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Filamin A: key actor in platelet biology

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Abstract:
Filamins (FLNs) are large dimeric actin binding proteins regulating actin cytoskeleton remodeling. In addition FLNs serve as scaffolds for signaling proteins such as tyrosine kinases, GTPases or phosphatases, as well as for adhesive receptors such as integrins. They thus connect adhesive receptors to signaling pathways and to cytoskeleton. There are 3 isoforms of FLNs (FLNa, FLNb, FLNc) originating from 3 homologous genes. FLNa has been the recent focus of attention because its mutations are responsible for a wide spectrum of defects, called filaminopathies A, affecting brain (peri-ventricular nodular heterotopia or PVNH), heart (valve defect), skeleton, gastro-intestinal tract or more recently, the megakaryocytic lineage. This review will focus on the physiological and pathological role of FLNa in platelets. Indeed, FLNa mutations alter platelet production from their bone marrow precursors, the megakaryocytes, yielding giant platelets in reduced number (macrothrombocytopenia). In platelet *per se*, FLNa mutations may either lead to impaired alphaIIbbeta3 integrin activation or in contrast, increased alphaIIbbeta3 activation, potentially enhancing the risk of thrombosis. Experimental work delineating the interaction of FLNa with its platelet partners, including $alpha_{IIb}beta_3$, the von Willebrand receptor GPIb-IX-V, the tyrosine kinase Syk and the signaling pathway of the collagen receptor GPVI will also be reviewed.

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Abstract

Filamins (FLNs) are large dimeric actin binding proteins regulating actin cytoskeleton remodeling. In addition FLNs serve as scaffolds for signaling proteins such as tyrosine kinases, GTPases or phosphatases, as well as for adhesive receptors such as integrins. They thus connect adhesive receptors to signaling pathways and to cytoskeleton. There are 3 isoforms of FLNs (FLNa, FLNb, FLNc) originating from 3 homologous genes. FLNa has been the recent focus of attention because its mutations are responsible for a wide spectrum of defects called filaminopathies A, affecting brain (peri-ventricular nodular heterotopia or PVNH), heart (valve defect), skeleton, gastro-intestinal tract or more recently, the megakaryocytic lineage. This review will focus on the physiological and pathological role of FLNa in platelets. Indeed, FLNa mutations alter platelet production from their bone marrow precursors, the megakaryocytes, yielding giant platelets in reduced number (macrothrombocytopenia). In platelet *per se*, FLNa mutations may either lead to impaired αIIbβ3 integrin activation or in contrast, increased αIIbβ3 activation, potentially enhancing the risk of thrombosis. Experimental work delineating the interaction of FLNa with its platelet partners, including αIIbβ3, the von Willebrand receptor GPIb-IX-V, the tyrosine kinase Syk and the signaling pathway of the collagen receptor GPVI will also be reviewed.

Introduction

Platelets are produced by megakaryocyte (MK) cytoplasmic fragmentation and are critical in the bleeding arrest. They adhere to vascular lesions, rapidly recruiting additional platelets till blood stops leaking. Excessive platelet accumulation at sites of atherosclerotic plaque rupture, leads to arterial thrombus formation and acute myocardial infarction, sudden death or ischemic stroke. Conversely, defective platelets lead to unstoppable hemorrhage. In the last decades, the challenge has been to identify molecular actors involved in platelet functions to identify potential drug targets. Recently, protein defects associated with signaling pathways dysfunction have been identified, such as filaminopathy A, a defect in Filamin A (FLNa). FLNa is a large dimeric actin-binding protein stabilizing actin filament networks and recently found involved in both platelet production and platelet activation. This review is focused on the structure and functions of FLNa during platelet activation and megakaryocytopoiesis. We also discuss the molecular mechanisms involving FLNa in platelet activation. Finally, we analyze the hemorrhagic or thrombotic consequences of FLNa mutations in patients.

FLNa structure

FLNs in mammals are a family of three members, FLNa, FLNb and FLNc originating from three distinct genes (*FLNA*, *FLNB*, *FLNC*) that are highly conserved. Human *FLNA* is located on the X chromosome whereas human *FLNB* and *FLNC* are located on chromosomes 3 and 7, respectively. FLN protein isoforms exhibit high sequence identity (70%) except for the two hinge regions.

FLNa monomers (280 kDa, 2646 amino acids) assemble as dimers in a V-shaped structure. The N-terminal region of each monomer is an actin-binding-domain (ABP) consisting of two calponin homology (CH) sequences (CH1 and CH2) followed by a rod segment consisting of 24 immunoglobulin-like repeats of 96 amino acids residues that adopt an immunoglobulin-like fold (Ig repeats) (Figure 1). The 24 Ig repeats are composed of seven β-strands (A-G).[1] Two calpain-sensitive hinge domains separate the 24 β pleated sheet Ig repeats into rod-1 (repeats 1-15: 58 nm) and rod-2 (repeats 16-23: 19 nm) and represent cleavage sites for Ca^{2+}-dependent protease calpain.[2] Rod-1 Ig-repeats (9-15) also bind F-actin but with a lower affinity than the ABD domain adding intrinsic flexibility to actin networks upon mechanical stress. While FLNa rod-1 binds only a small number of partners, Ig repeats (16-24) in rod-2 interact with most of FLNa partners (>90) including membrane receptors (GPIbα and integrin β3), signaling proteins (GTPases-related proteins) and transcription factors conferring an important signaling scaffold role in a large variety of cellular processes.[3] However, rod-2 does not bind F-actin. Rod-2 forms a structure that is more compact and more globular than rod-1, due to interaction between repeat pairs (16-17, 18-19 and 20-21). For example, strand A of Ig-repeat 20 is associated with the β strands C and D of Ig-repeat 21, thereby obstructing the integrin binding site.[4] This is an auto-inhibition mechanism which limits accessibility to FLNa integrin-binding site, which may represent a general mechanism regulating FLNa-partner interactions. In response to mechanical forces, FLNa Ig repeat domains undergo conformational changes allowing the exposure of binding sites for new interactions. Thus, in platelets FLNa may participate in mechanotransduction converting mechanical forces into signaling events. For example, disruption of Ig-repeats 20-21 interaction (requiring mechanotransduction) enhances FLNa β-strands C and D binding to integrin β-tails.[5] The C-terminal region (repeat 24) mediates dimerization into a V-shaped flexible structure that results in F-actin perpendicular branching essential for mechanosensory functions,[6] though other domains may participate in dimerization.[7] Finally, repeat 24 also interacts with other FLNa partners such as the small GTPases RalA, Rho,[8] or Cdc42[9] involved in particular in

actin cytoskeleton regulation and the endoplasmic reticulum calcium sensor Stromal Interaction Molecule1 (STIM1), regulating Ca^{2+} influx through Orai-1.[10]

Regulation of FLNa interaction with its partners.

FLNa interaction with its partners is regulated by phosphorylation, proteolysis, mechanical forces and competition between partners. Several kinases, cAMP-kinase, PKC (protein kinase C) and CaM-kinase II (Ca^{2+}/calmodulin-dependent protein kinase II) phosphorylate FLNa on S2152.[11-13] The physiological roles of this phosphorylation remain largely uncharacterized. FLNa phosphorylation on S2152 by the cAMP-dependent protein kinase (PKA) was reported to stabilize and protect FLNa against proteolysis by calpain.[14] Indeed, we recently described a new gene mutation in *PRKACG* encoding the γ regulatory subunit of PKA which was associated with a macrothrombocytopenia and with a functional defect of PKA activity leading to a decrease of FLNa phosphorylation at S2152 and to FLNa degradation.[15] Phosphorylation may also regulate FLNa interaction with GTP-binding proteins[16] or with actin filaments,[11] and may regulate the role of FLNa in integrin activation.[17]

Mechanical strain is yet another essential actor regulating FLNa interaction with its partners.[18-19] This mechanical stress-dependent mechanism may result from the lift of an auto-inhibitory pairing of Ig domains (Ig 20-21, 18-19, 16-17) limiting binding sites accessibility to FLNa partners. The pair Ig20-21 was the most studied. Recent *in vitro* studies of mechanically strained FLNa-cross-linked actin networks have provided further support for a potential force-sensing role of FLNa.[18] Ehrlicher et al examined the effect of mechanical strain on FLNa's interactions with two rod-2 binding partners, cytoplasmic β-tail integrin and FilGAP, a GTPase specific for Rac. In this model, strain increased β-integrin binding to FLNa whereas it caused the dissociation of FilGAP from FLNa indicating that FLNa rod2 is highly flexible and that physiological forces are sufficient to expose new cryptic sites. The dynamic force sensing of FLNa, also revealed in single-molecule experiments, was shown to be a highly dynamic process shifting the autoinhibited domain pair Ig20-21 towards the increased binding affinity of β-integrin to IgFLNa 21.[20] It remains to be established in resting platelets if and how the interaction of FLNa with β3 occurs. To answer this question, the presence or not of the autoinhibitory Ig20-21 domain pairing must be determined in platelets. Finally, competition is an important regulatory mechanism of FLNa interaction with its partners. For example, competition between FLNa and talin or kindlin-3 has been extensively studied with respect to αIIbβ3 integrin, and will be presented below.

FLNA mutations are associated with a wide spectrum of genetic diseases

FLNA mutations cause a wide spectrum of rare diseases including skeletal dysplasia, neuronal migration abnormality putatively linked to intellectual disability, cardiovascular malformation, myofibrillar myopathy and intestinal malrotation and obstruction (Table 1). *FLNA* mutations are distributed throughout the entire *FLNA* gene and give rise to abnormal mRNA splicing or protein truncations.[21-22] These disorders are called Filaminopathies A and the most frequent disease is the periventricular nodular heterotopia (PVNH), characterized by a neuronal migration defect leading to ectopic accumulation of neurons into nodules lining cerebral ventricles margins.[21-23] PVNH is predominantly observed in heterozygous females exhibiting loss-of-function mutations.[22,24] Filaminopathy A in males is most frequently associated with perinatal lethality, with only rare hemizygous males surviving post-birth.[21,24-26] Most PVNH mutations disrupt the *FLNA* reading frame and completely ablate FLNa expression with only a small number of missense mutations identified in the ABD domain (CH1). In other cases, the PVNH phenotype is associated with Ehlers-Danlos syndrome (EDS),[27] with vascular defects including aortic valve insufficiency and patent ductus arteriosus,[22] hydrocephalus,[28] frontonasal malformation,[29] nephrosis[30] or congenital intestinal pseudo-obstruction (CIPO).[26,31]

FLNA mutations are also associated with skeleton alterations such as frontometaphyseal dysplasia (FMD), Melnik-Needles syndrome (MNS) and otopalatodigital syndrome type 1 and type 2 (OPD1 and OPD2).[32] The OPD1 syndrome is characterized by facial malformation and generalized bone dysplasia.[33] The OPD2 syndrome is a more severe disorder, associated with mental retardation.[34] In contrast to PVNH, mutations leading to OPD are mostly localized in specific domains. The actin binding domain (CH2) may harbor gain-of-function mutations with increased actin-binding affinity. Rod domain repeats 3, 10 and 14/15 are the target of many missense mutations affecting partners binding and consequently FLNa functions.[32,35-36] Likewise, recurrent *FLNA* mutations in the ABD (p.E254K) associated with OPD2 and in the Ig10 (p.S1199L) associated with MNS have been observed in unrelated families,[32,36-37] strongly suggesting different FLNa functions and/or partners involved in bone and brain development, remaining to be identified.

Finally, *FLNA* was identified as the first gene responsible for myxomatous valvular dystrophy and four mutations have been clearly identified so far:[38-39] three missense point mutations within Ig repeats 1, 4 and 5 and a deletion encompassing Ig repeats 5 to 7. Interestingly all

mutations are predicted to impair binding of FLNa partners by alteration of the β-strands organization.

FLNa in hemostasis

Up until 2010, thrombocytopenia and hemorrhages have only occasionally been described in PVNH patients.[22] The role of FLNa in platelets and megakaryocytes (MKs) has been unraveled first in mice lacking FLNa[40-41], and in mouse embryonic stem cells (ESCs)[42] or in patients carrying *FLNA* mutations.[26-27,43-45] MK-platelet lineage-specific *FlnA*$^{-/-}$ mice exhibit both increased tail bleeding and severe macrothrombocytopenia due to accelerated platelet clearance.[41] FlnA-null MKs prematurely release large, fragile and vesiculate platelets cleared from circulation by macrophages.[41] Impaired megakaryopoiesis was also noted in two female patients, one with PVNH, the other not suffering from filaminopathy, but both exhibiting hemorrhages and macrothrombocytopenia (Table 2).[45] Their MKs were morphologically abnormal, including enlarged alpha granules, abnormal cytoplasm fragmentation and impaired proplatelet (PPT) formation, associated with uneven FLNa distribution. Altered demarcation membrane system (DMS) was also observed in patients,[45] and FlnA-null MKs,[46] the likely result of impaired FlnA/PACSIN2 interaction which regulates membrane tubulation in MKs and platelets and DMS formation, at least in mice.[46]

More recently, induced pluripotent stem cells (iPSCs) derived from heterozygous female patients with PVNH,[43-44] were used to assess variability in patients platelet counts.[43] In fact, two platelet populations differing in size were observed in these heterozygous female patients. Moreover, random X inactivation during hematopoiesis suggests the generation of two MK progenitors populations expressing exclusively either the *wt* or the *null* X-linked *FLNA* alleles in heterozygous PVNH female patients. In fact, the reprogramming of FLNa-*wt* and FLNa-*null* clones isolated from each female patient showed no difference in MK differentiation, up to proplatelet formation, which was highly defective in the iPSC-derived *FLNA*-null MKs.[43]

Platelets predominantly express FLNa. Mouse platelets lacking FlnA are large but discoid with impaired actin membrane attachment and GPIbα expression.[40] The platelet morphology studied in two PVNH female patients exhibiting *FLNA* premature termination mutations and a female patient with isolated macrothrombocytopenia and an *FLNA* missense mutation[45] showed two platelet populations. Some platelets were normal, others were giant with an abnormal granule distribution.[27,44-45] Note that in contrast to murine platelets, enlarged human platelets are round and no GPIbα decrease or degradation was observed suggesting that the

GPIbα-FLNa-actin axis is differently regulated in mice and humans. In human platelets, FLNa distribution was irregular with low or no FLNa in giant platelets while FLNa level was normal in normal size platelets. Surprisingly, in patients with putatively truncated FLNa, full-length FLNa was detectable but at variable levels among patients. In these patients, altered platelet functions therefore correlated with low full-length platelet FLNa levels. Moreover, platelet count correlated with wt FLNa level, with low counts associated with low FLNa levels and normal counts with normal FLNa levels.[44]

A different question is raised by missense FLNa mutants with mutations not affecting expression or stability, thereby presumably expressed in platelets at similar levels than WT FLNa. Could these FLNa mutants exhibit a dominant alteration of platelet function leading to thrombosis or hemorrhage? The first case was a missense *FLNA* mutation (pGlu1803Lys) in one heterozygous female patient exhibiting a gain-of-platelet-functions with increased adhesion on VWF in conditions of pathological shears.[44] This effect may be related to the location of the mutation within Ig-repeat 16, next to Ig-repeat 17, the FLNa-GPIb interaction domain.[47] One possibility is that this mutation disrupts the Ig-repeats 16/17 auto-inhibitory pairing, facilitating GPIb binding, as suggested by the FLNa model of Ehrlicher.[18] Another platelet gain-of-function effect was reported in a hemizygous male patient (therefore carrying a single X-linked *FLNA* allele) with an extended C-terminal region due to a stop codon mutation.[26] Accordingly, the platelets of this hemizygous patient expressed only the mutant FLNa protein. Despite αIIbβ3 normal expression, αIIbβ3 exhibited enhanced ligand binding (twice the normal values), exposing this patient to a potential thrombotic risk. Because mutant FLNa seemed to bind β3 on resting platelets, we postulate that the gain-of-function effect is likely to be due to an easier release of mutant FLNa from β3 than normal, facilitating talin recruitment and overactivation of αIIbβ3. This is thus the first observation in humans confirming a down-regulatory role for FLNa in αIIbβ3 activation, a key feature in platelet physiology and pathology.

FLNa partners in platelets

Because FLNa interacts with several receptors (GPIbα and αIIbβ3 integrin) essential to platelet adhesion and aggregation, and binds to signaling proteins such as Syk involved in GPVI functions and STIM1 (Figure 1), filaminopathy A patients and the corresponding mouse models provide opportunities to examine the role of FLNa in platelet functions and signaling.

GPIb-IX-V

GPIb-IX-V, a platelet-specific membrane glycoprotein, mediates adhesion of platelets to blood vessel wall injury sites by binding to VWF immobilized on the subendothelial collagen matrix. FLNa constitutively interacts with GPIb-IX-V, and positively modulates VWF receptor function.[48-49] This interaction involves FLNa repeat 17 and GPIbα subunit cytoplasmic tail (aa 556-577).[47,50-51] Other Ig repeats (4, 9, 12, 17, 19, 21 and 23) may also bind GPIbα, but double missense mutations of Ig repeat 17 were sufficient to completely abolish FLNa-GPIb-IX-V interaction.[1,51] Phe568 and Trp570, within the hydrophobic FLNa binding site of the GPIbα cytoplasmic tail,[48] appear essential to strengthening GPIb-IX-V anchorage to the membrane skeleton and cell adhesion to VWF under high-shear.[48] This was later confirmed with a GPIbα knock-in mouse model in which GPIbα cytoplasmic tail was deleted and replaced by the reporter protein β-galactosidase.[52] Blunting GPIbα-FLNa interaction led to defective adhesion, unstable membrane tethers and loss of membrane integrity at pathological shear rates, indicating that FLNa-GPIbα linkage ensures mechanical stability of the platelet plasma membrane.

FLNa has also been shown to be required for GPIbα trafficking. FLNa involvement in GPIb trafficking to the plasma membrane was initially observed in FLNa-defective melanoma M1 cells[53] where GPIb-IX reached the cell surface only when co-expressed with FLNa, GPIb-IX remaining in the cytoplasm in absence of FLNa. FLNa-GPIbα interaction was proposed to regulate post-translational GPIb-IX-V assembly and trafficking: the binding of FLNa to GPIbα would trigger GPIb-IX-V endoplasmic reticulum (ER) exit, allowing post-ER trafficking.[54] Moreover FLNa expression level also seems important for GPIbα trafficking.[43] This mechanism could explain the apparent discrepancy between PVNH patients platelets exhibiting normal or increased membrane GPIb-IX-V levels despite low FLNa levels, and FlnA-null mouse platelets expressing decreased or degraded GPIbα and no FlnA.[40-41] Of note, 20% normal FLNa in heterozygous patient platelets seemed sufficient for normal GPIb-IX-V platelet surface expression.[44]

The integrin αIIbβ3

Extensive studies have been carried out concerning the role of FLNa in the regulation of integrin activation. For example, FLNa was shown to interact with β7 tail integrin.[5] However, in platelets the role of αIIbβ3-FLNa interaction remains unclear. A recent elegant structural

study proposed a new model where Ig repeat 21 (and possibly Ig repeats 9, 12, 17 and 19) of FLNa clasps together αIIb and β3 CTs thereby stabilizing αIIbβ3 in an inactive state and preventing αIIbβ3 spontaneous activation.[55] In this model, FLNa was proposed to prevent talin interaction with β3 and to mediate the retention of the integrin in a resting state. However, FLNa-β3 interaction was never demonstrated in intact resting platelets. This finding was consistent with the observation that basal αIIbβ3 activation was not observed in resting platelets of the patient with a gain-of-platelet function[26]nor in FlnA-null mice[40] despite normal or increased expression levels of αIIbβ3 for patient and mice platelets, respectively. The apparent discrepancy between intact platelets and the recent model proposed by Liu et al[55] could come from the model these authors used, consisting of an Ig repeat 21 peptide alone, thus without the auto-inhibiting Ig repeat 20. This model would thus allow free accessibility to the FLNa integrin-binding site (see below: section structure). It remains to be clarified whether Ig repeat 21 is inhibited by Ig repeat 20 in platelets in physiological conditions and if this inhibition could be lifted by high shear conditions.

The molecular basis of the dynamic equilibrium between resting and activated αIIbβ3 is not completely understood. Resting platelets exhibit low affinity αIIbβ3 displaying minimal ligand-binding. After platelet activation, αIIbβ3 binds ligands with a high affinity and avidity. αIIbβ3 high affinity transition is regulated by talin, kindlin and FLNa direct interaction with β3 CT. Talin and kindlins (kindlin-3 in hematopoietic cells) are known integrin activators while FLNa is a β1 and β3 integrin inhibitor.[56-57] Altered integrin activation is observed in talin-null platelets or after kindlins overexpression or knock-out.[58-61] Likewise, αIIbβ3 activation was impaired in knock-in mice expressing a kindlin-3 mutant disrupting kindlin-3-αIIbβ3 interaction.[62] Kindlin-3 and talin seem to synergistically co-activate αIIbβ3 [60,63] but with distinct roles: talin would modulate αIIbβ3 affinity while kindlin-3 would modulate clustering and avidity.[64] FLNa binds to β3 CT residues 747-755 overlapping with amino acids 739-750 and 752-759, respectively talin and kindlin-3 binding sites. This supports the view that talin and kindlin-3 compete with FLNa for β3 CT interaction.[57,65-67] The model proposes that platelet activation induces FLNa release from its constitutive β3 CT binding site, allowing talin and kindlin-3 β3 recruitment and suggests that FLNa would be capable to dissociate from β3 under platelet activation. In the new model proposed by Liu et al,[55] FLNa would prevent talin and kindlin-3 binding to β3 CT but also membrane insertion of the membrane-proximal region of β CT. FLNa release would allow talin and kindlin-3 β3 CT

binding, but also let αIIbβ3 CT membrane-proximal region to insert into the membrane and undergo conformational rearrangement, and thus expose the ligand binding site. FLNa would thus represent a double lock for αIIbβ3 activation.

What is the regulatory mechanism for FLNa dissociation from αIIbβ3? Migfilin, another FLNa partner reported to regulate integrin activation has been proposed to play a role in this mechanism. Overexpression of migfilin significantly enhanced integrin activation[56] and permeable migfilin peptides in platelets were able to induce extensive αIIbβ3 activation. Structural analysis of the migfilin-FLNa complex identified Ig repeat 21 as the preferential migfilin binding site on FLNa (and to a lesser extent, Ig repeats 19 and 22)[68] while reciprocally FLNa bound migfilin N-terminal domain.[56] Accordingly, a model was proposed where migfilin binding to FLNa would lead to FLNa release from β CT, thus letting the β CT binding sites free for interaction with talin and kindlin-3.[56-57,68] However, if this model is attractive, it was not demonstrated in platelets. Conflicting results have been published regarding the presence or the absence of migfilin in platelets.[57,69] Likewise, in human platelets, while FLNa and β3 copy numbers match each other (88,000 and 90 000 molecules respectively), migfilin was undetected in proteomics analysis of human platelets,[70] reported at low levels for both migfilin mRNA and protein,[57] inconsistent with a role in FLNa-β3 displacement. Other candidates, not yet identified, could play such a role. Alternatively, FLNa-β3 dissociation could also be the result of an FLNa conformational change occuring during platelet activation, leading to decreased FLNa affinity for β3 CT and to its dissociation from β3 (Figure 2).

The negative control of αIIbβ3 activation by FLNa in platelets was clearly demonstrated in a rare case of a male patient associating PVNH with congenital intestinal pseudo-obstruction. This male patient was hemizygous for a mutant FLNa exhibiting an extended (100 amino acids) C-terminal region due to the stop codon mutation, and showed gain-of-platelet-function characteristics.[26] The mutant FLNa was shown to augment αIIbβ3 activation by facilitating talin and kindlin-3 recruitment to αIIbβ3 integrin. This was not due to absence FLNa-β3 CT interaction, which appeared normal. The current hypothesis is that either the mutant FLNa, despite its ability to constitutively bind to β3 CT, is more easily released than wild-type FLNa from β3 CT, or since the mutation is next to the dimerization domain of FLNa, interference with dimerization may ease mutant FLNa release. This observation thus unravels an unsuspected set of potential mechanisms for FLNa-β3 CT dissociation, requiring further exploration. Finally this observation raises another question pertaining to the risk of

spontaneous αIIbβ3 activation and thus of thrombosis in this patient, in particular if he develops pro-thrombotic pathologies such as atherosclerosis or diabetes mellitus.

The tyrosine kinase Syk

The tyrosine kinase Syk involved in the signaling of immunoreceptor tyrosine-based activation motif (ITAM) and ITAM-like-mediated signal receptors GPVI and C-terminal lectin-like receptor 2 (CLEC-2) is also an important FLNa partner, as shown elegantly in MK-specific $FlnA^{-/-}$ mice which exhibited altered collagen-induced platelet functions.[40] Platelet adhesion under arterial shear was impaired, as well as platelet aggregation and αIIbβ3 activation induced by GPVI-specific agonists such as the collagen-related peptide (CRP). Defective Syk phosphorylation confirmed FlnA is essential for GPVI signaling. In fact, platelet FlnA contributes to Syk targeting to the cytoplasmic membrane leaflet. Interestingly Syk was shown to bind FlnA rod1 Ig repeat 5, contrasting with most other partners binding rod2 Ig repeats 16-24.

FLNa was also shown involved in GPVI signaling in platelets from heterozygous female PVNH patients with macrothrombocytopenia and low FLNa levels.[44] Abnormal responses to collagen, including aggregation, secretion, GPVI signaling and thrombus formation under flow were the consequence of low levels of wt FLNa (≤ 50%). Partial Syk activation impairment in patient platelets was probably the consequence of defective Syk-FLNa association, required for Syk recruitment by GPVI-associated FcRγ chain.

New partners

PACSIN2

The adaptator PACSIN2 is one of the most abundant BAR/F-BAR proteins in platelets.[70-71]The interaction of PACSIN2 with FLNa has recently been described in human platelets, and involves FLNa Ig repeat 20 and PACSIN2-BAR domain. This interaction regulates membrane tubulation in MKs and platelets and DMS formation in MKs.[46]

STIM1

Among the other signaling partners, the endoplasmic reticulum calcium sensor STIM1 which regulates Store-Operated Calcium Entry (SOCE), a major mechanism for Ca^{2+} influx, has recently been shown to interact with FLNa.[10] This interaction is dependent on the

phosphorylation of FLNa at Ser^{2152} by cAMP-dependent protein kinase and on the FLNa dimerization domain, and seems to down-regulate SOCE.[10]

Other partners

Among the hundred known partners of FLNa, only the FLNa/STIM1 and PACSIN2 interactions, described above, were demonstrated in platelets. Other candidates as well do probably interact with FLNa in platelets. For example the GTPase FilGAP (described above in FLNa in the section regulation) and expressed in all tissues, was shown to bind FLNa through Ig repeat 23.[72] The dissociation of FilGAP from FLNa occurs when FLNa-actin networks are subjected to mechanical shear strains *in vitro*[18] which corresponds to fluid shear stress driven by blood flow. In this model, mechanical shear stress deforms actin filaments and consequently the two arms of FLNa which reduces FilGAP-FLNa binding.[72] It remains to be established whether this mechanism occurs in intact platelets and what is its function in the modulation of Rho and Rac activities.

FLNa binds constitutively to Rho family GTPases (Cdc42, RhoA and Rac1) via Ig repeat 24. In megakaryocytes, the interaction of RhoA with FLNa was recently reported to be dependent on αIIbβ3 and to modulate proplatelet formation (see below).[42]

FLNa in megakaryopoiesis

Macrothrombocytopenia and defective proplatelet formation were observed in FLNA mouse knock-out models and mouse embryonic stem cells (ESCs).[41-42] In contrast to ESCs where inefficient MK differentiation was observed, knockout mice showed a marked increase in bone marrow and spleen MKs.

A recent original iPSC model via reprogramming peripheral blood CD34$^+$ cells from two female patients harboring two intragenic deletions showed that in differentiated MKs, there was no defect in CD41$^+$/CD42$^+$ MKs percentage nor in mean fluorescence intensity for αIIbβ3, GPIX and GPIbα expression.[43] These observations suggested that in human MKs, trafficking to the cell surface of GPIbα or αIIbβ3 is FLNa-independent. In contrast, complete FLNa ablation in MKs was associated with defective proplatelets production.[43] MK differentiation onto fibrinogen, thus involving αIIbβ3 engagement and presumably FLNa release from αIIbβ3, led to increased actomyosin contractility associated with inappropriate GTPase RhoA activation, leading to defective platelet production. Downregulation of Rho A activity by FLNa-αIIbβ3 interaction is thus required for proplatelet formation. This was

confirmed by experiments overexpressing FLNa deletion mutants unable to bind αIIbβ3 and RhoA (Figure 3). This is the first report proposing a molecular mechanism for macrothrombocytopenia involving FLNa.[43] Defective FLNa-αIIbβ3 interaction may also explain thrombocytopenia in some patients with αIIb and β3 mutations.[73-74]

Today, there is increasing evidence that GPIbα-FLNa interaction not only maintains platelet membrane mechanical stability at high shear rates but also regulates platelet size. Indeed, the abnormal architecture of (giant) platelets associated with the inherited deficiency of GPIb-IX-V (Bernard Soulier syndrome) has been proposed to arise from the absence of GPIbα-FLNa interaction.[75] This finding was supported by the presence of giant platelets in BSS patients with CT-truncated GPIbα or by the expression of transmembrane and cytoplasmic domains of GPIbα in GPIbα-deficient mice, sufficient to partially rescue platelet size.[76] Similar to GPIbα-deficient mice, FLNa-deficient mice form giant platelets.[77] Likewise, the population of giant platelets (20%) observed in female PVNH patients was associated with a large decrease or a total absence of platelet FLNa whereas normal size platelets were associated with the presence of wt FLNa.[44-45] Finally, different levels of FLNa and GPIb expressed in cultured embryonic stem cells (ESCs) differentiated in MKs forming platelets confirmed FLNa and GPIb role in the production of normal size platelets[43] indicating that platelet size is regulated by GPIbα-FLNa interaction and macrothrombocytopenia may stem from deficiency in either protein (FLNa or GPIbα). Finally, a model in which GPIbα controls MK localization and transendothelial MK migration via the regulation of the Rho GTPases RhoA and Cdc42 has been recently described.[78] The role of FLNa for the transmission of signaling between GPIbα and Cdc42-RhoA remains to be explored.

The future

Recent studies on FLNa have changed our current view of the role of FLNa in platelets and MKs. The structure, the identification of its partners, the FLNa mutants and finally the mouse models of FLNa deficiency have helped better understand the role of FLNa in hemostasis and megakaryocytopoiesis but have also raised new questions.

Little is known about the molecular role of FLNa in megakaryocytopoiesis. We have seen that the absence of FLNa in MKs leads to their inability to produce proplatelets and that the downregulation of RhoA activity required for proplatelet formation is dependent on αIIbβ3-FLNa interaction.[43] To investigate how RhoA is regulated, it will be interesting to identify

which GAPs, GEFs (known to interact with FLNa), or other partners are able to modulate Rho activity. Another question relates to whether RhoA pathway can be considered as a potential new target for increasing platelet counts in patients with thrombocytopenia. Since FLNa absence in MKs leads to αIIbβ3-dependent over-activation of the RhoA pathway, the state of αIIbβ3 activation remains to be investigated. One surprise in the study was that deletion of the GPIb-binding domain did not alter proplatelet formation, in apparent contradiction with the reported effect of GPIb-FLNa interaction ablation causing giant platelets or thrombocytopenia in mice. One potential explanation is that data originated from an *in vitro* system lacking conditions required for final platelet generation, including shear. This could indicate that the role of FLNa-GPIb interaction in platelet formation is relevant at a late stage in platelet biogenesis. This possibility may be consistent with the observation that GPIbα was shown to coordinate MK polarization and transendothelial migration *in vivo* via the regulation of Cdc42 and RhoA activity.[78]

In platelets, the regulation of αIIbβ3 activation by FLNa remains unclear. The constitutive association of FLNa with β3 preventing spontaneous integrin activation has not been shown in platelets and the determinants of FLNa-αIIbβ3 interaction are not understood. Can FLNa-αIIbβ3 interaction be regulated by phosphorylation, similarly to FLNa-integrin β2 CT binding inhibition by Thr758 phosphorylation?[79] Another important but still elusive question is if FLNa is associated to αIIbβ3, how FLNa dissociates from β3? We have seen that the cytoskeletal adaptor protein migfilin is not the good candidate for promoting FLNa release from β3 CT in platelets, and thus the molecular mechanism remains unknown.

The mechanisms by which different missense FLNa mutations in patients affect platelet production and/or functions is an important question, in particular because of the potential risk of hemorrhage or thrombosis. For example, the role of the dimerization domain (FLNa Ig repeat 24) in platelet production and activation, and the role of FLNa Ig repeat 21 in αIIbβ3 activation must be elucidated. Addressing the question of the hemostatic status of patients with filaminopathies A needs to be considered more systematically. The expected benefits are of a medical, physiopathological and physiological nature. Moreover, it is quite possible, in the future, that we establish a relationship between the mutation type and its pathophysiological impact. If such a correlation were made, subsequent monitoring of patients should be adjusted according to the mutation involved. With regard to the pathophysiology of the MK-platelet impact of filaminopathy A, we believe that future investigations should provide clues to the mechanism of thrombocytopenia.

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Disclosure of conflicts of interest

The authors have declared that there are no relevant conflicts of interest

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Figure legends

Figure 1: Monomeric structure of FLNa and partners in platelets

The amino-terminal actin-binding domain contains two calponin-homology domains (CH1 and CH2) followed by 24 Ig repeats probably folded into antiparallel β-sheets. Two hinge domains separate the 24 Ig repeats into two rod domains (hinge 1: between Ig repeats 15 and 16 and hinge 2 between Ig repeats 23 and 24). The Ig repeats 9-15 in rod 1 facilitate F-actin-binding whereas Ig repeats 16-23 interact with different partners. Dimerization occurs through Ig repeat 24.

(A) FLNa interacts with the platelet receptor GPIbα through the Ig repeat 17, and with the recently described partners PACSIN2 and STIM1 in resting platelets. The interaction of FLNa with αIIbβ3 through the Ig repeat 21 was not demonstrated.

(B) After platelet activation, FLNa interacts with the platelet receptors GPIbα and with the tyrosine kinase Syk involved in GPVI functions through the Ig repeats 17 and 5, respectively, and with PACSIN2 and STIM1.

Figure 2: Model of the regulation of αIIbβ3 activation.

(A) In resting platelets, FLNa is constitutively associated to αIIbβ3 (β3 CT) through its Ig repeat 21. After platelet activation, FLNa would interact through Ig repeat 21 with an unknown FLNa partner, leading to FLNa release from βCT. In this model FLNa-free βCT binding sites would now allow recruitment of talin and kindlin to βCT required for αIIbβ3 activation.

(B) In resting platelets, FLNa is constitutively associated to αIIbβ3 (β3 CT) through its Ig repeat 21. The dissociation of FLNa from β3 could be the result of a conformational change of FLNa which occured during platelet activation and in the presence of shear leading to a decreased affinity and a dissociation of FLNa from β3.

Figure 3: FLNa and platelet biogenesis

(A) αIIbβ3/FLNa interaction after adhesion to fibrinogen is crucial to keep RhoA inactive, allowing acto-myosin reorganization and proplatelet formation.

(B) The GPIbα/FLNa interaction stabilizes platelets and regulates the size under shear. It is not excluded that this interaction is necessary for the regulation of GPIb donstream effectors RhoA/Cdc42 during transendothelial platelet biogenesis.

Table 1 : FLNa distribution and genetic diseases

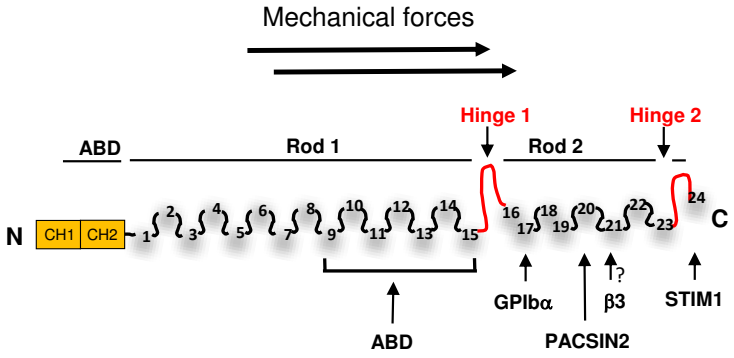
FLN subtype	Organ	Phenotype	Mutation domain distribution	Reference
FLNA	Brain	PVNH	Stochastic	21, 22, 23
FLNA	Skeleton	MNS	Ig10	32
FLNA	Skeleton	FMD	Ig 3, 9-10, 14-15,22-23 CH2 domain	32
FLNA	Skeleton	OPD	Ig 3, 10, 14, 15 CH2 domain	32, 36
FLNA	Heart	X-linked myxoid valvular dystrophy	Ig 1, 4, 5, 6-7	38, 39
FLNA	Intestine	CIPO	No-stop FLNA mutation: p(*2648Serext*100)	26, 31

Table 2 : Clinical parameters of the FLNa patients

Patients	Ref	Syndrome	Mutation	Platelet count	Bleding syndrome
P1: F	44, 45	PVNH	c.4573_4insA, p.Tyr1525X	80	Excessive/easy bruising tendency Menometrorrhagia
P2: F	43, 44, 45	PVNH	Deletion exons 31-32 to 48	238	Excessive/easy bruising Mucocutaneous hemorrhages Menometrorrhagia
P3: F	44, 45	Isolated thrombocytopenia	p.Glu1803Lys	40-60	Petechial hemorrhages Mucocutaneous hemorrhages Menometrorrhagia Bleeding after surgery
P4: F	43, 44	PVNH	Deletion exons 20 to 48	110	Metrorrhagia
P5: M	26	PVNH	c.7941_7942delCT p.2648Serext100	220-300	No bleeding
P6: F	27	PVNH	c.1621G>T p.Glu541Ter	Mild thrombocytopenia	?

Figure 1

A Resting platelets



B Activated platelets

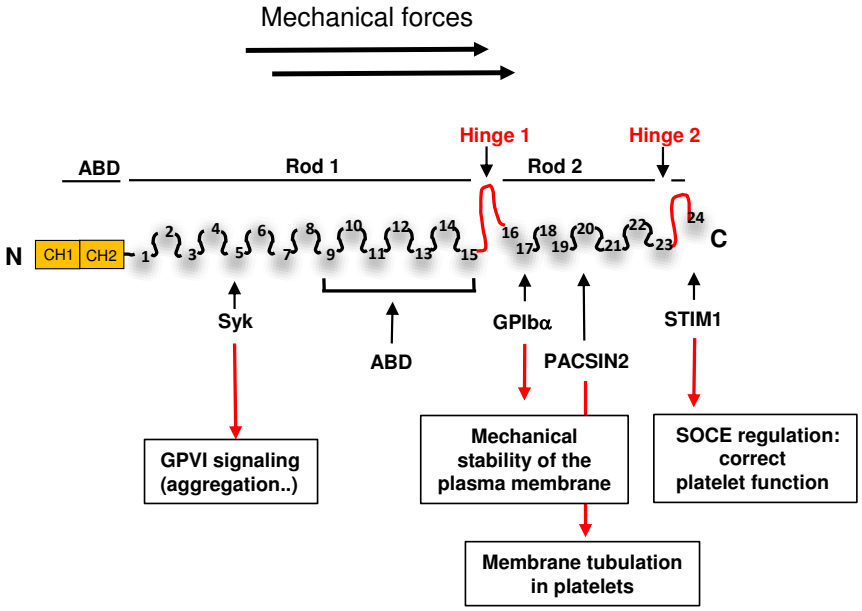


Figure 2

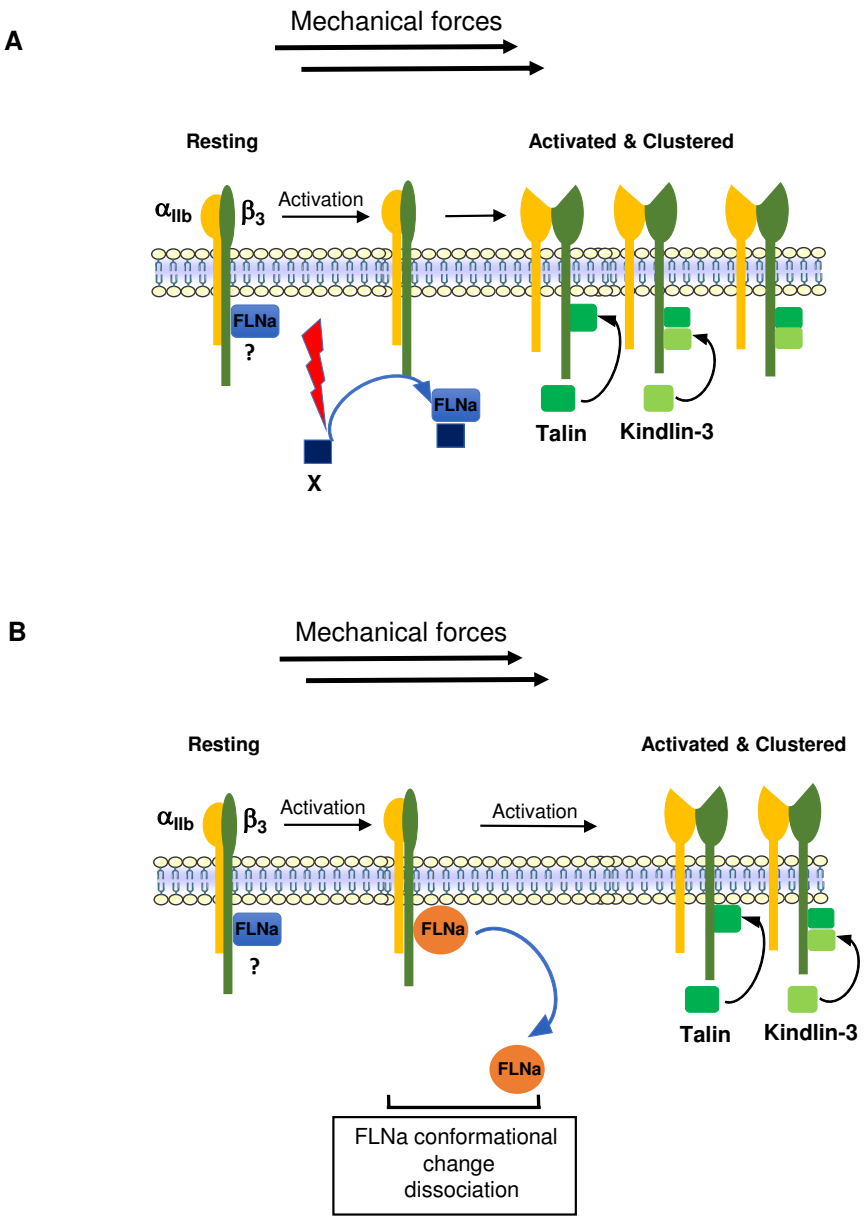
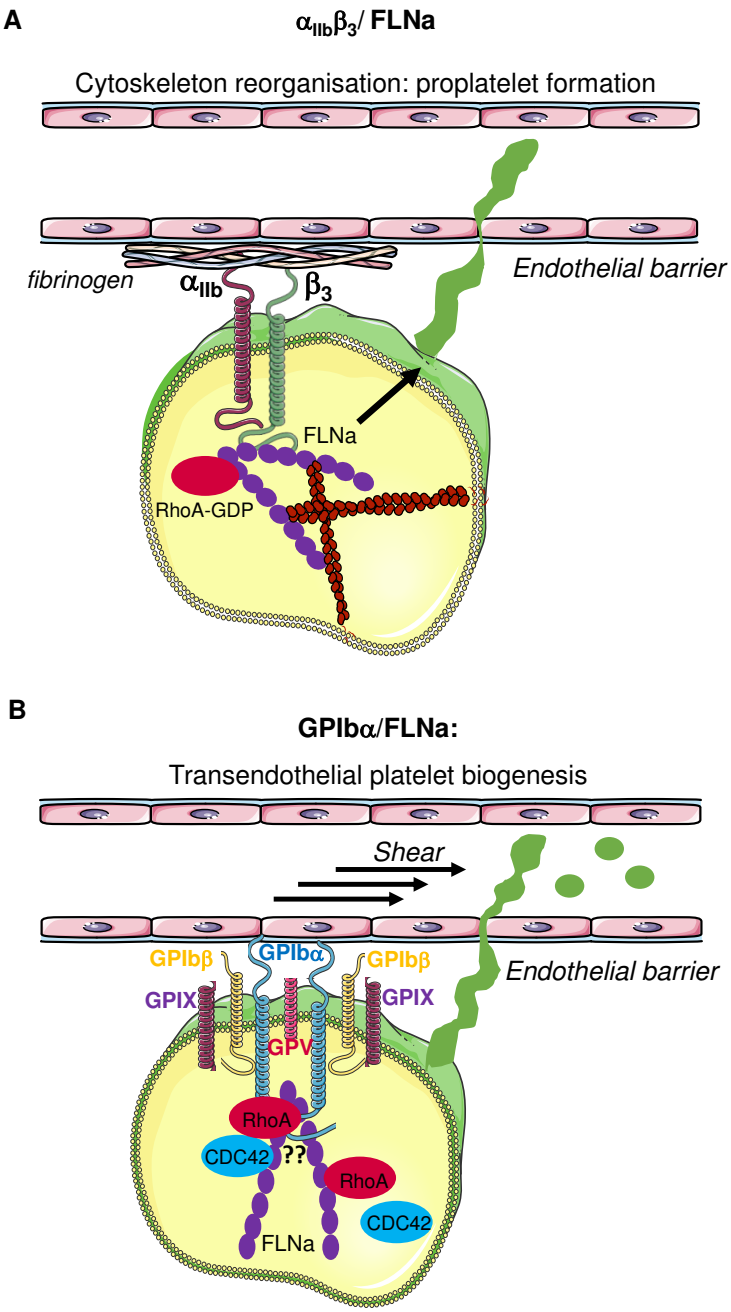


Figure 3



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Filamin A: key actor in platelet biology

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