slugs from a micropipette in previous studies, but we stimulated slugs by injecting cAMP from the micropipette into agar to diffuse it and avoid mechanical stimulation through contact between the micropipette and slugs (Fig. 4c and Supplementary Movie 5). This application of cAMP to a slug caused transient changes in the Flamindo2 signals and slug velocity (Fig. 4d). Thus, extracellular cAMP signals could modify slug movements, as reported previously, and induce cAMP production, although endogenous [cAMP]i waves were not detected in slugs.

### Development of acaA-null cells without [cAMP]i oscillations.

Our hypothesis that cAMP relay is dispensable for the collective cell migration of multicellular slugs is incompatible with the model that assumes cAMP relay plays key roles in organized collective cell migration in slugs. However, it is consistent with the fact that acaA-null cells lacking cAMP relay can aggregate and develop to form multicellular bodies when the expression of developmental genes is induced by exogenous and uniform cAMP pulses. To confirm whether acaA-null cells could develop and migrate as multicellular organisms without [cAMP]i oscillations, we monitored Flamindo2 signals during their development. When acaA-null cells were exposed to exogenous cAMP pulses,
Fig. 3 Simultaneous monitoring of [cAMP]_i and cell velocity at each developmental stage. Left graphs show time-course plots of [cAMP], (green solid lines) and cell velocity (black dashed lines). Individual cells were tracked, and Flamindo2 intensities within each cell and cell velocities were measured. The signals of Flamindo2 and cell velocities were averaged across several representative cells, and the averages of representative cells are plotted against time. The curves of Flamindo2 signals and cell velocities were smoothed by a running average over four data points. Right graphs show the cross-correlation between [cAMP]_i and cell velocity shown in the left graphs. a Early aggregation (n = 20 cells). b Aggregation stream (n = 14 cells). c Loose mound (n = 12 cells). d Tight mound (n = 10 cells). e Slug (n = 10 cells)
small clumps were formed by aggregation (Fig. 5a and Supplementary Movie 7). After terminating the exogenous cAMP pulses, the clumps deposited on agar started to elongate and then formed migrating slugs (Fig. 5b and Supplementary Movie 8). We found that the Flamindo2 signals from cell clumps formed by aggregation were unresponsive to external cAMP stimulation (Fig. 5c), and no obvious [cAMP]i oscillations were observed during the aggregation or slug stages (Fig. 5d, e). These observations suggest that oscillatory cAMP signaling is not essential for collective cell movements in migrating slugs.

**Transition of cAMP signaling dynamics occurs with the progression of development.** To reveal the temporal relationship between the transition of cAMP dynamics and development, we observed cAMP dynamics and tip formation simultaneously using Flamindo2 and ecmAO::mRFPmars (Fig. 6a and...
Supplementary Movie 9), because the tip of the mound is regarded as a prestalk region characterized by the high expression of the ecmAO gene33. [cAMP]i oscillated clearly from the loose to tight mound stage with no obvious expression of mRFPmars under the control of the ecmAO promoter (Fig. 6b, 0–120 min). When the tight mound begun to elongate and cells highly expressing ecmAO::mRFPmars were sorted into the tip (Fig. 6c), [cAMP]i oscillations became weaker and finally disappeared upon complete tip formation (Fig. 6b, 120–210 min). Considering the maturation time of mRFP has a time lag of ~90 min34, both the accumulation of prestalk cells to the tip and the loss of cAMP waves started to occur around the same time (Fig. 6b, c, ~150 min), suggesting that the transition of the [cAMP]i dynamics occurred simultaneously with the tip formation. To confirm whether the transition of cAMP signaling is a developmentally regulated event, we examined the [cAMP]i dynamics of a mutant lacking gfbA, which encodes the transcription factor G-box binding factor (GBF). GBF regulates late-development gene expression, and gfbA-null cells undergo developmental arrest at the loose mound stage without tip formation due to the lack of post-aggregative and cell-type specific genes35 (Supplementary Fig. 9). The results show that mutant cells had impaired transitions in cAMP dynamics, in which cAMP waves continued over 7 h and their propagation persisted 24 h after starvation with arrest in the loose mound stage (Fig. 6d–f). These results are consistent with a previous report that found mounds of a gfbA-null mutant showed optical density waves, although the pattern of the wave propagation was aberrant36. Thus, the cAMP dynamics transition from propagating waves to steady state was due to developmental regulation.

Fig. 5 Development of acaA-null cells without [cAMP]i oscillations. a Aggregation of acaA-null cells expressing Flamindo2 in DB with exogenous cAMP pulses under microscopic observation. Top panels, DIC images. Lower panels, fluorescent images of Flamindo2. Scale bar, 100 μm. b Slug formation of acaA-null cells expressing Flamindo2 on agar. Cells were washed and deposited on an agar plate after cAMP pulses. Upper panels, DIC images. Lower panels, fluorescent images of Flamindo2. Inside the fluorescent images, maximum intensity projections of Z-stacks are shown. Scale bar, 100 μm. c Time-course plot of Flamindo2 signals in cell clumps after 100 μM cAMP stimulation. The mean intensity of Flamindo2 in a 25 μm² region in the cell mass was measured, and the inverse of the fluorescence intensity of Flamindo2 is plotted on the y-axis (mean ± SD, n = 13 clumps). d Time-course plot of Flamindo2 signals in acaA-null cells during aggregation. The mean intensity of Flamindo2 in a 100 μm² region on the aggregation field shown in a was measured, and the inverse of the fluorescence intensity was plotted against time. e Time-course plot of Flamindo2 signals in acaA-null cells during slug formation. The mean intensity of Flamindo2 in a 100 μm² region in the cell mass shown in b was measured, and the inverse of the fluorescence intensity was plotted against time.
Discussion

In past studies, the details of cAMP signal dynamics have been examined only at the unicellular stage\(^{20,27}\). In contrast, our study investigated the dynamics of cAMP signaling throughout the development of Dictyostelium cells including the multicellular phase by using the cAMP indicator Flamindo2. Flamindo2 could detect the [cAMP] changes of Dictyostelium cells in response to external cAMP stimuli (Supplementary Fig. 2, Fig. 4), and purified Flamindo2 has an EC\(_{50}\) of 3.2 \(\mu\)M to cAMP and Hill coefficient of 0.95\(^{22}\), which covers the range of cytosolic cAMP levels.
measured biochemically in unstimulated and cAMP-stimulated Dictyostelium cells at all development stages. We estimated the cytosolic cAMP concentration to be ~0.3–12 μM, based on the values of cell volume and protein amount in previous reports. These estimates indicate that Flamindo2 is an appropriate tool for monitoring the cAMP signaling dynamics in Dictyostelium cells throughout their development. In the aggregation and mound stages, we observed cAMP wave oscillations and wave propagations in the cell populations (Figs 1, 2). The wave oscillations were tightly coupled with the cell movement (Fig. 3a–d). The phase relationship between the [cAMP] oscillation and cell velocity was dependent on the wave periods and not on the developmental stage (Table 1), suggesting that the relationship between cAMP production and chemotactic movement in response to the cAMP signal is maintained until the tight mound stage. The pattern of cAMP waves (Fig. 1a–d) and the cAMP oscillation period in the aggregation and mound stages (Table 1) agreed well with synchronous changes in the optical density of cell populations previously reported. The oscillation period decreased in loose mounds, but increased when tight mounds were formed (Table 1). These changes would be caused by two reasons; previous reports suggest that the decrease of the oscillation period can be explained by an increase in the cell density and extracellular cAMP, while the increase of the oscillation period can be explained by the expression of low-affinity cAMP receptors during the mound stage instead of high-affinity receptors expressed in the aggregation stage. It has also been suggested that the expression of low-affinity cAMP receptors causes changes in the cAMP wave geometry, which is agreement with the hypothesis that the expression of low-affinity cAMP receptor plays a key role in changing the cAMP wave propagation pattern at the stage from loose mounds to tight mounds (Fig. 1c, d). Furthermore, the changed wave geometry in the mound stages agrees with the classical model, which assumes prestalk cells are sorted on the top of the tight mound by chemotaxis toward cAMP signals. Overall, our findings show collective cell migration was coordinated with CAMP relay from the early aggregation to tight mound stages, which is consistent with the mechanism of collective cell migration in Dictyostelium cells.

Our observations revealed that cAMP, wave oscillations and wave propagations gradually weakened with slug formation and eventually disappeared (Fig. 2b, c), although cell velocity oscillations in slugs were consistent with those in early aggregation and mound stages (Fig. 3e and Supplementary Fig. 6c, d). Because a transient elevation of cAMP, in slug cells in response to external CAMP stimuli was observed (Fig. 4), we confirmed the vanishing of the cAMP oscillation was not due to impaired Flamindo2 function. Rather, our observations show that the transition of cAMP signaling dynamics occurs after slug formation and that any endogenous cAMP changes in slugs was below the detection limit of Flamindo2. We confirmed cAMP signaling transitions by investigating the dynamics of PIP3 signaling, which activates adenylyl cyclase and in turn produces cAMP. We monitored PIP3 levels on the plasma membrane using PHAKT/PKB–GFP and found periodic changes at the mound stages but not at the slug stage (Supplementary Fig. 8). This contrast is consistent with a previous report that tracked the PH domain of CRAC by GFP labeling. In addition, we found a correlation between the oscillations of cAMP, and PHAKT/PKB–GFP translocation (Table 1). Although the cells dissociated from slugs showed a transient translocation of PHAKT/PKB–GFP in response to CAMP stimuli, the continuous localization of PHAKT/PKB–GFP to the leading edge of cells in intact slugs was not inhibited by external CAMP stimuli or caffeine treatment (Supplementary Fig. 7). These observations suggest that the constant polarity of PIP3 levels on the cell membrane of slug cells depends on tonic CAMP signals and/or other signals such as cell–cell contacts, as indicated in a previous study. Thus, our findings demonstrate transitions from oscillations to steady state upstream of the CAMP signaling pathway during slug formation. Further, they raise the possibility that collective cell migration at the slug stage does not depend on oscillatory CAMP signaling for cell–cell communication, which challenges existing models. This hypothesis is supported by the fact that acaA-null mutant cells can aggregate and develop when prestimulated with uniform CAMP pulses or when PKA, which is downstream of the CAMP signaling pathway, is constitutively activated. Monitoring cAMP levels using Flamindo2 showed that acaA– cells could aggregate and form migrating slugs without cAMP, oscillations after the CAMP pulse treatment (Fig. 5d, e). Previous reports and our investigation using mutants lacking CAMP relay imply a development capacity without periodic CAMP signals. However, our approach using wild-type cells shows for the first time that the disappearance of periodic CAMP signals occurs even with normal developmental. Therefore, we concluded that oscillatory CAMP signaling is not necessary for collective cell migration at the slug stage. This conclusion does not exclude the possibility that CAMP signals affect slug movement. The existence of optical density waves, which act as an index of CAMP relay in slugs, is controversial. Therefore, it is possible that other experimental conditions would allow us to detect CAMP wave propagation using Flamindo2. Additionally, we found that cAMP, and slug movement are sensitive to external CAMP stimuli (Fig. 4d), indicating that collective cell migration could depend on any endogenous CAMP relay that occurs in slugs.

The simultaneous monitoring of cAMP, and cell sorting in Dictyostelium cells (Fig. 6a–c) suggested that the transition of CAMP signaling dynamics was a developmentally regulated event. This conclusion was confirmed in mutants that were developmentally arrested at the mound stage and showed no transition in CAMP signaling dynamics during development (Fig. 6d–f). In Dictyostelium cells, the expression pattern of genes essential for CAMP signaling is dramatically changed after slug formation (Supplementary Fig. 10). For example, the expression of high-affinity CAMP receptor CAR1 is high and seen in all cells during...
the aggregation stage, but becomes low after the mound stages43,44. In contrast, after slug formation, the expression of the high-affinity receptor cAR3 is seen in prespore cells, but the low-affinity receptors cAR2 and cAR4 are expressed in prestalk cells45,46. These expression changes are consistent with the sensitivity of the \([cAMP]i\) response to external cAMP stimulation at the multicellular phase being lower than at the unicellular phase (Fig. 4b and Supplementary Fig. 2c) and that the responsiveness of chemotaxis to cAMP gradients becomes weaker with mound formation47. The different sensitivities suggest changes in the cAMP signaling systems during multicellular formation. It has been reported that full-length CRAC is stably expressed under the control of a constitutively active promoter until the mound stage, but its expression is downregulated in slugs29. Therefore, our results suggest the active downregulation of molecules that mediate cAMP signaling and the developmental regulation of gene expression patterns during slug formation are involved in the transition of cAMP signaling dynamics. Additionally, one previous report showed that CAR1 is intrinsically internalized at the mound stage48, suggesting the possibility that the changes in protein localization and thus protein function also contributes to the transition of cAMP signaling dynamics.

Our study revealed the disappearance of oscillatory cAMP signaling after multicellular slug formation and suggests the presence of mechanisms other than cAMP relay for the organization of collective cell migration in slugs. One possible mechanism is that extracellular cAMP signals such as a steady gradient and/or oscillations in slugs guide the direction of multiple cell movements. Blocking the cAMP signal pathway by caffeine treatment results in arresting the slug migration18, although the morphology of the slug is maintained under caffeine treatment by cell–cell adhesions and the extracellular matrix (Fig. 7 and Supplementary movie 10). Furthermore, although the developmental arrest phenotype of acaA− cells can be rescued by the expression of constitutively active PKA19, mutant cells lacking acaA and accA, which encode ACA and ACB, respectively, cannot form normal multicellular bodies even if constitutively active PKA is expressed49. These findings indicate that cAMP is still required for the collective migration of slugs in spite of cAMP oscillations being absent in migrating slugs. In order to clarify the role of cAMP in slugs, molecular genetics approaches and more sensitive cAMP measurements are required. Additionally, it is possible that other chemoattractants such as pterin50 and anterior-posterior Ca2+ gradients in slugs51.
are also involved in the organization of collective cell migration in slugs. In addition to chemical cues, it is widely known that collective migration is regulated by physical guidance cues through cell-cell contacts in higher organisms during events such as epithelial wound healing and closure. Numerical simulations have shown that physical interactions between cells can organize collective cell migration in the absence of any external chemical signals. These studies suggest the possibility that the collective cell migration in Dictyostelium multicellular bodies is organized by physical guidance cues rather than chemical guidance cues. One possible explanation is provided by "contact following", which describes how cells follow other cells with which they have direct physical contact. In fact, mutant cells which lack chemotaxis toward cAMP due to chemical mutagenesis show an organized collective migration that is mediated by cell-cell adhesions. The adhesion proteins TrgB1/TrgC1 mediate cell alignment through head-to-tail cell contacts, leading to cell polarization including PILP localization and collective cell migration at later development stages. The effects of adhesion proteins suggest that cell-cell contacts play important roles in multicellular morphogenesis.

Overall, we demonstrated directly the disappearance of cAMP signal oscillations and propagations between cells at the multicellular phase of Dictyostelium. Our work calls for reconsideration of the role cAMP relay has on collective cell migration in Dictyostelium and proposes a possibility that alternative mechanisms to cAMP relay contribute to the organization of collective cell migration at the multicellular phase.

Methods

Cell strains and culture conditions. The Dictyostelium discoideum cell strains used in this study are as follows: Ax2 (wild type), acaA−, gca−, flgA−, flgB−, flgG− cells were obtained from the NbRP-nenkin Stock Center. Cells were grown axenically in HL5 Medium (Formedium, UK) in culture dishes or shaking flasks at 21 °C. Transfectants were maintained at 20 μg ml−1 G418 or 10 μg ml−1 BlastidacinS.

Plasmid construction and genetic manipulation. The plasmids used in this study are as follows: pECmAO-RFPmars, pHistonze2B-RFP, pHKG12neo_Flamindo2 was constructed by inserting the insertion of Flaimindo2 fragments (GenScript) into the pH112 and SpeI sites of pHK12neo by the In-Fusion technique (Clontech laboratories Inc.). pHK12neo_Citrine, and pHK12neo_Flamindo2. pHKG12neo_Flamindo2-GFP is from a laboratory stock. pHK12neo_Flamindo2 plasmid was obtained from the dictyBase laboratory stock. pEcmAO-RFPmars plasmid was obtained from the dictyBase laboratory stock. pHK12neo_Citrine, and pHK12neo_Flamindo2. pBIG_PHAkt/PKB-GFP is from a laboratory stock.

Immunoblot analysis. Cells were developed on a cellulose membrane (Econo-Prep, 30 μm) and harvested at 5 and 12 h after starvation. In this study, two methods were used to observe development on agar. To observe early aggregation, cells were plated on the entire surface of 2% water agar plates (2% w/v Difco Bacto-agar in ultrapure water) at a density of 5–7×106 cells cm−2 and incubated at 21 °C. To observe the mound and slug stages, 5 μl of cell suspension at a density of 2–4×107 cells ml−1 was deposited on 1.5% water agar and incubated at 21 °C for 6–15 h. To image development, a method described previously was used. Here, a piece of agar was cut out and placed upside down on a 35-mm glass bottom dish (12 mm diameter glass, Iwaki) or directly on a cover slip (thickness: 50, 100, or 150 μm) attached to the dish. The spacer was filled with liquid paraffin (Nacalai Tesque) to avoid light scattering. To prevent dessication during the observation, wet paper was placed in the dish and the agar piece was covered with liquid paraffin. In this condition, cells and multicellular bodies could move more freely and develop normally for more than 12 h under the microscope. The Z series of fluorescence images was taken by the confocal microscope at 10–30 s intervals.

In addition to the above methods, we also applied the technique ‘2D slug’ for efficient cell tracking in slugs because three-dimensional (3D) scroll movement of the slug and thickness of the tissue make it difficult to follow individual cell movements in normal slugs. One microlitre of cell suspension at a density of 4×107 cells ml−1 was deposited on 2% water agar plates together with 2 μl liquid paraffin. A coverslip was placed on top of the suspension, which was incubated at 21 °C for more than 15 h. The Z series of the fluorescent images was acquired at 15-s intervals for 20–30 min by the confocal microscope. The 2D slug, which has only few (~4) cell layers and thus enables us to follow cell movement easily, showed similar properties with normal slugs with respect to cell movement and proportion of cell types. We observed periodic cell movement with no obvious [cAMP] oscillation in both normal slugs and 2D slugs (Fig. 3e and Supplementary Fig. 6c, d).

Verification Flamind2 as an indicator of [cAMP]. Our experimental analysis of cell migration in multicellular bodies with no obvious [cAMP] oscillation showed similar properties with normal slugs with respect to cell movement and proportion of cell types. We observed periodic cell movement with no obvious [cAMP] oscillation in both normal slugs and 2D slugs (Fig. 3e and Supplementary Fig. 6c, d).
cAMP concentration (about 2–75 pmol mg\(^{-1}\) protein\(^{16,37}\)), protein amount per cell (7 × 10\(^{-8}\) mg cell\(^{-1}\) calculated based on the notations that 10\(^9\) cells equals about 1 g wet cells, which equals ~70 mg protein\(^{39}\) and cell volume (0.43 pl cell\(^{-1}\) \(^{38}\)). For example, 2 (pmol mg\(^{-1}\)) × 7 × 10\(^{14}\) (mg cell\(^{-1}\))=0.43 (pl cell\(^{-1}\)) = 0.32 μM.

Our calculation showed that the intracellular cAMP concentration in unstimulated and cAMP-stimulated Dictyostelium cells at all development stages is ~0.3–12 μM.

**Verification of proper function of Flamindo2 as the cAMP sensor at the slug stage.** Cells expressing Flamindo2 and ecmAO::mRFPmars were washed and deposited on a cellulose membrane filter (Advantec) at a density of 5 × 10\(^3\) cells cm\(^{-2}\) and incubated at 21 °C for 12 h to allow slug formation. The slugs were harvested in DB and dispersed into single cells by passage through a 25 G needle (Terumo) with a 1 ml syringe on ice\(^{68}\). Slugs were resuspended in DB at a density of 10\(^6\) cells ml\(^{-1}\), and 40 μl of the suspension was deposited onto a 12-mm glass bottom dish. CAMP stimulation and caffeine treatment were performed as described in the Method section titled ‘Verification Flamindo2 as an indicator of [cAMP] in Dictyostelium cells at the unicellular phase’. Fluorescent images were acquired by the confocal microscope at 5-1 s intervals during stimulation. Average fluorescent intensities of Flamindo2 in 5 μm\(^2\) regions positioned within the cytosol were measured at each time point. Cell types of slug-disaggregated cells were distinguished by the intensity of ecmAO:: mRFPmars.

We also performed the CAMP stimulation test on intact slugs by CAMP microinjection into water agar as shown in Fig. 4c. Cells were washed on 2% water agar plates until slug formation, and a piece of agar was cut out and placed upside down on a glass cover slip (thickness, 100 μm) attached to a glass bottom dish. The space was filled with liquid paraffin to avoid light scattering. A piece of 2% water agar with or without 4 mM CAMP was then put on the filter (Supplementary Fig. 11a). In the caffeine treatment experiments, the dishes were settled for 5 min before observation to allow the caffeine to permeate through the filter. Time-lapse images of slug migration were acquired at 30-μm intervals for 30 min by the confocal microscope.

**Monitoring the effect of caffeine treatment on slug migration.** Cells expressing Citrine were washed, and 5 μl of cell suspension at a density of 4 × 10\(^6\) cells ml\(^{-1}\) was deposited on a 35-mm glass bottom dish. Fifty microliters of 1 mM CAMP (final concentration: 100 μM) was applied to the dish under observation of the microscope. Fluorescent images were acquired by the confocal microscope at 5-s intervals during the stimulation.

**Data availability**

All data are presented in the manuscript or the supplementary materials. The source data underlying the graphs shown in the main figures are presented in the Supplementary Data 1–6. Other data supporting the findings of this study are available from the corresponding authors upon request. The plasmids generated in this study will be available at Dicty stock center (http://dictybase.org/StockCenter.html) and NBRP-nenkin (https://nenkin.nbrp.jp/locale/change/lang=en).

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References

1. Friedl, P. & Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. Nat. Rev. Mol. Cell Biol. 10, 445–457 (2009).
2. Ratnath, P. Collective cell migration. Annu. Rev. Cell Dev. Biol. 25, 407–429 (2009).
3. Haeger, A., Wolf, K., Zegers, M. M. & Friedl, P. Collective cell migration: guidance principles and hierarchies. Trends Cell Biol. 25, 556–566 (2015).
4. Weijer, C. J. Collective cell migration in development. J. Cell. Sci. 122, 3215–3223 (2009).
5. Tomchik, K. J. & Devreotes, P. N. Adenosine 3',5'-monophosphate waves in Dictyostelium discoideum: a demonstration by isotope dilution-fluorography. Science 212, 443–446 (1981).
6. Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B. & Devreotes, P. N. G protein signaling events are activated at the leading edge of chemotactic cells. Cell 95, 81–91 (1998).
7. Inasal, R. et al. CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in Dictyostelium. Cell 75, 1537–1545 (1994).
8. Meima, M. E. & Schaap, P. Fingerprinting of adenylyl cyclase activities during Dictyostelium development indicates a dominant role for adenylyl cyclase B in terminal differentiation. Dev. Biol. 212, 182–199 (1999).
9. Comer, F. I. & Parent, C. A. PI3-kinases and PTEN: how opposite tumouract. Cell 109, 541–544 (2002).
10. Weijer, C. J. Morphogenetic cell movement in Dictyostelium. Semin. Cell. Dev. Biol. 10, 609–619 (1999).
11. Siegert, F. & Weijer, C. Digital image processing of optical density wave propagation in Dictyostelium discoideum and analysis of the effects of caffeine and amonia. J. Cell. Sci. 93, 325–335 (1991).
12. Rietdorf, J., Siegert, F. & Weijer, C. J. Analysis of optical density wave propagation and cell movement during mound formation in Dictyostelium discoideum. Dev. Biol. 177, 427–438 (1996).
13. Dorman, D. & Weijer, C. J. Propagating chemotactract waves coordinate periodic cell movement in Dictyostelium slugs. Development 128, 4535–4543 (2001).
14. Stemfeld, J. & David, C. N. Cell sorting during pattern formation in Dictyostelium. Differentiation 20, 10–21 (1981).

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15. Traynor, D., Kessin, R. H. & Williams, J. G. Chemotactic sorting to CAMP in the multicellular stages of Dictyostelium development. Proc. Natl Acad. Sci. USA 89, 8303–8307 (1992).

16. Otte, A. P., Plomp, M. J., Arens, J. C., Janssens, P. M. & van Driel, R. Production and turnover of CAMP signals by prestalk and prespore cells in *Dictyostelium discoideum* cell aggregates. Differentiation 32, 185–191 (1986).

17. Early, A., Abe, T. & Williams, J. Evidence for positional differentiation of prestalk cells used for a morphogenetic gradient in Dictyostelium. Cell 83, 91–99 (1995).

18. Rietdorf, J., Siegert, F. & Weijer, C. J. Induction of optical density waves and chemotactic cell movement in Dictyostelium discoideum by Microinjection of CAMP Pulses. Dev. Biol. 204, 525–536 (1998).

19. Wang, B. & Kuspa, A. Dictyostelium development in the absence of CAMP. Science 277, 251–254 (1997).

20. Gregor, T., Fujimoto, K., Masaki, N. & Sawai, S. The onset of collective behavior in social amoebae. Science 328, 1021–1025 (2010).

21. Ohta et al. Nontrivial effect of the color-exchange of a donor/acceptor pair in the engineering of Förster resonance energy transfer (FRET)-based indicators. ACS Chem. Biol. 11, 1816–1822 (2016).

22. Odaka, H., Arai, S., Inoue, T. & Kitaguchi, T. Genetically-encoded yellow fluorescent CAMP indicator with an expanded dynamic range for dual-color imaging. PLoS ONE 9, e100252 (2014).

23. Ohta, Y., Furuta, T., Nagai, T. & Horikawa, K. Red fluorescent CAMP indicator with increased affinity and expanded dynamic range. Sci. Rep. 6, 12020 (2016).

24. Alvarez-Curto, E., Weening, K. E. & Schaap, P. Pharmacological profiling of the Dictyostelium adenylate cyclases ACA, ACR and ACG. Biochem. J. 401, 309–316 (2007).

25. Gerisch, G., et al. Development and differentiation in the cellular slime moulds pp. 105–124 (Elsevier, Amsterdam, 1977).

26. Van, E. S., Wessels, D., Soll, D. R., Borleis, J. & Devreotes, P. N. Tortoise, a novel mitochondrial protein, is required for directional responses of Dictyostelium in chemotactic gradients. J. Cell Biol. 152, 621–632 (2001).

27. Sgro, A. E. et al. From intracellular signaling to population oscillations: adenylate cyclases ACA, ACB and ACG. Curr. Biol. 25, 124–129 (2015).

28. Meili, R. et al. Chemoattractant mediated transient activation and membrane recruitment of a PH-domain-containing protein. J. Cell Sci. 112, 120–127 (2008).

29. Dornmann, D., Weijer, G., Parent, C. A., Devreotes, P. N. & Weijer, C. J. Visualizing PI3 kinase-mediated cell-cell signaling during Dictyostelium development. Curr. Biol. 12, 1178–1188 (2002).

30. Meili, R. et al. Chemoattractant mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to CAMP in *Dictyostelium*. EMBO J. 18, 2092–2105 (1999).

31. Funamoto, S., Milan, K., Meili, R., Firtel, R. A. Role of phosphatidylinositol 3’ kinase and a downstream pleckstrin homology domain–containing protein in chemotaxis of Dictyostelium discoideum. J. Biol. Chem. 273, 795–810 (2001).

32. Pitt, G. S., Brandt, R., Lin, K. C., Devreotes, P. N. & Schaap, P. Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in *Dictyostelium* cells lacking the aggregation adenyl cyclase (ACA). Genes Dev. 7, 2172–2180 (1993).

33. Early, A. E., Gaskell, M. J., Traynor, D. & Williams, J. G. Two distinct populations of prestalk cells within the tip of the migrating Dictyostelium slug with differing fates at culmination. Development 118, 353–362 (1993).

34. Bevis, B. J. & Glick, B. S. Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). Biochem. J. 346, 335–340 (1999).

35. Schnitzler, G. R., Fischer, W. H. & Firtel, R. A. Cloning and characterization of the engineering of fo.

36. ACS Chem. Biol. 11, 1816–1822 (2016).

37. Devreotes, P. N. & Weijer, C. J. Development and differentiation in the cellular slime moulds pp. 105–124 (Elsevier, Amsterdam, 1977).

38. Pitt, G. S., Brandt, R., Lin, K. C., Devreotes, P. N. & Schaap, P. Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in Dictyostelium cells lacking the aggregation adenyl cyclase (ACA). Genes Dev. 7, 2172–2180 (1993).

39. Early, A. E., Gaskell, M. J., Traynor, D. & Williams, J. G. Two distinct populations of prestalk cells within the tip of the migrating Dictyostelium slug with differing fates at culmination. Development 118, 353–362 (1993).

40. Bevis, B. J. & Glick, B. S. Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). Nat. Biotechnol. 20, 83–87 (2002).

41. Schnitzler, G. R., Fischer, W. H. & Firtel, R. A. Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in *Dictyostelium*. Genes Dev. 8, 502–514 (1994).

42. Sukumaras, S., Brown, J. M., Firtel, R. A. & McNally, J. G. lacG-NtwXF and gfp-NtwXF define key steps in the morphogenesis of Dictyostelium mounds. Dev. Biol. 200, 16–26 (1998).

43. Brenner, M. Cyclic AMP levels and turnover during development of the cellular slime mold *Dictyostelium discoideum*. Dev. Biol. 64, 210–223 (1978).

44. Waddell, D. R. Cell size in *Dictyostelium*. Dev. Genet. 9, 673–681 (1988).

45. Aubry, L. & Klein, G. Purification techniques of subcellular compartments for analytical and preparative purposes. Methods Mol. Biol. 346, 171–185 (2006).

46. Noorbakhsh, J., Schwab, D. J., Sgro, A. E., Gregor, T. & Mehta, P. Modeling oscillations and spiral waves in Dictyostelium populations. Phys. Rev. E 91, 062711 (2015).

47. Dornmann, D., Kim, J. Y., Devreotes, P. N. & Weijer, C. J. cAMP receptor affinity controls wave dynamics, geometry and morphogenesis in *Dictyostelium*. J. Cell Sci. 114, 2513–2523 (2001).

48. Parent, C. A. & Devreotes, P. N. Molecular genetics of signal transduction in *Dictyostelium*. Annu. Rev. Biochem. 65, 411–440 (1996).
Author contributions
H.H. conceived and designed the study, performed the experiments, analyzed the data, and wrote the manuscript. M.Y. designed the study, developed the software for cell tracking, and wrote the manuscript. Y.V.M. designed the study, performed the experiments, and wrote the manuscript. M.U. designed the study, contributed to the interpretation of the data analysis, and wrote the manuscript.

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