The atypical mitogen-activated protein kinase ERK3 is essential for establishment of epithelial architecture

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Chika Takahashi, Koichi Miyatake, Morioh Kusakabe, and Eisuke Nishida

From the Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Sakyō-ku, Kyoto 606-8502, Japan and AMED-CREST, Japan Agency for Medical Research and Development, 1-7-1 Otemachi, Chiyoda-ku, Tokyo 100-0004, Japan

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Epithelia contribute to physical barriers that protect internal tissues from the external environment and also support organ structure. Accordingly, establishment and maintenance of epithelial architecture are essential for both embryonic development and adult physiology. Here, using gene knockout and knockdown techniques along with gene profiling, we show that extracellular signal–regulated kinase 3 (ERK3), a poorly characterized atypical mitogen-activated protein kinase (MAPK), regulates the epithelial architecture in vertebrates. We found that in Xenopus embryonic epidermal epithelia, ERK3 knockdown impairs adherens and tight-junction protein distribution, as well as tight-junction barrier function, resulting in epithelial breakdown. Moreover, in human epithelial breast cancer cells, inhibition of ERK3 expression induced thickened epithelia with aberrant adherens and tight junctions. Results from microarray analyses suggested that transcription factor AP-2α (TFAP2A), a transcriptional regulator important for epithelial gene expression, is involved in ERK3-dependent changes in gene expression. Of note, TFAP2A knockdown phenocopied ERK3 knockdown in both Xenopus embryos and human cells, and ERK3 was required for full activation of TFAP2A-dependent transcription. Our findings reveal that ERK3 regulates epithelial architecture, possibly together with TFAP2A.

Extracellular signal-regulated kinase 3 (ERK3), also known as mitogen-activated protein kinase 6 (MAPK6), is an atypical MAPK family member that has been much less characterized than the classical MAPKs, such as ERK1/2, c-Jun N-terminal kinase (JNK), and p38 (1, 2). Genetic ablation of the Erk3 gene in mice has revealed that ERK3 plays important roles in fetal growth and lung maturation during embryogenesis (3). Additionally, ERK3 has recently emerged as a potential target for cancer therapy because (a) it promotes cancer cell migration, invasion, and chemoresistance (4–6), and (b) its overexpression and genomic mutations are observed in multiple human cancers (4, 7–9). However, molecular and cellular mechanisms by which ERK3 regulates embryogenesis or oncogenesis have not been fully investigated. Moreover, although ERK3 is evolutionarily conserved in vertebrates from fish to humans, its physiological role has not been examined in nonmammalian vertebrate classes.

Epithelia provide physical barriers that separate the internal environment from the external environment and support the structure of organs. Establishment and maintenance of epithelial architecture are essential for embryonic development and adult physiology, whereas impairment of epithelial architecture is a hallmark of cancer progression and metastasis. Transcription factor AP-2α (TFAP2A) plays a role in regulating the expression of epithelial genes, such as E-cadherin (encoding a major adherens junction protein) and keratin 14 (encoding a component of epithelial intermediate filaments) (10–17). Constitutive or conditional knockouts of Tfap2a in mice yield neural crest–related craniofacial defects, as well as malformation of epithelia-containing organs, such as the kidneys, ventral wall, and skin epidermis (18–22). Moreover, mutations in the human TFAP2A gene are found in patients with branchio-oculo-facial syndrome (23), a congenital developmental disorder characterized by defects in the craniofacial structures, neck skin, eyes, and ears, as well as less frequently occurring kidney malformation. These findings demonstrate the essential role of TFAP2A in diverse developmental processes. However, the regulatory mechanisms of TFAP2A are largely unknown.

In this study, our analyses in X. laevis embryos and human cancer cells demonstrate that ERK3 is crucial for maintaining epithelial cell junction integrity and epithelial tissue architecture in vertebrates. Our transcriptome analyses suggest that TFAP2A is involved in ERK3-dependent gene expression changes. Moreover, we demonstrate that TFAP2A, like ERK3, is required for epithelial cell junction integrity and
ERK3 expression was detected from the cleavage to tailbud human cancer cells. Thus, we conclude that ERK3 regulates ERK3 protein. We first examined the expression of and are 85 and 84% identical, respectively, to the human consist of 720 amino acids, are 95% identical to each other, pair of primers designed to detect both homeologs by real-time quantitative RT-PCR using a X. laevis nucleotide identity. ERK3X. laevis because it has two homeologous homeologous subgenomes, a long subgenome (L) and a short subgenome (S) (24, 25), it has two homeologous genes, ERK3A (mapk6.L) and ERK3B (mapk6.S), which share 95% nucleotide identity. X. laevis ERK3A and ERK3B proteins consist of 720 amino acids, are 95% identical to each other, and are 85 and 84% identical, respectively, to the human ERK3 protein. We first examined the expression of X. laevis ERK3 homeologs by real-time quantitative RT-PCR using a pair of primers designed to detect both ERK3A and ERK3B. ERK3 expression was detected from the cleavage to tailbud stages (Fig. 1A). We then examined the spatial expression patterns of ERK3 homeologs by whole-mount in situ hybridization using a probe synthesized from the coding region of ERK3A, which is expected to cross-hybridize with ERK3B due to 95% identity in sequences. ERK3 expression was detected in the animal (ectodermal) region at the late blastula stage (stage 9) and the late gastrula stage (stage 12) (Fig. 1B). At the neurula and tailbud stages (stage 19–33/34), ERK3 was highly expressed in the neural tissues, neural crest, and pronephros and moderately expressed in the somites and epidermis (Fig. 1B).

ERK3 is required for pronephros and epidermal development in X. laevis embryos

To investigate ERK3 function, we performed knockdown experiments with antisense morpholino oligonucleotides (MOs) against ERK3, ERK3 MO1 for ERK3A alone, ERK3 MO2 for ERK3B alone, and ERK3 MO3 for both ERK3A and ERK3B (Fig. 2A). Immunoblotting analyses showed that ERK3 MO1 and MO2 specifically blocked the translation of ERK3A and ERK3B mRNAs, respectively (Fig. 2, B and C). ERK3 MO3 blocked the translation of both ERK3A and ERK3B mRNAs (Fig. 2D). To first examine the role of pronephric ERK3 expression, we injected control MO, ERK3 MO1/2 (ERK3 MO1 plus MO2), or ERK3 MO3 into both ventral vegetal blastomeres (also called V2 blastomeres, from which the pronephros arises) (26) of 8-cell stage embryos. The injection of ERK3 MO1/2 or ERK3 MO3, but not that of control MO, caused edema formation (Fig. 3, A and B). As pronephros defects lead to increased water retention and edema in amphibian embryos (27), we next investigated expression of the pan-pronephros marker gene atp1b1 by whole-mount in situ hybridization. ERK3 knockdown led to a reduction of atp1b1 expression, suggesting that pronephros development was inhibited by ERK3 knockdown (Fig. 3, C and E). The reduction of atp1b1 expression in ERK3 morphants was partially rescued by overexpressing N-terminally Myc-tagged ERK3A and ERK3B (Myc-ERK3A and Myc-ERK3B) mRNAs, which were MO-resistant (Figs. 2E and 3C and D)). These results suggest that ventral vegetal ERK3 is required for pronephros development. Next, we investigated the role of ventral animal (i.e. presumptive epidermal) ERK3. The injection of ERK3 MO1/2 or ERK3 MO3 into the animal regions of all ventral blastomeres at the 4-cell stage led to epidermal disintegration at the tailbud stage (Fig. 3, F and G). At stage 23, ERK3 morphants began to show local cell detachment from the surface (Fig. 3F, black arrowheads). At stage 39, almost all ERK3 morphants were partly or completely crushed due to epidermal architecture disruption (Fig. 3G). In control MO-injected embryos, epidermal architecture disruption was not observed (Fig. 3, F and G). These results suggest that ERK3 is required for epidermal development.

Figure 1. ERK3 expression in X. laevis embryos. A, real-time quantitative RT-PCR analysis. The expression levels of ERK3 were normalized to those of odc in two independent experiments (#1 and #2). The normalized ERK3 expression level at stage 1 was defined as 1.0 in each experiment. B, whole-mount in situ hybridization analysis of ERK3 expression. ec, ectoderm; nc, neural crest; s, somite; nt, neural tube; ep, epidermis; pn, pronephros; b, brain; e, eye. Scale bars, 400 μm. Anterior is to the left (stage 19–33/34). Dorsal is up for the lateral views (stage 19–33/34). Shown are representative images of 6–10 embryos from one experiment using the ERK3A probe. Essentially the same results were obtained for 6–9 embryos from another experiment using the ERK3B probe.
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ERK3 is required for the formation of adherens and tight junctions in X. laevis embryonic epithelia

We hypothesized that the epidermal disintegration observed in ERK3 knockdown tailbuds might be caused by defective cell junctions in the early stages. To test this hypothesis, embryos after co-injection of CAAX-GFP mRNA with control MO, ERK3 MO1/2, or ERK3 MO3 were subjected to E-cadherin immunostaining at stage 13, when epidermal disintegration had not yet occurred in ERK3 morphants. The GFP signal of the plasma membrane-targeted CAAX-GFP revealed that epidermal epithelial cells in the control embryos were almost polygonal and had nearly straight cell–cell boundaries, but those in ERK3 morphants were less polygonal and had more curved cell–cell boundaries (Fig. 4A).

Moreover, junctional E-cadherin staining in epidermal epithelial cells almost completely disappeared in ERK3 morphants (Fig. 4A), suggesting that adherens junctions were severely defective. Consistent with this, the expression level of E-cadherin mRNA was down-regulated in ERK3 morphants (Fig. 4B). Next, embryos injected with control MO, ERK3 MO1/2, or ERK3 MO3 were subjected to immunostaining for ZO-1, a tight-junction protein, at stage 13. In control embryos, the ZO-1 signals in epidermal epithelial cells were detected as continuous straight lines (Fig. 4C). However, in ERK3 morphants, the signals were detected as discontinuous or curved lines (Fig. 4C). The number of gaps in ZO-1 staining in a single confocal section (Fig. 4C, yellow arrowheads), which could be indicative of the absence, apical shift, or basal shift of ZO-1 protein, was significantly higher in ERK3 morphants than in control embryos (Fig. 4D). Also, although ZO-1 mRNA expression was not significantly affected by ERK3 MO1/2 (Fig. 4E), the fluorescence intensity of junctional ZO-1 staining in a single confocal section was slightly but significantly lower in ERK3 morphants than in control embryos (Fig. 4E). These results suggest that the tight junctions were defective in ERK3 morphant. To assess the barrier function of the tight junctions, we used EZ-link Sulfo-NHS-LC-Biotin, a membrane-impermeable reagent that does not pass through the tight-junction barrier (28). The transverse sections of embryos revealed that externally added EZ-link Sulfo-NHS-LC-Biotin was diffused into the internal tissues in ERK3 morphants, suggesting...
Figure 3. ERK3 is required for pronephros and epidermal development in X. laevis embryos. A–G, phenotypes of ERK3 knockdown embryos. Lateral views are shown with anterior to the left and dorsal up. A–E, control MO (60 ng in A, 40 or 80 ng in B, 15 ng in C, and 40 ng in E), ERK3 MO1/2 (30 ng in A or 7.5 ng in C each of ERK3 MO1 and ERK3 MO2), or ERK3 MO3 (40 or 80 ng in B and 40 ng in E) was co-injected with the lineage tracer dextran-fluorescein into both (A and B) or the left (C and E) V2 blastomere(s) at the 8-cell stage. Embryos with pronephric fluorescence were further analyzed. A and B, phenotypes at stage 42. The black arrowhead indicates edema. The graph indicates the percentage of embryos with edema from four (A) or two (B) independent experiments. **, p < 0.01 by z test. Scale bars, 1 mm. C–E, expression of the pronephric marker gene atp1b1 at stage 33/34. pn, pronephros. Scale bars, 200 μm. C and D, in the rescue experiment, Myc-ERK3A and Myc-ERK3B mRNAs (1 ng each) were co-injected with ERK3 MO1/2. D, percentage of embryos with normal or aberrant atp1b1 expression in pronephros in four independent experiments. Severe, no expression; Mild, weak expression. **, p < 0.01 by Mann–Whitney U test. E, expression of atp1b1 was inhibited by ERK3 MO3 (n = 23/23) but not by control MO (n = 1/28) in three independent experiments. F and G, control MO (60 ng), ERK3 MO1/2 (20 ng each of MO1 and MO2), or ERK3 MO3 (60 ng) was injected into the animal regions of ventral blastomeres at the 4-cell stage. In three independent experiments, the epidermal architecture was disrupted by ERK3 MO1/2 (n = 14/38 at stage 23 and n = 37/38 at stage 39) and ERK3 MO3 (n = 36/40 at stage 39) but not by control MO (n = 0/39 at stages (St.) 23 and 39). Black arrowheads indicate initially observed, local disintegration of the epidermis. Scale bars, 500 μm.
the loss of the barrier function of the tight junctions (Fig. 4F).

Taken together, these results suggest that ERK3 knockdown leads to the disruption of adherens and tight junctions in *X. laevis* embryonic epidermal epithelia.

**ERK3 knockdown and ERK3 knockout both disturb epithelial architecture in human epithelial breast cancer cells**

So far, our results suggest that ERK3 regulates epithelial cell junction formation during epidermal development in *X. laevis*. 

**ERK3 regulates epithelial architecture**

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To determine whether the role of ERK3 is conserved in mammals, we used the human breast epithelial cancer cell line MCF7, in which ERK3 is highly expressed (according to the public online gene expression database BioGPS (http://www.biogps.org)). ERK3-specific siRNA1 and siRNA2 effectively decreased human ERK3 expression levels in MCF7 cells (Fig. 5A). Bright-field observation indicated that control siRNA–transfected cells, but not ERK3 siRNA–transfected cells, were able to reach confluence (Fig. 5B). ERK3 siRNAs did not affect cell proliferation (Fig. 5C), suggesting that the low confluence in ERK3 siRNA–transfected cells was not due to decreased cell numbers. 3D imaging revealed that control siRNA–transfected cells formed a flat monolayer epithelial sheet, whereas ERK3 siRNA–transfected cells piled up and formed dome-like shaped colonies (Fig. 5, B, E, G, and H). Moreover, junctional E-cadherin and ZO-1 signals in ERK3 knockdown cells were less sharp or less continuous than those in control cells (Fig. 5, D and F), suggesting that ERK3 is required for maintaining cell junction integrity to form a flat monolayer epithelial sheet.

We then generated two stable ERK3-knockout MCF7 cell lines by CRISPR/Cas9-mediated genome editing using ERK3-specific sgRNA (Fig. 6A). The two ERK3-knockout clones, which had frameshift mutations caused by different single-nucleotide insertions (Fig. 6B), did not express detectable levels of ERK3 protein (Fig. 6C). The ERK3-knockout cells exhibited lower confluence and a slightly slower proliferation rate than WT cells (Fig. 6, D and E). ERK3-knockout cells, but not WT control cells, piled up to form thickened sheets or colony-like structures (Fig. 6, D, H, I, and J), which were similar to but thinner than those formed by ERK3 knockdown cells (Fig. 5, B, E, G, and H). The milder defects in ERK3-knockout cells compared with ERK3-knockdown cells may be partly due to genetic compensation induced by gene knockout, but not by gene knockdown (29). Additionally, junctional E-cadherin and ZO-1 signals in ERK3-knockout cells were less sharp or less continuous than those in WT cells (Fig. 6, F and G), confirming again that ERK3 is required for cell junction integrity. Collectively, these results indicate that ERK3 is essential for maintaining epithelial cell junction integrity in human epithelial breast cancer cells as well as in the X. laevis embryonic epidermis.

**TFAP2A is a candidate factor for contributing to ERK3-dependent gene expression changes**

To study how ERK3 exerts its effects, we focused on changes in gene expression profiles during pronephros and epidermal development in X. laevis embryos. The animal caps (ACs) were dissected from embryos injected with control MO, ERK3 MO1/2, or ERK3 MO3 and were cultured alone or in the presence of activin plus retinoic acid (activin/RA) (Fig. 7A). Whereas epidermal cells can be induced from nontreated ACs, pronephric cells can be induced from activin/RA-treated ACs (30). Microarray analysis indicated that 825 genes in nontreated ACs were down-regulated >2-fold in common by both ERK3 MO1/2 and ERK3 MO3, as compared with control MO (Fig. 7B, orange circle). Additionally, in activin/RA-treated ACs, 294 genes were down-regulated >2-fold in common by both ERK3 MO1/2 and ERK3 MO3 (Fig. 7B, green circle). We focused on 123 genes that were commonly down-regulated by ERK3 MOs in both activin/RA-treated ACs and nontreated ACs, because they could act downstream of ERK3 in both pronephros and epidermal development. Then we performed a transcription factor–binding motif enrichment analysis by scanning promoter sequences with the online tool Pscan (http://159.149.160.88/pscan/). Due to the lack of available X. laevis genes in Pscan, we subjected mouse orthologs of the 123 genes to the analysis and found that the binding sequence of TFAP2A was highly enriched in their promoter sequences (Fig. 7C).

In *X. laevis*, tfap2a is expressed in the prospective epidermis and pronephros (16, 31) and is essential for epidermal development (16). Moreover, defects in the skin epidermis and kidneys can be detected in *tfap2a*-knockout mice and TFAP2A-mutated human patients with branchio-oculo-facial syndrome (19, 21–23, 32). These reports prompted us to speculate that TFAP2A might cooperate with ERK3 to regulate both pronephros and epidermal development in *X. laevis*.

**TFAP2A knockdown phenocopies ERK3 knockdown in X. laevis embryos and human epithelial breast cancer cells**

*X. laevis* has two *tfap2a* homeologs, *tfap2a.L* and *tfap2a.S*.

We examined the spatial expression patterns of *tfap2a* homeologs by whole-mount *in situ* hybridization using a probe complementary to the coding region of *tfap2a.L*, which is expected to cross-hybridize with *tfap2a.S* due to 95% identity in nucleotide sequences. As reported previously (16, 31), *tfap2a* expres-

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Expression was detected in the animal region (presumptive ectoderm) at stage 9 and in the ventral ectoderm (fated to be the epidermis) at stage 12 (Fig. 8). At the neurula and tailbud stages (stage 17–39), tfap2a was highly expressed in the neural crest, brain, and pronephros and moderately expressed in the epidermis (Fig. 8). Thus, tfap2a has a partially overlapping expression pattern with ERK3.

We next performed knockdown experiments using MOs against tfap2a, TFAP2A MO1 for tfap2a.L, and TFAP2A MO2 for tfap2a.S (Fig. 9A). Immunoblotting analyses showed that
TFAP2A MO1 and MO2 specifically blocked the translation of tfap2a.L and tfap2a.S mRNAs, respectively (Fig. 9, B and C). We then performed further microarray experiments on nontreated (i.e. epidermally differentiated) ACs derived from embryos injected with control MO, ERK3 MO1/2, or TFAP2A MO1/2 (TFAP2A MO1 plus MO2) (Fig. 10A). As compared with control, 3452 and 3636 genes were down-regulated >1.5-fold by ERK3 knockdown and tfap2a knockdown, respectively. A total of 1937 genes, which represent 56% of the 3452 genes and 53% of the 3636 genes, were down-regulated >1.5-fold in common by both ERK3 knockdown and tfap2a knockdown (Fig. 10B). Additionally, 2580 and 2838 genes were up-regulated >1.5-fold by ERK3 knockdown and tfap2a knockdown, respectively. A total of 1281 genes, which represent 50% of the 2580 genes and 45% of the 2838 genes, were up-regulated >1.5-fold in common by both ERK3 knockdown and tfap2a knockdown (Fig. 10B). These results indicate remarkable overlap between ERK3-regulated genes and TFAP2A-regulated genes. Moreover, the transcriptomic alterations in tfap2a knockdown ACs were highly correlated with those in ERK3 knockdown ACs (Fig. 10C, correlation coefficient 0.651; \( p < 0.01 \)). Notably, the correlation coefficient for cell junction-related genes (listed in Table S1) was higher than that for all genes (Fig. 10D, correlation coefficient 0.788; \( p < 0.01 \)). The number of cell junction-related genes down-regulated by tfap2a knockdown and ERK3 knockdown (represented by dots in the bottom left area of the graph) was higher than that up-regulated by tfap2a knockdown and ERK3 knockdown (represented by dots in the top right area of the graph) (Fig. 10D), indicating that many cell junction-related genes are positively regulated by ERK3 and TFAP2A. Taken together, these results suggest that ERK3 and TFAP2A have an overlapping role in regulating gene expression, especially cell junction–related genes.

We next observed the embryonic phenotypes induced by tfap2a knockdown. The ventral vegetal injection of TFAP2A MO1/2 led to edema as well as reduced atp1b1 expression (Fig. 11, A and B), similar to that of ERK3 MOs (Fig. 3, A–E). Moreover, the ventral animal injection of TFAP2A MO1/2 led to the disruption of adherens and tight junctions in epidermal epithelia at early stages (Figs. 4 (C–E) and 11 (D–F)), resulting in epidermal disintegration at the tailbud stage (Fig. 11C), similar to that of ERK3 MOs (Figs. 3 (F and G) and 4). These results indicate that tfap2a knockdown phenocopies ERK3 knockdown in X. laevis.

We also performed knockdown experiments with TFAP2A-specific siRNA1 and siRNA2, both of which effectively decreased human TFAP2A expression levels without affecting cell proliferation in MCF7 cells (Fig. 12, A and C). TFAP2A knockdown cells piled up to form thickened sheets or colony-like structures (Fig. 12, B, E, G, and H), which were similar to or thinner than those formed by ERK3 knockdown cells (Fig. 5, B, E, G, and H). The milder defects in TFAP2A knockdown cells compared with ERK3 knockdown cells suggest that the actions of ERK3 are not totally dependent upon TFAP2A. Additionally, junctional E-cadherin and ZO-1 signals in TFAP2A knockdown cells were less sharp or less continuous than those in control cells (Fig. 12, D and F). These results thus indicate that TFAP2A, like ERK3, is required for maintaining cell junction integrity to form a flat monolayer epithelial sheet in human epithelial breast cancer cells.

**ERK3 regulates epithelial architecture**

Next, we investigated using X. laevis embryos whether the pronephric and epidural phenotypes in ERK3 morphants could be rescued by tfap2a overexpression. The injection of tfap2a.L mRNA slightly but significantly rescued the reduction of both pronephric atp1b1 expression and epidural junctional E-cadherin protein in ERK3 morphants (Fig. 13). These results suggest that ERK3 regulates pronephros and epidermal development at least partly through TFAP2A.

The mRNA expression levels of tfap2a were not significantly affected by ERK3 knockdown (Fig. 14A), raising the possibility that ERK3 regulates TFAP2A in a posttranscriptional or posttranslational manner. We therefore examined whether ERK3 affects the transcription activity of TFAP2A by luciferase assays using the TFAP2A-luc reporter (3xAP2-Luc), which contains three tandem repeats of the consensus binding sequence of the TFAP2 family to drive the expression of luciferase (33, 34) (Fig. 14B). The TFAP2A-luc reporter activity was reduced to an average of 69% in ACs and to an average of 65% in whole embryos by ERK3 knockdown compared with that in the controls (Fig. 14C, green dots), suggesting that the endogenous transcription activity of the TFAP2 family is partly dependent upon ERK3 in X. laevis embryos. Additionally, the high TFAP2A-luc reporter activity achieved by injecting tfap2a.L mRNA was reduced to an average of 62% in ACs and to an average of 53% in whole embryos by ERK3 knockdown (Fig. 14C, blue dots), suggesting that TFAP2A requires ERK3 to exert its full transcription activity in X. laevis embryos. Moreover, in the human hepatoma cell line HepG2, in which TFAP2A is not detectably expressed (35), tfap2a.L overexpression increased the TFAP2A-luc reporter activity to real-time quantitative RT-PCR analyses. The expression levels of human ERK3 were normalized to those of human GAPDH. Shown are all data points from three or seven independent experiments. The bars represent the average ± S.D. (error bars). The normalized ERK3 expression level in control cells was defined as 1.0 in each experiment. **, \( p < 0.01 \) by Dunnett’s test. B, typical morphologies of MCF7 cells 3 days after transfection with the indicated siRNAs (20 nM). The data are representative of three images from three independent experiments. Scale bars, 100 μm. C, proliferation was assessed by cell counting. MCF7 cells (3.5 × 10^4) were transfected with the indicated siRNAs (20 nM) and counted 3 days after transfection. Shown are all data points from three independent experiments. Bars, average ± S.D. n.s., not significant by Dunnett’s test. D–H, MCF7 cells were transfected with the indicated siRNAs (20 nM), fixed 3 days after transfection, and subjected to triple staining with Hoechst (blue), Alexa Fluor 568 phalloidin (red), and an antibody against E-cadherin (D and E) or ZO-1 (F and G) (green). 3D images obtained by confocal microscopy were viewed from the apical (D and F), lateral (E), or apico-lateral (G) sides. D and E, representative images of 6–10 3D images from three independent experiments. F and G, representative images of 6–13 3D images from three independent experiments. D and F, higher magnification images of the regions outlined in white in the top panels are shown in the bottom panels. Images of Alexa Fluor 568 phalloidin staining are not shown. Scale bars, 20 μm. E, scale bars, 10 μm. H, the scatter plot represents the maximum thickness of the cell layers (or colonies) in the 3D images in D–G. The bars represent the average ± S.D. *, \( p < 0.05 \); **, \( p < 0.01 \) by Dunnett’s test.

**Figure 5. ERK3 knockdown induces thickened epithelia with defective adherens and tight junctions in MCF7 cells.** A, two different siRNAs targeting human ERK3 were effective in MCF7 cells. Cells were transfected with 20 nM control siRNA, ERK3 siRNA1 or ERK3 siRNA2, cultured for 3 days, and then subjected to real-time quantitative RT-PCR analyses. The expression levels of human ERK3 were normalized to those of human GAPDH. Shown are all data points from three or seven independent experiments. The bars represent the average ± S.D. (error bars). The normalized ERK3 expression level in control cells was defined as 1.0 in each experiment. **, \( p < 0.01 \) by Dunnett’s test. B, typical morphologies of MCF7 cells 3 days after transfection with the indicated siRNAs (20 nM). The data are representative of three images from three independent experiments. Scale bars, 100 μm. C, proliferation was assessed by cell counting. MCF7 cells (3.5 × 10^4) were transfected with the indicated siRNAs (20 nM) and counted 3 days after transfection. Shown are all data points from three independent experiments. Bars, average ± S.D. n.s., not significant by Dunnett’s test. D–H, MCF7 cells were transfected with the indicated siRNAs (20 nM), fixed 3 days after transfection, and subjected to triple staining with Hoechst (blue), Alexa Fluor 568 phalloidin (red), and an antibody against E-cadherin (D and E) or ZO-1 (F and G) (green). 3D images obtained by confocal microscopy were viewed from the apical (D and F), lateral (E), or apico-lateral (G) sides. D and E, representative images of 6–10 3D images from three independent experiments. F and G, representative images of 6–13 3D images from three independent experiments. D and F, higher magnification images of the regions outlined in white in the top panels are shown in the bottom panels. Images of Alexa Fluor 568 phalloidin staining are not shown. Scale bars, 20 μm. E, scale bars, 10 μm. H, the scatter plot represents the maximum thickness of the cell layers (or colonies) in the 3D images in D–G. The bars represent the average ± S.D. *, \( p < 0.05 \); **, \( p < 0.01 \) by Dunnett’s test.
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A

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activity (Fig. 14D). This increase was enhanced by the coexpression of WT ERK3A, but not by that of the catalytically inactive K49R/K50R mutant of ERK3A (KR) (36–38) (Fig. 14D), suggesting that ERK3 directly or indirectly stimulates the transcription activity of TFAP2A in a kinase activity–dependent manner. Additionally, ERK3 siRNAs, which were effective in HepG2 cells (Fig. 14E), slightly but significantly inhibited the tfap2aL overexpression–dependent TFAP2-L luc reporter activity (Fig. 14F, blue dots), but not the basal one (Fig. 14F, green dots), suggesting again that TFAP2A specifically requires ERK3 to exert its full transcription activity. Collectively, these results suggest that ERK3 cooperates with TFAP2A to regulate gene expression.

Discussion

In this study, we identified the crucial roles of the poorly characterized atypical MAPK ERK3 and the branchio-oculo-facial syndrome–related transcription factor TFAP2A in the establishment and maintenance of epithelial architecture. Although TFAP2A directly up-regulates E-cadherin transcription (12–14, 17), its regulatory mechanisms and its role in epithelial cell junction formation were unknown. Our results suggest that ERK3 acts in cooperation with TFAP2A to regulate gene expression changes.

**Figure 6.** ERK3 knockout induces thickened epithelia with defective adherens and tight junctions in MCF7 cells. A, genomic structure of the human ERK3 gene. Dark green, translated regions in exons; light green, untranslated regions in exons. sgRNA target sequences are underlined in blue. The black arrowhead indicates the predicted double-strand break (DSB) site between 3 and 4 nucleotides upstream of the PAM sequence (underlined in red). B, genomic sequencing of ERK3 knockout clones (Clone #1 and Clone #2) showing a one-base insertion in exon 3. C, ERK3 protein levels in WT and ERK3-knockout clones (#1 and #2) were determined by Western blotting. The data are from one experiment. D, typical morphologies of WT and ERK3-knockout cells (Clone #1 and Clone #2). The data are representative of two images from one experiment. Scale bars, 100 μm. E, proliferation was assessed by cell counting. WT and ERK3-knockout cells (3.5 × 10^5) were seeded in 6-well plates and counted after 3 days. The data points are from four independent experiments. The bars represent the average ± S.D. (error bars). *, p < 0.05; **, p < 0.01 by Dunnett’s test. F–J, WT or ERK3-knockout cells were seeded on coverglasses, cultured for 3 days, and subjected to triple staining with Hoechst (blue), Alexa Fluor 568 phalloidin (red), and an antibody against E-cadherin (F and I) or ZO-1 (G and H) (green). 3D images obtained by confocal microscopy were viewed from the apical (F and G), apico-lateral (H), or lateral (I) sides. F and I, show representative images of 4–7 3D images from three coverslips used in one experiment. G and H, show representative images of three 3D images from three coverslips used in one experiment. F and G, higher magnification images of the regions outlined in white in the top panels are shown in the bottom panels. Images of Alexa Fluor 568 phalloidin staining are not shown. Scale bars, 20 μm. I, scale bars, 10 μm. J, the scatter plot represents the maximum thickness of the cell layers (or colonies) in the 3D images in F–I. The bars represent the average ± S.D. *, p < 0.05; **, p < 0.01 by Dunnett’s test.
X. laevis and mammalian kidney development. Aberrant epithelial architecture could also be associated with other developmental defects observed in Erk3-knockout mice (3), Tfap2a-knockout mice (18–22), and TFAP2A-mutated human patients with branchio-oculo-facial syndrome (23, 32).

**Experimental procedures**

**Molecular cloning and plasmid construction**

The entire coding regions of *X. laevis* ERK3A (mapk6.L, ERK3B (mapk6.S), tfap2a.L, tfap2a.S, and atp1b1 (atp1b1.S) were amplified by PCR using late gastrula or neurula cDNA and primers designed based on deposited sequences (NCBI reference sequence numbers: ERK3A, XM_018253074.1; ERK3B, XM_018255437.1; tfap2a.L, NM_001087569.1; tfap2a.S, NM_001095572.1; and atp1b1 (atp1b1.S), NM_001086759.1). These amplified sequences were cloned into the pCS2/H11001 vector, the V5/Myc-tagged pCS2/H11001 vector, or the pcDNA3 vector. To generate a catalytically inactive mutant of *X. laevis* ERK3A, two lysine residues (Lys-49 and -50) were changed to arginine residues by site-directed mutagenesis according to previous studies (36–39).
All constructs and mutations were confirmed by DNA sequencing.

Embryo manipulation

*X. laevis* experiments complied with the Regulation on Animal Experimentation at Kyoto University and were approved by the Animal Experimentation Committee of Kyoto University. Frogs were bred and handled with care, according to an established protocol (47). *X. laevis* embryos were obtained by *in vitro* fertilization and cultured in 0.1 × MBS (1.0 mM HEPES, pH 7.4, 8.8 mM NaCl, 0.1 mM KCl, 0.24 mM NaHCO₃, 0.082 mM MgSO₄, 0.03 mM Ca(NO₃)₂, and 0.041 mM CaCl₂). Embryo injections were performed in 4% Ficoll in 0.1 × MBS. mRNAs or antisense MOs were injected into the indicated blastomeres at the indicated stage. All MOs were obtained from Gene Tools and designed to target the translational start site of each gene. The MO sequences were as follows: ERK3 MO1 targeting ERK3A, 5'-AAACTTTTCTGCCATTTTGGTGACC-3'; ERK3 MO2 targeting ERK3B, 5'-CATGGTGACGACTTTTCTTCCGCTC-3'; ERK3 MO3 targeting both ERK3A and ERK3B, 5'-TGAGGCTTTCAAACTTTTCGACC3'; TFAP2A MO1 targeting tfap2a.L, 5'-GCGATCC-TGCCATTTTGGTGACC-3'; TFAP2A MO2 targeting tfap2a.S, 5'-TTCCAGAGCATTTTCCATGGTGACC-3'; and a standard control MO (control MO), 5'-CCTCTTACCTCA-GTTACAATTTC-3'. For V2 cell-injection, the MOs were co-injected with the lineage tracer dextran-fluorescein to select embryos with pronephric fluorescence for further analyses. Images of the embryos were acquired using a stereomicroscope SZX16 (Olympus).

Real-time quantitative RT-PCR

Total RNA from *X. laevis* embryos was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Total RNA was extracted from cultured human cells with NucleoSpin RNA (MACHERY-NAGEL), and cDNA was synthesized by ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO). For real-time quantitative RT-PCR analyses, we used a 7300 real-time PCR system (Applied Biosystems) and SYBR Green PCR Master Mix (TAKARA). The gene expression levels were normalized to those of ornithine decarboxylase (*odc*) in *X. laevis* embryos or GAPDH in human cells. The sequences of primer pairs used were as follows: *X. laevis* odc, 5'-TGCAAATGGGAGAAGTGGTAAGGACTGATGATG (forward) and 5'-CATCAGTTGCTGATGGTGG (reverse); *X. laevis* ERK3, 5'-TGACATGTTGGGAGAAGTGGTAAGGACTGATGATG (forward) and 5'-CATCAGTTGCTGATGGTGG (reverse); and *X. laevis* TFAP2A, 5'-TGCAAATGGGAGAAGTGGTAAGGACTGATGATG (forward) and 5'-CATCAGTTGCTGATGGTGG (reverse).
GCTGCTGGATGT (forward) and 5’-TGAGGAGCTCTTG-GCGGTCT (reverse); X. laevis tfap2a, 5’-CTGTCTCTGCTC-AGCTCCAC (forward) and 5’-TCCCTCAGAGCCTTC-CTCC (reverse); X. laevis E-cadherin, 5’-TCACTCCTTGTG- TCGATTACGA (forward) and 5’-ATCAGAGTGGAGCA-GTTCAGAGA (reverse); X. laevis ZO-1, 5’-GAGGATGTGC- CTGACCAGAG (forward) and 5’-TTCGATTACGA (reverse); X. laevis E-cadherin, 5’-CTGTCTCTGCTC-AGCTCCAC (forward) and 5’-TCCCTCAGAGCCTTC-CTCC (reverse); human GAPDH, 5’-CCAGAACATCATCC-CTGCCTCTACT (forward) and 5’-GGTTTTTCTAGACGG- CAGGTCAGGT (reverse); human ERK3, 5’-GAAGCATCACAGCAACTGGC (forward) and 5’-ATCAGTAGGCTCATG- CTGGG (reverse); and human TFAP2A 5’-TTCTGTTCAGT- TCCGGGTG (forward) and 5’-GTGGAGACACTCGGGT-GGTG (reverse).

Whole-mount in situ hybridization

Whole-mount in situ hybridization on albino or WT X. laevis embryos was performed according to a standard protocol (47) and using a robot (InsituPro, Intavis). Briefly, embryos were fixed in MEMFA (100 mm MOPS (pH 7.4), 2 mm EGTA, 1 mm MgSO₄, and 3.7% formaldehyde) and dehydrated in meth-
an. After rehydration, the embryos were treated with 2.5 μg/ml proteinase K in PBS containing 0.1% Tween 20, incubated in 100 mM triethanolamine (pH 8.0), incubated in 100 mM triethanolamine containing 0.25% acetic anhydride, and then refixed in 4% paraformaldehyde in PBS. After washing, the embryos were hybridized with digoxigenin-labeled antisense probes and then subjected to staining with anti-digoxigenin (Roche Applied Science, catalog no. 11093274910, 1:2500). The color reaction was performed using BM purple solution (Roche Applied Science). The digoxigenin-labeled probes were synthesized from cDNAs corresponding to the coding regions of *X. laevis* ERK3A, ERK3B, tafpa2A.I, and atp1b1 (atp1b1.S).

**Immunoblotting**

*X. laevis* embryos were lysed in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1% Nonidet P-40, 10 mM NaF, 1 mM vanadate, 1 mM DTT, and 1% protease inhibitor mixture (Sigma, catalog no. 8340). The lysate was centrifuged, and the supernatant was used for immunoblotting. Cultured human cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.001% pyronine Y, and 0.05% β-mercaptoethanol. The lysate was boiled at 95°C for 15 min. The following antibodies were used at the indicated dilutions. The primary antibodies were mouse anti-Myc (Santa Cruz Biotechnology, 9E10, 1:2000), mouse anti-V5 (Invitrogen, catalog no. 46-0705; 1:2000), mouse anti-GFP (Clontech, XL-8; 1:4000), rabbit anti-ERK3 (Cell Signaling Technology, catalog no. 4067; 1:1000), and mouse anti-actin (NeoMarkers, catalog no. MS-1295-P0; 1:2000). Secondary antibodies used were anti-mouse IgG, horseradish peroxidase–linked F(ab')2 fragment (sheep) (GE Healthcare, catalog no. NA9310; 1:10000), and anti-rabbit IgG, horseradish peroxidase–linked F(ab')2 fragment (donkey) (GE Healthcare, catalog no. NA9340; 1:10000). Signals were detected with Western Lightning Plus-ECL Enhanced Chemiluminescence reagent (PerkinElmer Life Sciences) and LAS4000 (Fuji Film).

**Immunofluorescence staining**

*X. laevis* embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C for E-cadherin immunostaining or in Dent’s solution at −20°C for ZO-1 immunostaining. Fixed embryos were washed with PBS containing 0.1% Triton X-100 (PBST) and blocked with 10% goat serum in PBST at room temperature for 1 h. The primary antibodies used were anti-E-cadherin (DSHB, 5D3; 2.5 mg/ml) and anti-ZO-1 (Invitrogen, catalog no. 61-7300; 1:200). Bound primary antibodies were detected with Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, catalog no. A-11036; 1:250) or Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, catalog no. A-11031; 1:250). For immunofluorescence staining of MCF7 cells, the cells were fixed in 4% paraformaldehyde at room temperature for 10 min and then permeabilized with 0.2% Triton X-100 in PBST at room temperature for 10 min. After blocking with 3% BSA in PBS at room temperature for 1 h, the cells were incubated with 3% BSA in PBS containing anti-E-cadherin (Cell Signaling, catalog no. 3195; 1:200) or anti-ZO-1 (1:200) at 4°C overnight, washed with PBS, and then incubated with 3% BSA in PBS containing Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, catalog no. A-11034; 1:250) for 30 min at room temperature. Nuclei were simultaneously stained with Hoechst. To visualize F-actin, we used Alexa Fluor 568–conjugated phallolidin (Invitrogen, catalog no. A-12380). Cells were finally mounted in Mowiol. The confocal image was obtained by using a confocal laser-scanning microscope (Leica, TCS SP8). For the quantification shown in Figs. 4 (D and E) and 13D, images were acquired at a constant gain and offset for each sample. The fluorescence intensity values of junctional ZO-1 and E-cadherin in the graph were then calculated using TCS SP8 LAS X version 1.1 software (Leica) by subtracting the average fluorescence intensities of ZO-1 or E-cadherin in the midline drawn on the background cytoplasmic area from those in the cell–cell boundary, which was defined by appearance (Fig. 4E) or CAAX-GFP signals (Fig. 13D). For the 3D imaging in Figs. 5 (D–H), 6 (F–I), and 12 (D–H), cells were imaged with the XYZ scanning mode of a Leica TCS SP8 confocal laser-scanning microscope and at a constant gain and offset for each sample. 3D images were then generated by the 3D viewer in the TCS SP8 LAS X version 1.1 software.

**Examination of the tight-junction permeability barrier**

The indicated MOs were injected into the animal region of both ventral blastomeres at the 4-cell stage. After vitelline membrane removal at stage 23, the injected embryos were incubated with 1 mg/ml EZ-link Sulfo-NHS-LC-Biotin (Thermo Fisher, catalog no. 21335) in 0.1X MBS at 15°C for 10 min, fixed.
in 4% paraformaldehyde in PBS at 4 °C overnight, washed with PBS, cryoprotected with 7.5% gelatin and 15% sucrose in PBS, and embedded in OCT compound (Sakura). The embedded embryos were frozen in liquid N₂ and transversely sectioned at 14 µm using a CryoStar NX70 (Thermo Fisher). The sections were incubated at 4 °C overnight with streptavidin FITC diluted 1:500 in 5% BSA in PBS. All slides were washed with PBS and then mounted with Mowiol.
Cell culture

HepG2 cells and MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. For immunostaining, MCF7 cells were plated on noncoated glass coverslips and cultured.

Luciferase assay

To construct 3xAP2-Luc, three tandem repeats of the consensus binding sequence of the TFAP2 family (33, 34) were inserted into the pGL4.10 vector (Promega) with the minimal promoter. The Renilla luciferase vector pGL4.74 (Promega) was used for an internal control. In Fig. 14D, the indicated combinations of plasmids were transfected into HepG2 cells using Fugene HD (Promega). In Fig. 14F, the indicated plasmids and siRNAs were simultaneously transfected into HepG2 cells by reverse transfection using FuGENE HD transfection reagent and Lipofectamine RNAiMAX transfection reagent (Invitrogen), respectively. In Fig. 14C, the indicated combinations of plasmids, MOs, and tfap2a.L mRNA were injected into all blastomeres of 2-cell stage *X. laevis* embryos. Animal caps were dissected from the injected embryos at stage 9. Whole embryos (at stage 13), animal caps (at stage 13), and HepG2 cells (24 h after transfection for Fig. 14D or 48 h after transfection for Fig. 14F) were lysed in 1× Passive Buffer (Promega). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Signals were detected using the GloMax Multi + Detection System (Promega). Firefly luciferase activity derived from the 3xAP2-Luc vector was normalized to the internal control Renilla luciferase activity.

siRNA

Custom Silencer Select siRNAs specific for human ERK3 were purchased from Ambion. The sequences of the siRNAs were as follows (sense and antisense): ERK3 siRNA1 (48), 5’-G-GCUCUUUCGAUGACAUGTCTT-3’ and 5’-ACCUGAUAAC-AUGAAAAAGCGT-3’; ERK3 siRNA2 (48), 5’-GGAGUACA-UUGGAGACAGACTT-3’ and 5’-GUCUGUCUCAGUAGU-CUCCGTG-3’. Human TFAP2A-specific siRNAs (TFAP2A siRNA1 and TFAP2A siRNA2) were purchased from Ambion (catalog nos. s14003 and s14004, respectively). Silencer Select negative control No. 1 siRNA (Ambion, catalog no. 490843) was used as the control siRNA. In Figs. 5 and 12, all siRNAs were transfected into MCF7 cells by reverse transfection using Lipofectamine RNAiMAX transfection reagent (Invitrogen, catalog no. 13778150) according to the manufacturer’s instructions. MCF7 cells were then analyzed 3 days after transfection. In Fig. 14E, to examine the efficiency of siRNAs under the condition in which plasmid DNAs were co-transfected, the indicated siRNA and the mock plasmid were simultaneously transfected into HepG2 cells by reverse transfection using Lipofectamine RNAiMAX transfection reagent and FuGENE HD transfection reagent, respectively. Transfected HepG2 cells were harvested at 48 h after transfection.

Microarray analysis

Sampling schemes are shown in Figs. 7A and 10A. Briefly, control MO (80 ng), ERK3 MO1/2 (40 ng each of MO1 and MO2), ERK3 MO3 (80 ng), or TFAP2A MO1/2 (40 ng each of MO1 and MO2) were injected into the animal regions of all blastomeres at the 4-cell stage. The animal caps were dissected from the injected embryos at stage 9, cultured alone or in the presence of activin plus RA, and harvested at stage 15. For Fig. 7, one experiment was performed. For Fig. 10, two independent replicates were prepared. Total RNA was extracted using TRIzol reagent. The quality of the total RNA was assessed using an Agilent 2100 BioAnalyzer. cDNA synthesis and transcriptional amplification were performed using 250 ng of total RNA with the GeneChip 3′ IVT PLUS Reagent Kit (Affymetrix, catalog no. 902415). Fragmented and biotin-labeled cDNA targets were hybridized to the GeneChip *X. laevis* Genome 2.0 Array (Affymetrix) according to the manufacturer’s protocol. Hybridized arrays were scanned using an Affymetrix GeneChip scanner. Scanned chip images were analyzed with GeneChip operating software version 1.4 (GCOS). The probe set signal intensities in the raw data (CEL files) were normalized using a robust multiarray average algorithm and Expression Console software. Up-regulated genes and down-regulated genes were identified by statistical -fold change analyses using GeneSpring GX (Agilent Technologies). Correlation analyses were performed with R version 3.1.0. Because the genes that had low signals (<2.0) were considered not to be expressed by GeneSpring GX, we omitted these genes from further analyses. Gene Ontology term analyses were performed using the DAVID bioinformatics tool from the National Institutes of Health (http://david.abcc.ncifcrf.gov/). For transcription factor-binding motif enrichment analysis in Pscan, we used a mouse transcription factor–binding profile from the JASPAR database.

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**ERK3 knockout in MCF7 cells using CRISPR-Cas9 technology**

sgRNA was designed using the DNA2.0 gRNA Design Tool (https://www.dna20.com/eCommerce/cas9/input).
The oligonucleotide sequence encoding ERK3-specific sgRNA (5’-GCCATTGACATGTGGGCTGC-3’) was cloned into the eSpCas9(1.1)-2A-GFP vector, a modified version of the eSpCas9(1.1) vector (a gift from Feng Zhang, Addgene catalog no. 71814) (49), in which the eSpCas9(1.1) sequence was fused to the 2A-GFP fragment derived from the pSpCas9(BB)-2A-GFP (PX458) vector (a gift from Feng Zhang, Addgene catalog no. 48138) (50). MCF7 cells were transfected with the eSpCas9(1.1)-2A-GFP and sgRNA expression vector using Fugene HD (Promega) according to the manufacturer’s protocol. At 72 h after transfection, single GFP-positive cells were sorted using a FACS Aria II cell sorter (BD Biosciences) and seeded in culture dishes. The cloned cells were screened for CRISPR-edited indels using genomic PCR followed by sequencing. Genomic PCR was performed directly on 10–40 cells using KOD FX Neo (TOYOBO) and a set of primers flanking the CRISPR target site (forward, 5’-AGTAGAGATGGGGTTTCTCCG-3’; reverse, 5’-CCAGGAAGTGATGATACTGACA-3’).
to the manufacturer’s protocol. PCR products were purified with the QIAquick gel extraction kit (Qiagen) and used as a template for nested PCR using KOD FX Neo and a set of primers (forward, 5′-CCGGAATTCCGGGGTTTCTCCGGTTGGTCAGAC-3′; reverse, 5′-CCGCTCGAGCGGGATACTGACAGAATTATGCAAC-3′). The nested PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), cloned into the pCS2/H11001 vector at the EcoRI/XhoI site, and then sequenced.

Cell proliferation assay

For proliferation experiments, WT cells, CRISPR-edited knock-out cells, or siRNA-transfected cells were seeded at 3.5 × 10^5 cells/
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well in 6-well tissue culture plates (IWAKI). Cell proliferation was determined at 72 h after seeding by the direct counting of harvested cells under a microscope.

**Statistics**

The t test and z test were performed using Excel (Microsoft). The Tukey, Dunnett, and Mann–Whitney U tests were performed using SPSS (IBM). The results were considered significant when \( p \) was <0.05.

**Author contributions**—C. T. designed and carried out the experiments with the help of K. M. and M. K.; C. T., K. M., M. K., and E. N. wrote the manuscript; E. N. and M. K. supervised the project; all of the authors discussed the results and commented on the manuscript.

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