Doctoral thesis for the Degree of Doctor of Philosophy

# NEW ROLES OF FILAMINS IN CELL SIGNALING, TRANSCRIPTION AND ORGAN DEVELOPMENT

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# ABSTRACT

Filamins are large actin-binding proteins that stabilize delicate three-dimensional actin networks and link them to cellular membranes. They integrate cell architectural and signaling functions and are essential for cell locomotion and development. This thesis includes studies of two abundantly expressed filamin members, filamin A (FLNA) and B (FLNB).

FLNA has recently been shown to bind to the proteins that are related to cell motility and are implicated in diseases. The number of known FLNA interacting proteins is increasing, thus a complete understanding of the role of FLNA in diseases still requires intensive study. We identified hypoxia-inducible factor-1α (HIF-1α), a transcription factor, as a novel interacting partner of FLNA and studied the influence of their interaction on HIF-1α signaling in *FLNA*-deficient and *FLNA*-expressing human tumor cells. At hypoxia, cleavage of FLNA by calpain was induced. The cleaved C-terminal fragment interacted with HIF-1α and facilitated nuclear translocation and transactivation activity of HIF-1α. As a consequence, *FLNA*-deficient tumor cells produced less VEGF-A and exhibited an impaired ability to induce proliferation and migration of endothelial cells. In addition, we discovered that the interaction between FLNA and another transcription factor SMAD2 partially regulates c-MET expression. *FLNA*-deficient tumor cells expressed less c-MET and displayed impairments in c-MET signaling and hepatocyte growth factor-induced cellular migration. These results suggest that FLNA is important for cellular motility and may influence tumor growth by regulating angiogenesis and tumor metastasis in response to chemoattractants.

*FLNB* mutations in humans are associated with devastating congenital malformations. However, the causal role of *FLNB* in these genetic disorders is unknown. Using a gene-trapping technique, we generated a mouse model of *Flnb*-deficiency, which led to a high embryonic lethality. A few *Flnb*-deficient mice that reached term displayed severe skeletal malformations and disorganized microvasculature. *Flnb*-deficiency impaired the cell motility of embryonic fibroblasts, which may partly explain the observed developmental consequences. Generation of *in vivo* and *in vitro* models of *Flnb*-deficiency will advance our understanding of the biological importance of FLNB in organ development and disease progression.

Our studies provide clear evidence that cytoskeletal proteins such as filamins are involved in cell signaling, transcription and organ development.

**Keywords:** filamins; F-actin-binding proteins; cell movement; integrins; GTP phosphohydrolases; genetic diseases, inborn; mice, knockout; hypoxia-inducible factor 1; vascular endothelial cell growth factor A; proto-oncogene proteins c-met; Smad2 protein; hepatocyte growth factor; cartilage; osteogenesis; neovascularization; neoplasm metastasis

# LIST OF PUBLICATIONS

The thesis is based upon the following papers, referred to in the text by their roman numerals:

Paper I **Filamin A promotes VEGF-A activity through the HIF-1α-mediated hypoxic response**

Xiaowei Zheng*, Xianghua Zhou*, Meit Björndahl, Hidetaka Uramoto, Teresa Pereira, Lakshmanan Ganesh, Elizabeth G. Nabel, Yihai Cao, Jan Borén, Lorenz Poellinger, and Levent M. Akyürek

*Equal contribution to the paper

Under revision

Paper II **Filamin A regulates c-MET signaling via SMAD2**

Xianghua Zhou, Aslı Toylu, Neşe Atabey, Carl-Henrik Heldin, Gisela Nilsson, Jan Borén, Martin O. Bergö, and Levent M. Akyürek

Submitted

Paper III **Filamin B deficiency in mice results in skeletal malformations and impaired microvascular development**

Xianghua Zhou, Fei Tian, Johan Sandzén, Renhai Cao, Emilie Flaberg, Laszlo Szekely, Yihai Cao, Claes Ohlsson, Martin O. Bergö, Jan Borén, and Levent M. Akyürek

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# ABBREVIATIONS

| | |
|---|---|
| ABD | F-actin binding domain |
| ABP | Actin-binding protein |
| AOI, AOIII | Atelosteogenesis I and III phenotypes |
| AR | Androgen receptor |
| BD | Boomerang dysplasia |
| CH | Calponin homology |
| ES | Embryonic stem cells |
| F-actin | Filamentous actin |
| FILIP | Filamin A interacting protein |
| FILIP1L | Filamin A interacting protein 1-like |
| FLN | Human filamin protein |
| *FLN* | Human filamin gene |
| Fln | Mouse filamin protein |
| *Fln* | Mouse filamin gene |
| $Flnb^{+/+}$ | *Flnb*-wild type |
| $Flnb^{+/-}$ | *Flnb*-heterozygous |
| $Flnb^{-/-}$ | *Flnb*-deficient |
| FMD | Frontometaphyseal dysplasia |
| GAP | GTPase-activating protein |
| GEF | Guanine nucleotide-exchange factor |
| GTPase | GTP phosphohydrolase |
| HGF | Hepatocyte growth factor |
| HIF-1α | Hypoxia-inducible factor-1α |
| HRE | Hypoxia-response element |
| MAPK | Mitogen-activated protein kinase |
| MAP2K | Mitogen-activated protein kinase kinase |
| MAP3K | Mitogen-activated protein kinase kinase kinase |
| MEF | Mouse embryonic fibroblast |
| MNS | Melnick-Needles syndrome |
| OPD | Otopalatodigital syndrome |
| PAE | Porcine aortic endothelial cell |
| PAK1 | Serine/threonine kinase p21-activated kinase-1 |
| PVNH | Periventricular nodular heterotopia |
| SCT | Spondylocarpotarsal syndrome |
| TAD | Transactivation domain |
| TGF-β | Transforming growth factor-β |
| *VEGFA* | Vascular endothelial cell growth factor-A gene |
| VEGF-A | Vascular endothelial cell growth factor-A protein |
| VEGFR | Vascular endothelial cell growth factor receptor |
| VZ | Ventricular zone |
| XMVD | X-linked myxoid valvular dystrophy |

# TABLE OF CONTENTS

# 1 INTRODUCTION

Organization of the actin cytoskeleton mediated by actin-binding proteins (ABP) is not only essential for the formation and maintenance of cell shape but also dynamically regulates cellular morphology and locomotion in response to external stimuli [1]. The first filamin was discovered accidentally as a novel actin-binding protein during an attempt to isolate myosin from rabbit macrophages in 1975 [2]. It could efficiently precipitate purified muscle actin in solution [3] and was subsequently determined to be a potent filamentous actin (F-actin) crosslinking protein [4, 5]. Intracellularly, filamins crosslink cortical F-actin into a dynamic orthogonal network, thereby conferring membrane integrity and defending cells against mechanical stress. In addition to F-actin, filamins bind to numerous other proteins such as transmembrane receptors and signaling molecules, providing scaffolding functions and regulating multiple cellular behaviors. Due to their diverse functionality in humans, deleterious mutations in filamin genes can cause a wide range of developmental malformations in the brain, bone, limbs, and heart [6].

## 1.1 Structural properties of filamins

Vertebrate filamins are elongated dimeric V-shaped proteins with two large (240-280 kDa) polypeptide chains. Each monomeric chain of filamins comprises an F-actin-binding domain (ABD) at the N-terminus and a rod segment consisting of up to 24 homologous repeats of ~96 amino acid residues which adopt an immunoglobulin-like fold (Ig repeats). Two hinge domains interrupt the 24 Ig repeats into two rod domains between repeats 15 and 16 (hinge 1, H1) and separate the C-terminal dimerization domain from the actin-bindng region between repeats 23 and 24 (hinge 2, H2). The C-terminal dimerization domain associates two filamin monomers and anchors F-actin to the cell membrane through transmembrane receptors (Fig. 1).

ABD represents a key aspect of filamin subunit attachment to F-actin. The competitive binding of $Ca^{2+}$-calmodulin to ABD dissociates filamin from F-actin [7] and deletion of ABD from filamin subunits greatly diminishes its gelation activity in actin solution [8]. ABD consists of two calponin homology (CH) domains (CH1 & CH2) separated by a linker, which conform to other F-actin-binding proteins. The tandem organization of CH domains determines the binding capacity

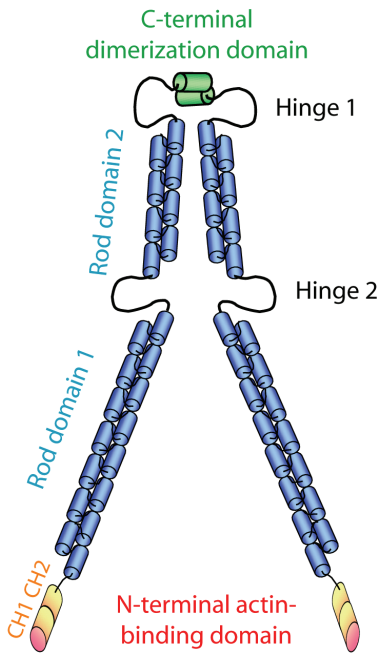


**Figure 1.** Structure of filamins includes the N-terminal actin-binding domain, C-terminal dimerization domain, and 24 Ig repeats disrupted by hinge 1 and 2 domains.

of two adjacent actin molecules and thus may determine the binding specificity towards F-actin [9]. A conserved hydrophobic region of the CH1 domain corresponding to amino acids 121 – 147 of filamin is the primary binding site for F-actin [10]. The amino acid residues of actin which bind to filamin overlap with those of several other ABPs including α-actinin, myosin, tropomyosin and caldesmon [11]. This explains how filamin can compete with other proteins for binding to actin. Both ABD and actin undergo structural rearrangements upon binding to each other which implies that binding of an ABD could affect the shape of F-actin.

The long-range Ig repeats provide not only additional affinity towards F-actin but also intrinsic flexibility of actin networks in response to mechanical stresses. Ig repeats 9–15 containing an F-actin-binding domain are necessary for high avidity F-actin binding. The flexibility of Ig repeats 1–8 enables repeats 9–15 to find the proper alignments of actin monomers in F-actin downstream from the initial binding sites. The overall linearity and flexibility of rod domain 1 can accommodate the twist in the helix groove of F-actin to facilitate binding. Ig repeats 16–24 do not bind F-actin, which renders nearly a third of filamin subunit contour length available for reversible unfolding and partially accounts for the prestress-mediated increase in elasticity of F-actin networks [8]. They are also potentially free to interact with other partner proteins, which may explain why most partner interactions are observed at this site. The two hinge regions of human filamin have increased amino acid diversity and their length enables the whole rod to be more flexible and even to crosslink widely dispersed actin filaments at perpendicular angles. For instance, H1 is required for maintaining the viscoelastic properties of actin networks against stresses [12]. Moreover, the hinge regions represent proteolysis sites that are cleaved by $Ca^{2+}$-dependent protease calpain, producing the 200 kDa N-terminal and 90 kDa C-terminal fragments [13]. This suggests a regulatory mechanism on filamin functions.

Dimerization amplifies the actin-binding avidity of filamin monomers and permits cross-linking of perpendicular F-actin into T-, X- or L-shaped junctions. Ig repeat 24 is necessary and sufficient to confer the self-association of two filamin subunits and inclusion of the preceding H2 region increases the efficiency of dimerization [14]. Each filamin subunit binds to only one F-actin. When the unbound filamin subunit engages the second F-actin positioned in the correct orientation, the apparent affinity of the interaction increases markedly [8]. The dissociation constant for the Ig repeat 24 dimer is on the order of the cellular filamin concentration, which implies that regulation of monomer-dimer equilibrium could be functionally important [15].

## 1.2 Filamin family

Filamins in mammals comprise a family of three members: filamin A (FLNA), filamin B (FLNB) and filamin C (FLNC). The three filamin genes are highly conserved and filamin proteins show about 70% overall amino acid identity, with greater divergence being observed at the two hinge regions with 45% identity [9]. Both human *FLNA* and mouse *Flna* genes are located on the X chromosome, whereas human *FLNB* and *FLNC* are on autosomal chromosomes 3 and 7, respectively, and mouse *Flnb* and *Flnc* at chromosomes 14 and 6, respectively. *FLNA* mutations can cause X -chromosome-linked genetic diseases

which differentiate them from the diseases caused by *FLNB* and *FLNC* mutations. All three filamins are widely expressed during development. In adults, FLNA is the most abundant isoform and FLNC expression is mainly restricted to skeletal and cardiac muscle cells [16].

### ***1.2.1*** *Filamins form both homodimers and heterodimers*

All three isoforms of filamins can form homodimers via their C-terminal dimerization domain. Even proteolytically cleaved C-terminal fragments of filamins can keep the dimer form [17, 18]. However, different filamin isoforms have been observed to be expressed simultaneously in many cell types, including endothelial cells and pericytes [19]. Considering the very similar protein structures of filamin isoforms, their co-existence in the same cell raises an interesting question: can these isoforms form heterodimers? In fact, FLNA and FLNB have been shown to be highly expressed in both the leading processes and somata of migratory neurons during corticogenesis and to form FLNA-FLNB heterodimers [20]. Both the co-expression of FLNA and FLNC in Z-bodies of the early myotubes and the gradual replacement of FLNA by FLNC from the developing myofibrils suggest a transitory heterodimerization [18]. Furthermore, the heterodimer formation between FLNB and FLNC have been confirmed by mixing recombinant C-terminal fragments of different filamin isoforms *in vitro* [14]. The heterodimerization of filamin isoforms adds another layer of complexity to filamin family. It raises the possibility that individual filamin isoforms serve compensatory functions for the other family members or extend their functionality through isoform-specific binding to cell surface proteins.

### ***1.2.2*** *Splice variants increase the diversity of filamins*

The diversity of filamin family is increased by the alternative splicing. Most of the internal deletions or substitutions occur at rod domain 2, which seems to be inessential for the actin-binding, but may affect the binding to other interacting partners. The H1 domain is lacking in some splice variants of human FLNB [21] and FLNC [22], as well as in mouse Flnc. Since H1 confers flexibility to the dimeric filamin molecule, the absence of H1 may affect the orthogonal crosslinking pattern. Chicken filamin that lacks the H1 region promotes actin crosslinking into parallel bundles [23]. The expression of splice variants of filamins exhibits tissue specificity and different cellular localization. In the thyroid, the predominant isoform is FLNB containing H1, but FLNC lacking H1 [21, 22]. In myotubes, deletion of H1 is required for the localization of FLNB at the tips of actin stress fibers [24]. This may, therefore, result in a functional discrepancy between filamin isoforms, such as FLNB lacking H1 accelerates the differentiation of myoblast cells into myotubes compared to the canonical isoform [24]. This raises the possibility that these variants have specialized functions within different tissues or cells, and play an unprecedented role in the subtle regulation of actin dynamics and organization.

## 1.3 Filamins crosslink F-actin

Two main types of actin-crosslinkers, the Arp2/3 complex and FLNA, are associated with branched actin networks. The Arp2/3 complex anchors the pointed ends of newly formed filaments to the existing filament at angles of 70 degrees and thus allows the elongation of free barbed ends

[25]. The Arp2/3 complex has been considered to be the main actin-filament crosslinker in cortical cytoplasm. However, Arp2/3 complex-mediated branching of F-actin is metastable, can dissociate, and is insufficient by itself to maintain the mechanical stability at the leading edge of cells. In contrast, FLNA mediates a more robust F-actin network. Most likely, the Arp2/3 complex initiates F-actin branching and subsequently FLNA stablilizes the dendritic network [26].

FLNA crosslinks actin-filament efficiently and a single FLNA dimer per actin filament is sufficient to induce gelation [27]. The actin networks built by filamin comprise a mostly perpendicular F-actin organization. Interestingly, the type of actin filament organization depends on the molar ratio of filamin to actin. An increase in this ratio leads to tighter F-actin networks [28]. The formation of parallel bundles of actin filaments is prompted when this ratio is high (from 1:10 to 1:50), while a ratio of 1:150 to 1:740 leads to the formation of orthogonal actin networks, depending on the source of filamin [4, 29]. This suggests a dual role of filamin in controling simultaneously both the architecture and mechanics of F-actin networks [30]. Filamin localizes to both actin-rich lamellae and actin stress fibers of adherent cells [31]. At the leading edge of motile cells, FLNA is present at the X, Y, and T junctions of the three-dimensional orthogonal networks of short actin filaments [26]. This is the region where fast remodeling of the actin cytoskeleton is required; however, reasonably high stiffness is also required to allow for net pushing forces produced by polymerizing actin against the cell membrane [32]. The combined orthogonal architecture and stiffness could readily be provided by low filamin concentrations. In contrast, stable F-actin bundles lying on the ventral side of adherent cells require high contractility, which can be provided in part by high filamin concentrations. The filamin concentration at the cellular edge is indeed not as high as the local concentration in F-actin bundles [26, 31]. This implies that the spatial distribution, level of expression, and even the activation for actin-binding of filamin may contribute to stabilization and remodeling of cortical actin during cell motility.

### 1.4 Filamins regulate cell migration

Cell migration is essential for both embryonic development and homeostasis. It also influences many pathological processes, including vascular disease, chronic inflammatory disease, and tumor formation and metastasis. This highly orchestrated migration process involves the following steps: polarization and protrusion in the direction of movement, formation of adhesion complexes to stabilize the protrusion, and retraction and release of the attachments at the rear. The turnover and reorganisation of the actin cytoskeleton are the driving forces of cell migration. Filamins exist in various motile cells at both the leading edge and the rear of polarized cells. They influence both cell protrusion and retraction by directly regulating actin cytoskeletal remodeling. In addition, filamins bind to a large number of diverse proteins and, so far, more than 70 filamin-binding partners have been reported [33]. Many filamin-interacting proteins have great functional importance on the regulation of cell motility. Thus, the existence of filamins in the leading edge and the rear of cells may bring the interacting proteins in proximity to the sites where migration occurs and mediate their effects on cell migration.

### *1.4.1 Filamins mediate integrin signaling*

The ability of adhesion receptors to transmit biochemical signals and mechanical forces across cell membranes depends on interactions with the actin cytoskeleton. Filamins are well-positioned to mediate matrix-cytoskeletal signaling pathways by virtue of their dual binding to both F-actin and the major transmembrane adhesion receptors, integrins. Filamins interact with the cytoplasmic tails of multiple β-integrins: integrin $\beta_1$, which regulates endothelial cell motility, binds to both FLNA at repeats 21–24 [34] and FLNB [35]; integrin $\beta_2$, which mediates leukocyte extravasation, binds to FLNA at repeat 21 [36]; integrin $\beta_3$, which induces platelet aggregation, binds to FLNA; integrin $\beta_7$, which mediates lymphocyte migration and homing, binds to FLNA at repeat 19–21 [37].

FLNA regulates cell spreading via integrin $\beta_1$ in human gingival fibroblasts and kidney cells. FLNA is recruited to the cell membrane immediately following stimulation of integrins by the extracellular matrix. As a result, FLNA may promote integrin–ligand interactions by binding the cytoplasmic domain of the integrin $\beta_1$ and facilitate ligand binding through inside-out signaling [38]. The effects between integrins and filamins are reciprocal. The mechanical stress delivered through integrin $\beta_1$ recruits both FLNA and F-actin to integrin $\beta_1$-containing focal adhesion [39]. It also selectively activates the p38 pathway that improves *FLNA* promoter activity and the prolongation of its mRNA half-life in fibroblasts [38]. In contrast, blocking function of integrin $\beta_1$ reduces cell spreading and localization of FLNA to cell extensions. Reciprocally, *FLNA*-deficiency in human tumor cells reduces expression of endogenous integrin $\beta_1$ in the cell membrane [40]. Furthermore, the reduced FLNA expression impairs integrin $\beta_1$–collagen binding [41].

Although FLNA is necessary for efficient cell migration, the formation of a strong link between integrin and filamin impairs migration. The influence of this interaction on cell migration seems to be a fine-tuning process regulated by both the affinity of filamin to integrins and the other integrin-binding partners. In fact, the increased binding of FLNA to integrin $\beta_7$ and $\beta_{1A}$ tails impairs migration without altering focal adhesion formation or fibronectin matrix assembly [37, 42]. These effects on cell migration are ascribable to a reduction in transient membrane protrusions and cell polarization. This suggests that changing the filamin-binding affinity of β-integrin can alter membrane protrusion and cell polarization and thus cell migration [37]. It can simultaneously affect the binding capacity of other integrin partners such as talin [43] and α-actinin [36] which use binding sites on integrins that overlap with those of FLNA. FLNA may compete with these proteins for binding to integrin tails and, therefore, allow integrin-filamin interactions to impact talin-dependent integrin activation [37, 43]. The phosphorylation of integrins can serve as molecular switches for regulating binding between filamins and other integrin regulators. For instance, the phosphorylation of integrin $\beta_2$ can inhibit FLNA binding but allow the binding of 14-3-3 adaptor protein, thereby inducing T-cell adhesion [44].

Collectively, the binding between filamin and integrin requires an equilibrium: a sufficient degree of integrin–filamin binding stabilizes cell–matrix adhesions, while excessive binding of filamin prevents efficient actin remodeling and cell motility [41].

### *1.4.2 Filamins coordinate signaling of small GTPases*

Following integrin binding of extracellular matrix ligands, the small GTPases are activated, leading to actin polymerization and the formation of lamellipodia and filopodia. The branched actin networks are particularly important for the formation of lamellipodia that is believed to be the actual motor that pulls cells forward. Filopodia originate from the pre-existing lamellipodial actin network that is prevented from capping and, as a result, can elongate at the leading edge of the lamellipodia.

In mammalian cells, the generation of actin-based dynamic motile structures is regulated by the small GTPases of the Rho family. FLNA interacts with both small GTPases including Rac, Rho, Cdc42 and RalA [45] and factors upstream and downstream GTPases mostly at repeats 23 and 24 of the C-terminal region. These interactions present a delicate regulatory mechanism on the activity of GTPases. The interaction between FLNA and RalA may recruit filamin into the filopodial cytoskeleton and is necessary for RalA-mediated filopodial protrusion [45]. FLNA interacts with the serine/threonine kinase p21-activated kinase-1 (PAK1), a downstream effector of Rac1 and Cdc42. Reciprocally, FLNA is phosphorylated by PAK1 and PAK1 is activated by binding to FLNA which is necessary for PAK1-mediated membrane ruffling [46]. Another way to regulate PAK1 activity by FLNA is via its interaction with sphingosine kinase 1. This kinase catalyzes the phosphorylation of sphingosine to produce the potent lipid mediator sphingosine-1-phosphate which directly stimulates PAK1 kinase [47]. FLNA also forms a complex with ROCK, a Rho downstream effector and this complex co-localizes at protrusive cell membranes [48]. As the upstream regulators of GTPases, guanine nucleotide-exchange factors (GEFs) activate GTPases by promoting their exchange from GDP to GTP. FLNA associates with both Rac GEF Trio [49] and Rho GEF Lbc [50] and may regulate the spatial positioning of actin assembly. In opposition to GEFs, a variety of GTPase-activating proteins (GAPs) suppress GTPase activity by returning them to the inactive GDP-bound state. FLNA may inactivate Rho during the earliest phases of cell spreading by virtue of its ability to promote accumulation of p190RhoGAP in lipid rafts [51]. A specific GAP for Rac, FilGAP, has also been shown to interact with FLNA. This interaction is required for FilGAP to inactivate Rac, to suppress leading edge protrusion and to promote cell retraction [52].

In summary, FLNA anchors GTPase signaling factors in proximity to the cell membrane where the conversion between GDP and GTP occurs, acts as a scaffold for these factors and coordinates their actin -remodeling activities. During cell protrusion, Trio activates Rac and stimulates actin assembly through PAK and other pathways. Simultaneously, Rho is inactivated by the accumulation of P190RhoGAP in the cell membrane [51]. During cell retraction, activation of Rho by Lbc stimulates ROCK activity. Active ROCK phosphorylates and stimulates FilGAP to inactivate Rac. In addition, ROCK phosphorylates and activates myosin II to promote contractile activity [30].

### *1.4.3 Filamins regulate migration of multiple cell types*

Besides integrins and GTPases, a number of other filamin interacting partners can regulate the cell motility via filamin-mediated cell signaling in a broad range of

circumstances.

In cerebral cortical neurons, *FLNA* mutations prevent cellular migration and cause human periventricular nodular heterotopia (PVNH) [53]. Therefore, studies have been particularly focused on the functional importance of filamin on neuron migration. In the developing neocortex, most excitatory neurons travel from the ventricular zone (VZ) to the cortical plate, which is an essential process in corticogenesis. The precise regulation of FLNA level in VZ appears to control the initiation of neuron migration. FLNA-interacting protein (FILIP), which is restricted to the VZ in the developing neocortex, induces FLNA degradation in VZ. This suggests a switch mechanism to control the start of neocortical cell migration from VZ [54-56]. The mitogen-activated protein kinases (MAPKs) have recently been suggested as another switch mechanism to regulate FLNA level [57, 58]. MAPKs are activated via signaling cascades involving MAPK kinases (MAP2K) that are in turn activated by MAP2K kinases (MAP3K). One of the MAP2Ks, MKK4 (SEK1) physically interacts with FLNA [59] and mediates MEKK4 (one of the MAP3Ks) signaling [57]. *MEKK4* knockout mice [57] frequently develop PVNH with similar phenotypes to those caused by *FLNA* mutations. *MEKK4* deficiency both enhances FLNA expression in the developing forebrain and induces the FLNA phosphorylation on Ser2152 that confers resistance to calpain cleavage [46] and further elevates FLNA level. Overexpression of FLNA prevents neurons from migrating away from the VZ surface [57]. These results are consistent with the inhibiting function of high FLNA level on β integrin function [37] and emphasize the point that either excessively low or excessively high FLNA levels impair neuron migration.

In the vasculature, FLNA may play important roles in development and pathological processes. FLNA directly binds to tissue factor which contributes to the regulation of blood vessel development in early embryogenesis and displays an independent manner of regulating cell adhesion and spreading [60]. Recent evidence supports a role for FLNA on fully differentiated vascular wall cell types. The G protein-coupled $P2Y_2$ nucleotide receptor-mediated spreading and migration of aortic smooth muscle cells is impaired when this receptor loses the interaction with FLNA [61]. ECSM2, an exclusively endothelial-specific surface protein, interacts with FLNA and regulates endothelial chemotaxis and tube formation [62]. These studies implicate a role for FLNA in angiogenesis via modulation of the actin cytoskeleton.

In tumors, FLNA may play roles in both regulation of vasculature and cancer cell metastasis. Inhibiting angiogenesis has been a major therapeutic strategy for cancer treatment. In endothelial cells, endostatin, an inhibitor of angiogenesis, upregulates the expression of FILIP 1-like (FILIP1L). Targeted expression of an active *FILIP1L* mutant in tumor vasculature inhibits tumor growth *in vivo* potentially through inhibition of FLNA-bridged cytoskeletal remodeling and cell migration [63]. Moreover, FLNA regulates tumor cell metastasis. The oncogenic protein caveolin-1 interacts with FLNA and promotes both transcription and AKT-mediated Ser2152 phosphorylation of FLNA in human breast cancer cells. This specifies a novel mechanism for FLNA in mediating the effects of caveolin-1 on IGF -1-induced cancer cell migration [64]. On the other hand, the local association of FLNA with carcinoembryonic antigen-related cell adhesion molecule strengthens

the cell-cell contact and thus presents a negative effect on the tumor cell metastasis [65].

### 1.5 Filamins regulate transcriptional activity

There is recent evidence for the involvement of filamins in transcriptional regulation. One way for filamins to regulate transcriptional activity is through the activation or retention of transcription factors in cytoplasm. FLNA interacts with SMAD2 and SMAD5, positively regulating their receptor-induced phosphorylation and thus nuclear accumulation [66]. However, the binding between FLNA and either PEBP2β/CBFβ, the subunit of the heterodimeric transcription factor PEBP2/CBF [67, 68], or p73α, a transcription regulator [69], retains them in the cytoplasm and implicates transcriptional repression.

Interestingly, a small fraction of full length FLNA resides in the nucleus of human skin fibroblasts and HeLa cells, where it participates in DNA damage response through a nuclear interaction with BRCA2 [70]. This unanticipated finding raises the possibility that FLNA may affect transcription factors in the nucleus. It has been demonstrated that the regulation of FOXC1 transcriptional activity is mediated through multiple interactions with both FLNA and the transcriptional regulator PBX1 [71]. The full length FLNA efficiently carries PBX1 into the nucleus where the FOXC1-PBX1 transcriptional inhibitory complex forms.

However, nuclear transport of FLNA is still controversial. In androgen-dependent prostate cancer cells, a 90 kDa proteolytic fragment cleaved by calpain is actually localized to the nucleus. In contrast, in its androgen-independent subline, FLNA fails to be cleaved and remains cytoplasmic [72]. Whether the small amount of full length FLNA in the nucleus is below the limit of detection is unknown. But this observation of only cleaved FLNA in nucleus is consistent with the finding that the calpain-cleaved fragment facilitates nuclear translocation of androgen receptor (AR), a transcription factor that regulates sexual differentiation. Nuclear presence of the FLNA fragment interferes with the interdomain interactions and coactivator binding of the AR and thus represses the transactivation activity of AR [73, 74].

By interacting with transcription factors, filamins may regulate the expression of growth factors that induce cell motility via autocrine or paracrine signaling. In Paper I of this thesis, we discovered a novel interaction between FLNA and hypoxia-inducible factor-1α (HIF-1α). HIF-1α/aryl hydrocarbon receptor nuclear translocator complex mediates the adaption of mammalian cells to low levels of oxygen by activating a network of target genes [75, 76]. At normoxia, HIF-1α protein is targeted for degradation by the von Hippel-Lindau tumor suppressor protein acting as an E3 ubiquitin ligase following hydroxylation of proline residues [77]. Upon exposure to hypoxia, the hydroxylase activity is inhibited, resulting in the stabilization of HIF-1α protein. HIF-1α is then translocated into the nucleus by a process that is not completely understood, to bind to the hypoxia-response element (*HRE*) and activate transcription of target genes, including vascular endothelial cell growth factor A (*VEGFA*) [78]. HIF-1α has two transactivation domains (TAD). The capacity of the N-terminal and C-terminal TAD is differentially regulated by $O_2$ tension, where the N-TAD constitutes an oxygen-dependent degradation box and the C-TAD functions in a strictly hypoxia-inducible

fashion [79]. In contrast to the repressive impact of cleaved FLNA on AR transcriptional activity, we found that this fragment facilitates nuclear translocation of HIF-1α and promotes its transactivation activity. Consequently, it induces the production of VEGF-A that acts as an important chemoattractant to regulate endothelial cell migration and proliferation.

FLNA may also regulate the expression of transmembrane receptors by interacting with transcription factors, thus altering the cellular response to the extracelluar signals including growth factors. In Paper II of this thesis, we found that c-MET expression and phosphorylation are impaired in *FLNA*-deficient human tumor cells. c-MET tyrosine kinase is a cell surface receptor for hepatocyte growth factor (HGF), a pleiotropic cytokine that induces promigratory and mitogenic signals [80]. The mature form of the c-MET receptor is a heterodimeric protein consisting of a 45 kDa extracellular α-subunit linked by disulfide bonds to a 145 kDa β-subunit [81]. The β-subunit spans the membrane and contains a catalytic kinase domain as well as a number of tyrosine phosphorylation sites in its cytoplamic region. Binding of HGF to c-MET triggers transphosphorylation of two tyrosine residues (Tyr1234 and 1235) in the kinase domain followed by phosphorylation of two other tyrosine residues (Tyr1349 and 1356) in the multiple docking site. A group of signaling molecules and/or adaptor proteins is then recruited to the cytoplasmic domain and multiple docking sites of c-MET. This action leads to the activation of several subsequent signaling cascades that form a complete network of intra- and extracellular responses. Activation of c-MET by HGF induces diverse biological events, such as scattering, invasion, proliferation and branching depending on the different combinations of signaling pathways and/or differences in magnitude of responses [82]. In human cancers, c-MET germ-line missense, activating and somatic mutations have been identified [83], but the most frequent occurrence is the aberrant expression of c-MET associated with metastatic phenotype [81]. In our study, we found that c-MET expression is up-regulated by FLNA partially through the action of transcription factor SMAD2. FLNA binds to SMAD2 and facilitates its transport into nucleus [66] where SMAD2 activates c-MET by binding to a putative SMAD binding element in the *c-MET* promoter [84]. This indicates a novel mechanism by which FLNA regulates c-MET signaling and thus the cellular response to HGF.

### 1.6 Filamins are associated with human genetic diseases

The versatile functions of filamins in regulating cell motility and signaling suggested that their mutation may cause a large spectrum of human disorders. Indeed, null and specific missense mutations in *FLNA* or *FLNB* cause a wide range of developmental malformations of the brain, bone, limbs, and heart in human [6].

Null mutations in *FLNA* cause an X-chromosome-linked brain malformation known as PVNH. Neurons in PVNH patients fail to undergo radial migration from the VZ to form the six-layered neocortex during fetal development [53]. X-linked PVNH is mainly confined to females, indicating a predominant prenatal lethality for males who are hemizygous for *FLNA* mutations [85]. PVNH can result from abnormal mRNA splicing or truncation of the FLNA protein into different sizes [53, 86, 87], leading to loss-of-function mutations. FLNA levels are higher in the brains of mouse embryos and human fetuses, but

reduced at postnatal ages [53]. As FLNA is essential for cell migration, mutations of FLNA in neurons may impair neuronal migration to appropriate anatomical sites during development and thus provide a plausible explanation for the occurrence of PVNH [6]. However, not all clinical observations can be explained by the impact of cell migration. For instance, a number of non-central nervous system phenotypes are recognized in PVNH patients, including a high incidence of cardiovascular abnormalities such as patent ductus arteriosus, minor cardiac malformations, aortic aneurysms, premature strokes, and apparent hypercoagulable state in females [53, 88]. Very rarely live born males have lethal vascular defects and intractable haemorrhage [6]. Current understanding of the molecular mechanisms underlying these cardiovascular symptoms is still quite poor.

Clustered missense mutations in *FLNA* have been identified in a diverse spectrum of congenital malformations in humans, including otopalatodigital syndrome (OPD), frontometaphyseal dysplasia (FMD) and Melnick-Needles syndrome (MNS) [89]. The syndromes certainly overlap between OPD, MNS and FMD with generalized dysplasia involving craniofacial structures, digits and long bones. However, the clusters of genetic mutations associated with various syndromes are strikingly segregated in FLNA protein [6]. The patterns of *FLNA* mutations, X-chromosome inactivation and phenotypic manifestations indicate that they have gain -of-function effects [89, 90]. Mutations clustered in the CH2 domain are predicted to increase the actin binding of FLNA, thereby either disorganizing actin or creating toxic products that function in a dominant-negative fashion. However, FLNA repeats 10, 14 and 15, which contain many of the missense mutations in OPD spectrum disorders, are less characterized in terms of their functions and binding partners [6]. Remarkably, the phenotypes of OPD, MNS and FMD caused by missense mutations are entirely distinct from PVNH. Almost none of these disorders are associated with PVNH or any other definable cerebral disturbance in neuronal migration [6]. It suggests that distinct developmental mechanisms underlie the two groups of disorders.

Recently, X-linked myxoid valvular dystrophies (XMVD) in the heart have also been linked to specific mutations in *FLNA* [91]. XMVD are frequently the cause of valvular disease and anomalies, including mitral valve prolapse, and mitral and aortic regurgitation. However, no signs of PVNH, OPD, FMD, or MNS are found in these patients. FLNA may contribute to these myxomatous changes in the cardiac valves by regulation of transforming growth factor-β (TGF-β) signaling through its interaction with SMADs [66, 92]. Defective signaling cascades that involve members of the TGF-β superfamily have been described in impaired remodeling of cardiac valves during development.

Similar to mutations in *FLNA*, *FLNB* mutations produce diverse phenotypes depending on the nature and location of the mutation. Nonsense mutations of *FLNB* can result in autosomal recessive spondylocarpotarsal syndrome (SCT), which is characterized by short stature and vertebral, carpal and tarsal fusions [93]. Some SCT patients with *FLNB* mutations exhibit narrowing of retinal vessels, in addition to severe skeletal malformations [94]. SCT patients are homozygous with respect to the mutations which result in truncations or absence of FLNB protein. Missense mutations cause autosomal dominant Boomerang dysplasia (BD), Larsen syndrome and

atelosteogenesis I and III phenotypes (AOI, AOIII). BD is a perinatal lethal osteochondro-dysplasia, characterized by the absence or hypo-ossification of the limb bones and vertebrae [95]. Larsen syndrome is a genetically heterogeneous disorder characterized by multiple joint dislocations, craniofacial abnormalities and accessory carpal bones. AOI and AOIII are autosomal dominant lethal skeletal dysplasias with vertebral abnormalitites, disharmonious skeletal maturation, poorly modelled long bones and joint dislocations [93]. Intense FLNB expression at the cleavage furrow of dividing chondrocytes indicates that some mutations in *FLNB* confer a defect in chondrocyte division, possibly accelerating apoptosis [93].

It is interesting to note that the phenotypes of *FLNB* mutation-caused disorders have some overlap with the OPD spectrum diseases caused by *FLNA* mutations. This may be explained by the fact that FLNA and FLNB are both widely expressed and are highly similar in both structure and molecular interactions. The potential genetic and functional redundancy and compensation between these two filamin isoforms thus add another layer of complexity to our understanding of the human diseases caused by filamin mutations [6].

## 1.7 Cellular and animal models of filamin deficiency

The actin-gelation function of filamins was originally discovered using *in vitro* purified actin solutions. The identification of *FLNA*-deficient human melanoma cells has dramatically prompted the field of filamin biology research to determine both the actin-binding properties and the interacting partners of FLNA in living cells. Very recently, mouse models of filamin deficiency for all three isoforms have been reported [35, 96-101]. Subsequently, primary cells including fibroblasts, endothelial cells and chondrocytes have been extracted from embryos [35, 97, 98, 100]. The generation of both *in vivo* mouse models and primary cell lines will definitely advance our understanding of the molecular functions and biological consequences of filamins during embryonic development. This will facilitate deciphering of human congenital disorders caused by filamin mutations.

### *1.7.1 Human melanoma cells deficient for FLNA*

From seven human melanoma cell lines, Cunningham *et al.* identified three lines lacking FLNA protein due to markedly reduced *FLNA* mRNA levels [102]. Asymmetrical spreading to form lamellae is exhibited by *FLNA*-containing cell lines, but not those that are *FLNA*-deficient. Cells from all *FLNA*-deficient lines display extensive, continuous blebbing of the plasma cell membrane, poor pseudopod protrusion and impaired motility. A few stable *FLNA*-expressing cell lines have been generated by transfecting an LK444 vector containing the full-length *FLNA* coding sequence driven by the human β-actin promoter into one of the three *FLNA*-deficient lines (designated M2). *FLNA*-restored cells migrated up to five times more than M2 cells through a porous membrane in response to a gradient of chemoattractant. One of the *FLNA*-transfected lines, designated M2A7 (or A7), possesses a physiological molar ratio of FLNA to actin and is widely used in the studies of FLNA [102].

### *1.7.2 Mouse models of filamin deficiency*

In 2006, one chemically-induced and one genetically-modified mouse model of *Flna*

deficiency were reported from independent laboratories. In a screen for dominant eye mutations induced by N-ethyl-N-nitrosourea, a nonsense mutation which maps to the *Flna* gene (Y2388X in Ig repeat 22), designated *Dilp2*, results in the absence of Flna [99]. This loss-of-function mutation causes mild skeletal abnormalities in female carrier mice. In males, high lethality occurs because of incomplete septation of the heart during gestation and is accompanied by other cardiac, skeletal and palate defects. The genetically-modified mouse model was produced by a conditional knockout strategy by cross-breeding *Flna*-floxed females with β-actin *Cre* males. In this model, an early truncation at amino acid 121 in CH1 domain results in complete loss of *Flna*. *Flna* heterozygous knockout females are viable, but no male hemizygous embryos reach term [100]. Embryonic lethality is caused by severe cardiac structural defects involving ventricles, atria, and outflow tracts, as well as widespread aberrant vascular patterning. These abnormalities in cardiac morphogenesis are the most common phenotypes exhibited in both models of *Flna* deficiency. The migration of *Flna*-deficient fibroblasts is not affected, but a cell motility-independent function of Flna in cell-cell contacts and adherens junctions during organ development has been shown in endothelial cells [100]. The observed palatal and sternal defects in the chemically-induced model share some similarities with the clinical symptoms observed in human OPD syndrome associated with *FLNA* missense mutations. However, the major human phenotype PVNH caused by *FLNA* loss-of-function has not been observed in chemically-induced *Flna* knockout mice. This may be explained by the compensatory action of Flnb in the absence of Flna, as FLNA and FLNB are co-expressed and form heterodimers within neurons during periods of neuronal migration [20].

The high sequence identity between FLNA and FLNB makes it motivating to compare biological functions of filamin isoforms in *in vivo* models. In Paper III of this thesis, we generated a mouse model of *Flnb* deficiency. We observed embryonic lethality and severe skeletal malformations, including scoliotic and kyphotic spines, lack of intervertebral discs, fusion of vertebral bodies, and reduced hyaline matrices in extremities, thorax and vertebrae in a few homozygous *Flnb*-deficient mice that reached term. The coupled malformations in the vasculature included rudimentary vascular appearance particularly in the central nervous system and disorganized microvascular patterning around the vertebral column, although these vascular malformations were less severe as compared with those seen in *Flna*-deficient mice [97]. Following our report, three additional *Flnb*-deficient mouse models have been published from independent laboratories [35, 96, 98]. A detailed discussion comparing these models is given in the Results and Discussion.

Similar to *Flnb* mutant mice, *Flnc* mutant mice have severely reduced birth weights [101] and die shortly after birth, owing to respiratory failure. Flnc expression is specific to cardiac and skeletal muscle. Despite expression of Flnc expression in the heart, they do not show cardiovascular developmental phenotypes. However, consistent with the expression of Flnc in skeletal muscle, the mutant mice show fewer muscle fibers and primary myotubes, indicating defects in primary myogenesis. This model suggests that Flnc has a crucial role in muscle development and maintenance of muscle structural integrity.

# 2 AIM OF THE THESIS

Filamins have been recently recognized as versatile signaling scaffolds via interactions with a large number of cellular proteins with great functional diversity. In this thesis, I intended to identify novel interacting partners of filamins and filamin-regulated signaling pathways that could reveal novel functions of filamins and provide a better understanding of filamin-associated molecular signaling. Filamin mutations cause a wide range of human genetic diseases. To understand the cellular and molecular mechanisms underlying these human diseases, I aimed to generate mice and cell lines deficient in filamins.

This thesis is organized into three scientific reports, and intended to address the following questions:

**Paper I:**

How does FLNA modulate cellular response to hypoxia by regulating HIF-1α transcriptional activity?

**Paper II:**

How does FLNA influence c-MET signaling and HGF-induced cell migration?

**Paper III:**

Can *Flnb* deficiency mouse model mimic *FLNB* mutation-caused human diseases? What is the mechanism underlying *Flnb* deficiency-induced malformations?

# 3 METHODOLOGICAL CONSIDERATIONS

**Cell culture**

The following cell lines and primary cells were used in this thesis: *FLNA*-deficient M2 and *FLNA*-expressing A7 human melanoma cells, COS-1 fibroblasts, PAE cells expressing either VEGF receptor 1 (VEGFR1) or VEGFR2, NIH3T3, primary *Flna* wild-type and knockout, *Flnb* wild-type, heterozygous and homozygous knockout mouse embryonic fibroblasts. All cells were cultured in a 5% $CO_2$ incubator at 37ºC. Oxygen tension was either 140 mm Hg (21% $O_2$ [vol/vol], normoxia) or 7 mm Hg (1% $O_2$ [vol/vol], hypoxia).

**Gene expression: RT-PCR and real-time RT-PCR**

Total RNA was extracted from either cells or tissues. cDNA was synthesized from total RNA by reverse transcription and submitted to either conventional or real-time PCR.

RT-PCR followed by agarose gel electrophoresis and ethidium bromide staining was used to measure *c-MET* (Paper II), and *Flna* and *Flnb* (Paper III) transcripts. *18S* rRNA transcript was used as internal control.

Real time RT-PCR was used to measure human *VEGFA*, *PGK* and *GLUT3* transcripts (Paper I). Expression of *β-actin* or *18S* rRNA was included as internal loading control.

**Protein quantification: Western blotting and ELISA**

In Western blotting, either whole cell, nuclear or cytosolic extracts from cells or tissues were used. In Paper I, protein levels of FLAG- or HA-tagged FLNA and HIF-1α, and endogenous HIF-1α and FLNA were measured. Actin was used as the internal loading control for whole cell extracts, YY1 for nuclear extracts, and paxillin for cytosolic extracts. Immunoprecipitation was employed to concentrate low-level HIF-1α protein before immunoblotting. Protein levels of c-MET, phosphorylated-c-MET (p-c-MET), AKT, p-AKT, ERK1/2, p-ERK1/2, SMAD2, and p-SMAD2 were measured in Paper II. Either actin or GAPDH were used as the internal loading control for whole cell extracts. In Paper III, protein levels of Flnb and RhoA were measured, whereas actin was used as the internal loading control for whole cell extracts.

ELISA was used to quantify secreted VEGF-A level in culture medium of M2 and A7 cells (Paper I) and GTP-bound form of RhoA and Rac from cell extracts of embryonic fibroblasts (Paper III).

**Protein expression: histological analysis**

Immunohistochemical or immunofluorescence staining were used to detect the localization and expression pattern of proteins in either cells or tissues. In Paper I, intracellular localization of FLNA in M2, A7, and COS-1 cells was visualized with TRITC-conjugated secondary antibody. In Paper III, formaldehyde-fixed and paraffin-embedded sections were stained for Flnb, CD31, and LYVE-1 (a lymphatic endothelial cell marker). Whole mount CD31 staining was used to visualize mouse embryonic vasculature.

Non-immunohistochemical staining techniques included phalloidin staining to visualize F-actin of endothelial cells (Paper I) and MEF (Paper III), X-gal staining to detect expression of inserted *β-geo* gene which represents the endogenous expression of Flnb in frozen *Flnb*-heterozygous

or homozygous knockout embryos or tissues (Paper III).

**Protein-protein interaction: yeast two-hybrid screening and co-immunoprecipitation**

In yeast two-hybrid screening, a GAL4-based system was used to screen a human T-cell cDNA library to identify novel proteins interacting with HIF-1α. The N-terminal 984-bp (1-328 aa) fragment of human HIF-1α cloned into pGBKT7 was used as bait (Paper I).

Co-immunoprecipitation was used to examine the interaction between FLNA and HIF-1α and to map the domains of HIF-1α that interact with the C-terminus of FLNA in Paper I, and to examine the interaction between FLNA and SMAD2 in Paper II.

**Cell migration and invasion: Boyden chamber and wound-healing assays**

In Boyden chamber assays, a cell suspension in serum free or low serum medium was added into the upper chamber and a chemoattractant was added into the lower chamber, which is isolated from upper chamber by a porous membrane. In Paper I, PAE cells migrated towards M2- or A7-conditioned culture medium. In Paper II, M2 or A7 cells migrated towards gradient of HGF. In Paper III, *Flnb* wild-type or knockout MEF migrated towards 10% serum medium. The number of cells that migrated through the holes was counted as an index of cell migration. In Paper II, the number of M2 or A7 cells that migrated through a Matrigel-coated membrane towards an HGF gradient was used as an index of cell invision.

In Paper II, a wound-healing assay was used to measure cell motility of *Flnb* wild-type or knockout MEF in addition to Boyden chamber assay. In this assay, no gradient of chemoattractant was applied. Cells in this assay must disrupt cell-cell contacts during migration, while in the Boyden chamber assay, cells are not in contact with each other.

**Cell growth: proliferation and colony formation assays**

The proliferation assay referred to anchorage-dependent cell growth, in which adherent cells were seeded on cell culture dishes and counted daily for up to 4 days. In Paper I, PAE cells were cultured in M2- or A7-conditioned medium. In Paper II, M2 or A7 cells were cultured in medium with or without HGF supplement.

The colony formation assay referred to anchorage-independent cell growth. In Paper II, M2 or A7 cells were cultured in agarose gel containing HGF and the number and size of colonies were counted at day 10.

**Regulation of protein expression: plasmid and siRNA transfection**

Plasmid vectors were transfected into cells to overexpress the following proteins. In Paper I, plasmids encoding HA-FLNA C-terminus were transfected into M2 cells. Plasmids encoding HA-tagged C-terminal FLNA and Flag-tagged HIF-1α were co-transfected into NIH3T3 cells to study FLNA–HIF-1α interaction. Plasmids encoding either pFLAG-mHIF-1α (1–390 aa), pFLAG/GAL4-mHIF-lα (392–622 aa), or pFLAG/GAL4-mHIF-lα (531–822 aa) were co-transfected with HA-tagged FLNA C-terminus into 293 cells to map the domains of HIF-1α that interact with FLNA C-terminus. In Paper II, plasmids encoding SMAD2 and FLNA were transfected into M2 or A7 cells.

pGFP-HIF-1α was transfected into M2, A7, or COS-1 cells to visualize intracellular localization of HIF-1α (Paper I).

To modify cleavage of FLNA by calpain, the full-length of FLAG-tagged wild-type FLNA was used for mutagenesis studies to produce calpain-resistant FLNA constructs including pFLNA-ΔH1 encoding FLNA lacking H1 region, mut1 construct encoding FLNA with Q1760G single amino acid mutaion, and mut2 construct encoding FLNA with both Y1761G and T1762G mutaions. These plasmids were transfected into M2 cells to study the consequence of calpain cleavage (Paper I).

siRNA FLNA was transfected to knockdown FLNA expression in A7 cells (Paper I).

**Promoter activity: luciferase assay**

Luciferase reporter assays were used to measure promoter activity which reflects transcriptional activity. In Paper I, luciferase-expressing plasmids driven by either *HRE*- or *VEGFA*-promoter were transfected into M2, A7, or COS-1 cells. To normalize transfection efficiency, a *β-gal* reporter plasmid was co-transfected. In Paper II, a plasmid vector driven by the *c-MET*-promoter which encodes firefly luciferase was transfected into M2 cells. In this assay, transfection efficiency was controlled with pRL-SV40 encoding *Renilla* luciferase.

A GAL4 reporter assay was used to detect the N-TAD and C-TAD activity of HIF-1α (Paper I). In this assay, a GAL4-responsive luciferase plasmid was co-transfected with either pFLAG-GAL4/mHIF-1α (531–584 aa) encoding N-TAD or pFLAG-GAL4/mHIF-1α (772–822 aa) encoding C-TAD into M2 and A7 cells.

**Production of *Flnb*-deficient mice and genotyping**

The mouse ES cell line (BCB085 from strain 129/Ola) with an insertional mutation in *Flnb* caused by a *β-geo*-containing gene-trapping vector (pGT1lxf) was obtained from BayGenomics. The insertional mutation occurred in intron 20 which encodes the immunoglobulin-like domain repeat 16 just after hinge domain 1. The ES cells were injected into C57BL/6 blastocysts to create chimeric mice which were then bred with wild-type C57Bl/6 mice to generate heterozygous *Flnb*-deficient mice. All animal experiments were approved by the local animal ethical committee. Genomic DNA (10–20 μg) from tail biopsies or yolk sacs of embryos was genotyped by PCR.

**Bone and cartilage analyses**

In Paper III, a mixture of alcian blue and alizarin red was used to stain cartilage as blue and bone as red color in mouse embryos or in pups. Dual X-Ray absorptiometry was performed to measure areal bone mineral density of the mid-diaphyseal area of tibia *ex vivo*. Peripheral quantitative computerized tomography (pQCT) was performed to determine trabecular volumetrical bone mineral density *ex vivo*.

**Statistical analysis**

At least three independent samples were included in each study to perform statistical analysis. Mean ± SD values were given. For comparison of one dependent variable between two groups, Student's *t*-test was used.

# 4 RESULTS AND DISCUSSION

In this thesis, we identified a novel interaction of FLNA with a hypoxia responsive transcriptional factor, HIF-1α (Paper I), discovered a novel mechanism for FLNA in regulating c-MET-mediated intracellular signaling (Paper II), and established for the first time the *Flnb* knockout mouse model (Paper III).

## 4.1 FLNA promotes VEGF-A activity through the HIF-1α-mediated hypoxic response (Paper I)

FLNA, classically recognized as a cytoskeletal protein, has been recently shown to interact with a number of transcription factors and regulate their nuclear translocation [66, 73]. Here, we identified a novel interaction between FLNA and HIF-1α, and provided new evidence for the involvement of cytoskeletal proteins in hypoxic transcriptional regulation. In *FLNA*-deficient cells, we demonstrated an impaired functional activity of HIF-1α compared with *FLNA*-positive cells, resulting in decreased *VEGFA* promoter activity and VEGF-A secretion.

### *4.1.1 FLNA-deficient cells exhibit impaired nuclear localization and transactivation activity of HIF-1α at hypoxia*

We first identified FLNA as a HIF-1α-binding protein in a yeast two-hybrid screen and confirmed the interaction by a series of co-immunoprecipitation studies. The interaction sites were mapped to the N -terminus of HIF-1α (1–390 aa) and C-terminus of FLNA.

Nuclear accumulation of HIF-1α is regulated by hypoxia due to either facilitated nuclear import or inhibition of nuclear export [103]. It has been previously reported that FLNA is involved in the nuclear translocation of other transcription factors such as androgen receptor [73]. To determine the effect of *FLNA* deficiency on the nuclear localization of HIF-1α, we overexpressed HIF-1α in *FLNA*-deficient and -expressing cells and studied its intracellular distribution by confocal microscopy. Upon hypoxia treatment, *FLNA*-expressing cells exhibited an exclusively nuclear distribution of HIF-1α while there was no dramatic change in HIF-1α distribution in *FLNA*-deficient cells. These observations indicated that nuclear translocation of HIF-1α and/or the nuclear retention of HIF-1α at hypoxia was impaired in cells lacking FLNA. Moreover, endogenous HIF-1α protein levels were higher in the cytoplasm of *FLNA*-deficient cells when compared with *FLNA*-expressing cells, whereas *FLNA*-expressing cells presented a higher level of HIF-1α protein in the nucleus at hypoxia. The degradation of the HIF-1α protein is another major process in the regulation of HIF-1α signaling. We demonstrated that the half-life of the HIF-1α protein was approximately the same between *FLNA*-deficient and -expressing cells, indicating that FLNA does not interfere with HIF-1α degradation. These results demonstrate that FLNA facilitates nuclear localization of HIF-1α at hypoxia while not affecting its stability at normoxia.

We studied the impact of FLNA on the transactivation function of HIF-1α by determining the *HRE* activity in cells kept at normoxia or hypoxia. The response to hypoxia-dependent induction was much higher in *FLNA*-expressing cells compared

to *FLNA*-deficient cells, indicating that FLNA increases the transactivation function of HIF-1α. We further proved that the hypoxia-induced transactivation activity of HIF-1α N-TAD and even C-TAD was impaired in *FLNA*-deficient cells, although a direct interaction between C-terminal HIF-1α and FLNA was not shown. This indicates that FLNA can potentiate the transactivation activity of HIF-1α, which is independent of its impact on nuclear localization of HIF-1α. FLNA may participate in the recruitment of coactivator proteins to these HIF-1α transactivation domains. In fact, a direct interaction of FLNA with pVHL that binds to N-TAD has been shown [104].

### *4.1.2 Hypoxia-induced cleavage of FLNA increases nuclear localization and function of HIF-1α*

We detected both the full-length and cleaved C-terminal fragment of FLNA in the nucleus, however, the full-length FLNA was predominantly present in the cytosol. The cleaved fragment of FLNA was bound to HIF-1α in the nucleus at hypoxia. We found that hypoxia-inducible generation of the nuclear form of FLNA was calpain-dependent, which is in agreement with earlier studies showing that calpain efficiently cleaves FLNA at the H1 region [13] and that hypoxia upregulates calpain activity [105]. Using confocal microscopy, we observed that FLNA was colocalized with HIF-1α in the nuclear compartment of cells treated with hypoxia. These results show that the calpain-cleaved fragment of FLNA is a nuclear protein that, in contrast to full-length FLNA, is upregulated by hypoxia.

To investigate the effects of the cleaved FLNA fragment on HIF-1α signaling, we treated cells with calpain inhibitor and studied the subcellular localization of HIF-1α. Inhibition of calpain activity at hypoxia resulted in decreases in nuclear localization of HIF-1α and HRE activity in a dose-dependent manner, indicating that the cleaved form of FLNA is required to achieve maximal transactivation by HIF-1α. Overexpression of the C-terminal fragment of FLNA spanning the rod-domain repeats 20–24 was sufficient to increase hypoxia-inducible transcriptional activity of HIF-1α in *FLNA*-deficient cells. FLNA can also be cleaved by caspases, producing fragments of similar size [106]. To differentiate fragments cleaved by calpain versus caspase, we created single amino acid mutations within the H1 domain which specifically abolished cleavage of FLNA by calpain. Overexpression of this mutated FLNA protein reduced mRNA and protein levels of the HIF-1α target gene, *VEGFA*. Taken together, these results suggest that the cleaved fragment of FLNA plays an important role in regulating HIF-1α signaling.

### *4.1.3 FLNA-deficient cells show impaired promoter activity and secretion of VEGF-A*

In response to hypoxia, VEGF-A expression is induced through the increased transcription following the binding of HIF-1α to the *HRE* in the *VEGFA* promoter [107]. As *FLNA* deficiency impaired HIF-1α signaling, the promoter activity and secretion of VEGF-A were reduced in *FLNA*-deficient cells at both normoxia and hypoxia. Moreover, siRNA-mediated inhibition of FLNA production resulted in reduced secretion of VEGF-A. Expression of other HIF-1α target genes, such as *PGK* and *GLUT3*, was also reduced following transfection with siRNA FLNA. Consequently, conditioned medium obtained from *FLNA*-expressing cells, which con-

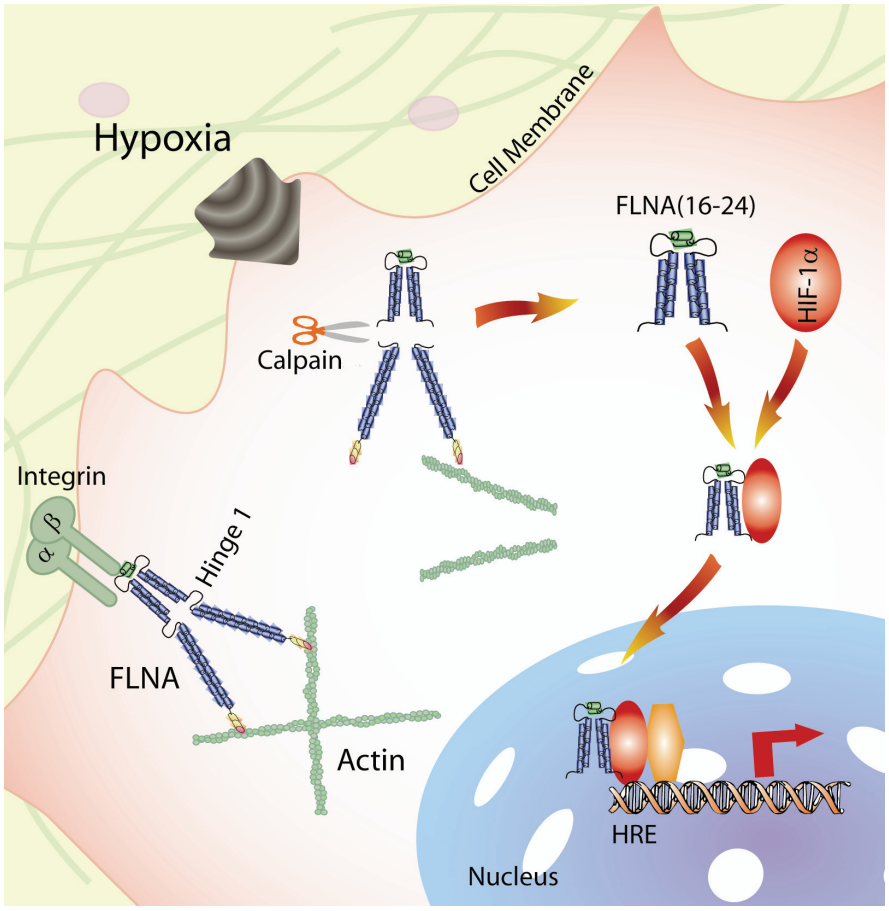


**Figure 2.** Schematic illustration of the proposed crosstalk between FLNA and HIF-1α signaling. A cleaved fragment of FLNA by calpain in hypoxia enhances the nuclear transport and transactivation of HIF-1α by protein – protein interaction, resulting in increased levels of expression of HIF-1α target genes, including *VEGFA*.

tained more VEGF-A, induced the morphology, proliferation, and migration of the endothelial cells expressing VEGFR2 but not VEGFR1. It has been suggested that VEGFR2 is the receptor that transduces functional signals of VEGF-A [108]. VEGF-A is crucial for tumor cells to recruit endothelial cells and to induce angiogenesis, thus the lack of FLNA may limit tumor growth *in vivo*.

In summary, our results show that the calpain-dependent cleavage product of FLNA is upregulated by hypoxia and is located in the nuclear compartment of cells. Based on these observations, we propose a model in which FLNA mediates a previously unrecognized mechanism of regulation of HIF-1α function and thus control of VEGF-A activity (Fig. 2). Under hypoxic conditions, calpain activity is increased and a higher level of the cleaved C-terminal fragment of FLNA is present in the nucleus. This fragment facilitates the nuclear localization of HIF-1α by enhancing either nuclear import or nuclear retention of HIF-1α by protein – protein interaction within either the cytoplasm or nucleus. The consequence of this interaction is an enhanced transactivation function of HIF-1α, resulting in increased levels of expression of HIF-1α target genes, including *VEGFA*.

### 4.2 FLNA regulates c-MET signaling via SMAD2 (Paper II)

A number of interacting partners of FLNA have been shown to modulate tumor cell metastasis by regulating FLNA-bridged

cell remodeling and migration [63-65]. We explored a novel mechanism by which FLNA regulates HGF-driven tumor cell migration and invasion by activating its receptor, c-MET.

### *4.2.1 FLNA deficiency decreases c-MET expression and downstream signaling*

c-MET activation through overexpression of the protein due to transcriptional upregulation is associated with high risk of tumor metastasis [109]. First, we demonstrated that *FLNA*-deficient tumor cells expressed significantly lower levels of *c-MET* mRNA and active form of c-MET compared to *FLNA*-expressing cells. To examine the downstream signaling pathways, we studied the phosphorylation of c-MET, which is fundamental for the broad biological functions involving c-MET kinase activity. We found that the phosphorylation of Tyr1234/1235 was reduced upon HGF stimulation in *FLNA*-deficient tumor cells compared to *FLNA*-expressing tumor cells. The phosphorylation of Tyr1234/1235 is required for full catalytic activity of c-MET to phosphorylate tyrosines outside the catalytic domain [110]. The decreased phosphorylation on these tyrosines may thus impair phosphorylation in the multidocking sites and the recruitment of the downstream signaling components.

Among the several major c-MET downstream signaling pathways, the PI3K-AKT pathway is associated with cell motility, while the RAS pathway via ERK/MAPK mediates HGF-induced cell scattering and proliferation. We found that *FLNA*-expressing cells responded to HGF treatment with a sustained activation of AKT while *FLNA*-deficient cells did not respond at all. On the other hand, we found that the constitutive levels of phosphorylated ERK1/2 were lower in *FLNA*-expressing cells compared to *FLNA*-deficient cells, which is in agreement with other studies [111]. However, in response to HGF, *FLNA*-expressing cells exhibited a remarkable transient increase in ERK1/2 phosphorylation. As sustained activation of ERK1/2 is required for transducing c-MET signals, the transiently induced ERK1/2 activation that we observed may be not a major effect, but rather indicate that *FLNA*-expressing cells are more sensitive than *FLNA*-deficient cells to HGF stimulation. These data imply that FLNA specifically regulates HGF-induced cell motility.

### *4.2.2 FLNA deficiency impairs HGF-induced cell migration and invasion*

Melanomas that occur in *Hgf* transgenic mice display a striking correlation between high metastatic potential and levels of overexpressed c-Met [112]. The reduced expression of c-MET expression and PI3K-Akt signaling that we observed in *FLNA*-deficient melanoma cells may indicate a reduced metastatic ability of these cells. We showed that both the migration and invasion of *FLNA*-deficient tumor cells towards a gradient of HGF were impaired. Neither the proliferation of *FLNA*-deficient nor *FLNA*-expressing melanoma cells was altered by HGF treatment. These results are consistent with an earlier study showing that HGF efficiently supports melanocyte growth *in vitro* only in combination with basic-fibroblast growth factor while HGF itself is sufficient to stimulate the motility of normal human melanocytes [113]. In the colony formation assay, *FLNA*-deficient cells formed smaller colonies without affecting their number in the presence of HGF. These data suggest that the impact of FLNA on anchorage-independent cell growth is mediated by different pathways and mechanisms.

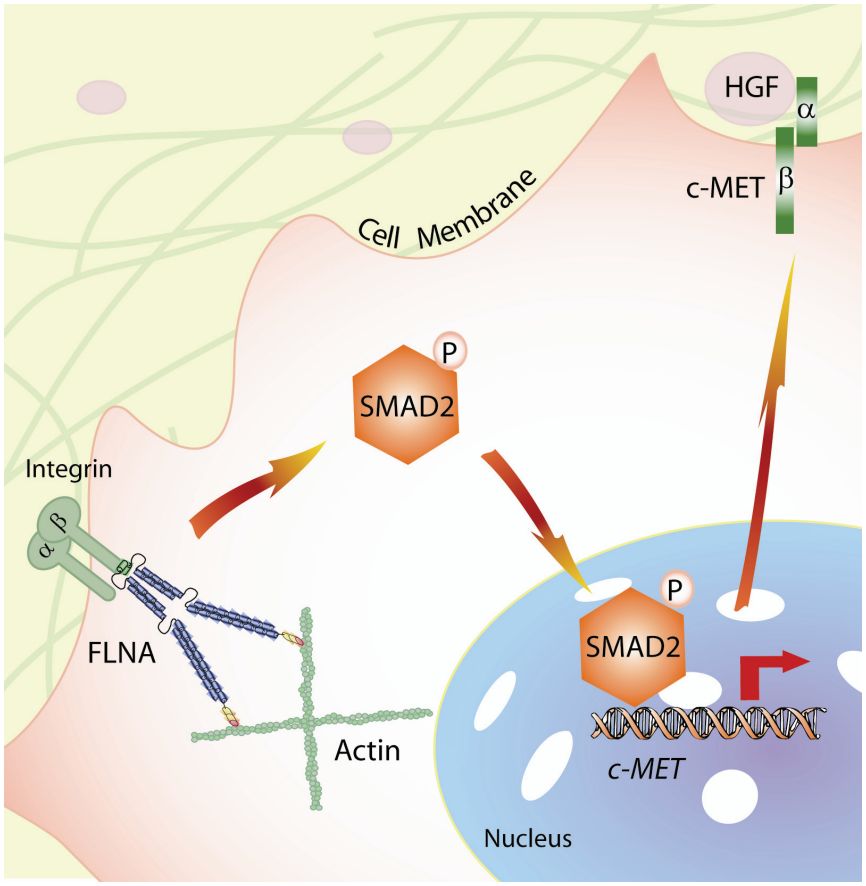


**Figure 3.** Schematic illustration of the proposed crosstalk between FLNA and c-MET signaling. FLNA induces c-MET expression and signaling by increased phosphorylation of SMAD2 and mediates HGF-induced cell migration.

#### *4.2.3 FLNA and SMAD2 cooperatively regulate c-MET expression and function*

It has been shown that FLNA binds to SMAD2, facilitates its transport into nucleus [66] and that SMAD2 regulates *c-MET* transcription by binding to its promoter [84]. Compared with *FLNA*-expressing cells, *FLNA*-deficient cells showed a decreased level of phosphorylated SMAD2. We confirmed the physical interaction of FLNA with SMAD2. The reduced promoter activity of *c-MET* in *FLNA*-deficient cells was significantly increased by transfection of plasmid vectors driving either *SMAD2* or *FLNA* alone, or in combination. Finally, we observed that *FLNA*-deficient tumor cells exhibited enhanced migration after overexpression of either SMAD2 or FLNA.

#### *4.2.4 Flna-deficient mouse embryonic fibroblasts exhibit reduced c-Met levels and impaired migration*

To confirm the impact of Flna on the relative levels of endogenous c-Met and phosphorylated Smad2 in another cell line, we studied c-Met activity in immortalized wild-type and *Flna*-deficient MEF and assayed these cells for HGF-induced migration. Similar to *FLNA*-deficient cells, *Flna*-deficient MEF exhibited a low level of active c-Met and Smad2. The migratory ability of *Flna*-deficient MEF was impaired.

In summary, FLNA induces c-MET expression via increased phosphorylation of SMAD2 and subsequent downstream c-MET signaling cascades. As a consequence of *FLNA* deficiency, HGF-induced tumor cell migration is impaired (Fig. 3).

### 4.3 *Flnb* deficiency in mice results in skeletal malformations and impaired microvascular development (Paper III)

To better understand how *FLNB* mutations cause human skeletal malformations, we produced *Flnb*-deficient mice and cell lines, and analyzed the consequences of *Flnb* deficiency on cell migration, the actin cytoskeleton, as well as embryonic and postnatal development. We show that *Flnb* deficiency leads to both severe skeletal malformations that recapitulate human *FLNB* disorders and impairments in microvascular development.

#### *4.3.1 Flnb deficiency causes embryonic lethality*

We generated *Flnb* knockout mice with 129/Ola × C57BL/6 background by a targeted disruption of *Flnb* in intron 20 using a gene-trapping technique. The mutant transcript was readily detected in both *Flnb* heterozygous and homozygous embryos indicating that a truncated protein lacking the C-teminus was likely formed. Unfortunately, we were unable to detect the truncated protein because the antibody recognized only the C-terminus of Flnb. Rare $Flnb^{-/-}$ mice that survived postnatally a few weeks developed severe skeletal malformations, while $Flnb^{+/-}$ mice exhibited normal growth rate and appeared healthy and fertile.

Following our first report of $Flnb^{-/-}$ mice, three other models were published. Zheng *et al.* used the same strategy as ours in their *Flnb* knockout model [98], while early disruptions at exon 3 and intron 3 were created in the models reported by Lu *et al.* [35] and Farrington-Rock *et al*. [96], respectively. These disruptions resulted in complete loss of Flnb protein. Although in all these models $Flnb^{-/-}$ mice died early in embryonic or postnatal stages, our $Flnb^{-/-}$ mice displayed most severe lethality, which was reminiscent of the *FLNB* mutation-caused perinatal lethal disorder, BD [95].

#### *4.3.2 Flnb-deficient mice develop severe skeletal malformations*

Our $Flnb^{-/-}$ pups exhibited fusions of vertebral bodies due to the lack of intervertebral discs, which was evident already at E13.5. These vertebral anomalies likely explain their short trunk and rigid posture due to the kyphosis and scoliosis. Moreover, reduced hyaline cartilage in the ribs, metacarpal bones, phalanges and tarsal bones in our $Flnb^{-/-}$ mice may result in additional fusions. These phenotypes strongly suggest that Flnb is crucial for cartilage development. The short stature and fusions of vertebral, carpal and tarsal bones are evident in the human SCT disorder caused by the nonsense *FLNB* mutation [93].

Furthermore, we noticed that tibiae from $Flnb^{-/-}$ mice were shorter. They also displayed reduced bone mineral in the mid-diaphyseal area of tibiae and reduced cortical thickness, but the trabecular volume was not changed. This implies that *Flnb* deficiency may cause abnormal skeletal ossification in addition to the defective cartilage development.

Cartilage and bone development is initiated by differentiation of mesenchymal cells to chondrocytes. Differentiated chondrocytes have two distinct fates: one is to remain as chondrocytes to form articular cartilage; the other is to mature into hypertrophic chondrocytes to function as a template for long bone during endochondral ossification [114]. We found that Flnb was strongly expressed in the chondrocytes of developing vertebral bodies. Thus, we

speculate that Flnb acts on chondrocyte differentiation, which could therefore affect both cartilage development and bone ossification.

Zheng *et al.* described in their model that chondrocytes between sternebrae, which are supposed to form cartilage, undergo ectopic hypertrophy starting at P5 [98]. This results in the abnormal ossification of the cartilage and eventually leads to fusions of vertebrae and sterna. Farrington-Rock *et al.* reported a similar phenotype of cartilage mineralization without any difference in femur length at P60 [96]. They detected neither any gross change in the organization of the growth plate cartilage of long bone nor in the lengths of the hypertrophic, proliferative, resting, or reserve zones of the growth plate. The ectopic hypotrophy in *Flnb*$^{-/-}$ mice is attributed to increases in Runx2 activity. Flnb inhibits Runx2 activity, which is partially mediated through the Tgf-β-Smad3 pathway, likely by controlling phosphorylated Smad3 [98]. These proposals of ectopic cartilage mineralization give a reasonable explanation for the lack of cartilage in *Flnb*$^{-/-}$ adult mice. However, they cannot completely explain the impaired ossification in the long bones nor the embryonic and early postnatal lethality observed in our and Lu *et al.*'s models. Instead of the excessive mineralization, the *Flnb*$^{-/-}$ mice of Lu *et al.* exhibited overall delayed calcification and ossification which was obvious at E18.5 [35]. Similar to the shortened tibiae in our *Flnb*$^{-/-}$ model, the ulna and radius of their *Flnb*$^{-/-}$ mice were shortened and less calcified. They attributed delayed ossification to a delay in the transition from prehypertrophic to hypertrophic chondrocytes during differentiation caused by an ectopic shift of chondrocyte progenitors from the hypertrophic zone to the resting zone. Moreover, the increased apoptosis in hypertrophic zone may explain the decreased bone width.

Most probably, Flnb functions as an important switch to determine the fate of differentiated chondrocytes to either remain as chondrocytes or undergo hypertrophy and ossification. In this way, Flnb may control both cartilage development and endochondral bone formation (Fig. 4).

### ***4.3.3*** *Flnb deficiency impairs vascular development*

Strong expression of Flnb in vascular endothelial cells encouraged us to study vascular development in *Flnb*$^{-/-}$ mice in detail. We observed a general impairment of microvascular structures in *Flnb*$^{-/-}$ embryos at E13.5. Strikingly, the perivertebral vasculature exhibited a disorganized network. In addition, larger blood vessels, such as the middle cerebral artery, were also rudimentary. Vascular pathologies in general have not been described in human disorders caused by *FLNB* mutations, except that some SCT patients display a narrowing of the retinal vessels [94]. However, cardiovascular malformations are very common in human diseases caused by *FLNA* mutations. Considering the high structural similarity between FLNA and FLNB, and their ability to form heterodimers, loss of FLNB may potentially impair some functions of FLNA in the cardiovascular system.

Blood vessel supply plays a key role in cartilage and bone development. Hypertrophic chondrocytes secrete VEGF together with other factors to attract blood vessels, which is required for the replacement of cartilage by bone and for balancing the number of osteoblasts and osteoclasts [114]. The impaired vasculature in *Flnb*$^{-/-}$ mice may retard bone development by decreasing the supply of nutrients or

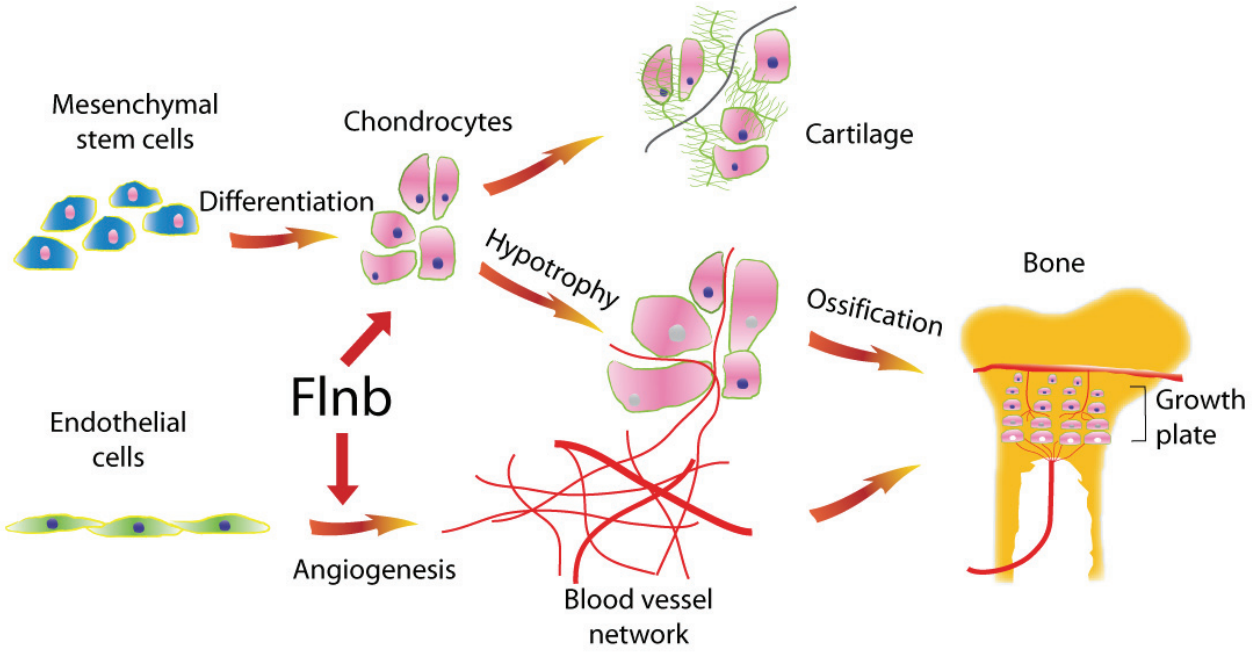


**Figure 4.** Impact of Flnb on development of cartilage, bone, and vasculature. Flnb may act on differentiated chondrocytes and determine their fate to form either cartilage tissue or bone. Flnb also regulates vascular development and thus contributes to osteogenesis.

regulatory factors into the growth plate (Fig. 4).

### *4.3.4 Flnb-deficiency reduces migration of embryonic fibroblasts*

To better understand the cellular and molecular mechanisms of Flnb in skeletal and vascular development, we studied the migration of MEF extracted from *Flnb*$^{-/-}$ embryos at E13.5. Migration of *Flnb*$^{-/-}$ MEF was markedly reduced both in a Boyden chamber assay and a wound healing assay. The *Flnb*$^{-/-}$ MEF exhibited more disorganized actin filaments in response to serum stimulation, suggesting an important role of Flnb in regulating actin network remodeling during cell migration. As FLNA interacts with GTPases and regulates cell migration [45], we questioned whether Flnb could have a similar function. We discovered a reduced level of activated RhoA in *Flnb*$^{-/-}$ MEF than in *Flnb*$^{+/+}$ MEF, which indicates an impairment in the contraction of actin fibers. Lu *et al.* isolated primary chondrocytes from *Flnb*$^{-/-}$ mice [35]. As it has been shown that FLNB interacts with integrin $\beta_1$ [24], they found that Flnb colocalizes with integrin $\beta_1$ along the chondrocyte cell periphery and induces the activity of integrin $\beta_1$. Consequently, *Flnb*$^{-/-}$ chondrocytes display impaired adhesion and spreading on several extracellular substrates. Taken together, impaired cell adhesion and migration may partly explain the skeletal and vascular developmental disorders caused by *Flnb* deficiency.

*Flnb*$^{-/-}$ mice reported by different groups all displayed severe skeletal malformations, similar to those seen in human genetic diseases, although there were discrepancies on whether the malformations developed in the cartilage, bone, or both. In addition, we observed some vascular defects in our *Flnb*$^{-/-}$ mice. Phenotypic discrepancies between the mouse models

may be partly explained by the differing lengths of truncated Flnb produced and the different mouse strains used. A longer truncated Flnb can probably lead to a partial gain-of-function effect in addition to the loss-of-function effect. Such combined effects have also been observed in *FLNA*-mutation patients who exhibit PVNH as well as the features of skeletal malformations [115-118]. Similarly, human disorders caused by nonsense and missense *FLNB* mutations have overlapping phenotypes. A potential *FLNB* gain-of-function could possibly alter the function of its interacting partners, such as FLNA. Therefore, $Flnb^{-/-}$ mouse models that mimic human diseases caused by *FLNB* mutations will considerably advance our understanding of these complex phenotypic appearances.

# 5 CONCLUDING REMARKS

This thesis reveals that (1) FLNA interacts with HIF-1α and promotes its nuclear translocation and transcriptional activity, (2) FLNA enhances c-MET expression and signaling, and (3) the *Flnb* knockout mouse model mimics *FLNB* mutation-caused human malformations and provides a mechanistic explanation.

1. A hypoxia-inducible fragment of FLNA increases nuclear localization of HIF-1α and its transactivation potential. *FLNA* deficiency in tumor cells results in reduced secretion of VEGF-A, thereby limiting their ability to induce the migration and proliferation of endothelial cells. This provides a novel mechanism for regulating HIF-1α transcriptional activity. Furthermore, our data suggest that FLNA can induce secretion of growth factors from tumor cells by regulating the activity of transcription factors and thus influence the behavior of other cells.

2. FLNA enhances c-MET expression partially by means of interaction with SMAD2. *FLNA* deficiency in tumor cells leads to decreases in *c-MET* signaling and cellular responses to HGF stimulation. These data provide a novel understanding of c-MET signaling. Furthermore, our data suggest that FLNA can induce expression of transmembrane receptors in tumor cells by regulating transcription factors and thus potentiate their cellular response to external stimuli.

*3.* *Flnb* deficiency is associated with embryonic lethality, severe skeletal malformations, and impaired vascular development. These phenotypes mimic human *FLNB* genetic disorders. The impaired migration of *Flnb*-deficient fibroblasts provides a plausible explanation for the severe malformations, in which Flnb may be important for proper migration of cells into appropriate anatomical locations during development.

# 6 PERSPECTIVES

Since the discovery of filamins as actin-binding proteins, a few decades have past and the focus of filamin studies has been changing. Gradually, it was found that filamins can interact with many other molecules of great functional importance. They emerged as essential scaffolding proteins that also play roles in cell signaling. Later, filamins were shown to be associated with severe human genetic diseases which further explored their functional importance. Very recently, mouse models of filamin deficiency have been generated. Together these findings contribute to further our scientific understanding, while also raising important new issues:

1. The number of filamin-interacting proteins is still increasing. Particularly, many interesting binding partners of FLNB will be identified as FLNB has not been studied as intensively as FLNA. Filamin-deficient mice and isolated primary cells will help us to identify signaling pathways regulated by filamins and then to explore their new binding partners.

2. Missense filamin mutations cause a wide spectrum of human disorders. Whether these disorders are caused by structural perturbations of filamins or by alterations of extrinsic interactions remains to be clarified. Filamin-interacting proteins binding to the regions that manifest deleterious filamin mutations in humans are poorly characterized. Production of recombinant filamin proteins mutated at the same sites as manifested in human disorders will become important tools. Screening of interacting proteins that bind to either wild-type or mutated recombinant filamins will identify functionally important mechanisms.

3. Alternative splicing variants of filamins are rarely taken into account in most studies, which may explain certain functional discrepancies associated with filamins.

4. The post-translational modification of filamins has been considered as a crucial factor in regulating their binding to actin and other interacting partners. Various kinases including protein kinase A/B/C [119-121], ribosomal S6 kinase [122], phosphatases such as calcineurin [123], as well as proteases such as calpain [124, 125], caspase [126] and granzyme B [107] have been reported to modify filamins. A deep understanding of their functional importance still requires intensive study and will lead to the development of new strategies to modulate the functions of filamin.

5. Filamins expressed in different cellular compartments may carry out distinct functions. For instance, it is unknown whether the nuclear existence of either full length or proteolytic fragment of filamins can affect the nuclear actin network. FLNA has been reported to be expressed on the cell surface [127] and high titers of anti-filamin antibody have been found in sera from patients [128] and mice [129]. These observations add more complexity to the biology of filamins.

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