Formin 1 and Filamin B physically interact to coordinate chondrocyte proliferation and differentiation in the growth plate

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Received December 23, 2013; Revised March 20, 2014; Accepted April 17, 2014

Filamin B (FlnB) is an actin-binding protein thought to transduce signals from various membrane receptors and intracellular proteins onto the actin cytoskeleton. Formin1 (Fmn1) is an actin-nucleating protein, implicated in actin assembly and intracellular signaling. Human mutations in FLNB cause several skeletal disorders associated with dwarfism and early bone fusion. Mouse mutations in Fmn1 cause aberrant fusion of carpal digits. We report here that FlnB and Fmn1 physically interact, are co-expressed in chondrocytes in the growth plate and share overlapping expression in the cell cytoplasm and nucleus. Loss of FlnB leads to a dramatic decrease in Fmn1 expression at the hypertrophic-to-ossification border. Loss of Fmn1-FlnB in mice leads to a more severe reduction in body size, weight and growth plate length, than observed in mice following knockout of either gene alone. Shortening of the long bone is associated with a decrease in chondrocyte proliferation and an overall delay in ossification in the double-knockout mice. In contrast to FlnB null, Fmn1 loss results in a decrease in the width of the prehypertrophic zone. Loss of both proteins, however, causes an overall decrease in the width of the proliferation zone and an increase in the differentiated hypertrophic zone. The current findings suggest that Fmn1 and FlnB have shared and independent functions. FlnB loss promotes prehypertrophic differentiation whereas Fmn1 leads to a delay. Both proteins, however, regulate chondrocyte proliferation, and FlnB may regulate Fmn1 function at the hypertrophic-to-ossification border, thereby explaining the overall delay in ossification.

INTRODUCTION

Filamins are actin-binding proteins with multiple receptor and intracellular interactors that serve to regulate cytoskeleton-dependent cell proliferation, differentiation and migration (1–6). Null alleles of Filamin B (FLNB loss of function) in humans cause recessive spondylocarpotarsal synostosis syndrome (SCT; OMIM 272460), characterized by dwarfism and premature fusion of the vertebral, carpal and tarsal bones (7). Autosomal dominant mutations of FLNB (gain of function including missense, in-frame deletions or insertions) cause a group of skeletal dysplasias, including Larsen syndrome (LS; OMIM 150250), atelosteogenesis I and III (AOI and AOIII; OMIM 108720 and 108721) and boomerang dysplasia (BD; OMIM 112310) (8–10). AOI, AOIII and BD exhibit severe phenotypes and often feature undermodeled bones or ossification initiation failure (7,9,10).

Loss of FlnB function in mice mirrors the two major skeletal phenotypes seen in humans. Mice develop dwarfism with delayed bone formation in the long bones and early bone fusion of the vertebral, carpal and tarsal bones (11–14). Our recent work has suggested that FlnB inhibition impairs chondrocyte proliferation, thereby providing an explanation for the slowing of skeletal development and shortened stature. FlnB loss also leads to early prehypertrophic differentiation, which might contribute to the premature bone phenotypes but would

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not explain the delay in ossification. Several receptors have been implicated in this pathway. FlnB-binding Smad receptors can regulate the transcription factor Runx2, which promotes chondrocyte hypertrophy (13). Additionally, FlnB-binding integrin receptors can trigger the PI3K/Akt pathway to activate cell cycle proteins and chondrocyte proliferation (15). A larger question remains as to what downstream modifiers of the actin cytoskeleton might regulate these processes and why the premature prehypertrophic differentiation seen with FlnB inhibition is not associated with earlier rather than delayed ossification in the long bones.

In the present study, we used a two-hybrid screen to identify potential FlnB interactors. We found that Flnb bound Formin 1 (Fmn1) and that these two proteins were co-expressed in the growth plate and co-localized in the cell cytoplasm and nucleus. Loss of Flnb led to down-regulation of Fmn1 at the hypertrophic-to-ossification transition border. Formins are actin-nucleating proteins involved in various cellular functions such as cell polarity, cytokinesis, cell migration and serum response factor transcriptional activity. Loss of Fmn1 and Flnb function in the double-knockout mice caused more severe skeletal shortening than seen with either knockout alone and led to a delay in ossification. The shortened stature was associated with a significant reduction in chondrocyte proliferation but paradoxically, an increase in chondrocyte differentiation. Staining bone for calcium and phosphate, however, indicated a delay in ossification. Flnb inhibition resulted in loss of Fmn1 expression along the hypertrophic-to-ossification border, suggesting that these two proteins may coordinate chondrocyte transition to osteocyte formation. Disruption of this transition may potentially explain the overall delay in bone formation.

RESULTS

Flnb interacts and co-localizes with Fmn1

To identify potential interactors downstream of filamin function, we first performed a yeast two-hybridization screen using FLNA and FLNB as baits, with a mouse embryonic day 12.5 library as prey. Initial screening using the C-terminal fragments FLNA(aa 2167–2648) and FLNB(aa 2111–2592) identified a potential interaction with the Fmn1 (FH1, aa 639–744) fragment (Fig. 1A). To confirm their binding, we used directed co-immuno precipitation analysis with a GFP-tagged FMN1 (FH1), which pulled down the myc-tagged C-terminal fragments of both FLNA and FLNB (Fig. 1B). We have previously shown that both Flna and Flnb are expressed in ATDC5 cells (15). Fmn1 mRNA could similarly be detected in both naive ATDC5 chondroprogenitors and chondrocytes following induction of differentiation with ITS (insulin-transferrin-sodium selenite media supplement, Fig. 1C). Co-immunostaining with specific antibodies showed that Fmn1 co-localized with both Flna and Flnb, predominantly in the cytoplasm and to a lesser extent in the nucleus (Fig. 1D). In tissue, Fmn1 was widely found in the proliferative, prehypertrophic and, to a lesser degree, hypertrophic zones but was most highly expressed along the growth plate–bone border (hypertrophic-to-ossification transition zone) at P1 age. Flnb was similarly expressed in the proliferative and prehypertrophic zones of the growth plates and showed relatively weaker expression within the hypertrophic zone. Loss of Flnb leads to a loss of Fmn1 expression along the hypertrophic chondrocyte-to-osteocyte border (Fig. 1E, white arrow). However, we found that the FlnB expression pattern did not change in the growth plates of Fmn1−/− mice. Further, no discernible changes in Fmn1 expression were observed with loss of Flnb and vice versa, either in ATDC5 chondrocytes or whole tissue (Fig. 1F). Collectively, these studies show that Fmn1 and Flnb physically interact within the cytoplasm and possibly the nucleus of chondrocytes and that Flnb might regulate Fmn1 function along the growth plate–bone border and thereby influence the chondrocyte hypertrophy to ossification transition.

Fmn1 and Flnb regulate growth plate development and skeletal ossification

Given the observed physical binding and their expected roles in skeletal development, we generated Fmn1−/− + Flnb−/− double-knockout mice. Neither Flnb nor Fmn1 were detected on western blot in the double-knockout mice (Fig. 2A). The average survival rates of homozygous mice deficient for Flnb, Fmn1 and Flnb + Fmn1 were 3.7, 96.7 and 1.9%, respectively. The gross body size of both Fmn1−/− and Flnb−/− mice were reduced at 2 months of age. The reduced weight in Flnb−/− mice was more significant than that in the Fmn1−/− mice, whereas the Fmn1−/− + Flnb−/− mice showed greater reduction in body weight compared with either knockout mouse alone (Fig. 2B). The smaller size and loss in body weight in the double null mice corresponded to a greater shortening in the growth plate, which could be appreciated as early as E16.5 (Fig. 2C).

To determine the potential changes in skeletal development that could contribute to the reduction in size and weight with loss of these actin-associated proteins, we performed Alizarin red (bone) on E16.5 (Fig. 3A) and von Kossa staining for phosphate on P1 and P7 WT and mutant mice (Fig. 3B). The bone density in cortical bone was measured indirectly through phosphate intensity in von Kossa staining. Loss of Flnb and Fmn1 led to a delay in calcification (analogous to bone formation) within the double-knockout mice compared with control and was more severe than loss of function of the individual proteins alone during early postnatal ages. Overall, these findings suggest that Fmn1 and Flnb inhibition acts synergistically in impairing chondrocyte development and formation of the growth plate, leading to a delay in ossification and long bone growth.

Loss of Fmn1 and Flnb inhibits chondrocyte proliferation

Our prior studies had shown a progressive decline in the numbers of proliferating chondrocytes with loss of Flnb (12,15). Given the observed impairments in skeletal size and delay in ossification, we wanted to determine whether a similar proliferative decline was seen in the Fmn1−/− + Flnb−/− mice. We used the transcription factor Sox9 as an early marker of less differentiated proliferating chondrocytes. Col2a1 expression from proliferating chondrocytes is closely correlated with high levels of SOX9 RNA and protein (16). We also used a cell cycle mitotic marker phospho-histone H3 (PH3) to gauge the number of actively proliferating cells (17–19). Consistent with prior findings, Sox9 immunostaining was reduced in E16.5 Fmn1−/− and Flnb−/− mice alone. We observed an even greater reduction in both Sox9+ and PH3+ chondrocytes with inhibition of both
Figure 1. Filamin B interacts and co-localizes with Formin 1. (A) Yeast two-hybridization analysis shows that both human Filamin A (FLNA) and Filamin B (FLNB) preys interact with Formin 1 (FMN1) bait. The fragments used for the interaction experiments are graphically shown. An interaction between the bait and prey is gauged by level of blue intensity generated through the X-gal reaction. (B) Immunoprecipitation analysis shows that GFP-tagged FMN1 can immunoprecipitate Myc-tagged FLNA and FLNB. C. RT-PCR shows Fmn1 mRNA expression in naïve ATDC5 cells and following induction of differentiation with ITS (insulin–transferrin–selenium). (D) FlnA-Fmn1 and FlnB-Fmn1 double staining shows that both FlnA-Fmn1 and FlnB-Fmn1 are highly co-localized in the cytoplasm, and in multiple nuclear foci (white arrows) in ATDC5 cells. (E) Fluorescent photomicrograph of P1 WT radial growth plate shows that Fmn1 (Dylight488, fluoroscein) and FlnB (Dylight594, rhodamine) are widely expressed in chondrocytes across the proliferative, prehypertrophic and hypertrophic zones. Fmn1 expression is especially strong in chondrocytes along the growth plate–ossification border, generally at the end of the hypertrophic zone (white arrow). Fmn1 expression is significantly reduced at the border (white arrow) of the hypertrophic zone and region of ossification in the radial growth plate of P1 FlnB−/− mice. FlnB expression is not affected by loss of Fmn1−/−. (F) FlnB inhibition does not alter Fmn1 expression in stably transfected, null FlnB ATDC5 cell lines. Similarly, Fmn1 loss of function in chondrocytes does not show any change in FlnB expression. Scale bars = 20 µm for D and 200 µm for E.
actin-associated proteins, compared with wild-type and loss of either protein alone (Fig. 4A and B). Taken collectively, these markers suggest a reduction in proliferative rates that would contribute to the delay in skeletal formation.

Loss of Fmn1 and FlnB causes shortening of the proliferative zone and lengthening of the hypertrophic zone

During development within the growth plate, proliferating chondrocytes progress from the proliferative to the prehypertrophic and finally, the hypertrophic zone. To determine where chondrocyte differentiation was affected with concomitant loss of Fmn1 and FlnB, we stained the growth plates with Collagen2 (Col2a1, proliferative zone marker) and Collagen10 (Col10a1, hypertrophic zone marker) (20–22). Although the ratio of the Col2a1+ length to growth plate length was unchanged in Fmn1−/− and FlnB−/− mice, it was decreased in the Fmn1−/− + FlnB−/− mice (Fig. 5A and B). Conversely, the ratio of Col10a1+ length to growth plate length was increased in Fmn1−/−, greater in the FlnB−/− mice and greatest in the Fmn1−/− + FlnB−/− mice (Fig. 5A and C). The relative length of Col10a1+ zone was dramatically increased with loss of Fmn1 and FlnB, suggestive of early chondrocyte differentiation in the hypertrophic zone at the expense of chondrocyte proliferation in the prehypertrophic and proliferative zones.

Fmn1 and FlnB knockout display opposite changes of Pthr1+ zone

The relative thickening in the Col10a1+ zone was indicative of premature chondrocyte hypertrophy and contrasted with
the delay in bone formation in Fmn1\(^{-/-}\) + FlnB\(^{-/-}\) mice. To gain a better understanding of the differences in chondrocyte transition from proliferative to prehypertrophic-to-hypertrophic states, we stained the growth plates with the prehypertrophic zone-specific marker, parathyroid hormone 1 receptor (Pthr1) (23). The ratio of Pthr1\(^+\) zone/growth plate length was increased significantly in FlnB\(^{-/-}\) mice but did not change much in Fmn1\(^{-/-}\) or Fmn1\(^{-/-}\) + FlnB\(^{-/-}\) compared with WT mice, indicating delayed calcification in Fmn1\(^{-/-}\) + FlnB\(^{-/-}\). The delay in calcification is more severe in the double null mice compared with the Fmn1\(^{-/-}\) or FlnB\(^{-/-}\) mice. Results are quantified by graph to the left and show differences in staining intensity of the cortical bones among these mice, as measured by mean gray value from Image J. Scale bar = 3.6 mm in A, and 200 µm in B.

**DISCUSSION**

Filamins and formins regulate changes in the actin cytoskeleton. Mutations in either gene cause bone malformations in patients or mice (1–3,5,24–27). Prior work has shown that FlnB loss causes dwarfism and bone fusion in vertebral bones (12), whereas Fmn1 loss causes oligodactyly, an aberrant fusion of the carpal digits (25). In the current study, we find that these two actin-associated proteins physically interact and co-localize well in the cytoplasm and nucleus of chondrocytes. The dual loss of function leads to enhanced shortening of the long bones and delay in ossification, in part owing to diminished proliferative activity. Concomitantly, loss of FlnB + Fmn1 leads to a widened hypertrophic zone and shortened proliferative zone. The prehypertrophic zone is shifted toward the epiphysis with loss of FlnB as opposed to the diaphysis with Fmn1 inhibition, suggesting differential regulation of the prehypertrophic zone. However, loss of FlnB leads to inhibition of Fmn1 at the hypertrophic-to-ossification border, suggesting that these two proteins may serve a shared pathway in this transition. These studies demonstrate a coordinated interaction between FlnB and Fmn1 in regulating normal chondrocyte proliferation and differentiation.
Physical interaction between Fmn1 and FlnB in chondrocytes

While filamins have been thought to transduce signals onto the actin cytoskeleton following receptor activation, the downstream mechanisms that modulate this process are not entirely known. The actin-binding proteins have been known to bind Rho GTPases (i.e. RhoA, Cdc42 and Rac1 being the most studied), which serve as effectors of actin cytoskeletal dynamics. We now find that FlnA and FlnB can bind Fmn1, which is part of the formin family of proteins. The formin family consists of 25 members, which are highly conserved and play important roles in modulating actin assembly and polarization, cytokinesis and migration (24–31). Formins contain two highly conserved formin homology domains, FH1 and FH2, which regulate stress fiber formation through a proline-rich domain, previously implicated in actin nucleation and elongation (24,26–28,31). The interaction between filamins and Fmn1 at the highly conserved FH1 domain suggests that FlnA or FlnB likely can interact with various formin proteins, thereby establishing a mechanism for diverse regulation. Additionally, most formin proteins are RhoGTPase effector proteins, such that many contain an N-terminal Rho GTPase-binding domain that will interact with the C-terminal Dia autoregulatory domain (24,26–28,32). Activation of the Rho family members releases this interaction and leads to onset of FH1- and FH2-dependent processes. The current studies suggest that there are both shared and independent functions attributable to Fmn1 and FlnB interactions. It remains to be seen whether other distinct FlnB-associated phenotypes are regulated through separate formin homology proteins.

Regulation of chondrocyte proliferation by Fmn1 and FlnB

Both Fmn1−/− and FlnB−/− show similar defects in chondrocyte proliferation. Loss of function of either protein causes similar trends in long bone and growth plate shortening, and the loss of both Fmn1−/− + FlnB−/− in mice leads to a more severe phenotype, suggesting some synergistic effect. This

Figure 4. Fmn1–FlnB loss of function (Fmn1−/− + FlnB−/− ) impairs chondrocyte proliferation in mice. (A) Fluorescent photomicrographs of Sox9 immunostaining (Dylight594, rhodamine) in E16.5 radius show that the number of Sox9+ chondrocytes in the proliferation and prehypertrophic zones is reduced in both Fmn1−/− and FlnB−/− mice. An even greater reduction in positively labeled cells is seen in the Fmn1−/− + FlnB−/− mice. Quantification is graphically summarized below. (B) Fluorescent photomicrographs of phospho-histone H3 immunostaining (PH3, Dylight488, fluorescein) in E16.5 radius shows that the number of PH3+ chondrocytes is similarly reduced in both the Fmn1−/− and FlnB−/− mice. An even greater reduction in positively labeled cells is seen in the Fmn1−/− + FlnB−/− mice. Quantification is graphically summarized below. Scale bars = 200 μm. *P < 0.05, **P < 0.01, ***P < 0.001, n = 3 samples per group.
Figure 5. Fmn1–FlnB loss of function (Fmn1 \(^{-/-}\) + FlnB \(^{-/-}\)) causes widening of the hypertrophic zone in the growth plate of long bones. (A) Fluorescent photomicrographs show collagen2 (Col2a1, Dylight488 fluorescein) and collagen10 (Col10a1, Dylight594 rhodamine) double staining in P1 radius. The ratio of Col2a1 \(^+\) width to the growth plate does not change much in Fmn1 \(^{-/-}\) mice and FlnB \(^{-/-}\) mice but is significantly reduced in the Fmn1 \(^{-/-}\) + FlnB \(^{-/-}\) double-knockout mice. Florescent photomicrographs for Col10a1 \(^+\) immunostaining of the P1 radius, on the contrary, show progressively increased widening of the Fmn1 \(^{-/-}\) mice, FlnB \(^{-/-}\) mice and Fmn1 \(^{-/-}\) + FlnB \(^{-/-}\) double-knockout mice. (B) Quantitative analyses of the Col2a1 and Col10a1 immunostaining are graphically summarized. Scale bar in A = 200 μm. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6. Fmn1 and FlnB differentially regulate the width of Pthr1 \(^+\) prehypertrophic zones in the long bones of knockout mice. (A) Fluorescent photomicrographs show that the width of the prehypertrophic zone as gauged by parathyroid hormone 1 receptor immunostaining (Pthr1, Dylight594 rhodamine) relative to the absolute growth plate length decreases in P1 Fmn1 \(^{-/-}\) mice, increases in FlnB \(^{-/-}\) mice and displays no significant change in Fmn1 \(^{-/-}\) + FlnB \(^{-/-}\) mice compared with wild-type mice. In addition, Pthr1 \(^+\) immunostaining appears to be shifted to the right adjacent to the diaphysis (white arrows) in Fmn1 \(^{-/-}\) mice and Fmn1 \(^{-/-}\) + FlnB \(^{-/-}\) mice, but not in FlnB \(^{-/-}\) or WT mice. (B) The quantitative analyses are graphically summarized for the images in A. Scale bar in A = 200 μm. *P < 0.05, **P < 0.01, ***P < 0.001, n > = 3 samples per group.
bone shortening coincides with a reduction in staining for the early proliferative chondrocyte marker Sox9 and the mitotic marker PH3. A reduction in proliferative rates would be seen with increased chondrocyte cell death, a prolongation in the cell cycle and/or a shift in cell fate (enhanced differentiation would lead to slowing of proliferation). We did not observe any increase in cell death within the growth plates of the various mutant mice (unpublished observations). Our prior studies had shown that within neural progenitors, loss of FlnA led to a prolongation of the cell cycle and delay in neural differentiation (33). The delay was in part related to impaired degradation of specific cell cycle proteins, thereby implicating filamins in the regulation of vesicle trafficking-dependent protein degradation. Conversely, loss of FlnB led to a more rapid transition through the cell cycle and promoted chondrocyte prehypertrophic and hypertrophic differentiation, leading to a progressive slowing in proliferative rates (15). The current work suggests that Fmn1 influences these same pathways, as Fmn1 inhibition causes a decline in chondrocyte proliferation and leads to a widened hypertrophic zone (but not a widened prehypertrophic zone). The observations that FlnA and FlnB functionally interact with one another to form heterodimers (34) and both proteins can bind Fmn1 raise the likelihood that Fmn1 serves as an effector of FlnA/B function. The exact means by which FlnA and FlnB concurrently regulate activation of formins and possibly protein degradation to give rise to the observed proliferative phenotypes requires further investigation.

### Regulation of chondrocyte differentiation by Fmn1 and FlnB

The current work implicates FlnB and Fmn1 in the regulation of chondrocyte differentiation. In our initial characterization of embryonic bone development, we had observed a shortening of the Col10a1+ hypertrophic zone in E16.5 null FlnB embryos compared with age-matched WT (12). This early change was thought secondary to the decreased proliferation and consequent decrease in the number of differentiating chondrocytes. With continued development, loss of either FlnB or Fmn1 proteins ultimately leads to relatively widened Col10a1+ zone (the ratio of Col10a1+ length/growth plate length), indicating an effect owing to premature hypertrophic differentiation (15). Prior studies have suggested that RhoA inhibits the transition from proliferation to hypertrophic differentiation (35). In general, filamins and formins are thought to bind RhoGTPases such as RhoA, with RhoGTPase activation dependent on filamins. Subsequent formin activation is thought to be dependent on RhoGTPase activation (36,37). Taken in this context, FlnB might regulate RhoA activation and consequently Fmn1 activation, thereby effecting hypertrophic differentiation. Given the opposing phenotypes seen with loss of FlnA (delayed differentiation) and FlnB (increased differentiation), it also remains to be seen whether FlnA has an opposing effect on RhoA activation, compared with FlnB.

While FlnB and Fmn1 share many similar phenotypes governing chondrocyte differentiation, they also exhibit some differences, suggesting that these proteins also have unrelated functions. We found that Pth1r labeling of prehypertrophic zone was thinned and shifted to the diaphysis side in Fmn1+/- mice, but thickened and shifted to the epiphysis side in FlnB+/- mice. Prior studies have also shown premature widening of the prehypertrophic zone of FlnB null mice by Indian hedgehog (Ihh) and parathyroid hormone protein receptor (PTHrP) (12). These results indicate that although Fmn1+/−/ mice and FlnB+/- mice show similar bone shortening phenotypes and enhanced hypertrophic differentiation, the two proteins might play different roles in regulating proliferative-to-prehypertrophic transition and/or prehypertrophic-to-hypertrophic transition of differentiating chondrocytes. The observation of ectopic Pth1r+ cells in the hypertrophic zone in Fmn1+/− mice highly suggests that the prehypertrophic-to-hypertrophic transition is more likely affected. The inability to completing this transition might cause the transiting cells to be both Pth1r and Col10a1 positive. The expression patterns of the Pth1r+ cells suggest that FlnB might play more roles in controlling proliferative-to-hypertrophic zone transition, whereas Fmn1 might play a greater role in promoting prehypertrophic-to-hypertrophic transition.

Increased chondrocyte differentiation and widening of the hypertrophic zone with loss of FlnB or Fmn1 function would not be expected to cause a delay in bone ossification. The current studies do show that loss of FlnB or Fmn1 impairs chondrocyte proliferation, and decreased chondrocyte production could retard long bone growth. However, we also observed a delay in bone calcification and transition from cartilage to bone across various embryonic and postnatal ages. One possible explanation lies in an inhibition of hypertrophic-to-ossification transition with loss of either actin-associated protein. Given that Fmn1 exhibits very high expression along the hypertrophic-to-ossification border, it may be required for hypertrophic chondrocyte and/or osteoblast function. A role for Fmn1 in effecting hypertrophic chondrocyte development is supported by the observation that Fmn1 loss causes a thickened Col10a1+ zone whereas the prehypertrophic differentiation is unaffected. That FlnB loss induces dramatic down-regulation of Fmn1 expression at the hypertrophic-to-ossification border also might explain why premature differentiation in FlnB−/− mice can cause delayed bone formation but not accelerated bone formation (12).

### Regulation of chondrocyte migration by Fmn1 and FlnB

Although little is known about why the hypertrophic-to-ossification transition is impaired in Fmn1+/− mice and FlnB−/− mice, one important factor might involve cell migration. Chondrocytes migrate in a defined direction, depending on the signals they receive and the local microenvironment. Directed cell migration requires dramatic changes in the cell shape and adhesion to the extracellular matrix. Both filamins and formins regulate cell migration through these same processes (2,3,29,38–41). Our previous work implied that the longitudinal migration can be affected by abnormal phosphorylation of integrin beta1 seen in FlnB−/− mice (12). The spreading defects and focal adhesion formation defects in Fmn1+/− mice with oligodactyly (25,29) similarly suggest that Fmn1 also regulates chondrocyte migration. Both filamins and formins can function under integrin receptor signaling (38–43), which is dependent on binding to the extracellular matrix and directs RhoGTPase activation in regulating cell migration. In this context, filamins, formins and RhoGTPases would be expected to alter migration of chondrocytes through the actin cytoskeleton in response to extracellular matrix-cell adhesions (2,27,29,44). Further studies focusing on cell migration as they pertain to the chondrocyte-to-osteoblast...
transition could provide a better understanding of the delay seen in bone ossification observed with loss of Fmn1 or FlnB function.

CONCLUSION

The current studies provide a novel downstream effector of FlnB function, namely Fmn1. Our data indicate that Fmn1 loss, like FlnB inhibition, leads to long bone shortening and a delay in skeletal bone formation. Fmn1 expression is dramatically reduced with FlnB loss at the hypertrophic zone to ossification border whereas FlnB expression is unchanged with Fmn1 loss. These results suggest that Fmn1 function is regulated to some extent by FlnB and that FlnB acts upstream of Fmn1. Disruption of the hypertrophic-to-ossification transition would provide a potential explanation for the delay in ossification. Finally, formin interactions with filamin provide one of the first direct mechanisms by which filamins can regulate actin assembly and disassembly, potentially through the RhoGTPases.

MATERIALS AND METHODS

Mice breeding, tissue isolation and bone decalcification

The FlnB−/− mice were generated and bred as previously reported (12). The wild-type allele was detected by PCR amplification using the primer pair 5′-AGATTACACCGAAGGTG-3′ and 5′-CCCTGGCTAAATGGCA-3′, and the mutated allele by using 5′-AGTGTGCTAGTTGAGC-3′ and 5′-GATCCCTCAGGAATCGT-3′. The Fmn1−/− mice were gifted by Dr Philip Leder, and the same genotyping method was used (25). Tissues were isolated after euthanization and fixed with 4% parafomaldehyde or 10% tri-chloroacetic acid (TCA) for different staining. Owing to the typical tissue pattern of the various zones in the growth plate and the availability of animals, different ages of animals [Embryonic 16.5 day (E16.5), postnatal day 1 (P1) and postnatal day 7 (P7) mice] were used for growth plate analysis. At least three mice per experimental variable were used. All mouse studies were performed under approval from the Institutional Animal Care and Use Committees of Harvard Medical School and Beth Israel Deaconess Medical Center, in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Yeast two-hybridization assay

A yeast two-hybrid screen was performed according to standard protocol and previously described (34). The Matchmaker yeast two-hybrid system (cat. # K1615–1; Clontech, Mountain View, CA, USA) was used. The coding sequence of the C-terminal FLNA (2167–2648, human) and C-terminal FLNB (2111–2592, human) were cloned into the bait vector pNLex. The cloning primers are as follows: 5′-CCCTGGCTAAATGGCA-3′ and 5′-GGGACCAACAGGTTGTTGAGC-3′ for C-terminal FLNA; 5′-CCCTGGCTAAATGGCA-3′ and 5′-GGGACCAACAGGTTGTTGAGC-3′ for C-terminal FLNB. For confirmation through directed yeast two hybrid, the coding sequence of the C-terminal FMN1 (639–744, FH1 domain, mouse) was cloned into the prey vector pjG45. The cloning primers are as follows: 5′-CCCTGGCTAAATGGCA-3′ and 5′-GGGACCAACAGGTTGTTGAGC-3′ for C-terminal FLNB. The FlnB cloning primers are as follows: 5′-CTGTTGCTCTGACGTTGC-3′ and 5′-AGTGGTCCTAATTGGCAGA-3′.

For immunostaining of Fmn1, FlnB, Col2a1, Col10a1 and Pthr1, tissues were further decalcified by 5% TCA solution containing 1% HCl and 1% acetic acid for 7 days. Cultured cells were fixed using 10% (w/v) ice-cold TCA for 20 min. For immunostaining of Sox9 and PH3, the samples were fixed with 4% paraformaldehyde. After washing in PBS, fixed samples were permeabilized with 0.5% Triton X-100 and blocked with 5% normal horse serum for 2 h. Tissue was incubated with the primary antibodies for 1 h at room temperature or overnight at 4°C. The Dylight488- and Dylight594-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were incubated for 1 h at room temperature. Samples were further counterstained with 100 ng/ml Hoechst 33342 (Life Technologies, Grand Island, NY, USA). Images were obtained with an LSM5 Pascal confocal microscope (Zeiss, Germany). The primary antibodies (for immunostaining and some also for western blotting) were as follows: mouse anti-Fmn1 monoclonal antibody (Cat.# Ab3092, Abcam, Cambridge, MA, USA); rabbit anti-FlnB polyclonal antibody (Gifted by Dr Kao, CWRU); mouse anti-Col2a1 (Cat.# Ab3092, Abcam); rabbit anti-Col10a1 monoclonal antibody [kindly gifted by Dr Horton and Dr Lunstrum, Shriners Hospital for Children, Portland, OR, USA (22)]; rabbit anti-Sox9 polyclonal antibody (O9–1, gift of Professor Dr Michael Wegner, Institute of Biochemistry, Friedrich-Alexander-University, Erlangen-Nurnberg, Germany); rabbit anti-PH3 polyclonal antibody (1 : 250, Cat.# 06–570, Millipore, Billerica, MA, USA).

ATDC5 cell culture and ITS-induced differentiation and RT-PCR analysis

Mouse ATDC5 chondrocyte cells were gifted by Dr Gori (45) and were cultured in modified DMEM medium containing 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Cat.# 15140, Invitrogen). Unless otherwise
specified, ATDC5 cells were passaged every two days. To induce differentiation, ITS (insulin–transferrin–sodium selenite media supplement, Cat.# 1107457001, Roche, Germany) was added to the culture medium for 30 days, and then, the ATDC5 cells were collected for RT-PCR analysis. Total RNA samples were prepared from ATDC5 cells and ITS (insulin–transferrin–sodium selenite media supplement)-treated ATDC5 cells. The RNA was extracted from adult tissues using TRIzol (Invitrogen). Reverse transcription and PCR were performed as conventional methods by using SuperScript III First Strand Kits (Cat.# 18080–400, Invitrogen). The cDNA was amplified by PCR for 30 cycles. Primers were selected encompassing the intron sequences. The primer sequences used for Fmn1 RT-PCR analysis were as follows: forward, 5′ gcctaggtggactaagagac 3′; reverse, 5′ agggatactctgctgtcag 3′. The Gapdh was used as internal control, and the primers were as follows: forward, 5′ ggcaaaaagagagctttgctgc 3′; reverse, 5′ aagagttgatgtggcttcccg 3′. PCR cycling was performed at 95°C for 2 min followed by 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and finally 72°C for 6 min, by using the EmeraldAmp MAXPCR master mix (Takara, Cat.# RR320A, Japan). PCR products were analyzed by 1.5% agarose gel.

Immunoprecipitation and western blotting

Two hundred and ninety-three T-cell samples were collected either immediately or 36 h after EGFP-FMN1 and/or Myc-FNBL transfection. Modified RIPA buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl, 1.0% Triton X-100; 0.5% sodium deoxycholate and 0.1% SDS), with proteinase inhibitor cocktail and 20 mM EDTA, was used for cell lysis. Conventional immunoprecipitations were performed by using Protein-A/G ultralink resin beads according to the protocol provided by the manufacturer (Cat.# 53132, Thermo Scientific, Rockford, IL, USA). Proteins were separated in 8 or 10% SDS–polyacrylamide gels and transblotted to PVDF membrane. The primary antibodies that were used in this study are as follows: mouse anti-Myc antibody (Cat.# M4439, Sigma, USA) and goat-anti-GFP antibody (Cat.# 600101215, Rockland, USA). The primary antibodies that were used in this study are as follows: mouse anti-Myc antibody (Cat.# M4439, Sigma, USA) and goat-anti-GFP antibody (Cat.# 600101215, Rockland, USA). Immunoblot analysis was performed with primary antibodies based on manufacturers’ guides or suggested dilutions. Blots were detected by using LumiGOLD ECL western blotting detection kit (Cat.# SL100309, SignaGen, Rockville, MD, USA).

Statistical analyses

Results were expressed as the mean ± s.d. of n experiments (n ≥ 3, or specified in figure legends). Statistical analysis was performed with Student’s t-test, with P < 0.05 considered significant.

ACKNOWLEDGEMENT

We thank Dr Hung-Ying Kao (Case Western Reserve University, Cleveland, OH, USA) for gifting the FLNB antibody, Dr Horton and Dr Lunstrum (Shriners Hospital for Children, Portland, OR, USA) for gifting the rabbit anti-Col11a1 antibody and Dr Michael Wegner (Institute of Biochemistry, Friedrich-Alexander-University, Erlangen-Nurnberg, Germany) for gifting the Sox9 antibody. We also thank Dr Gori (Massachusetts General Hospital) for gifting the ATDC5 cells.

Conflict of Interest statement. None declared.

FUNDING

This work was supported in part by the National Institutes of Health (NS063997-01 to V.L.S.). This work was also supported in part by the Empire State Stem Cell Fund (the New York State Department of Health Contract #C024324 to V.L.S.). The opinions expressed here are solely those of the authors and do not necessarily reflect those of the Empire State Stem Cell Board, the New York State Department of Health, or the State of New York.

REFERENCES


