Filamin-interacting proteins, Cfm1 and Cfm2, are essential for the formation of cartilaginous skeletal elements

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Mutations of Filamin genes, which encode actin-binding proteins, cause a wide range of congenital developmental malformations in humans, mainly skeletal abnormalities. However, the molecular mechanisms underlying Filamin functions in skeletal system formation remain elusive. In our screen to identify skeletal development molecules, we found that Cfm (Fam101) genes, Cfm1 (Fam101b) and Cfm2 (Fam101a), are predominantly co-expressed in developing cartilage and intervertebral discs (IVDs). To investigate the functional role of Cfm genes in skeletal development, we generated single knockout mice for Cfm1 and Cfm2, as well as Cfm1/Cfm2 double-knockout (Cfm DKO) mice, by targeted gene disruption. Mice with loss of a single Cfm gene displayed no overt phenotype, whereas Cfm DKO mice showed skeletal malformations including spinal curvatures, vertebral fusions and impairment of bone growth, showing that the phenotypes of Cfm DKO mice resemble those of Filamin B (Flnb)-deficient mice. The number of cartilaginous cells in IVDs is remarkably reduced, and chondrocytes are moderately reduced in Cfm DKO mice. We observed increased apoptosis and decreased proliferation in Cfm DKO cartilaginous cells. In addition to direct interaction between Cfm and Filamin proteins in developing chondrocytes, we showed that Cfm is required for the interaction between Flnb and Smad3, which was reported to regulate Runx2 expression. Furthermore, we found that Cfm DKO primary chondrocytes showed decreased cellular size and fewer actin bundles compared with those of wild-type chondrocytes. These results suggest that Cfms are essential partner molecules of Flnb in regulating differentiation and proliferation of chondrocytes and actin dynamics.

INTRODUCTION

Chondrocytes provide an essential function in the skeletal system by producing and maintaining the cartilaginous matrix. In addition, chondrocytes play critical roles at several stages of endochondral ossification in skeletal development. Chondrocyte development is regulated by multiple cell-extrinsic and cell-intrinsic factors, including growth factors and transcription factors (1,2). Interestingly, various studies have shown the importance of actin dynamics for chondrocyte differentiation (3).
Inhibitors of actin polymerization stimulate chondrocyte differentiation in cultured mesenchymal cells and murine embryonic stem cells (4,5). Importantly, abnormalities of the actin cytoskeletal system lead to various human chondrodysplasias. Actin cytoskeleton organization, arranged and rearranged by the assembly and disassembly of actins, is regulated by a large number of actin-binding proteins.

Filamin, an actin-binding protein, plays an important role in skeletogenesis. Filamins function in stabilization of the actin cytoskeleton, linkage of the actin network with cellular membranes and mediation of the interactions between actin and transmembrane receptors (6). In addition to filamentous actin, Filamins have been reported to interact directly with >90 other binding partners with great functional diversity (7). In mammals, the Filamin family consists of three members: Filamin A (Flna), Filamin B (Flnb) and Filamin C (Flnc). The amino acid sequences of Filamins are very similar to each other, and share well-conserved actin-binding domains (8). Flna and Flnb are ubiquitously expressed, although Flnc is restricted to heart and skeletal muscles (9). Filamins are critical for the development of multiple human organs (10,11). Mutations in Flnb were found in five cases of human skeletal malformations including autosomal recessive spindlylocaportarsal syndrome (OMIM:#272460), autosomal dominant boomerang dysplasia (OMIM:#112310), Larsen syndrome (OMIM:#150250), atelosteogenesis type I (OMIM:#108720) and atelosteogenesis type III (OMIM:#108721) phenotypes (11–14). Consistent with abnormalities in human patients with Flna mutation, defects of Flnb in mice produce skeletal malformations, including vertebral fusions, scoliosis, kyphosis and shortening of the distal appendages (15–18). Clustered missense mutations in Flna have been identified in a diverse spectrum of congenital malformations in humans, including otopalatodigital syndrome type 1 (OMIM:#311300), otopalatodigital syndrome type 2 (OMIM:#304120), frontometaphyseal dysplasia (OMIM:#305620) and Melnick-Needles syndrome (OMIM:#309350) (10,19,20). Similar to the abnormalities in human patients with Flna mutations, mice without Flna showed cardiac malformations and skeletal defects of the sternum and palate (21). Based on these observations, the importance of Filamin in skeletal development is well recognized. However, the precise molecular mechanisms underlying Filamin function in skeletal development are largely unknown.

To identify important molecules involved in skeletal system development, we performed a comparative microarray analysis between undifferentiated and differentiation-induced ATDC5 cells, a murine chondroprogenitor cell line (22). We identified multiple uncharacterized genes whose expression significantly increased in differentiated ATDC5 cells. In our previous report, we showed that Obif (Osteoblast induction factor, Tnem119), one of these uncharacterized genes, is essential for bone formation in association with osteoblastogenesis in mice (23). In the same screen, we also identified Cfm2 (Fam101a, Refilina), which encodes a 204 amino acid protein of an unknown function in vivo. A Cfm2 paralog, Cfm1 (Fam101b, RefilinB), is expressed broadly in the developing central nervous system, including the forebrain and midbrain, however, Cfm1 expression in the skeletal system has not been analyzed. Cfm1 homozygous mutant mice (Cfm1<sup>+/−</sup> mice) have been shown to be viable and without any apparent developmental defects (24), probably due to functional redundancies between Cfm1 and Cfm2. To elucidate Cfm functions in vivo, we generated Cfm2 mutant mice by targeted gene disruption. A single gene knockout of Cfm2 showed no obvious phenotype, whereas Cfm1/Cfm2 double-knockout (Cfm DKO) mice displayed severe malformations due to premature ossification. Interestingly, these cytoskeletal defects of Cfm DKO mice were similar to those of Filamin mutant mice. In addition, we found that Cfm proteins co-localize with Flna along actin bundle-like structures in ATDC5 cells. These results led to the idea that Cfm proteins control Filamin functions and suggest that loss of Cfm proteins modify actin dynamics in developing cartilaginous cells.

Our results suggest that Cfm genes regulate cell survival and cell proliferation, and modulate the cyto- and nucleoskeleton through regulating actin dynamics by interacting with Filamins in cartilaginous cells. The current study shows that Cfm genes play an essential role in the formation of cartilaginous skeletal elements, leading to clarification of the molecular basis for Filamin functions in the skeletal system formation.

RESULTS

Expression of Cfm1 and Cfm2

In the course of our microarray screening for genes specifically upregulated upon chondrocyte differentiation (22), we found that mouse Cfm2 transcripts markedly increased in ATDC5 cells upon differentiation. Mouse Cfm2 encodes a 204 amino acid protein with an unknown function in vivo. Hirano et al. identified Cfm1, using a screen to find genes that play roles in initial regionalization of anterior neuroectoderm (24). Mouse Cfm1 encodes a 216 amino acid protein with no known functional domains. There are two conserved domains, termed CR1 and CR2, in both Cfm1 and Cfm2 (24). The CR1 domain of mouse Cfm1 has 100% identity with that of mouse Cfm2. The CR2 domain of mouse Cfm1 has 43% identity with that of mouse Cfm2 (Fig. 1A).

To investigate Cfm2 expression during mouse skeletal development, we performed in situ hybridization analysis using a Cfm2 probe. Cfm2 expression was first detected in the marginal zone of the vertebral primordia at embryonic day 12.5 (E12.5), consistent with Collagen2 (Col2), a gene expressed in proliferating and prehypertrophic chondrocytes (Fig. 1B; Supplementary Material, Fig. S1A). Cfm2 expression was subsequently observed during skeletal development in the cartilaginous elements including the vertebral bodies, carpal bones, femora, ribs and caudal vertebrae (Fig. 1C–G and Supplementary Material, Fig. S1B). At E18.5, Cfm2 expression increased in the layers of proliferating and prehypertrophic chondrocytes (Supplementary Material, Fig. S1C). Furthermore, Cfm2 expression was also observed in intervertebral disc (IVD), including nucleus pulposus (NPs) and annulus fibrosus (AFs), during skeletal development (Fig. 1H and I).

Cfm1 was not expressed in the vertebral primordia at E12.5, but was expressed in the peripheral nerves (Fig. 1J). At E15.5, Cfm1 expression was observed in developing ribs and NPs in IVDs, consistent with Aggrecan, a gene expressed in proliferating chondrocytes and permanent cartilage (Fig. 1K and L; Supplementary Material, Fig. S1B). At E18.5, Cfm1 expression was detected in proliferating and prehypertrophic chondrocytes, consistent with Col2 and Ihh, in vertebral bodies (Fig. 1M and
Figure 1. Structure and expression of Cfm1 and Cfm2. (A) The amino acid homologies between mouse Cfm1 and Cfm2 proteins. The CR1 domain (black) and the CR2 domain (shaded) are shown. The mouse Cfm1 CR1 domain has 100% identity with that of mouse Cfm2. The mouse Cfm1 CR2 domain has 43% identity with that of mouse Cfm2. (B–I) In situ hybridization analysis of Cfm2 at E12.5 (B), E15.5 (C–G), P0 (H) and P4 (I). (B) Cfm2 expression was first detected in the marginal zone of the vertebral primordia at E12.5. Cfm2 expression was observed during skeletal development, including in the vertebral bodies (C), carpal bones (D), femora (E), ribs (F), caudal vertebrae (G), NPs, AFs and VBGP (H and I). (J–N) In situ hybridization analysis of Cfm1 at E12.5 (J), E15.5 (K and L), E18.5 (M) and P0 (N). At E12.5, Cfm1 was first detected in the peripheral nerve (J). Subsequently, Cfm1 expression was found in ribs (K), NPs (L–N), and VBGP (M and N) during skeletal development. NP, nucleus pulposus; AF, annulus fibrosus; VBGP, vertebral body growth plate. Scale bars represent 200 μm (B and J) and 100 μm (C–I, K–N).
Supplementary Material, Fig. S1C). Although we did not detect Cfm1 expression in other developing cartilage, contrary to Cfm2 expression at E15.5, by in situ hybridization analysis, we found that both Cfm1 and Cfm2 were expressed in the adult cartilage using quantitative real-time PCR (Q-PCR) analysis (Supplementary Material, Fig. S2A and B). At postnatal Day 0 (P0), Cfm1 is preferentially expressed within NP in IVD (Fig. 1N). Taken together, these results demonstrated that Cfm1 genes are predominantly expressed in proliferating and prehypertrophic chondrocytes, and in nucleus pulposus cells.

**Single Cfm gene mutants showed no obvious phenotype**

To examine a possible functional role of Cfm2 in development, we generated Cfm2-deficient mice by targeted gene disruption. We deleted the first exon that contains a start codon of the Cfm2 open reading frame (Supplementary Material, Fig. S3A). We confirmed that the 24 kDa full-length Cfm2 protein was undetectable by western blots using an antibody against the Cfm2 N-terminus (residues 1–110) in the femur from Cfm2–/– mice (Supplementary Material, Fig. S3B). Cfm2–/– mice were born in the expected Mendelian ratio and were indistinguishable in appearance from control littermates (Supplementary Material, Fig. S3C and D). Body weight, body length and tibial length at 12 weeks were unaltered between control and Cfm2–/– mice (Supplementary Material, Fig. S3E–G). There was no significant difference in survival between Cfm2–/– and wild-type mice (data not shown).

Cfm1–/– mice were previously established, and it was reported that they exhibited no obvious phenotype in the developing brain (24). As is the case with Cfm2–/– mice, Cfm1–/– mice exhibited no overt phenotype (Supplementary Material, Fig. S3H–L), and survival was not significantly different between Cfm1–/– mice and wild-type mice (data not shown). The lumbar disks from Cfm2–/– and Cfm1–/– mice were unaltered when compared with those of wild-type mice (Supplementary Material, Fig. S3M).

To examine the compensatory expression of Cfm genes in mice development, we analyzed the expression level of Cfm1 in the lung, which expresses substantial levels of Filamins and Cfms, in Cfm2-deficient mice. Although the expression level of Cfm1 in Cfm2-deficient mice was slightly upregulated compared with that of wild-type mice, it was not statistically significant (Supplementary Material, Fig. S4A). Reciprocally, we analyzed the expression level of Cfm2 in the lung in Cfm1-deficient mice compared with that of wild-type mice. We found that the expression level of Cfm2 in Cfm1-deficient mice was also slightly upregulated compared with that of wild-type mice, but was not statistically significant (Supplementary Material, Fig. S4B).

**Cfm DKO mice showed severe skeletal malformations in the spine**

Since we suspected functional redundancies between Cfm1 and Cfm2 for development of the skeletal system, we generated Cfm DKO mice to investigate Cfm function. Cfm DKO neonatal mice had an equal number of male and female mice, and the frequency of each genotype was close to the expected Mendelian ratio (Fig. 2A).

We next examined the effect of the loss of Cfm genes during growth after birth. Newborn Cfm DKO mice showed slightly stunted growth compared with newborn wild-type mice, whereas there was no obvious difference in vertebral columns between control and Cfm DKO mice at P0 using Alcian blue and Alizarin red staining for cartilage (blue) and bones (red) (Fig. 2B). To confirm that the loss of Cfm genes affects skeletal formation, we analyzed Cfm DKO mice at P4 using a micro-computed tomography (mCT) instrument. We observed the small size vertebra, vertebral body osteopenia and shortening of the distance between vertebral bodies (Fig. 2C), and the pups of Cfm DKO mice showed kyphoscoliosis and decreased body weight, and height at P28 in Cfm DKO mice (Fig. 2D and E). The fusions of vertebral bodies were observed throughout the vertebral column in the skeletal preparation at P28 (Fig. 2F). The crown-rump (CR) length, femoral length and width of Cfm DKO mice were significantly shorter than those of wild-type mice at 8 weeks (Fig. 2G). Taking into consideration these results and Cfm gene expression, we focused on the vertebral columns in Cfm DKO mice. In mCT analysis at 8 weeks, the spaces for IVD and intervertebral foramina were absent and the structures of spinous processes could not be identified in Cfm DKO mice (Fig. 2, H and I). Since individual vertebrae could not be distinguished, the vertebral column appeared as one unified block in Cfm DKO mice.

We further examined the process of abnormal vertebra formation at P14. Cfm DKO mice showed kyphosis, and the IVDs were almost absent (Supplementary Material, Fig. S5A and B). The cartilages of sternums in Cfm DKO mice prematurely mineralized at P14 (Supplementary Material, Fig. S5C). In the thoracic region, some of the transverse processes were absent or fused in Cfm DKO mice (Fig. 3A). The mineralization of vertebral bodies was advanced in Cfm DKO mice (Fig. 3B and C). IVDs often showed notable shrinkage in the thoracic regions of Cfm DKO mice (Fig. 3B). The transverse processes were hypoplastic in the lumbar region, and IVDs displayed notable shrinkage throughout this region in Cfm DKO mice (Fig. 3C). The average of lumbar IVD height (L1–L4) in Cfm DKO mice was significantly shorter than that in wild-type mice (Fig. 3D). Histological analysis revealed that NP cells in Cfm DKO mice were decreased remarkably at 3 weeks (Fig. 3E). NPs and AFs of IVDs were diminished, and vertebral bodies were misshapen in Cfm DKO mice at 12 weeks (Fig. 3F). However, thoracic disks and lumbar disks of Cfm1–/–/Cfm2–/– and Cfm1–/–/Cfm2–/– mice were similar to those of wild-type mice at 4 weeks (Supplementary Material, Fig. S6).

To investigate abnormalities in other systems such as cardiovascular, central nervous and urogenital organs, we analyzed brains, hearts and kidneys in Cfm DKO mice at 4 weeks (Supplementary Material, Fig. S7A–F). As far as we examined, there was no substantial difference between wild-type and Cfm DKO mice (Supplementary Material, Fig. S7A, C and E). To further investigate tissue abnormalities, we analyzed sections of the brain, heart and kidney, in Cfm DKO mice, and confirmed that there was no substantial difference between wild-type and Cfm DKO mice (Supplementary Material, Fig. S7B, D and F). Moreover, there was no significant difference in survival between wild-type and Cfm DKO mice (data not shown).

**Chondrocyte maturation is accelerated in Cfm DKO mice**

To precisely determine if abnormal mineralization results from the absence of Cfm genes, we analyzed the expression of skeletal
Figure 2. Cfm DKO mice are small and have fused vertebrae. (A) In the intercross of Cfm1^{1/-} mice and Cfm2^{2+/-} mice, the frequency of Cfm DKO mice was close to the expected Mendelian ratio. (B) Newborn Cfm DKO mice showed slightly stunted growth compared with wild-type mice. (C) The small size vertebra, vertebral body osteopenia, and shortening of the distance between vertebral bodies were observed in Cfm DKO mice at P4 using micro-computed tomography (mCT). (D) Cfm DKO mice were smaller compared with wild-type mice at P28. (D and E) The body weight and height of Cfm DKO mice were significantly smaller than those of wild-type mice at P28 (n = 3), and severe scoliosis is indicated in Cfm DKO mice (D, arrow). (F) Fusions were present throughout the vertebral column of Cfm DKO mice. Severe scoliosis was observed. Skeletal preparations were stained with Alcian blue (cartilage) and Alizarin red (bone). (G) The CR length, femoral length and femoral width of Cfm DKO mice were significantly shorter than those of wild-type mice at 8 weeks (n = 5). (H–I) mCT analysis revealed severe vertebral column abnormalities in Cfm DKO mice at 8 weeks. Cfm DKO mice exhibited severe malformations in the vertebral column, including vertebral fusions, scoliosis (H) and kyphosis (I). The spaces for IVD are absent in both ventral and lateral views (H, arrowheads and I, asterisks). Intervertebral foramina are also absent in Cfm DKO mice (I, arrows). Some of the spinous processes are absent in the lumbar region (I, arrowheads). DKO, Cfm DKO mice; CR length, crown-rump length. Staining was with Alcian blue (cartilage) and Alizarin red (bone) (B and F). Scale bars represent 2 cm (B and D) and 3 cm (F). Error bars show the SEM. *P < 0.03.
hypoplastic in the lumbar region (C, arrowheads), and IVDs displayed notable shrinkage throughout this region in mice (B, arrowheads and C, asterisks). IVDs often showed notable shrinkage in the thoracic regions of some of the transverse processes were absent (A, arrows) or fused (A, asterisks) in mice (B, arrowheads) and lumbar IVD height. Scale bars represent 50 μm (E) and 200 μm (F). Error bars show the SEM.

**Complete loss of Cfm genes affects chondrogenesis in the growth plate and bone growth**

Since Cfm genes were strongly expressed in growth plates of the longitudinal bones and vertebral bodies and Cfm DKO mice showed growth retardation (Fig. 1E, H, I, M, N and Fig. 2D–G), we examined a possible role of Cfm genes in chondrocytes and bone growth. The CR length and tibial length of Cfm DKO mice were significantly shorter than those of wild-type, Cfm+/−/Cfm2−/− and Cfm1−/−/Cfm2−/− mice at 4 weeks (Fig. 5A). Histological analysis of proximal tibiae from 4-week-old mice revealed that a 20–30% reduction in growth plate thickness in Cfm DKO mice (Fig. 5B and C). Although the parameters of the resting zone (RZ) in Cfm DKO mice were unaltered from those of wild-type mice, the thickness of the proliferating zone (PZ) and the hypertrophic zone (HZ) were reduced in Cfm DKO mice (Fig. 5B and C). Proliferating cells were properly oriented along the long axis of the bone, whereas the number of cells and cells per column were reduced in Cfm DKO mice (Fig. 5B and D). A reduction of the number of hypertrophic cells was observed in Cfm DKO mice (Fig. 5D).

**Cfm DKO mice exhibited decreased bone formation**

Since the bone of vertebral bodies showed a moth-eaten appearance in Cfm DKO mice at P21 in mCT analysis (Supplementary Material, Fig. S8A), we investigated Cfm gene expression in the mouse bone tissue. We observed that Cfm genes were expressed at low level in the mouse calvariae using Q-PCR analysis (Supplementary Material, Fig. S2A and B). To assess the effect of Cfm gene loss for bone formation, we performed a quantitative mCT analysis. The bone volume/tissue volume was unaltered between wild-type mice and Cfm DKO mice. However, the trabecular number of Cfm DKO mice significantly decreased, whereas the trabecular separation of Cfm DKO mice significantly increased compared with that of wild-type mice (Supplementary Material, Fig. S8B and C). Therefore, Cfm1 and Cfm2 may also have some functions in osteoblasts or osteoclasts.
**Cfm genes are required for cartilaginous cell proliferation and apoptosis**

The reduced number of cartilaginous cells including NP cells, AF cells and chondrocytes suggested that Cfm1 and Cfm2 might protect against apoptosis. To assess this possibility, we performed TUNEL assays using the spines of Cfm DKO mice at P28. TUNEL-positive cells increased remarkably in the NP and AF, and moderately in the VBGP of Cfm DKO mice (Fig. 6A–G). These results suggest that increased apoptosis in Cfm DKO mice cause defects of the IVD and of growth plate development. We investigated the effect of Cfm gene loss on cell proliferation rates using primary chondrocytes from the rib growth plates of Cfm DKO mice at P24. The proliferation rate in Cfm DKO chondrocytes significantly decreased compared with that in control chondrocytes (Fig. 6H).

**Cfms interact with Filamins in chondrocytes**

To identify the mechanism underlying Cfm-mediated skeletogenesis, we performed a yeast two-hybrid screen using a cDNA library prepared from mouse whole embryo at E11 and a full-length Cfm2 construct as bait. In this screen, we found that several independent clones encoding Flna interact with the Cfm2 bait, consistent with a recent study that reported that Cfm1 and Cfm2 bind to Filamins (Flna, Flnb and Flnc) (25). In addition, Filamin-deficient mice (Flna and Flnb-deficient mice) displayed skeletal malformations that are similar to Cfm DKO phenotypes (15–17,21). Filamin proteins regulate the cytoskeletal network by cross-linking actins and integrate cell signaling, transcription and organ development (26). Flna and Flnb are expressed in chondrocytes (11). Our results and data from a previous study proposed the possibility that the interaction of Cfm and Filamin proteins is important for skeletal development. To investigate this possibility, we focused on Filamin and Cfm function in chondrocytes. First, to investigate the subcellular localization of Cfm and Filamin proteins, we transfected plasmids expressing FLAG-tagged Cfm1 or Cfm2 along with plasmids-expressing HA-tagged Flna into a chondroprogenitor cell line, ATDC5. We found that Cfm proteins co-localized with Flna along actin bundle-like structures in ATDC5 cells.

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**Figure 4.** Premature ossification is attributed to chondrocyte hypertrophy in Cfm DKO mice. (A and B) Transcripts of the markers for endochondral ossification were detected by in situ hybridization in sections of the sterna and ribs in wild-type and Cfm DKO mice at P1 (A) and P4 (B). (A) Arrowhead indicates the disappearance of Col2 in Cfm DKO mice. The chondrocytes between sternebrae 3 and 4 are undergoing terminal differentiation in Cfm DKO mice, as demonstrated by the fusion of the Col10-expressing cell layers (arrow). (B) OPN was strongly expressed in Cfm DKO mice (arrowhead). Arrows show precocious expression of OPN, Runx2 and Col1 in Cfm DKO mice. (C) Schematic representation of precocious ossification of the sternum in Cfm DKO mice. (D) The sternum in Cfm DKO mice at P5 stained with toluidine blue. Arrow indicates the area was replaced with bone. DKO, Cfm DKO mice. Scale bars represent 100 μm (A and D) and 200 μm (B).
To examine the expression level of Filamin in Cfm deficiency, we analyzed the lungs of Cfm1<sup>2</sup>/2, Cfm2<sup>2</sup>/2 and Cfm DKO mice using Q-PCR. However, the expression level of Flna and Flnb was unaltered between wild-type and Cfm-deficient mice (Supplementary Material, Fig. S9A and B).

Loss of Cfm genes affects actin bundle formation and cell morphology of chondrocytes

Our results demonstrated that Cfm1 and Cfm2 regulate chondrocyte survival and proliferation, and interact with Filamins. Cfms interacted with Filamins to organize perinuclear actin networks and regulated nuclear shape (25,27). Therefore, we examined whether Cfm1 and Cfm2 play intrinsic roles in actin filament networks and nuclear shape. We found that primary rib chondrocytes from wild-type mice often formed actin bundles, whereas those from Cfm DKO mice formed fewer actin bundles (Fig. 7D). We found that the cell surface area and the length of the nuclear long axis of chondrocytes from Cfm DKO mice significantly decreased compared with those of wild-type mice (Fig. 7E). These results suggest that Cfm promotes actin bundles and regulates cell shape through interaction with Filamins in chondrocytes.

Cfms are involved in the interaction of Flnb and Smad3

Since Flnb interacts with Smad3 and inhibits phosphorylation of Smad3, phospho-Smad3 increases in Flnb-deficient mice, leading to Runx2 hyperactivity (18). Thus, fewer phospho-Smad3-HDAC4 complexes bind Runx2, and abnormal differentiation of chondrocytes occurs in Flnb-deficient mice (18). This report and results in the current study raise the possibility that Cfm proteins associate with Flnb, influence the interaction between Flnb and Smad3, and regulate Runx2 function. To assess this hypothesis, we first performed an immunoprecipitation assay using primary chondrocytes isolated from ribs of P3 wild-type and Cfm DKO mice. We found that Cfm DKO rib chondrocytes contain less Flnb-Smad3 complex than those of wild-type chondrocytes (Supplementary Material, Fig. S10). This result suggests that Cfm is required for the interaction
between Flnb and Smad3 in chondrocytes, and that the Flnb-Cfm-Smad3 complex may play an important role in chondrogenesis. Next, based on the previous report on Flnb mutant mice (18), we examined the amount of phosphorylated Smad3 in Cfm DKO chondrocytes. The amount of phosphorylated Smad3 in Cfm DKO chondrocytes was unaltered when compared with that in wild-type chondrocytes (Supplementary Material, Fig. S10). These results suggest the presence of Smad3-mediated and non-Smad3-mediated mechanisms for skeletal abnormalities in Cfm DKO mice. For instance, fewer actin bundles might explain the abnormalities in Cfm DKO mice.

Cfm-deficient fibroblast had no effect on cellular migration during embryonic development

Cell migration is an actin-dependent process. We tested whether the absence of Cfm would have an influence on cellular migration. The migration degree of primary fibroblasts isolated from E14.5 embryos was unaltered between wild-type mice and Cfm DKO mice using the Boyden chamber assay (Supplementary Material, Fig. S11A). Morphology of Flna and actin filament of primary fibroblasts was unaltered between wild-type and Cfm DKO mice (Supplementary Material, Fig. S11B). These observations suggest that the absence of Cfm genes had no effect on fibroblast cellular migration during embryonic development.

DISCUSSION

In the current study, we found by microarray analysis that Cfm2 gene expression increased during chondrocyte differentiation and that Cfm2 is expressed predominantly in cartilaginous tissues, including the developing IVD and cartilage, by in situ hybridization.

Cfm2 encodes a 204 amino acid protein without a known functional domain, showing high homology with Cfm1 protein. This indicates that Cfm1 and Cfm2 genes are paralogous. Both Cfm1 and Cfm2 are evolutionarily well conserved among humans, mice, chickens, Xenopus and zebrafish. There are two motifs termed CR1 and CR2, which are conserved throughout these species, in both Cfm1 and Cfm2 proteins (24).

To investigate the biological function of Cfm2 during skeletal development, we generated Cfm2-null mice by targeted gene disruption. Cfm2-null mice displayed no overt phenotype. It was previously reported that Cfm1 was uniquely expressed in the developing forebrain and midbrain, however, Cfm1 expression in the skeletal system has not been analyzed (24). In the current study, we found that Cfm1 is also expressed in cartilaginous tissues in the vertebral cartilage and IVD during skeletal development. Since we assumed functional redundancies between Cfm1 and Cfm2, we generated Cfm double-mutant mice. Cfm DKO mice exhibited severe skeletal malformations, as characterized by scoliosis, kyphosis, IVD defects, vertebral fusion in the spine and longitudinal bone growth retardation. We showed that these malformations are due to accelerated chondrocyte maturation, reduced chondrocyte proliferation and increased apoptosis in chondrocytes.

How do Cfm proteins play a role in skeletal formation? We found that Cfm proteins interact with Filamin, consistent with data published recently (25). Filamin is a ubiquitously expressed actin-binding protein that has been implicated in many cellular processes including cell proliferation, cell migration and signaling pathways that mediate organogenesis in multiple tissues (8,28). We examined the phenotypic similarity between Cfm DKO mice and Filamin-deficient mice. We observed that Cfm DKO mice displayed skeletal abnormalities similar to the malformations in Flnb-deficient mice related to scoliosis, kyphosis, vertebral fusion in the spine and shortening of the distal appendages (15–18). In addition, the malformations in Cfm DKO mice are similar to those in mutational congenital anomalies of Flnb including autosomal recessive spondyloepatrotarsal syndrome, autosomal dominant boomerang dysplasia, Larsen syndrome and atelosteogenesis I and III phenotypes (11–14,29,30). These results suggest the possibility that Cfm and Flnb complex function mainly in skeletogenesis. Contrarily, there are phenotypic similarities and differences between Filamin-deficient mice and Cfm DKO mice. Flna mutant mice showed cardiac and skeletal malformations, including sternum and palate defects (21). Multiple lines of evidence showed that Flna deficiency in humans disrupts not...
only skeletal development but also development of other tissues including the neuronal and cardiac systems. Missense mutations in human \textit{Flna} produced the severe thoracic hypoplasia, irregular ribs and scoliosis known as Melnick-Needles syndrome (19). Null mutations in human \textit{Flna} cause X-chromosome-linked brain abnormalities known as periventricular nodular heterotopia (PVNH; OMIM:#300049). Neurons in PVNH patients fail to undergo radial migration from the ventricular zone to form the six-layered neocortex during fetal development (26,31). In contrast, we observed that \textit{Cfm} DKO mice exhibited only skeletal malformation. Thus, these data suggest that \textit{Cfm} proteins function with \textit{Flnb} proteins predominantly in the developing skeletal system.

Transforming growth factor-beta (TGF-\(\beta\)) is one of the key regulators in skeletal development, and Smad2 and Smad3 mediate intracellular signaling of TGF-\(\beta\) (32). It has been reported that \textit{Flnb} binds to diverse proteins, including Integrin \(\beta1\) and Smad3 (16,18). \textit{Flnb} interacts with Smad3, inhibiting phosphorylation of Smad3, and leading to suppression of chondrogenesis (18). This previous study indicated that \textit{Flnb} normally prevents excessive Smad3 phosphorylation. In the current study, we examined the possibility that \textit{Smad3} is unable to interact with \textit{Flnb} in \textit{Cfm}-deficient chondrocytes; however, the \textit{Smad3} phosphorylation level in \textit{Cfm}-deficient chondrocytes was unaltered as compared with that in wild-type chondrocytes.

\textit{Smad3}-deficient mice showed malformations including kyphosis, and decreased proteoglycan and collagen content in the IVDs; however, those mice did not exhibit scoliosis and vertebral fusions, which are observed in both \textit{Flnb}-deficient mice and \textit{Cfm} DKO mice (15–18,33). It was reported that TGF-\(\beta1\) stimulation upregulated \textit{Cfm1} protein expression, but \textit{Cfm2} protein and mRNA were below detection level in an epithelial cell line (25). In contrast, our current study shows that both \textit{Cfm1} and \textit{Cfm2} proteins cooperatively function in chondrogenesis and IVD formation in vivo, therefore, loss of the \textit{Cfm} genes perturb cartilaginous cell development. Further studies are necessary to determine the exact roles of \textit{Flnb}, Smads and \textit{Cfms} in the TGF-\(\beta\) pathway in skeletal development.

\textit{Cfm1} protein was implicated in the regulation of the perinuclear actin network and nuclear shape through interaction with \textit{Flna} from a study using cultured mouse NIH 3T3 fibroblasts (25,33). In the current study, the primary chondrocytes isolated from \textit{Cfm} DKO mice showed fewer actin filament bundles, a decrease of the cell surface area, and shortening of the nuclear long axis compared with those of the primary chondrocytes isolated from wild-type mice. The data presented in this study and previous reports raise the possibility that \textit{Cfm} proteins associate with actin filaments, and loss of \textit{Cfm} proteins in cells reduces the level of filamentous actin in chondrocytes. Our results suggest that \textit{Cfm} proteins

**Figure 7.** \textit{Cfms} interact with Filamins in chondrocytes and control actin dynamics in chondrocytes. (\textbf{A} and \textbf{B'}) Co-localization of \textit{Cfm1}, \textit{Cfm2} and \textit{Flna} in mouse chondrogenic ATDC5 cells. Constructs expressing FLAG-tagged \textit{Cfm1} and \textit{Cfm2} were transfected into ATDC5 cells with constructs expressing HA-tagged \textit{Flna}. Localization of FLAG-tagged proteins was observed using an anti-FLAG antibody (green), an anti-HA antibody (red) and DAPI (blue). \textit{Cfm1} and \textit{Cfm2} co-localized with \textit{Flna} in mouse chondrogenic ATDC5 cells (\textbf{A'} and \textbf{B'}, arrows). (\textbf{C}) \textit{Flna} and \textit{Flnb} interact with \textit{Cfm2} and \textit{Cfm1} in primary chondrocytes. Endogenous \textit{Flna} was immunoprecipitated with an anti-\textit{Flna} antibody. Immunoprecipitated \textit{Cfm2}, \textit{Cfm1} and \textit{Flnb} were detected by western blot analysis using anti-\textit{Cfm2}, anti-\textit{Cfm1} and \textit{Flnb} antibodies. Endogenous \textit{Flnb} was immunoprecipitated with an anti-\textit{Flnb} antibody. Immunoprecipitated \textit{Cfm2}, \textit{Cfm1} and \textit{Flnb} were detected by western blot analysis using anti-\textit{Cfm2}, anti-\textit{Cfm1} and \textit{Flnb} antibodies. (\textbf{D} and \textbf{E}) Immunofluorescent analysis of primary cultured chondrocytes of ribs from wild-type and \textit{Cfm} DKO mice at 8 weeks. Primary rib chondrocytes from wild-type mice often formed actin bundles, whereas those from \textit{Cfm} DKO mice less formed actin bundles. (\textbf{D}) Cells were stained with rhodamine phalloidin (red) and DAPI (blue). (\textbf{E}) Cell surface area and length of nuclear long axis of chondrocytes from \textit{Cfm} DKO mice significant decreased compared with those of wild-type mice (WT chondrocytes; \(n=122\), \textit{Cfm} DKO chondrocytes; \(n=88\)). DKO, \textit{Cfm} DKO mice. Scale bars represent 5 \(\mu\)m (\textbf{A} and \textbf{B'}) and 10 \(\mu\)m (\textbf{D}). Error bars show the SEM. *\(P<0.03\).
control Filamin-mediated stabilization of actin filaments in chondrocytes.

We observed abnormal skeletogenesis in Cfm DKO mice. How does loss of Cfm genes affect the development of the skeletal system? We observed that TUNEL-positive cells increased in the cartilaginous cells including NP cells, AF cells and chondrocytes in the Cfm DKO mice. These results are reminiscent of the increasing chondrocyte apoptosis in Flnb-deficient mice (16). In addition, we found that primary chondrocytes in Cfm DKO mice exhibit decreased proliferation rate. Our results suggest that the balance of proliferation and apoptosis was disrupted, followed by the perturbation of cartilaginous cell development in Cfm DKO mice.

Our results showed that in the absence of Cfm1 and Cfm2, the stabilization of actin networks, the regulation of cartilaginous cell proliferation and survival are impaired, and cartilaginous skeletal formation is retarded. Thus, both Cfm1 and Cfm2 play critical roles in the molecular function of Filamin complex for skeletal formation is retarded. Thus, both Cfm1 and Cfm2 play critical roles in the molecular function of Filamin complex for

Moreover, lamin A/C is connected to the cytoskeleton through linkers of the nucleoskeleton to the cytoskeleton (LINC) complexes (36,37), and potentially link the actin cytoskeleton to lamin A (38). Lmna mutant mice displayed skeletal defects consistent with Hutchinson-Gilford progeria syndrome (OMIM: #176670), including a marked reduction in growth rate, osteoporosis and kyphosis (39–41). These malformations are similar to Cfm DKO mice defects. Gay et al. proposed a hypothetical model of actin perinuclear structure stabilization by the Cfm/Flna/Actin/LINC complex (27). Our results suggest that the Cfm1 and Cfm2/Filamins/Actin/LINC/lamins complex plays an important role in the formation of nucleoskeleton during cartilaginous tissue formation and chondrogenesis. Thus, our current study elucidates the molecular mechanisms of Filamins in skeletal development in vivo. In future studies, it will be important to assess how Cfm proteins coordinate and maintain the actin-binding partners.

MATERIALS AND METHODS

In situ hybridization

In situ hybridization was performed as described previously (42) with a probe containing a 910 bp fragment of Cfm2 cDNA amplified using primers 5′-AGATCTCTAGGTAGCTCCAGTGC-3′ and 5′-TGGGATCCATCATACGAAAGGGAAGA-3′, and a 990 bp fragment of Cfm1 cDNA amplified using primers 5′-AGAGAAAAAGGGAAGGCTCCAGTCT-3′ and 5′-AGGCTCTACCCTGGCCAAGA-3′. We used cartilaginous gene primer sets described previously (43).

Q-PCR analysis

Q-PCR analysis was performed using SYBR Green ER Q-PCR Super Mix (Invitrogen) and Thermal Cycler Dice Real Time System single MRQ TP870 (Takara Bio) according to the manufacturer’s instructions. Quantification was performed by Thermal Cycler Dice Real Time System software Ver. 2.0 (Takara Bio). The following sets of PCR primers were used: 5′-GACACCCCGCAGCAATAGACTG-3′ (Cfm1, left) and 5′-GGGGGATTTGGAGAGGAGAGG-3′ (Cfm1, right), 5′-TGCCCATCCGGCCTGAGCCGGA-3′ (Cfm2, left) and 5′-CGCTACCTACATGGTGCAGATGTC-3′ (Cfm2, right), 5′-ACCAGAATCTACAAAGAAGG-3′ (Flnb, left) and 5′-GGCGTATGGGTATCTACCAGTGAAT-3′ (Flnb, right), 5′-ATCTCCGCTAGCCCTCCTACTGTC-3′ (Flna, left) and 5′-GTTTCTCATTGCAAAACCCCTTG-3′ (Flna, right), and 5′-ACTG GCATGGCCTCAGTGGTCTCTA-3′ (GAPDH, left) and 5′-TCAGTACCCAGATTCGCCCTTC-3′ (GAPDH, right).

Generation of Cfm2 mutant mice

We obtained Cfm2 genomic DNA clones from a screen of the 129S6 (129/SvEv Taconic) mouse genomic DNA library (Stratagene). We subcloned a 7.5 kb SpI–EcoRI fragment and a 6.0 kb SpI–EcoRI fragment from Cfm2 genomic clones into a modified pPNT vector (44,45), and transfected the linearized targeting construct into the 129S6 embryonic stem cell line. The culture, electroporation and selection of 129S6 were carried out as previously described (46). Embryonic stem cells that were heterozygous for the targeted gene disruption were microinjected into C57BL/6 blastocysts to obtain chimeric mice.

Anti-Cfm2 antibody production

By using PCR, a cDNA encoding the N-terminal portion (residues 1–110) of mouse Cfm2 (N-Cfm2) was amplified and subcloned into pGEX4T-1 (GE Healthcare). The fusion was expressed in Escherichia coli strain DH5α and purified with glutathione Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions. An antibody against N-Cfm2 was obtained by immunizing rabbits with the purified GST-N-Cfm2. The rabbit antiserum against N-Cfm2 was pre-absorbed with GST-Sepharose and affinity purified with an immunizing fusion protein-bound Sepharose column.

Genotyping of wild-type and mutant type allele

Genotyping was performed by PCR, using primers to detect the Cfm2 wild-type allele (5′-CCCCCTCCCACTTTTGGGCA ACT-3′ and 5′-GAACCCAGGGTCAAGATCTGCCCT-3′), which amplify a 261 bp fragment, and the Cfm2 mutant allele (5′-CCCCCTCCCACTTTTGGGCAACT-3′ and 5′-GCCAA GCTGGTATTGGCCCTG-3′), which produce a 434 bp band. Genotyping was performed by PCR, using primers to detect the Cfm1 wild-type allele (5′-TACACAGTGAATGCGAGGGCC AGCT-3′ and 5′-CTGTCATACGCTGGTACTGCT-3′), which amplify a 530 bp fragment, and the Cfm1 mutant allele (5′-TACACAGTGAATGCGAGGGCCAGCT-3′ and 5′-GCCCT TCTATCGCTTGACAGGT-3′), which produce a 638 bp band.

Histological analysis

To prepare sections for toluidine blue (Sigma-Aldrich) and safranin-O (Nacalai Tesque) staining, we fixed mice with 4% paraformaldehyde in PBS buffer, decalcified vertebral columns and
limbs in Morse’s solution (47) and embedded in Tissue-Tec Optimum Cutting Temperature. Frozen sections (18–25 μm thick) were stained with toluidine blue, and subjected to histochemical analysis. Analysis of the growth plates was performed as described by previously (48). To prepare sections of the brain, heart and kidney, we fixed the tissues with 4% paraformaldehyde in PBS buffer and embedded in Tissue-Tec Optimum Cutting Temperature. Frozen sections (10 μm thick) were stained with toluidine blue and subjected to histochemical analysis.

Generation of Cfm DKO mutant mice

The Cfm DKO mice were generated by intercross of Cfm1−/− and Cfm2−/− mice (Acc. No. CD80025K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) in the C57BL/6 genetic background (49) and Cfm2 heterozygous mutant (Cfm2+/−) mice in the 129/SvEv genetic background, or intercross of Cfm2−/− mice in the 129/SvEv genetic background and Cfm1 heterozygous mutant (Cfm1+/−) mouse in the C57BL/6 genetic background.

Staining of bone and cartilage

After euthanasia, mice were skinned, and all tissues were eviscerated. Mice were fixed in 95% ethanol for 3–5 days, and then stained with 0.15% Alcian blue (Sigma A3157) dissolved in glacial acetic acid plus 75% ethanol and with 0.5% Alizarin red (Sigma A5533) dissolved in 2% KOH.

mCT analysis

Quantitative mCT analysis was conducted as described previously (43). Briefly, Quantitative mCT was performed with an mCT system (μCT-40; ScancoMedical). CT scans were performed at the distal metaphysis to calculate trabecular parameters, and at mid-shaft to calculate cortical thickness in 8-week-old mice. The craniocaudal scan lengths were 1.2 mm in 8-week-old mice in the distal metaphysis, and the scan length was 0.24 mm at the mid-shaft. The analysis of the vertebral columns was performed with an RmCT system (Rigaku). The exposure parameters were 90 kV and 100 μA, or 90 kV and 150 μA.

TUNEL assay

Frozen vertebral columns were sectioned to a thickness of 20–30 μm and fixed with 4% paraformaldehyde in PBS for 15 min. The TUNEL assay was performed using Click-iT® TUNEL Alexa Fluor® imaging Assay (Invitrogen) according to the manufacturer’s protocol.

Primary cultured chondrocytes preparation and proliferation assay

Primary cultured chondrocytes were isolated from growth plates of the rib cartilage from P24 wild-type mice and Cfm DKO mice using a modification of the previously published protocol (50). Briefly, chondrocytes were isolated using 0.2% collagenase P (Roche). Isolated cells were maintained in RPI-1640 (Sigma-Aldrich) supplemented with ascorbic acid (50 μg/ml) and 10% FBS. We seeded them in a 12-well plate at a density of 8 × 10^5 cells per well for the proliferation assay. At each time point, cells were harvested and counted. Duplicate measurements were performed on three-independent wells for each time point.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed as described previously (51). Briefly, the ORF of the mouse Cfm2 cDNA (full length) was inserted into the pGBK7 bait vector and transformed into the AH109 yeast strain. We screened 3.5 × 10^6 transformants from a mouse E11 cDNA library (Takara Bio) according to manufacturer’s instructions for the Matchmaker System 3 (Takara Bio).

Plasmid constructs

A plasmid-encoding full-length Cfm2 was amplified using a Fam101a clone (3110032G18Rik; GenBank accession No. NM028443) as a template and inserted into the N-terminal FLAG-tagged pCAGGSII expression vector to produce pCAG-FLAG-Cfm2. A plasmid-encoding full-length Cfm2 was amplified using a Fam101b clone (150005K14Rik; GenBank accession No. NM029658) as a template and inserted into the N-terminal FLAG-tagged pCAGGSII expression vector to produce pCAG-FLAG-Cfm1. A plasmid-encoding full-length Flna was amplified using an Flna clone (IMAGE clone C25A9), rabbit polyclonal anti-Cfm1 (Scorpion Biotechnology, Inc., N-16), rabbit polyclonal anti-Filamin A (Abcam, ab51217), goat polyclonal anti-Filamin B (Santa Cruz Biotechnology, Inc., N-16), rabbit monoclonal anti-HA (Roche, 3F10), rabbit polyclonal anti-Flag (Sigma-Aldrich), rabbit polyclonal anti-Flag (Sigma-Aldrich) and 10% FBS. We seeded them in a 12-well plate at a density of 8 × 10^5 cells per well for the proliferation assay. At each time point, cells were harvested and counted. Duplicate measurements were performed on three-independent wells for each time point.

Cell culture and transfection

ATDC5 cells were maintained in F-12 with 5% FBS, 10 μg/ml human transferrin (Sigma-Aldrich) and 3 × 10^{-8} M sodium selenite (Sigma-Aldrich). Transfection of plasmid DNA was performed using Lipofectamine LTX (Invitrogen) for ATDC5 cells according to the manufacturer’s instruction. Cells were cultured for 24 h. For immunostaining, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min at room temperature, and subsequently incubated with blocking solution for 1 h. Cells were immunostained with a primary antibody in blocking solution for 2 h at room temperature and subsequently incubated with a secondary antibody solution for 1 h at room temperature. Alexa Fluor 488 (1:500) or Cy3 (1:400)-conjugated IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies.

Antibodies

The following primary antibodies were used for immunostaining: mouse monoclonal anti-FLAG M2 (Sigma-Aldrich, F1804), rat monoclonal anti-HA (Roche, 3F10), rabbit polyclonal anti-Filamin A (Abcam, ab51217), goat polyclonal anti-Filamin B (Santa Cruz Biotechnology, Inc., N-16), rabbit monoclonal anti-Smad3 (Cell Signaling Technology, C67H9), rabbit monoclonal anti-phospho-Smad3 (Ser423/425) (Cell Signaling Technology, C25A9), rabbit polyclonal anti-Cfm2 and guinea pig polyclonal anti-Cfm1.
Anti-Cfm antibody production
An antiserum against the N-terminal portion (residues 39–142) of mouse Cfm1 (N-Cfm1) was raised by immunizing guinea pig with purified GST-N-Cfm1, then, the antiserum were reacted with the fusion protein-coupled CNBr-activated Sepharose 4B (GE Healthcare). This sepharose 4B was washed with PBS and eluted with 0.1 M glycine buffer (pH2.5) to obtain a purified antibody against N-Cfm1. The eluted antibody was neutralized by 20 × PBS, and further dialyzed in PBS at 4°C.

Immunoprecipitation and western blotting
For the immunoprecipitation and immunoblotting for the interaction between endogenous Cfm and Filamins proteins, protein samples from E18.5 chondrocytes in vertebral bodies were lysed in a lysis buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1% NP40, one protease inhibitor cocktail tablet (Roche)). Lysates and antibodies were incubated for 16 h with protein G-Sepharose (GE Healthcare), washed four times with wash buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1% NP40) and resolved by SDS–PAGE. Western blot analysis was performed using a semidry transfer cell (iBlot system; Invitrogen) with iBlot Gel Transfer Stack PVDF (Invitrogen). Signals were detected using ECL Plus Western Blotting Detection System (GE Healthcare). For the immunoprecipitation and immunoblot analysis of the interaction between endogenous Smad3 and Flnb proteins without Cfm proteins, primary chondrocytes from rib cages of wild-type and Cfm DKO mice at P3 were isolated using 0.2% collagenase P (Roche) and were seeded in a 6 cm dish. Analysis and detection procedures were mentioned above.

Morphometric measurement and immunofluorescence microscopy
Primary cultured chondrocytes were isolated from growth plates of the rib cartilage of wild-type and Cfm DKO mice at 8 weeks. Cells were spread on a 3.5 cm dish and incubated for 48 h. Immunocytostaining was performed as described above. To visualize actin filaments, cells were incubated with phalloidin Rhodamine (Cytoskeleton, Inc). DAPI (Sigma-Aldrich) was applied to stain nuclei. The specimens were observed under a laser confocal microscope (LSM710, Carl Zeiss). Cell surface area and nuclear long axis were measured using AxioVision rel.4.8 (Carl Zeiss).

Migration assay
Primary fibroblasts were isolated from Cfm DKO and wild-type embryos at E14.5. Migration assays were performed on cells in passages 2–3. Cell migration was examined using BD Falcon Cell Culture Insert containing polycarbonate filters with 8 μm pore size (BD Sciences), essentially as described (15). 3 × 10^4 cells were seeded in the upper chamber in 2% FBS DMEM, and the chamber was lowered into a well containing 10% FBS DMEM, used as a chemoattractant. After 4 h of incubation at 37°C, the filters were collected and cells migrating to the lower surface were fixed in methanol, stained with Giemsa’s azure eosin methylene blue solution (MERCK) and counted by using a light microscope.

Statistics
Statistical analysis was performed using Student’s t-test for comparisons between two groups and analysis of variance with Tukey–Kramer test for comparisons among three groups, unless otherwise described. All data are expressed mean ± SEM.

Study approval
All procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments and the Animal Research Committee of Osaka Bioscience Institute, and by the Recombinant DNA Committee (3380-3) and the Animal Care Committee (24-05-1) of Osaka University.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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