The chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) controls cellular quiescence by hyperpolarizing the cell membrane during diapause in the crustacean *Artemia*

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Cellular quiescence, a reversible state in which growth, proliferation, and other cellular activities are arrested, is important for self-renewal, differentiation, development, regeneration, and stress resistance. However, the physiological mechanisms underlying cellular quiescence remain largely unknown. In the present study, we used embryos of the crustacean *Artemia* in the diapause stage, in which these embryos remain quiescent for prolonged periods, as a model to explore the relationship between cell-membrane potential (V_mem) and quiescence. We found that V_mem is hyperpolarized and that the intracellular chloride concentration is high in diapause embryos, whereas V_mem is depolarized and intracellular chloride concentration is reduced in postdiapause embryos and during further embryonic development. We identified and characterized the chloride ion channel protein cystic fibrosis transmembrane conductance regulator (CFTR) of *Artemia* (Ar-CFTR) and found that its expression is silenced in quiescent cells of *Artemia* diapause embryos but remains constant in all other embryonic stages. Ar-CFTR knockdown and GlyH-101–mediated chemical inhibition of Ar-CFTR produced diapause embryos having a high V_mem and intracellular chloride concentration, whereas control *Artemia* embryos released free-swimming nauplius larvae. Transcriptome analysis of embryos at different developmental stages revealed that proliferation, differentiation, and metabolism are suppressed in diapause embryos and restored in postdiapause embryos. Combined with RNA sequencing (RNA-Seq) of GlyH-101–treated MCF-7 breast cancer cells, these analyses revealed that CFTR inhibition down-regulates the Wnt and Aurora Kinase A (AURKA) signaling pathways and up-regulates the p53 signaling pathway. Our findings provide insight into CFTR-mediated regulation of cellular quiescence and V_mem in the *Artemia* model.

Cellular quiescence is a sleep-like and reversible state in which growth, proliferation, and other cellular activities are arrested. This state is thought to be homogenous and induced by diverse anti-mitogenic signals (1). Quiescent cells re-enter the cell cycle in response to physiological stimuli (2). Reactivation of quiescent cells is crucial for tissue repair and regeneration and plays a key role in growth, development, and health of higher multicellular organisms (3–5). Cellular quiescence also explains how some species produce embryos in a state of diapause or dormancy, which often enables their offspring to survive remarkably harsh environmental conditions (6, 7). Development, reproduction, and metabolic activities are highly suppressed in this state, meaning that embryos are protected until the environmental conditions become favorable, at which point the cell cycle resumes (8). Entry of cells into quiescence is often associated with dramatic changes in metabolism (9) and cell-cycle regulation. In addition, the cell membrane potential (V_mem) becomes hyperpolarized (10, 11). V_mem, which refers to the voltage across the plasma membrane, is a key bioelectric property of nonexcitable cells (12). Hyperpolarization of the resting V_mem is accompanied by decreased proliferation of astrocytes isolated from the developing spinal cord and hippocampus of rats (13, 14). These observations led us to speculate that V_mem plays a critical role in cellular quiescence. However, there have been few reports on the relationship between cellular quiescence and V_mem.

Ion channels establish the resting V_mem of all cells by determining the gradients of major ions, such as sodium, potassium, calcium, and chloride, across the cell membrane (15). Chloride, which is the most abundant anion in all organisms, has multiple functions, including regulation of cell volume, signal transduction, cell death/survival, and intracellular pH and is believed to contribute to V_mem (16, 17). Cystic fibrosis transmembrane...
Conductance regulator (CFTR)\textsuperscript{2} is an ATP-gated chloride channel belonging to the ABC transporter superfamily (18). CFTR provides a pathway for reversible chloridion movement across cell membrane and regulates the rate of chloridion flow (18). Structural studies described that CFTR is composed of a regulatory R domain and two repeated domains, each of which consists of a hydrophobic membrane-spanning domain (MSD) and a cytosolic hydrophilic region for binding to ATP (NBD) (19). Increasing evidence indicates that CFTR not only is a secretory chloride channel but also modulates a wide range of other membrane transport proteins, including epithelial Na\textsuperscript{+} channels (ENaCs), Ca\textsuperscript{2+}-activated chloride channels, outwardly rectifying chloride channels, and the anion (Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−}) exchanger (20–23). Mutations of the CFTR gene affecting its chloride channel function can lead to dysregulated fluid transport in the pancreas, lung, liver, and other organs, resulting in cystic fibrosis (24–26).

In this study, we used *Artemia*, a small crustacean, as a model system to investigate regulation of cellular quiescence. *Artemia* produce encysted gastrula embryos that rapidly enter a state of dormancy, called diapause, when environmental conditions are unfavorable (27, 28). A number of cross-sectional studies suggest an association between *Artemia* and dormancy (29–34).

The present study demonstrates that *Vₘₚ*, was hyperpolarized and the intracellular chloride concentration was high in diapause embryos, whereas *Vₘₚ* was depolarized and the intracellular chloride concentration was reduced in postdiapause embryos and during further embryonic development. CFTR of *Artemia* (Ar-CFTR) was silenced in diapause embryos, leading to inhibition of chloride outflow and consequently hyperpolarization. Transcriptome analysis indicated that proliferation, differentiation, and metabolism were suppressed in diapause embryos and that the Wnt and AURKA signaling pathways were down-regulated and the p53 signaling pathway was up-regulated upon inhibition of CFTR. Our findings provide evidence that modulation of *Vₘₚ* by CFTR regulates cellular quiescence.

**Results**

**Cells in Artemia diapause embryos are quiescent and hyperpolarized**

*Artemia* possesses two independent reproductive pathways, ovoviviparous and oviparous, as a strategy to survive fluctuating environmental conditions. Under favorable conditions, embryos mature in the ovisac (uterus) and are released into the environment as free-swimming nauplius larvae via the ovoviviparous pathway (Fig. 1A, panels 1 and 2). By contrast, under unfavorable conditions, mature females produce and release encysted embryos that exist in a state of obligate dormancy, called diapause, via the oviparous pathway (Fig. 1A, panels 3 and 4). Cells in diapause embryos can remain quiescent and not divide for many years (27). Signals received from favorable environmental stimuli terminate diapause. This induces embryos to enter a state, followed by hatching of swimming nauplii after 18–24 h (Fig. 1A, panels 5–7). Western blotting analysis showed that expression of cell proliferation markers, phosphorylation histone H3 at Ser\textsuperscript{10} (pH3S10) and phosphorylation of retinoblastoma at Thr\textsuperscript{146} (pRBTS146), was greatly reduced in diapause and postdiapause embryos (Fig. 1B). This finding suggests that the cell cycle has ceased at the diapause and postdiapause stages but not at other developmental stages. Furthermore, immunofluorescence analysis showed that the proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA) were expressed in postdiapause embryos but not in diapause embryos (Fig. 1C). These results indicate that cells are quiescent in *Artemia* diapause embryos.

This study focused on *Vₘₚ* in *Artemia* during diapause formation. We isolated cells from embryos at each developmental stage (Fig. S1) and measured *Vₘₚ* using DiBAC\textsubscript{4}(3), a *Vₘₚ*-sensitive dye (Fig. 1D). Cells were considered depolarized when *Vₘₚ* became less negative and hyperpolarized when *Vₘₚ* became more negative. *Vₘₚ* was relatively constant during ovoviviparous development; however, cells were extremely hyperpolarized from the prediapause stage to the diapause stage and depolarized in the postdiapause and nauplii stages.

We measured the intracellular chloride concentration at each developmental stage using MQAE, a chloride-sensitive fluorescent dye. The fluorescence intensity of this dye decreases as the intracellular chloride concentration increases. Almost no fluorescence was observed in cells of diapause embryos (Fig. 1E), indicating that the intracellular chloride concentration was high. Together, these results indicate that cells in diapause embryos are quiescent and hyperpolarized with a high intracellular chloride concentration.

**Identification and characterization of Ar-CFTR**

To investigate the regulation of *Vₘₚ* during diapause formation, we characterized Ar-CFTR. A candidate for Ar-CFTR was identified using the tblastn algorithm with human CFTR as the input. The full-length Ar-CFTR mRNA is 4924 bp, with a 4635-bp ORF encoding a protein of 1545 amino acids (aa) (Fig. S2). The calculated molecular mass and pI of Ar-CFTR are 173.02 kDa and 7.43, respectively. Structure analysis showed that Ar-CFTR contains two MSDs, MSD1 (aa 316–581) and MSD2 (aa 981–1255); two nucleotide-binding domains, NBD1 (aa 625–825) and NBD2 (aa 1302–1522); and a regulatory domain, R (aa 638–963), which are conserved in typical CFTRs (Fig. S3A).

The amino acid sequence of the R domain of Ar-CFTR shows low overall sequence similarity (<30%) with those of the R domains of CFTRs in other species. However, the amino acids predicted to interact with serine and play a role in phosphorylation-dependent activation of this channel in vivo are conserved in Ar-CFTR, including Ser\textsuperscript{686}, Ser\textsuperscript{726}, Ser\textsuperscript{727}, and Ser\textsuperscript{768} in the KXS/RXXS motif (Fig. S3B). Phylogenetic analysis
suggested that Ar-CFTR is grouped with CFTR expressed in the arthropod Daphnia pulex (Fig. S3C).

Polyclonal antibodies specific to Ar-CFTR detected a band of ~173 kDa in Artemia. The mRNA level of Ar-CFTR at each developmental stage was analyzed by real-time quantitative PCR (qPCR). mRNA expression of Ar-CFTR was ~2–4-fold lower in diapause embryos than in embryos at other stages of the ovoviviparous pathway (Fig. 2A). Similarly, protein expression of Ar-CFTR was ~7–10-fold lower in diapause embryos than in embryos at other developmental stages according to analysis performed using ImageJ (Fig. 2B). Furthermore, immunofluorescence analysis showed that Ar-CFTR was barely detected in diapause embryos but was observed at the cellular membrane in prediapause embryos, postdiapause embryos, and hatched nauplii (Fig. 2C). Thus, down-regulation of Ar-CFTR may be important for cellular quiescence during diapause formation in Artemia.

**Ar-CFTR plays a critical role in regulation of cellular quiescence and diapause formation by modulating $V_{\text{mem}}$**

To elucidate the role of Ar-CFTR in the regulation of diapause formation in Artemia, Ar-CFTR was knocked down by RNAi. A dsRNA designed based on the Ar-CFTR cDNA sequence was injected prior to ovarian development. Real-time qPCR and Western blotting analyses revealed that the mRNA and protein levels of Ar-CFTR were more than 80% lower in animals injected with Ar-CFTR dsRNA (Ar-CFTRi) than in those injected with GFP dsRNA (GFPi) (Fig. 3, A and B). An average of 75% of ovoviviparous Artemia injected with Ar-CFTR dsRNA released diapause embryos, whereas all of those injected with GFP dsRNA produced free-swimming nauplii (Fig. 3C). Western blotting and immunofluorescence analyses showed that expression of pH3S10 and pRbT356 was reduced upon Ar-CFTR knockdown (Fig. 3, D and E). Furthermore, the diapause-specific proteins p26 and artemin (35, 36) were enriched upon Ar-CFTR knockdown (Fig. 3D).

Next, we investigated whether knockdown of Ar-CFTR induces hyperpolarization by increasing the intracellular chloride concentration. As expected, analysis of the fluorescence intensity of MQAE showed that Ar-CFTR knockdown increased the intracellular chloride concentration (Fig. 3F). Moreover, the fluorescence intensity of DiBAC$_4$(3) was mark-
CFTR controls cellular quiescence in Artemia model

Figure 2. Molecular characterization and expression pattern of Ar-CFTR during development. A, real-time qPCR analysis of Ar-CFTR mRNA expression at each developmental stage (numbers indicated in Fig. 1A legend). mRNA expression of Ar-CFTR was normalized against that of tubulin. **, p < 0.01. B, Western blotting analysis of Ar-CFTR protein expression at each developmental stage. Tubulin was used as a loading control. Relative band intensities were quantified using ImageJ. **, p < 0.01. C, immunofluorescence analysis of Ar-CFTR at each developmental stage. Bar, 50 μm. BF, brightfield.

edly reduced upon Ar-CFTR knockdown (Fig. 3G), indicating that silencing of Ar-CFTR induces hyperpolarization.

To further verify the function of Ar-CFTR, we soaked nauplius-destined Artemia adults in 40‰ seawater containing 30 μM GlyH-101, a CFTR inhibitor. 30 μM was chosen according to a result of a dose-dependent experiment (Fig. S4A). Average 85% of ovoviviparous Artemia adults treated with GlyH-101 produced diapause embryos, whereas control Artemia adults treated with 30 μM DMSO released nauplii (Fig. 4A). Analysis of the chloride concentration and $V_{\text{mem}}$ showed that inhibition of Ar-CFTR by GlyH-101 induced hyperpolarization by preventing chloride outflow (Fig. 4, B and C). The induced hyperpolarization was validated by analysis of a patch clamp recording (Fig. S5). Inhibition of Ar-CFTR by GlyH-101 also decreased the intracellular potassium and sodium concentrations, which were lower in diapause embryos than in prediapause and postdiapause embryos (Fig. S6). These findings indicate that CFTR regulates $V_{\text{mem}}$ and thereby controls cellular quiescence during diapause formation. In addition, GlyH-101 treatment decreased expression of pH3S10, pRbT356, Ki67, and PCNA but increased expression of p26 and artemin (Fig. 4, D and E).

To characterize the molecular signatures of quiescent cells in diapause embryos, we treated decapsulated postdiapause embryos with 100 μM GlyH-101 to suppress Ar-CFTR activity during hatching. 100 μM was chosen according to the results of a dose-dependent experiment (Fig. S4B). Development of GlyH-101–treated embryos was arrested at the pre-emergence stage, before resumption of cell division (Fig. S4A). However, swimming nauplii hatched from control postdiapause embryos treated with DMSO after 24 h. In addition, removal of GlyH-101 resulted in average of 80% released nauplii, a rate that is similar to the natural hatching rate of Artemia in its natural environment (Fig. S5A). The results indicated that Ar-CFTR is required for termination of cellular quiescence. Fluorescence analysis using the bioelectricity reporter DiBAC4(3) and the chloride-sensitive dye MQAE revealed that $V_{\text{mem}}$ remained hyperpolarized, and the chloride concentration remained high upon continuous treatment with GlyH-101 (Fig. 5, B and C). However, $V_{\text{mem}}$ was depolarized, and chloride outflow was increased following removal of GlyH-101 (Fig. 5, B and C). Western blotting analysis revealed that levels of pH3S10 and pRbT356 increased after the pre-emergence stage and were high in nauplii in the control group. However, the levels of these two proliferation markers were low in the presence of GlyH-101 and increased after removal of GlyH-101 (Fig. 5D). Together, these results indicate that Ar-CFTR controls cellular quiescence during diapause formation by mediating chloride outflow and thereby modulating $V_{\text{mem}}$.

Molecular signatures of quiescent cells in diapause embryos

To characterize the molecular signatures of quiescent cells and analyze downstream signaling pathways of CFTR, RNA-Seq of diapause embryos was performed. Genes that were differentially expressed between nauplius-destined ovoviviparous embryos, diapause-destined ovoviviparous embryos (prediapause), diapause embryos, and postdiapause embryos are presented in a heat map (Fig. 6A). In total, 9573, 9458, and 6356 genes were differentially expressed between nauplius-destined ovoviviparous embryos and diapause embryos, between prediapause and diapause embryos, and between postdiapause and diapause embryos, respectively (Fig. 6B). In addition, expression of 3167 genes, including markers of diapause (p26, artemin, and ArHsp22) and cell division (Ki67 and PCNA), was higher or lower in diapause embryos than in ovoviviparous, prediapause, and postdiapause embryos. These results reveal the key molecular characteristics of quiescent cells in diapause embryos. Gene ontology (GO) enrichment analysis was performed of genes that were differentially expressed between diapause and postdiapause embryos. This revealed that expression of genes associated with proliferation, differentiation, and metabolism
wassuppressed in diapause embryos (Fig. 6C). Genes related to metabolism, including those associated with oxidative phosphorylation, the carbohydrate metabolic process, the cholesteryl metabolic process, polysaccharide digestion, the fatty–acyl-CoA metabolic process, the aerobic electron transport chain, and autophagy, as well as genes related to cell proliferation, including those associated with the mitotic cell cycle, RNA polymerase II transcription factor activity, and translation regulator activity, were down-regulated in diapause embryos. By contrast, genes associated with chitin binding, cuticle development, negative regulation of cellular metabolic processes, and lipid storage were up-regulated in diapause embryos. Analysis of related pathways and genes showed that the Wnt and AURKA signaling pathways were down-regulated, and the p53 signaling pathway was up-regulated in diapause embryos (Fig. S7).

Pathways upstream and downstream of CFTR-regulated hyperpolarization in MCF-7 cells

To identify the signaling pathways by which CFTR-regulated hyperpolarization controls cellular quiescence, we treated MCF-7 cells with 10 μM GlyH-101 to inhibit CFTR. 10 μM was chosen according to a result of a dose-dependent experiment (Fig. S4C). Immunofluorescence analysis of the proliferation marker Ki67, EdU incorporation analysis, and Western blotting analysis of pH3S10, pRbT356, and the diapause-specific proteins p26 and artemin upon injection of Ar-CFTR and GFP dsRNAs. Tubulin was used as a loading control. Relative band intensities were quantified using ImageJ. **, p ≤ 0.01. E, immunofluorescence analysis of Ki67 and PCNA upon injection of Ar-CFTR and GFP dsRNAs. Bar, 50 μm. F, intracellular chloride concentration upon injection of Ar-CFTR and GFP dsRNAs. The average fluorescence intensity (I_{average}) was quantified (bottom panel) (n = 15 cells). The color bar indicates the relative level of chloride concentration. Bar, 5 μm. G, V_{mem} upon injection of Ar-CFTR and GFP dsRNAs. The average fluorescence intensity (I_{average}) was quantified (bottom panel) (n = 15 cells). The color bar indicates the relative level of hyperpolarization. Bar, 5 μm. BF, brightfield. n = 40 Artemia adults injected with dsRNA.
V.mem was patched in the whole-cell configuration and ranged from $-46$ to $-94$ mV after treatment with GlyH-101 for 24 h and returned to $-42$ mV following removal of GlyH-101 (Fig. 7E). The patch clamp tracing also showed that GlyH-101 inhibited the chloride currents in MCF-7 cells (Fig. S9). In contrast to MCF-7 cell line, after GlyH-101 treatment, the hyperpolarization and proliferation inhibition were not observed in the U251 cell line (which has an extremely low level of CFTR expression) (Fig. S10). In addition, CFTR knockdown in SKOV3 and HT-29 could induce cellular quiescence in the both of these cell lines (Fig. S11). The results may indicate that inhibition of CFTR leads to hyperpolarization, a high intracellular chloride concentration, and quiescence in mammalian cells.

To interrogate the pathways downstream of CFTR-regulated hyperpolarization in quiescent cells, RNA-Seq of GlyH-101–treated MCF-7 cells was performed. The transcriptome profiles of three replicates of MCF-7 cells treated with GlyH-101 for 0, 1, and 24 h were compared. We performed cluster analysis of differentially expressed genes (DEGs) in the entire transcriptome and observed the overall expression gene pattern in GlyH-101–treated cells. The gene expression pattern markedly differed between cells treated with GlyH-101 for 24 h and untreated cells (Fig. S12). In total, 2749 genes were differentially expressed between cells treated with GlyH-101 for 1 h and untreated cells, whereas 8021 genes were differentially expressed between cells treated with GlyH-101 for 24 h and untreated cells (Fig. 8A). According to ranking of the fold changes in gene expression and analysis of the gene signatures of quiescent cells in the molecular function, metabolic processes, and pathways categories, 31 typical DEGs were identified (Fig. 8B and Table S1). GO enrichment analysis of DEGs is shown in Fig. 8C. Genes involved in the p53 signaling pathway and negative regulation of cell proliferation were up-regulated in cells treated with GlyH-101 for 24 h, whereas genes involved in cell division, the Wnt and AURKA signaling pathways, and many metabolic pathways were down-regulated. The analysis of transcriptomes in Artemia diapause and GlyH-101–treated MCF-7 cells showed some similar signatures of cellular quiescence based on GO term enrichment, including the genes of p53 and Wnt signaling pathways (Fig. S13). Our results demonstrate that CFTR may regulates V.mem and cellular quiescence in both Artemia and MCF-7 cells.

**Discussion**

Artemia is an ideal model system to study the regulation of cellular quiescence because embryos can remain in diapause for prolonged periods, and the cell cycle resumes during development of postdiapause embryos. The present study demonstrates that V.mem was hyperpolarized and the intracellular chloride concentration was high in diapause embryos and that...
this was controlled by Ar-CFTR. Thus, Ar-CFTR–regulated hyperpolarization seems required for cellular quiescence during diapause formation in Artemia. Transcriptome analysis indicated that proliferation, differentiation, and metabolism were suppressed, the Wnt and AURKA signaling pathways were down-regulated, and the p53 signaling pathway was up-regulated in diapause embryos and GlyH-101–treated MCF-7 cells.

A hyperpolarized $V_{\text{mem}}$ tends to be quiescent and does not typically undergo mitosis; conversely, a depolarized $V_{\text{mem}}$ tends to have mitotically active (14, 37, 38). Here, $V_{\text{mem}}$ was low in prediapause embryos (mitotic cells), hyperpolarized in diapause embryos (quiescent cells), and depolarized in postdiapause embryos and hatched nauplii (mitotic cells). This implies that hyperpolarization induces cellular quiescence during diapause formation in Artemia.

Fluctuations in the chloride concentration contribute to cell polarity, and this appears to be linked to the cell cycle and regulates the proliferation of various cell types (39, 40). ClC-3, a member of the voltage-gated chloride channel protein family, modulates proliferation of rat basilar arterial smooth muscle cells by suppressing Akt/GSK-3β signaling pathway, leading to down-regulation of cyclin D1 and cyclin E (41). Furthermore, the chloride channel blocker 5-N’-2-(3-phenylpropylamino) benzoic acid inhibits re-entry of quiescent (G0) NIH-3T3 fibroblasts into the cell cycle (42). However, other voltage-gated ion channels, such as the CFTR, may also play a role in cellular quiescence during diapause formation.
channels, such as potassium, calcium, and sodium channels, which contribute to hyperpolarization, also regulate cell proliferation (43–45). In neural stem cells, conditional deletion of ENaCs results in the absence of inward sodium flow, which induces hyperpolarization and reduces the number and proliferation of cells in the subependymal zone (46). An increase in potassium channel activity corresponding to transient hyperpolarization is involved in proliferation and cell cycle progression; cells reversibly arrest in G0/G1 phase upon inhibition of ATP-sensitive potassium channels (47, 48). Evidence has shown that CFTR affects other ion channels and ions. For example, application of a CFTR blocker was reported to reduce the inward rectifier potassium current and sodium current by 43% and 82% (49). CFTR inhibits ENaCs by increasing the intracellular chloride concentration in Xenopus oocytes (50). The inward potassium and sodium currents were decreased upon inhibition of Ar-CFTR by GlyH-101, and these currents were lower in diapause embryos than in prediapause and postdiapause embryos. Our results indicate that CFTR regulates the intracellular chloride concentration and influences the potassium and sodium currents and thereby modulates $V_{\text{mem}}$ and controls cellular quiescence in Artemia embryos.

We previously reported that the PLK1–MEK–ERK–RSK signaling pathway regulates cellular quiescence in diapause embryos (29, 30). Here, transcriptome analysis showed that expression of AURKA, the upstream kinase of PLK1–MEK–ERK–RSK, was very low in diapause embryos, indicating that the AURKA signaling pathway was suppressed. Wnt signaling helps to maintain quiescence of hematopoietic stem cells, which may underlie why it is required to preserve the self-renewal capability of these cells (51, 52). In the current study, expression of dishevelled (DVL) and transcription factor (TCF), which are components of the Wnt signaling pathway, was significantly decreased in diapause embryos, indicating that suppression of Wnt signaling is also critical for diapause formation in Artemia. p53 is downstream of CFTR and plays a role in quiescence of various cell types (53). Consistently, p53 was upregulated in diapause embryos and down-regulated in prediapause and postdiapause embryos. Moreover, liver kinase B1 (LKB1), a kinase of AMPK that is upstream of CFTR (54, 55), was highly expressed in diapause embryos, indicating that activated AMPK suppresses expression of CFTR and up-regulates expression of p53 during diapause formation in Artemia. Taken together, these results suggest that the AURKA–PLK–MEK–ERK–RSK, LKB1–AMPK–CFTR–p53, and Wnt signaling pathways are simultaneously required for regulation of cellular quiescence in diapause embryos.

Clinical drug therapy for cancer is far from optimal, as indicated by the high death rate of cancer patients. Standard therapies mainly target the tumor bulk but often fail to eradicate the quiescent cancer cells that may play important roles in resistance to chemoradiotherapy (56, 57). Effective targeting of the
quiescent cancer cells may represent a far more efficient focus toward the complete eradication of a tumor and prevention of tumor relapse. Our findings suggest that CFTR could also be used as one of the targets in the clinical treatment of cancer.

Materials and methods

Animals

Diapause cysts of parthenogenetic Artemia were harvested from Gahai Lake, Qinghai Province, China. Animal experiments were approved and performed in accordance with the institutional guidelines for animal care of animal ethics committee of Zhejiang University.

The cysts of parthenogenetic Artemia were hatched in 20‰ artificial seawater at 25 °C under continuous illumination. On the third day of the nauplii hatching from the cysts, chlorella powder (Fuqing King Dnarmsa Spirulina, China) was supplied to the seawater as food. When the nauplii developed into larvae, they were separated into two groups, one of which was reared in 40‰ artificial seawater with a photoperiod of 16 h of light and 8 h of dark per day to release the nauplii (ovoviviparity). The other group was reared in 80‰ artificial seawater with a photoperiod of 5 h of light and 19 h of dark per day to release dormant cysts (oviparity).

Western blotting analysis

Proteins were extracted using TRIzol reagent (Invitrogen). After homogenizing the sample with TRIzol reagent, chloroform was added, and the sample was centrifuged at 12,000 × g for 15 min. We then used isopropanol to precipitate proteins from the lower aqueous layer. The precipitate was washed with 0.3 M guanidine hydrochloride solution and dissolved with 2% SDS solution. Approximately 25 μg of protein from each sample was separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were blocked for 1.5–2 h at room temperature in 5% skim milk solution and incubated with the following antibodies: anti–Ar-CFTR, anti–P26 and anti-Artemin were raised in rabbits (Hangzhou HuaAn Biotechnology Company, Hangzhou, China);
anti-H3 (Cell Signaling, Boston, MA); anti-p-(Ser10) H3 (Epitomics), anti-Rb (Epitomics); anti-p-(Thr356) Rb (Abcam, London, UK); anti-CFTR (Abcam); and anti-tubulin-α (Sigma–Aldrich).

Detection was performed using BM Chemiluminescence Western blotting kits (Roche) according to the manufacturer’s instructions.

Molecular cloning of parthenogenetic Artemia CFTR cDNA

From a genomic database of *Artemia franciscana* built at Artemia Reference Center at Ghent University (Ghent, Belgium), we obtained a CFTR candidate (*Ar-CFTR*) using a homologous tblastn search with the human CFTR (GenBank™ accession no. NP_000483.3) as the input. The nucleotide sequence of *Ar-CFTR* was submitted to the GenBank™ under submission number MH822146. Total RNA was extracted from diapause embryos and nauplii using TRIzol reagent according to the manufacturer’s instructions, 1 μg of which was used as a template to reverse-transcribe first-strand cDNA.

Phylogenetic analysis of Ar-CFTR

The evolutionary history was inferred using the Neighbor-Joining method (58). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (59). The analysis involved 15-amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 1247 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (60).

Real-time quantitative PCR

RNA extraction and reverse-transcription were performed according to the methods mentioned above. Specific primers of *Ar-CFTR* and internal control Tubulin (RT-*Ar-CFTR* forward ATATCCCGTCAACGCTCTCAT, and RT-*Ar-CFTR* reverse TCGGTTTTTCACCTCCTTACTAC for *Ar-CFTR*; and Tubulin forward GCAGTGGTCTACAAGGTTTC and Tubulin reverse ATCAAAACGAAGGCTGGCGGTG for tubulin) were designed according to cDNA. PCRs were performed using SYBR Green (TOYOBO, Osaka, Japan) on the Bio-Rad MiniOpticon™ real-time PCR system in triplicate. The list of primers used for real-time qPCR analysis of MCF-7 cells were shown in Table S2. Statistical analysis was performed using one-way analysis of variance followed by Tukey’s all pair comparison test using the STATISTICA software. Differences where the *p* value was equal to 0.05 were considered significant, and a *p* value of 0.01 was considered extremely significant. A histogram was made using the SigmaPlot 10.0 software.
**CFTR controls cellular quiescence in Artemia model**

**Knockdown of CFTR in Artemia, SKOV3, and HT-29 cells**

For dsRNA preparation, the PET-T7 plasmid containing two inverted T7 promoter sites flanking the multiple-cloning sites was used as the expression vector. To obtain the reconstructed plasmid expressing Ar-CFTR dsRNA, a 326-bp fragment in the coding region of the Ar-CFTR gene was amplified with specific primers (ds-Ar-CFTR forward GCTCTAGAAATCCAAGTCTCCAAGAAAAG and ds-Ar-CFTR reverse GGAATTCACCCCAAGGCCCCATA, where XbaI and EcoRI sites are underlined) and subcloned into PET-T7 at the XbaI and EcoRI sites. The recombinant plasmid was transformed into *Escherichia coli* DH5α, sequenced to confirm the inserted nucleotide sequence, and then transformed into *E. coli* HT115 cells to express the dsRNA. The plasmid expressing GFP dsRNA was constructed as described previously and was used as a negative control. The dsRNA was produced and purified as described previously by Yodmuang et al. (61). *Artemia* adults (nauplii destined) were injected with 1 µg of Ar-CFTR dsRNA or GFP dsRNA by using an Ultra-MicroPump II instrument equipped with a Micro4TM MicroSyringe pump controller (World Precision Instruments). After injection, the animals were cultured in 40% artificial seawater. One week later the samples were collected.

**CFTR siRNA** (sense, GCCUGUGCUGUAAACUGAUGGCUAA; and antisense, UUAAGCAUCGUUUAACAGACAGC) were designed, and SKOV3 and HT-29 cells were transfected with CFTR siRNA and the scramble. Cell transfections were performed with Lipofectamine 3000 (Invitrogen) as recommended by the manufacturer.

**Immunofluorescence**

*Artemia* samples were fixed with 4% paraformaldehyde and embedded with paraffin. Tissue slides (5 µm thick) were treated with 0.25% Triton X-100 for 10 min. The slides were blocked in antiserum dilution buffer containing 1% BSA and 0.1% Triton X-100 for 1 h and then incubated with appropriate antibodies at 4 °C overnight. The slides were incubated in a secondary FITC-conjugated 594 antibody (Invitrogen) for 2 h, and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sangon Biotech, Shanghai, China). All confocal images were collected by using a Zeiss LSM 710 (Carl Zeiss) confocal laser-scanning microscope equipped with a 1.4-numerical aperture objective lens.

**EdU-labeled assay**

MCF-7 cells were treated with 10 µM GlyH-101 (MedChem Express, Monmouth Junction, NJ) for 24 h, and then immunofluorescence was performed as previously described using Ki67 antibodies (Abcam) with a secondary FITC-conjugated 594 antibody. DNA synthesis activity in GlyH-101–treated cells was studied using the EdU-DNA synthesis assay. Cell-LightTM EdU Apollo® 643 in vitro imaging kit (RiboBio, Guangzhou, China) was used according to the supplier’s protocol. EdU was added to the culture media at 50 µM, and after 2 h, the cells were fixed in 4% paraformaldehyde for 30 min, permeated with 0.25% Triton X-100 for 10 min, and stained with 10 µM Apollo 643 for 30 min. The nuclei were counterstained with DAPI, and the cells were analyzed by confocal laser-scanning microscope.

**Membrane potential measurements**

For live cell staining, the cysts were crushed in 1× PBS using Disposable PELLET PESTLES (Kimble Chase Life Science and Research Products) gently by hand and blown a few times to spread the cells apart. After 2–3 min standing, the upper phase was centrifuged at 1500 rpm for 5 min, and the precipitation was washed twice with 1× PBS. *Vmem* was measured using the membrane potential–sensitive dye DiBAC4(3) (Invitrogen). Upon transmembrane depolarization, the DiBAC4(3) enters the cell and binds to protein molecules, acquiring enhanced fluorescence. By contrast, fluorescence intensity is reduced when the membrane is hyperpolarized. After incubation with 0.5 µM DiBAC4(3) for 30 min at 37 °C, the cells were analyzed by Zeiss 510 (Carl Zeiss) confocal laser-scanning microscope with excitation at 488 nm and emission measured at 518 nm.

**Chloride concentration measurements**

Live cells were incubated with 8 mM MQAE (Invitrogen) for 1 h at 37 °C and rinsed with Ringer’s standard solution. The MQAE fluorescence was analyzed by Zeiss 510 confocal laser-scanning microscope with excitation at 353 nm and emission measured at 460 nm.

**Potassium and sodium concentration measurements**

Live cells were incubated with 10 µM ANG-2/AM (Asante Potassium Green-2 AM; Abcam) or 10 µM CoroNa (CoroNa™ Green; Invitrogen) for 30 min at 37 °C under a dark condition, followed by washing twice with 1× PBS, and the cells were analyzed by Zeiss 510 confocal laser-scanning microscope with excitation at 488 nm and emission measured at 540 nm.

**Cell culture**

MCF-7 cells were cultured in eagle’s minimum essential medium (EMEM) (Genom, Hangzhou, China), SKOV3 cells were cultured in Dulbecco’s modified Eagle’s medium (Genom), and HT-29 cells were cultured in 1640 (Genom) at 37 °C in 5% CO2, supplemented with 10% fetal bovine serum (Gibco), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma–Aldrich).

**Cell-cycle analysis**

Fixed cells were treated with 100 µg/ml RNase A (Sigma) and stained for 30 min with 50 µg/ml propidium iodide (Sigma) at 4 °C. Cell cycle analysis was performed on a Beckman Coulter flow cytometer (FC-500MCL). The analysis of quantification depending on the raw data was manually adjusted by the FITTING parameter.

**Patch-clamp recording**

Data acquisition and recording methods were as those reported previously (62). Whole-cell patch-clamp recordings were performed on the AXON 700B. Cells were superfused with solution containing 160 mM NaCl, 2.5 mM KCl, 5 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 8 mM d-glucose, pH 7.4, with NaOH.

**Cell proliferation assay**

Cell proliferation was assessed using CCK-8 kit (Beyotime Biotechnology, Shanghai, China). The cells were planted in...
96-well plates (1000 cells/well) in triplicate. After 24 h, the cells were treated with GlyH-101 or transfected with siRNA for days and used the Multiskan EX plate reader (Thermo Fisher Scientific) to quantify the viable cells by measuring absorbance at 450 nm.

RNA-Seq analysis

The mRNA-Seq experiments were performed by Novogene (Beijing, China). mRNA-Seq library was prepared for sequencing using standard Illumina protocols. Briefly, total RNA from MCF-7 with or without GlyH-101 treatment was isolated using TRIzol reagent and treated with RNase-free DNase I (New England Biolabs) to remove any contaminating genomic DNA. mRNA extraction was performed using Dynabeads oligo(dT) (Invitrogen). Double-stranded complementary DNAs were synthesized using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. The cDNAs were then fragmented by nebulization, and the standard Illumina protocol was followed thereafter to create the mRNA-Seq library. For the data analysis, base calls were performed using CASAVA. Reads were aligned to the genome using the split read aligner TopHat (v2.0.7) and Bowtie2, using default parameters. HTSeq was used for estimating their abundances. Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq R package (1.18.0). GO enrichment analysis of differentially expressed genes was implemented by the GOSeq R package, in which gene length bias was corrected. GO terms with corrected p value less than 0.05 were considered significantly enriched by differential expressed genes.

The Artemia mRNA-Seq experiments were performed by Sangon (Shanghai, China). Total RNA from nauplius-destined ovoviviparous embryos, prediapause embryos, diapause embryos, and postdiapause embryos was extracted as above described. RNA integrity was evaluated with a 1.0% agarose gel. Thereafter, the quality and quantity of RNA were assessed using a Nano Photometer® spectrophotometer (IMPLEN) and an Agilent 2100 Bioanalyzer (Agilent Technologies). Sequencing libraries were generated using VAHTSTM mRNA-Seq V2 Library prep kit for Illumina® following the manufacturer’s recommendations, and index codes were added to attribute sequences to each sample. Paired-end sequencing of the library was performed on the HiSeq XT sequencers (Illumina, San Diego, CA). FastQC (version 0.11.2) was used for evaluating the quality of sequenced data. Unigenes were blasted against NCBI Nr (NCBI non-redundant protein database), SwissProt, TrEMBL, Conserved Domain Database, Pfam, and KOG (Eukaryotic Orthologous Groups) databases (E-value < 1e-5). At the same time, TransDecoder (version 3.0.1) was used to predict CDS sequences of the unaligned Unigenes.

Statistics and quantification

The sample size for each experiment is indicated in the figure legend. The data are presented as the means ± S.D. For two groups of data, a two-tail Student’s t test was used. For three or more groups, the data were analyzed using a one-way analysis of variance. p values less than 0.05 were considered significant, and p values less than 0.01 were considered to be highly significant. For information on quantification of fluorescence (DiBAC4(3)/MQAE), we used 15 cells for immunofluorescent observation. All data represent the averages of at least three independent experiments.

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The chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) controls cellular quiescence by hyperpolarizing the cell membrane during diapause in the crustacean Artemia

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