

# Protein 4.1, a component of the erythrocyte membrane skeleton and its related homologue proteins forming the protein 4.1/FERM superfamily

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**Abstract:** The review is focused on the domain structure and function of protein 4.1, one of the proteins belonging to the membrane skeleton. The protein 4.1 of the red blood cells (4.1R) is a multifunctional protein that localizes to the membrane skeleton and stabilizes erythrocyte shape and membrane mechanical properties, such as deformability and stability, via lateral interactions with spectrin, actin, glycophorin C and protein p55. Protein 4.1 binding is modulated through the action of kinases and/or calmodulin-Ca<sup>2+</sup>. Non-erythroid cells express the 4.1R homologues: 4.1G (general type), 4.1B (brain type), and 4.1N (neuron type), and the whole group belongs to the protein 4.1 superfamily, which is characterized by the presence of a highly conserved FERM domain at the N-terminus of the molecule. Proteins 4.1R, 4.1 G, 4.1 N and 4.1 B are encoded by different genes. Most of the 4.1 superfamily proteins also contain an actin-binding domain. To date, more than 40 members have been identified. They can be divided into five groups: protein 4.1 molecules, ERM proteins, talin-related molecules, protein tyrosine phosphatase (PTPH) proteins and NBL4 proteins. We have focused our attention on the main, well known representatives of 4.1 superfamily and tried to choose the proteins which are close to 4.1R or which have distinct functions. 4.1 family proteins are not just linkers between the plasma membrane and membrane skeleton; they also play an important role in various processes. Some, such as focal adhesion kinase (FAK), non-receptor tyrosine kinase that localizes to focal adhesions in adherent cells, play the role in cell adhesion. The other members control or take part in tumor suppression, regulation of cell cycle progression, inhibition of cell proliferation, downstream signaling of the glutamate receptors, and establishment of cell polarity; some are also involved in cell proliferation, cell motility, and/or cell-to-cell communication.

**Key words:** Erythrocyte - Membrane skeleton - Protein 4.1 - FERM family

## Introduction

The general role of the erythrocyte membrane skeleton is well known. It maintains the shape and the membrane mechanical properties of this cell. In other tissues, membrane-associated cytoskeletal proteins are also involved in the control of cell-cell and cell-matrix attachments. They also have roles to play in the organization of specialized membrane domains. The main component of the red blood cell membrane skeleton is spectrin. This fibrous polypeptide acts as a scaffold in the membrane skeleton meshwork. The spectrin tetramers are attached to the membrane by the protein ankyrin, which is linked through its ank-repeat region to band 3 dimers and protein 4.1, which forms a ternary complex with glycophorin C and the membrane-associated guanylate kinase p55 [for review see 12]. Other proteins of the

membrane skeleton, such as actin, tropomyosin, tropomodulin and dematin form the junction with spectrin at its nodes.

This review is devoted to protein 4.1, a multifunctional membrane protein which stabilizes the spectrin-actin association. This 80-kDa protein, which is essential for normal cell shape and integrity, provides connections between the skeleton and the plasma membrane in erythrocytes, but also plays an important role in several cell functions in many other tissues/organs, including the bone marrow, cerebellum, lungs, testes and thymus [130]. Protein 4.1 is also a prototype of a large superfamily of proteins, the members of which share a common more or less homologous domain

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**Abbreviations:** FERM - four.one protein, ezrin, radixin, moesin (name of domain), NBL4 - novel band 4.1-like 4, NF2 - neurofibromatosis type 2, PDZ - postsynaptic density-95/discs large/zona occludens-1 domain, PIP2 - phosphatidylinositol 4,5-bisphosphate, PS - phosphatidylserine, PTPH - protein tyrosine phosphatase, PTPMEG - protein tyrosine phosphatase, megakaryocyte

named the FERM domain. It includes all homologues of protein 4.1 and the ERM proteins - ezrin, radixin and moesin. Talin and some types of protein tyrosine phosphatase, such as PRP-Meg and PTPH1, are also part of this family [77]. The aim of this paper is to review recent data on this large family of proteins, which play a role in the structure and regulation of the membrane skeleton and cytoskeleton, and are also involved in cell adhesion, and in some cases in the regulation of the cell cycle.

It should be noted that we have chosen to review the proteins which are close to 4.1R or which have distinct functions, since to present the characteristics of all members of the 4.1 superfamily in one review would be a very difficult task, because in humans, for instance, there are about 50 genes now known to encode FERM domains (Table 1) which appear in many proteins such as JAK kinases, unconventional myosins and some GEFs among others.

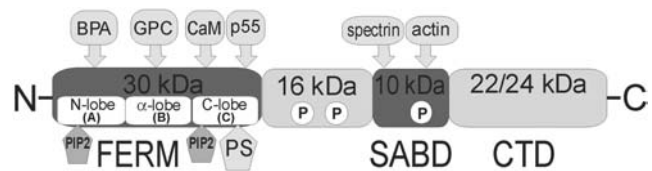
### The structure of protein 4.1

Human protein 4.1 consists of 864 amino acid residues (molecular weight: 97017 Da) and is a product of human gene *epb41* (<http://www.expasy.org/uniprot/> (UniProtKB/Swiss-Prot: P11171)).

It should be noted that there are more residues in isoforms containing exons 17A and 17B and fewer in isoforms translated from ATG2 or ATG 3. Mentioning of all these isoforms could complicate our review, so we have decided to choose atypical entries as examples of presented structures and proteins. Protein 4.1R consists of four structural and functional domains (Fig. 1) [22].

#### The FERM domain

The very important N-terminal (30 kDa) domain, also known as the FERM domain, is present in a wide range of proteins called the ERM family (Fig. 2A) [20]. This domain is encoded by exons 4 to 12 and is responsible for the binding of protein 4.1R to other proteins such as glycophorin C [37, 52], protein p55 [78, 79], and calmodulin [93]. The analysis of the crystal structure of the ERM domain led to the identification of 3 subdomains, the N-,  $\alpha$ - and C-lobes [49], called also A, B, and C lobes, which form a clover leaf structure. Each lobe contains a distinct region or regions that are responsible for specific binding of different membrane proteins and each of them represents a compactly folded structure. The N-lobe contains the band 3 binding motif; the sequence LEEDY encoded by exon 5 is the binding site for the sequences IRRRY and LRRRY present in the cytoplasmic domain of band 3 [65, 127]. This lobe has a fold resembling ubiquitin [31, 124]. The next lobe (named the  $\alpha$ -lobe because it is formed by four  $\alpha$ -helices) contains the glycophorin C-binding region and resembles acyl-CoA binding proteins [31, 124]. The C-



**Fig. 1.** Schematic representation of the domain structure of protein 4.1R, on the basis of [4, 49, 126, 127]. Domains: CTD - carboxyl terminal domain, FERM - 4.1-ezrin-radixin-moesin domain, SABD - spectrin-actin-binding domain. Receptors: BPA - band 3, CaM - calmodulin, GPC - glycophorin C, p55 - protein p55, P - phosphate group, PIP2 - phosphatidylinositol 4,5-bisphosphate, PS - phosphatidylserine.

lobe, containing the p55, CaM, PS and PIP2-binding region, with two  $\beta$ -sheets and an  $\alpha$ -helical end, has a fold related to pleckstrin homology domain/phosphotyrosine-binding domain (PTB) [31, 124]. This binding site is located in the region encoded by exon 10 and associated with a positively charged, 39-amino acid residue motif (D5 domain) within p55 [80, 94]. With all these binding sites, the 30 kDa domain contributes to the formation of the ternary complex of 4.1R, glycophorin C and p55 [1, 80]. The C-lobe, C-terminal part of FERM domain was also shown to interact with phosphatidylserine (PS) [2, 112] and calmodulin; the latter requires the presence of  $\text{Ca}^{2+}$ . The  $\text{Ca}^{2+}$ -sensitive calmodulin binding sites are situated between the  $\alpha$ - and C-lobes, and the  $\text{Ca}^{2+}$ -insensitive between the C- and N-lobes, in the regions encoded by exons 11 and 9, respectively [49, 93]. The FERM domain is required during erythropoiesis for the stable retention of glycophorin C and p55 in the membrane [119].

#### The 16 kDa domain

The 16 kDa domain, which lacks any known binding activities, separates the FERM domain and the next, 10 kDa domain. Its function is not yet clearly defined. It was recorded that two serine residues, Ser<sup>312</sup> and Ser<sup>331</sup>, could be phosphorylated by protein kinase A and protein kinase C. This means the 16 kDa domain possibly affects 4.1R interactions with band 3, glycophorin C, spectrin and actin [59, 78].

#### The SABD domain

The 10 kDa domain, encoded by exons 13, 16 and 17, binds spectrin, actin [23], U2AF<sup>35</sup> and importin- $\alpha$ , a protein that is necessary for the nuclear import of protein 4.1R [38, 76]. This domain also contains residues such as Ser<sup>467</sup> and Tyr<sup>418</sup>, which can be phosphorylated by various kinases [58, 78]. The primary structure of this domain is highly conserved in many vertebrates, indicating conservation of its function [140], except for 4.1N homologue of SAB domain, which is different and does not bind spectrin and actin [40]. Also *Drosophila* 4.1

**Table 1.** Human genes that are known to encode FERM domain IPR000299 located on Ensembl ([http://www.ensembl.org/Homo\\_sapiens/domainview?domainentry=IPR000299](http://www.ensembl.org/Homo_sapiens/domainview?domainentry=IPR000299))

Gene ID	Gene name	Genome location	Description (if known)
ENSG00000159023	EPB41	Chromosome 1: 29.09m	Protein 4.1 (Band 4.1) (P4.1) (EPB4.1) (4.1R). [Source:Uniprot/SWISSPROT;Acc:P11171]
ENSG00000162434	JAK1	Chromosome 1: 65.07m	Tyrosine-protein kinase JAK1 (EC 2.7.10.2) (Janus kinase 1) (JAK-1). [Source:Uniprot/SWISSPROT;Acc:P23458]
ENSG00000152104	PTPN14	Chromosome 1: 212.60m	Tyrosine-protein phosphatase non-receptor type 14 (EC 3.1.3.48) (Protein-tyrosine phosphatase pez). [Source:Uniprot/SWISSPROT;Acc:Q15678]
ENSG00000152527	PLEKHH2	Chromosome 2: 43.72m	pleckstrin homology domain containing, family H (with MyTH4 domain) member 2 [Source:RefSeq_peptide;Acc:NP_742066]
ENSG00000088179	PTPN4	Chromosome 2: 120.23m	Tyrosine-protein phosphatase non-receptor type 4 (EC 3.1.3.48) (Protein-tyrosine phosphatase MEG1) (PTPase-MEG1) (MEG). [Source:Uniprot/SWISSPROT;Acc:P29074]
ENSG00000115109	EPB41L5	Chromosome 2: 120.49m	Band 4.1-like protein 5. [Source:Uniprot/SWISSPROT;Acc:Q9HCM4]
ENSG00000169994	MYO7B	Chromosome 2: 128.09m	MYO7B protein. [Source:Uniprot/SPTREMBL;Acc:Q6PIF6]
ENSG00000006607	FARP2	Chromosome 2: 241.94m	FERM, RhoGEF and pleckstrin domain-containing protein 2 (FERM domain including RhoGEF) (FIR). [Source:Uniprot/SWISSPROT;Acc:O94887]
ENSG00000114541	FRMD4B	Chromosome 3: 69.30m	FERM domain-containing protein 4B (GRP1-binding protein GRSP1). [Source:Uniprot/SWISSPROT;Acc:Q9Y2L6]
ENSG00000163629	PTPN13	Chromosome 4: 87.73m	Tyrosine-protein phosphatase non-receptor type 13 (EC 3.1.3.48) (Protein-tyrosine phosphatase 1E) (PTP-E1) (hPTPE1) (PTP-BAS) (Protein-tyrosine phosphatase PTPL1) (Fas-associated protein-tyrosine phosphatase 1) (FAP-1). [Source:Uniprot/SWISSPROT;Acc:Q12923]
ENSG00000145555	MYO10_HUMAN	Chromosome 5: 16.72m	Myosin-10 (Myosin X). [Source:Uniprot/SWISSPROT;Acc:Q9HD67]
ENSG00000129595	EPB41L4A	Chromosome 5: 111.51m	Band 4.1-like protein 4A (NBL4 protein). [Source:Uniprot/SWISSPROT;Acc:Q9HCS5]
ENSG00000007944	MYLIP	Chromosome 6: 16.24m	Ubiquitin ligase MYLIP (EC 6.3.2.-) (Myosin regulatory light chain-interacting protein) (MIR). [Source:Uniprot/SWISSPROT;Acc:Q8WY64]
ENSG00000079819	EPB41L2	Chromosome 6: 131.20m	Band 4.1-like protein 2 (Generally expressed protein 4.1) (4.1G). [Source:Uniprot/SWISSPROT;Acc:O43491]
ENSG00000092820	VIL2	Chromosome 6: 159.11m	Ezrin (p81) (Cytovillin) (Villin-2). [Source:Uniprot/SWISSPROT;Acc:P15311]
ENSG00000153303	FRMD1	Chromosome 6: 168.20m	FERM domain containing 1 [Source:RefSeq_peptide;Acc:NP_079195]
ENSG00000001631	KRIT1	Chromosome 7: 91.67m	Krev interaction trapped protein 1 (Krev interaction trapped 1) (Cerebral cavernous malformations 1 protein). [Source:Uniprot/SWISSPROT;Acc:O00522]
ENSG00000120899	PTK2B	Chromosome 8: 27.24m	Protein tyrosine kinase 2 beta (EC 2.7.10.2) (Focal adhesion kinase 2) (FADK 2) (Proline-rich tyrosine kinase 2) (Cell adhesion kinase beta) (CAK beta) (Calcium-dependent tyrosine kinase) (CADTK) (Related adhesion focal tyrosine kinase) (RAFTK). [Source:Uniprot/SWISSPROT;Acc:Q14289]
ENSG00000188179	XP_932590.1	Chromosome 8: 49.99m	PREDICTED: similar to Band 4.1-like protein 5 [Source:RefSeq_peptide_predicted;Acc:XP_942094]

Table 1. (cont.)

ENSG00000169398	PTK2	Chromosome 8: 141.74m	Focal adhesion kinase 1 (EC 2.7.10.2) (FADK 1) (pp125FAK) (Protein-tyrosine kinase 2). [Source:Uniprot/SWISSPROT;Acc:Q05397]
ENSG00000096968	JAK2	Chromosome 9: 4.98m	Tyrosine-protein kinase JAK2 (EC 2.7.10.2) (Janus kinase 2) (JAK-2). [Source:Uniprot/SWISSPROT;Acc:O60674]
ENSG00000137076	TLN1	Chromosome 9: 35.69m	talin 1 (TLN1), mRNA [Source:RefSeq_dna;Acc:NM_006289]
ENSG00000070601	FRMPD1	Chromosome 9: 37.64m	FERM and PDZ domain containing 1 [Source:RefSeq_peptide;Acc:NP_055722]
ENSG00000172159	FRMD3	Chromosome 9: 85.05m	FERM domain containing 3 [Source:RefSeq_peptide;Acc:NP_777598]
ENSG00000095203	EPB41L4B	Chromosome 9: 110.97m	Band 4.1-like protein 4B (EHM2 protein) (FERM-containing protein CG1). [Source:Uniprot/SWISSPROT;Acc:Q9H329]
ENSG00000070159	PTPN3	Chromosome 9: 111.18m	Tyrosine-protein phosphatase non-receptor type 3 (EC 3.1.3.48) (Protein-tyrosine phosphatase H1) (PTP-H1). [Source:Uniprot/SWISSPROT;Acc:P26045]
ENSG00000151474	FRMD4A	Chromosome 10: 13.73m	FERM domain-containing protein 4A. [Source:Uniprot/SWISSPROT;Acc:Q9P2Q2]
ENSG00000170324	FRMPD2	Chromosome 10: 49.03m	FERM and PDZ domain containing 2 isoform 2 [Source:RefSeq_peptide;Acc:NP_001017929]
ENSG00000126391	NP_114110.1	Chromosome 11: 64.91m	No description
ENSG00000137474	MYO7A	Chromosome 11: 76.53m	Myosin-7A (Myosin VIIa). [Source:Uniprot/SWISSPROT;Acc:Q13402]
ENSG00000137710	RDX	Chromosome 11: 109.61m	Radixin. [Source:Uniprot/SWISSPROT;Acc:P35241]
ENSG00000152767	FARP1	Chromosome 13: 97.59m	FERM, RhoGEF and pleckstrin domain-containing protein 1 (Chondrocyte-derived ezrin-like protein). [Source:Uniprot/SWISSPROT;Acc:Q9Y4F1]
ENSG00000139926	FRMD6	Chromosome 14: 51.03m	FERM domain-containing protein 6. [Source:Uniprot/SWISSPROT;Acc:Q96NE9]
ENSG00000073712	PLEKHC1	Chromosome 14: 52.39m	Pleckstrin homology domain-containing family C member 1 (Kindlin-2) (Mitogen-inducible gene 2 protein) (Mig-2). [Source:Uniprot/SWISSPROT;Acc:Q96AC1]
ENSG00000054690	NP_065766.1	Chromosome 14: 67.07m	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1 [Source:RefSeq_peptide;Acc:NP_065766]
ENSG00000070778	PTPN21	Chromosome 14: 88.00m	Tyrosine-protein phosphatase non-receptor type 21 (EC 3.1.3.48) (Protein-tyrosine phosphatase D1). [Source:Uniprot/SWISSPROT;Acc:Q16825]
ENSG00000171877	FRMD5	Chromosome 15: 41.95m	No description
ENSG00000171914	-novel-	Chromosome 15: 60.73m	talin 2 (TLN2), mRNA [Source:RefSeq_dna;Acc:NM_015059]
ENSG00000091536	MYO15A	Chromosome 17: 17.95m	Myosin-15 (Myosin XV) (Unconventional myosin-15). [Source:Uniprot/SWISSPROT;Acc:Q9UKN7]
ENSG00000068137	PLEKHH3	Chromosome 17: 38.07m	No description
ENSG00000204325	XR_001390.1	Chromosome 17: 71.13m	myosin XVB pseudogene, transcript variant 33 (MYO15B), misc RNA [Source:RefSeq_dna;Acc:XR_000970]
ENSG00000082397	EPB41L3	Chromosome 18: 5.38m	Band 4.1-like protein 3 (4.1B) (Differentially expressed in adenocarcinoma of the lung protein 1) (DAL-1). [Source:Uniprot/SWISSPROT;Acc:Q9Y2J2]

Table 1. (cont.)

ENSG00000105397	TYK2	Chromosome 19: 10.32m	Non-receptor tyrosine-protein kinase TYK2 (EC 2.7.10.2). [Source:Uniprot/SWISSPROT;Acc:P29597]
ENSG00000105639	JAK3	Chromosome 19: 17.79m	Tyrosine-protein kinase JAK3 (EC 2.7.10.2) (Janus kinase 3) (JAK-3) (Leukocyte janus kinase) (L-JAK). [Source:Uniprot/SWISSPROT;Acc:P52333]
ENSG00000088367	EPB41L1	Chromosome 20: 34.14m	Band 4.1-like protein 1 (Neuronal protein 4.1) (4.1N). [Source:Uniprot/SWISSPROT;Acc:Q9H4G0]
ENSG00000186575	NF2	Chromosome 22: 28.33m	Merlin (Moesin-ezrin-radixin-like protein) (Neurofibromin-2) (Schwannomin) (Schwannomerlin). [Source:Uniprot/SWISSPROT;Acc:P35240]
ENSG00000169933	NP_055543.1	Chromosome X: 12.07m	PDZ domain containing 10 [Source:RefSeq_peptide;Acc:NP_055543]
ENSG00000147065	MSN	Chromosome X: 64.80m	Moesin (Membrane-organizing extension spike protein). [Source:Uniprot/SWISSPROT;Acc:P26038]
ENSG00000147234	FRMPD3	Chromosome X: 106.66m	Novel protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q5JV73]
ENSG00000165694	NP_919253.1	Chromosome X: 131.04m	No description

does not have this domain. The minimum consensus sequence which is necessary for interactions with spectrin and actin (critical to the mechanical integrity of the erythrocyte) is a 21-amino acid sequence consisting of the residues Lys<sup>407</sup>-Glu<sup>427</sup> [26]. This motif, which is responsible for strong spectrin binding, is situated in the middle of a 59-amino acid region and activates the spectrin-actin complex formation [27, 113], therefore playing a crucial role in the mechanical stabilization of the membrane [26].

### The CTD domain

The C-terminal domain of protein 4.1 (CTD) is a 22/24 kDa domain, encoded by exons 18-21. The 2 kDa molecular weight difference between protein 4.1Ra and 4.1Rb is a result of a conversion of asparagine Asn<sup>502</sup> to Asp<sup>502</sup>, which takes place during erythrocyte aging [61]. As a consequence, the mobility of 4.1R in SDS gel is changed so that two isoforms can be distinguished. The 22/24 kDa domain is not stable and is rapidly processed to a 15 kDa fragment by chymotrypsin [116]. This smaller fragment is extremely stable, and is composed of 5-6  $\beta$ -sheets and an  $\alpha$  helix preceding the C-terminal helix. In nonerythroid cells, the 22/24 kDa domain is known to interact with some proteins that form tight junctions, such as occludin, zonula occludens-1 (ZO-1) and zonula occludens-2 (ZO-2) [83]. Others suggest that the erythroid C-terminal domain can bind the polypeptide eIF3-p44 subunit of the eukaryotic translation factor 3 (eIF3) complex [60]. Amino acid residues 525-622 of the C-terminus of protein 4.1R and a region (residues 54-321) of eIF3-p44 are essential for this interaction. This association suggests a possible importance of pro-

tein 4.1 as a linker between the cytoskeleton network and the translation apparatus [60].

The 4.1R-null mice have erythrocytes with abnormal morphology, lowered membrane stability and reduced expression of other skeletal proteins such as spectrin and ankyrin. It suggests that loss of 4.1R compromises membrane skeleton assembly in erythroid progenitors [119]. Thus, there are two separate functions for 4.1R: mechanical stabilization of the membrane and membrane protein targeting/retention.

### The role of 4.1R in the organization of the membrane skeleton

The ability of 4.1R to bind various proteins is essential for the organization of the red cell membrane skeleton. The membrane skeleton network is created by numerous horizontal interactions of the spectrin-actin network with protein 4.1, adducin, tropomyosin, tropomodulin, dematin and protein p55 [127]. Protein 4.1 is also necessary for the vertical connections of the skeleton underlying the red blood cell membrane with the protein precursors present in the membrane, such as band 3 (in this case, the linker is ankyrin) [81] or glycophorin C [77] (Fig. 2A). All these interactions are essential in maintaining the biconcave disc shape of the erythrocyte and the mechanical properties of the membrane [86, 96]. The affinity of 4.1 to the other membrane proteins can be regulated by calmodulin, which can bind to both Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent binding sites in protein 4.1 [93]. The interaction of a 10-kDa domain of protein 4.1 with the actin and spectrin  $\beta$ -subunit is very important for the membrane stability of erythrocytes, and it accelerates spectrin-actin interactions [96]. Interactions

with both proteins can be modulated by various factors, such as the binding of calmodulin- $\text{Ca}^{2+}$  to protein 4.1R [92, 129] or the phosphorylation of protein 4.1R [59, 103]. In the presence of  $\text{Ca}^{2+}$ , calmodulin reduces the affinity of protein 4.1R for the spectrin-actin complex and decreases the membrane mechanical stability [92]. Similarly, the phosphorylation of protein 4.1 reduces its ability to associate with spectrin by about 5-fold, and may increase the relaxation of the skeleton and the flexibility of the membrane structure [30]. Protein 4.1R can be phosphorylated by a variety of kinases *in vivo* and *in vitro*, including casein kinase, tyrosine kinase, PKA and PKC [59, 103]. It was shown that phosphorylation of 4.1R by protein kinase C decreases its ability to form a ternary complex with spectrin and actin. Phosphorylation induces the dissociation of glycophorin C from the membrane skeleton. Therefore the final effect of this process is a marked decrease in the mechanical stability of the erythrocyte membrane [78].

Protein 4.1 also interacts with glycophorin C and protein p55 [37, 80, 96]. These three protein interactions, glycophorin C-protein 4.1, glycophorin C-p55 and p55-protein 4.1, constitute the ternary complex in the erythrocyte membrane [1, 79]. The p55 and glycophorin C binding sites were identified in the N-terminal 30-kDa domain of protein 4.1R. They are encoded by exons 8 (responsible for 4.1R binding glycophorin C) and 10 (responsible for 4.1R binding p55) [94]. The binding of protein 4.1 to p55 increases the affinity of the interaction between p55 and glycophorin C, which implies a very important role of protein 4.1R in the regulation of the stability of the ternary protein complex (glycophorin C, protein 4.1 and p55) involved in the "vertical" interactions of the membrane skeleton with the membrane bilayer [79, 94]. Protein 4.1 deficiency in the red blood cell membrane causes a lack of glycophorin C and leads to an elliptocytic erythrocyte morphology. Protein 4.1 seems to be required for the retention of normal amounts of glycophorin C in the erythrocyte membrane [107].

In addition, protein 4.1 also binds to the cytoplasmic domain of band 3 via its N-terminal 30-kDa domain [19], and through this interaction, modulates band 3 binding to ankyrin, decreasing the affinity of band 3 for ankyrin [3]. The association with calmodulin and  $\text{Ca}^{2+}$  reduces the binding affinity to band 3 [92]. These interactions stabilize the mechanical resistance of the erythrocyte membrane [3]. Although most non-erythroid cells lack band 3 and glycophorin C in their membranes, they contain adhesion molecules, such as cadherins and CD44, which could interact with protein 4.1 and its non-erythroid analogues [95]. Protein 4.1 binds to the cytoplasmic domain of CD44 in a manner similar to binding to band 3. Similarly, CD44-protein 4.1 interaction is also decreased by  $\text{Ca}^{2+}$  and calmodulin [95]. CD44 was found to associate with ankyrin [74]. Although protein 4.1 and ankyrin bind to distinctly different regions of the

cytoplasmic domain of CD44, the binding of protein 4.1 prevents subsequent ankyrin binding, probably playing an important role in the regulation of ankyrin binding to CD44 [95].

Apart from the binding of membrane proteins, protein 4.1 was shown to interact directly with membrane phospholipids, mainly phosphatidylserine (PS), through the N-terminal 30 kDa domain of protein 4.1, as characterized by Sato and Onishi [112]. The PS binding site was found in the sequences encoded by exons 10 and/or 11 [2]. The positively charged YKRS motif (residues 442-445) proved important for the initiation of interaction between protein 4.1 and the negatively charged surface of PS [2]. Furthermore, a tight hydrophobic interaction of fatty acyl chains with the cluster of hydrophobic residues near the YRKS motif was suggested [2]. Association with PS prevents the interaction of 4.1 with any of the membrane proteins [2]. The localization of a PS binding site in the FERM domain, which is present in most of the protein 4.1 superfamily members, could be a clue for the biological significance of the domain.

The recently published data showed that 4.1R bound to PIP2-containing liposomes through its FERM domain and this binding induced a conformational change in this domain [4]. Binding of PIP2 to 4.1R selectively modulated the ability of 4.1R of interaction with its different partners, e.g. PIP2 significantly enhanced binding of 4.1R to glycophorin C *in situ* in the membrane [4]. This is in contrast to the FERM domain of focal adhesion kinase (FAK), which does not bind PIP2 [19].

### Protein 4.1R in nucleated cells

The existence of multiple isoforms of 4.1R in nucleated cells indicates that it has a much more sophisticated role there than in red blood cells. 4.1R epitopes were detected at different intracellular sites, and the association of 4.1R with various cellular proteins has been reported [60, 82, 94], suggesting that 4.1R may be involved in many processes in nucleated cells. Proteins interacting with 4.1R and possessing predominant extranuclear localization and function, such as actin, were detected also in the nucleus. There is a strong possibility that 4.1R can take part in the organization of the nuclear architecture.

The identification of signals involved in intracellular localization of 4.1R proteins revealed that of the seven isolated 4.1R cDNAs, four localized predominantly to the nucleus and the other three to the cytoplasm [75]. A comparative analysis of the exon composition of the naturally occurring 4.1R cDNAs showed that all 4.1R molecules contained a conserved region encoded by constitutive exons, named the "core region", which determines the nuclear localization, which is able to confer nuclear targeting to a cytosolic reporter. The nuclear localization signal was identified in the alternative exon 16. Exon 5-

encoded sequences blocked nuclear entry of the core region, resulting in 4.1R isoforms being predominantly distributed to the cytoplasm. Exon 5 was also able to confer cytoplasmic localization to a nuclear reporter. In the case of simultaneous expression of exons 5 and 16, the nuclear targeting effect of exon 16 was dominant over the inhibitory effect observed due to the expression of exon 5 [75]. Further studies identified the amino acid sequence responsible for the cytoplasmic localization of 4.1R isoforms expressing exon 5. Exon 5 encodes a hydrophobic, leucine-rich sequence (L<sup>26</sup>LKRVCEHLNLL) that is very similar to the sequences of nuclear export signals (NESs) [76]. This sequence adopts an  $\alpha$ -helical conformation that is similar to the topology employed for the NESs of other proteins with two conserved leucine residues (L<sup>34</sup> and L<sup>36</sup>), which in NESs are critical for 4.1R cytoplasmic localization. The leucine-rich sequence is required for 4.1R binding to the export receptor CRM1 in a RanGTP-dependent fashion [76]. The leucine-rich sequence in 4.1R probably controls the distribution and function of a specific set of 4.1R isoforms. Although the physiological role of 4.1R isoforms containing the leucine-rich sequence remains still unknown, they are candidates for being part of the information transmission system between the cytoplasm/plasma membrane and the nucleus. *In vivo* and *in vitro* biochemical analyses indicate an association between 4.1R and microtubules. Analysis of the distribution of 4.1R in human T cells shows that both exogenous and endogenous 4.1R colocalize with the microtubule network [100]. Transfection experiments of T-cell 4.1R cDNAs in conjunction with confocal microscopy showed the colocalization of exogenous 4.1R isoforms with the tubulin skeleton. A 22-amino acid sequence in the constitutive core region, which contains 7 repeats of leucine residues, was essential for tubulin binding [100]. The 4.1R interaction with interphase microtubules seems to play a role in the organization of microtubule architecture and the mitotic spindle poles.

Protein 4.1R may also play a key role at the centrosome, contributing to the maintenance of a radial microtubule organization [101]. Specific 4.1R isoforms disturbed the microtubule architecture but did not affect the actin cytoskeleton. In microtubule depolymerizing-repolymerizing assays, 4.1R-transfected cells showed a similar ability to depolymerize and nucleate microtubules to that of untransfected cells. In microtubule-depolymerized transfected cells and during the initial steps of microtubule regrowth, centrosomal 4.1R colocalized with gamma-tubulin. This colocalization was not observed when the microtubules became dissociated. Further studies showed that 4.1R was present in isolated centrosome preparations, and that it remained in the center of microtubule asters [101]. Further studies might prove that protein 4.1R plays an essential role in the regulation of many processes in non-erythroid cells.

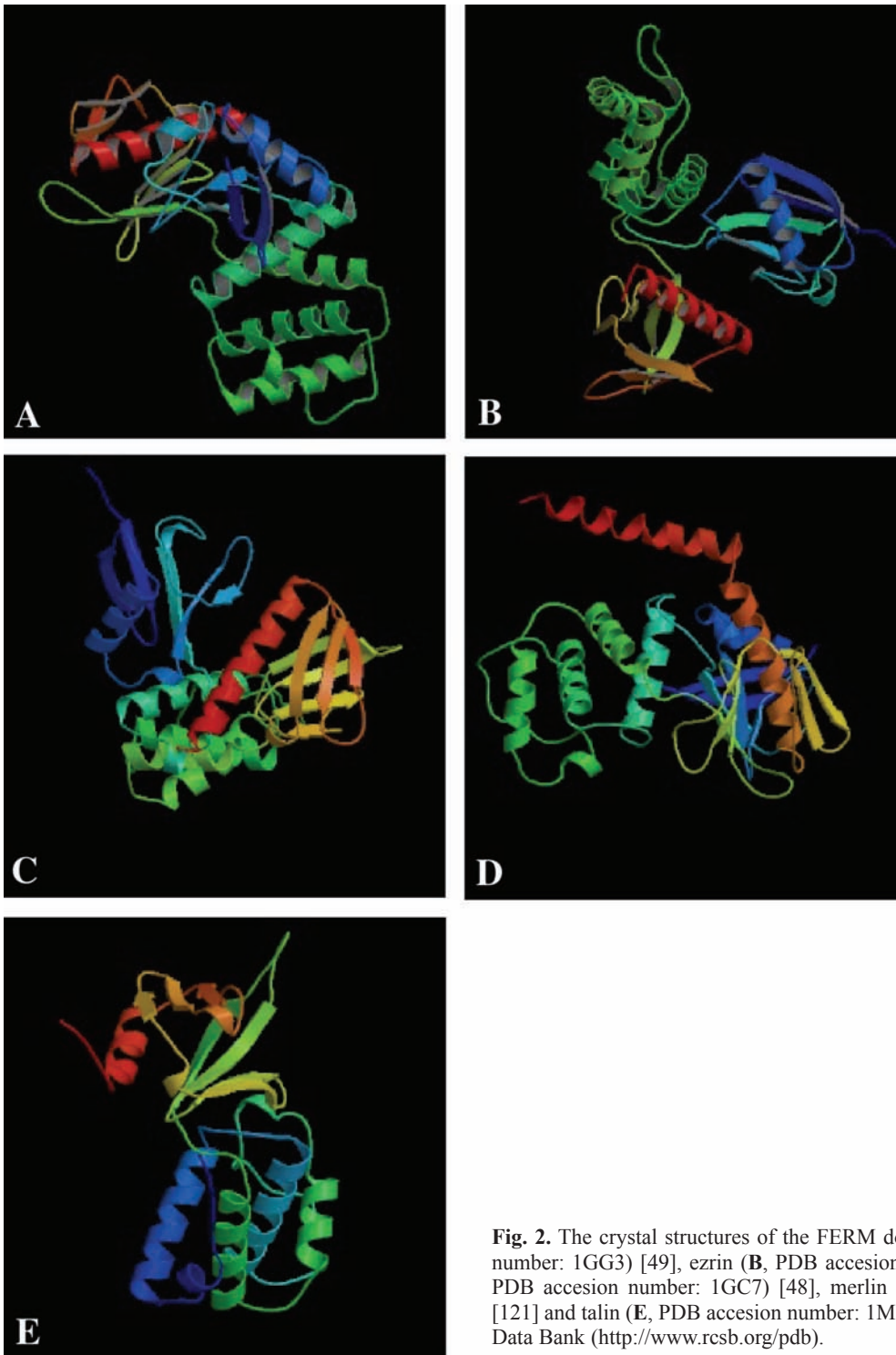
## The protein 4.1 superfamily

All the members of the protein 4.1 superfamily contain a region known as the FERM (or ERM) domain at the N-terminus of the molecule (Fig. 2). Its consensus sequence is as follows: W - LIV- x(3) - KRQ- x - LIVM- x(2) - QH- x(0,2) - LIVMF- x(6,8) - LIVMF- x(3,5) - F - FY- x(2) - DENS [(PROSITE Database of protein families and domains (<http://www.expasy.org/prosite/>) identifier: PS00660; FERM domain signature 1 (PATTERN)]. The widely distributed FERM family contains more than 40 identified members. The family is generally divided into five subfamilies on the basis of protein sequence similarity: (1) protein 4.1 molecules, (2) ERM proteins, (3) talin-related molecules, (4) PTPH (protein tyrosine phosphatase) proteins, and (5) NBL4 (novel band 4.1-like 4) proteins [128]. In the talin subfamily, one member with two isoforms has been identified so far. Proteins from subfamilies 4 and 5 lack actin-binding domains. Lately, new members of this family, such as URP1 [137] or willin [47], have been found.

## The protein 4.1 family

The protein 4.1 family includes four well-defined members: erythroid protein 4.1 (4.1R), the best-known and characterized member, 4.1G (general), 4.1N (neuronal), and 4.1 B (brain) [95, 134, 142]. Besides three highly conserved domains, proteins from this family contain several unique domains: U1, U2 and U3 [36] (Fig. 3), their function remains unknown. Actually the "true" 4.1 proteins are characterized in vertebrates by their FERM, SAB and CTD regions. Sequence alignment of the 4.1R, 4.1G, 4.1N and 4.1B reveals that a high level of sequence identity extends beyond the end of FERM domain, and into the 16 kDa region [97]. Indeed, the CTD is the unique defined characteristic domain of all true 4.1 proteins since the *Drosophila* 4.1 homologue (D4.1), localized to the septate junctions of epithelial cells and encoded by the coracle gene, lacks the SAB domain [32].

The presence of similar domains makes 4.1 homologues candidates for participation in analogous processes to 4.1R [58]. Unfortunately, although the YKRS motif of the FERM domain is highly conserved in all three proteins (4.1G, 4.1B and 4.1N), there is no data on their interaction with PS. All the members of the protein 4.1 family are highly expressed in human nervous tissue. The results of real-time PCR experiments indicate that mRNA for 4.1R is mainly present in the bone marrow, cerebellum, lungs, testes and thymus. 4.1G mRNA is widely expressed in the brain, spinal cord and testes, while 4.1N mRNA is found in the brain, spinal cord and adrenal gland, and 4.1B mRNA in the testes, brain, spinal cord and kidneys



**Fig. 2.** The crystal structures of the FERM domains of: 4.1R (A, PDB accession number: 1GG3) [49], ezrin (B, PDB accession number: 1NI2) [122], radixin (C, PDB accession number: 1GC7) [48], merlin (D, PDB accession number: 1ISN) [121] and talin (E, PDB accession number: 1MIX) [35]. Reproduced from Protein Data Bank (<http://www.rcsb.org/pdb>).

[130].

Protein 4.1N (UniProtKB/Swiss-Prot: Q9H4G0), also known as type I brain 4.1 (genes KIAA0338 or EPB41L1), is a neuronal homologue of erythrocyte membrane cytoskeletal protein 4.1 (4.1R). It contains 881 amino acid residues (molecular mass 98503 Da), and respectively shares 70, 36, and 46% identity with 4.1R in the defined membrane-binding, spectrin-actin-

binding, and C-terminal domains. It should be mentioned that 4.1N SAB domains are actually quite divergent from other such domains and does not bind spectrin/actin [40]. Protein 4.1N can stabilize the plasticity of the neuronal membrane via interactions with the spectrin-actin-based skeleton, integral membrane channels and receptors, and membrane-associated guanylate kinases [104]. Protein 4.1N is expressed in



almost all the central and peripheral neurons of the mammalian body, and is detected in embryonic neurons at the earliest stage of post-mitotic differentiation. The regions of synaptic contact between neurons potentially playing a role in synaptic architecture and function are enriched in this homologue [136]. And not only this one. Antibodies to each of 4.1R, 4.1G, 4.1N and 4.1B recognized polypeptides in postsynaptic density preparations, it means that specific isoforms of all four 4.1 proteins are components of postsynaptic densities [115]. Each of these proteins coisolate with postsynaptic complexes enriched in NMDA (N-methyl-D-aspartate) receptors [21]. In the context of the earlier discussion of 4.1 phosphorylation we should mention that Collins *et al.* showed that postsynaptic 4.1 proteins are phosphorylated *in vivo* [21].

Protein 4.1B (human, UniProtKB/Swiss-Prot: Q9Y2J2) or type II brain 4.1 (genes KIAA0987, EPB41L3) is also a neuronal, brain-enriched homologue with a molecular mass of 145 kDa (1087 amino acid residues, molecular weight: 120678 Da); it was localized in neuronal populations in the mouse brain, especially in the Purkinje cells of the cerebellum, pyramidal cells in the hippocampal regions CA1-3, thalamic nuclei, and the olfactory bulb [97, 104]. In addition, it was found in the heart, lungs, kidneys, intestine and testes. This homologue is subcellularly localized at the plasma membrane in regions of cell-cell contact [97]. Recent data concerning the structure and distribution of three members of the protein 4.1 family, 4.1B, 4.1R and 4.1N, in the mouse kidney indicates potential functions for 4.1 proteins in the kidneys, such as organization of signaling complexes, response to osmotic stress, protein trafficking, and control of cell proliferation [18, 104].

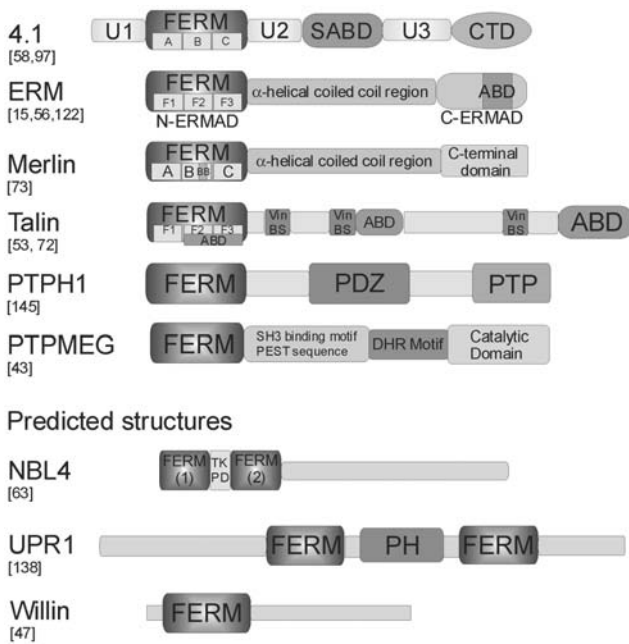
Protein 4.1G (human, UniProtKB/Swiss-Prot: O43491) (EPB41L2), located on human chromosome 6q23, is a widely expressed homologue found in brain tissue, especially in cultured hippocampal neurons. It also occurs in the heart, placenta, skeletal muscles, lungs, kidneys, pancreas and gonads [98, 102]. The complete nucleotide sequence of 4.1G cDNA predicts a 113-kDa protein that exhibits three regions of high homology to protein 4.1R (1005 amino acid residues, molecular weight: 112588 Da). In COS cells, protein 4.1G was found in the cytosol, perinuclear area and centrosomes [98].

Recently, Ni *et al.* discovered a novel human form named 4.1O (ovary), containing 553 amino acid residues [90]. The 4.1O gene consists of 14 exons and 13 introns and was located on chromosome 9q21-9q22 using bioinformatic analysis [90]. Recent data indicate however, that 4.1O is not a member of the true 4.1 family and is actually a member of poorly characterized family of FERM and transmembrane domain proteins (two in vertebrate genomes, one in invertebrates) [6].

### The ERM family

The ERM family includes ezrin, radixin, moesin and merlin. The proteins of the ERM family are close homologues with about 75% sequence identity [5, 127, 133]. Radixin (~80 kDa), ezrin (~82 kDa) and moesin (~75 kDa) are very closely related to the protein 4.1 superfamily. They possess N-terminal FERM (ERM) domain (Fig. 2B-D) consisting of about 300 residues, also called N-ERMAD (N-terminal ERM association domain), and an about 100-residue long C-terminal domain C-ERMAD (C-terminal ERM association domain), where the last 34 residues form an F-actin-binding site (ABD) [134]. The domains are separated by an  $\alpha$ -helical, coiled coil region (CRR). Additionally, two actin-binding sites have been identified in the middle and N-terminal domains (Fig. 3) [82, 111]. The ERM domains share about 30% sequence homology with the 30 kDa domain of 4.1R. Merlin is structurally similar to the ERM proteins, but instead of an actin-binding domain (ABD), it contains a C-terminal domain (CTD), just like the proteins from the 4.1 family (Fig. 3). Homologues of the ERM family of proteins have also been found in genetically-tractable model organisms, like *Drosophila melanogaster* and *Caenorhabditis elegans*. However, only one gene in flies [137] and two nearly identical genes in nematodes were found [135]. Activated ezrin, radixin and moesin are probably involved in the linking of actin filaments to CD43, CD44, ICAM1-3 cell adhesion molecules, various membrane channels and receptors, such as the Na<sup>+</sup>/H<sup>+</sup> exchanger-3 (NHE3), cystic fibrosis transmembrane conductance regulator (CFTR), and the  $\beta$ 2-adrenergic receptor [14, 133]. With the help of the PCR method, some other members of the 4.1 superfamily were identified. Seven cDNA clones encoded novel NBL 1-7 proteins [128]. Two of them, NBL6 and NBL7, demonstrated high similarity to the corresponding sequences of the ERM family.

**Ezrin.** Ezrin, also called cytovillin or p81, was first isolated and identified as a protein localized under the plasma membrane of chicken intestinal microvilli [14]. The name of ezrin came from Ezra Cornell University, where it was first purified. The ezrin gene is located on human chromosome 6 (13 exons). Ezrin obtained from the human placenta was subsequently recognized as a good substrate for some tyrosine kinases *in vivo* [14]. Human ezrin (UniProtKB/Swiss-Prot: P15311) contains 585 amino acid residues, and its molecular weight is 69268 Da. The N-terminal 296 residues of ezrin fold into a N-ERMAD domain that associates with the C-terminal 107-residue C-ERMAD domain containing an F-actin binding site [36]. The ERM domain contains binding sites for several proteins - CD44, EBP50 (PDZ-containing phosphoprotein) and Rho-GDI (Rho pathway regulator) - and also for the signaling phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) [15]. Between



**Fig. 3.** A comparison of the structural domains present in the main members of the protein 4.1 superfamily, references in brackets. Domains: A, B, C - FERM subdomains (lobes), ABD - actin binding domain, BB - blue box, CTD - carboxyl terminal domain, DHR - Drosophila hormone receptor, FERM - 4.1-ezrin-radixin-moesin domain, F1, F2, F3 - FERM subdomains, PDZ - postsynaptic density-95 /discs large/zonula occludens-1 domain, PH - pleckstrin homology domain, PTP - protein tyrosine phosphatase domain, SABD -spectrin-actin-binding domain, TKPD - tyrosine kinase phosphorylation domain, U1-3 - unique regions, VinBS - vinculin binding site.

the main domains, there is a 172-residue long  $\alpha$ -helical domain containing the binding site for the RII-regulatory subunit of PKA [28], and a very short, 7-residue long polyproline segment. It is suggested to play a role in the Rho signaling pathways, *e.g.* in the activation of Rho through the sequestration of Rho-GDI [125]. Ezrin is mostly associated with the membrane and various membrane and cytoskeletal components, such as CD44, EBP50 and, of course, F-actin [15]. It was also shown to associate with PIP2-containing liposomes via its N-terminal domain. Using internal deletions and C-terminal truncations, the PIP2 binding site was located to amino acid residues 12-115 and 233-310, in regions containing a KK(X)(n)K/RK motif, which is conserved in the ERM family [7]. Ezrin is involved in the control of cell surface topography through the regulation of the cortical cytoskeleton structure. It also takes part in the regulation of acid secretion in the parietal cells of gastric glands [50, 51].

**Radixin.** Radixin was identified as a barbed-end-capping, actin-modulating protein. It was found in cell-to-cell adherens junctions from rat liver and in the cleavage furrow in mitotic cells [132]. This protein is very similar to ezrin - 75% overall identity and 85% identity

for the N-terminal domain [34]. The gene for radixin is located on human chromosome 11 (11 exons) [138]. Human radixin (UniProtKB/Swiss-Prot: P35241) consists of 583 amino acid residues (molecular weight: 68564 Da). Its  $\alpha$ -helical domain is an extremely long (24 nm in length from N- to C-terminus), linear, stable rod (amino acid residues 311-469). The  $\alpha$ -helical radixin domain residues are available for electrostatic interactions and surface salt bridges that contribute synergistically to its thermal stability [56, 57]. This domain is required for regulating the formation of the functional cortical layer, including attachment of actin filaments to the membrane and possibly organization of the membrane transporters and channels [57].

The regulation of radixin function occurs via conformational changes in the protein structure. The open and closed forms represent the active and inactive molecules [64]. Intramolecular associations between ERM and CTD domains inhibit the membrane- and actin-binding activities of radixin. The protein itself is thought to acquire an inactive globular closed form, as shown using low-angle shadowing electron microscopy [64]. Phosphorylation of radixin and/or PIP2 binding leads to an elongated, open form with two associated globular structures on its two ends; these can form actin-membrane linkages. Both closed and open conformations of radixin can coexist in a population [57]. Similarly to ezrin and moesin, activated radixin has been shown to attach actin microfilaments to CD 43, CD 44 and ICAM 1-3 cell adhesion molecules and to various membrane channels and receptors, such as cystic fibrosis transmembrane regulator (CFTR),  $\text{Na}^+/\text{H}^+$  exchanger 3 (NHE3) or  $\beta$ 2-adrenergic receptor [14, 133].

**Moesin.** Moesin (UniProtKB/Swiss-Prot: P26038), a membrane-organizing extension spike protein (576 amino acid residues,  $M_r = 67689$  Da for human moesin), was first discovered as an extracellular heparin-binding protein. It was isolated from bovine uterus smooth muscle cells. Later, moesin was shown to occur intracellularly [71]. The genes for moesin reside on human chromosome X (12 exons). Moesin is composed of the same domains as the other members of the ERM family (Fig. 3). Following the N-terminal FERM domain is a 200-residue long region predicted to be largely  $\alpha$ -helical, and a C-terminal tail domain consisting of 100 residues [71]. Its FERM domain consists of three subdomains similar to A, B, C subdomains of 4.1 FERM domain. The first, named F1 (residues 5-82), has a structure similar to ubiquitin, and contains five  $\beta$ -sheet strands with an  $\alpha$ -helix running across it. The next subdomain, F2 (residues 96-195), shows similarity to acylCoA-binding protein, and is almost  $\alpha$ -helical with a short linker region of 30 residues between the second and third of four helices. The final, F3 (residues 204-296), is composed of two antiparallel  $\beta$ -sheets followed by an  $\alpha$ -helical fragment

with a sequence similarity to the pleckstrin homology (PH), phosphotyrosine-binding (PTB), and enabled/VASP homology 1 (EVH1) domains [31]. Moesin and radixin are 90% identical in the region of residues 1-297, and are considered isoforms with a similar function. Protein 4.1 is much more distantly related, showing only 16.5% sequence identity in the structural alignment with moesin [31]. Moesin is the quantitatively dominant ERM protein in human blood lymphocytes, monocytes and neutrophils [117], and the only one in human platelets [88]. In *Drosophila*, only one protein from the ERM family was identified - Dmoesin [84]. Dmoesin plays a role in the localization of determinants in the oocyte posterior cortex.

**Merlin.** Merlin (595 amino acid residues,  $M_r = 69690$  Da for human merlin), *i.e.* moesin-ezrin-radixin-like protein (UniProtKB/Swiss-Prot: P35240), is also called NF2 or schwannomin (for its role in preventing schwannoma formation) [109, 131]. This protein is encoded by 17 exons with alternative splicing of the exon producing 2 major isoforms [46]. Isoform 1, encoded by exons 1-15 and 17, is a 595-amino acid residue protein that is composed of three domains (Fig. 3): N-terminal FERM domain that precedes a long  $\alpha$ -helical, proline-rich region and is followed by a unique C-terminal domain [46, 66]. The FERM domain of merlin contains 3 subdomains, labeled A (residues 1-98), B (residues 112-212), and C (residues 212-311), and has a unique Blue Box (BB, residues 177-183) [73]. The FERM domain of merlin is responsible for membrane binding in a PIP2-dependent manner [48]. C-terminal domain of merlin is different from CTDs of true 4.1 proteins and is actually recognised by the hidden Markov model (HMM) for ERM CTDs [6]. Isoform 2 is a 590-amino acid residue protein formed as a result of an insertion in exon 16 that provides a new stop codon. This isoform differs from isoform 1 only at the C-terminus. Merlin lacks the conserved actin-binding site at its C-terminus, characteristic for the other members of the FERM family. However, it has alternative actin-binding sites inside the ERM domain [140]; these interact with cytoskeletal actin. The two isoforms differ in their regulation of this interaction. Like the other ERMs, isoform 1 can exist in the closed state where the N- and C-terminal regions undergo an intramolecular interaction, masking the conserved actin-binding site, which must be disrupted (by phosphorylation or interaction with phosphoinositides) to produce the open state, in which this interaction is possible [54, 141]. Isoform 2 always occurs in the open state and is presumably available for interaction with actin and other proteins. The ability of merlin to change conformational states with a different capacity for intermolecular interactions could be important in the signaling by the Rho pathway, which regulates the reorganization of the actin cytoskeleton [125]. Merlin is involved in regulating cell proliferation and motility by integrating the

growth-promoting and arrest signals initiated by CD44 and hepatocyte growth factor receptor activation, and probably plays a role in integrating multiple cell-signaling pathways [105]. Merlin can also indirectly associate with the skeleton through its interaction with  $\beta$ II spectrin [114]. As in the other ERM family proteins, the FERM domain of merlin is responsible for membrane binding in a PIP2-dependent manner [48].

**Talin.** The talin family in mammals consists of two gene products: TLN1, coding for a 2541-amino acid residue long protein (UniProtKB/Swiss-Prot: Q9Y490), and TLN2, coding for a protein one amino acid residue shorter (UniProtKB/Swiss-Prot: Q9Y4G6). Both isoforms are multifunctional proteins with a molecular mass of about 270 kDa [106], localized in a variety of mammalian cells; they are found in cell-extracellular matrix adherens-type junctions, in focal adhesions of adherent cells, in skeletal and cardiac muscle cells [24] and in the membrane ruffles of migrating cells [16], and are concentrated in the undercoat of cell-to-substrate adherens junctions [11, 16, 17]. Talin plays an important role in initiating actin filament growth in motile cell protrusions [25]. This protein is responsible for linking the cytoplasmic domains of integrins to the actin-based cytoskeleton, and is involved in vinculin, integrin and actin interactions [11]. In platelets, talin redistributes from the cytoplasm to the membrane upon activation, which leads to secretory events and platelet coagulation via integrins [10]. Analytical ultracentrifugation of native talin, chemical cross-linking with glutaraldehyde and SDS-PAGE analysis showed that in its native biological state, talin forms an antiparallel rod-like homodimer with an average length of 51 nm [41]. The N-terminal region of talin contains a FERM domain (47 kDa, amino acid residues 86-410) (Fig. 2E). It shares 20% sequence identity with protein 4.1 and 23% with ezrin [104]. The FERM domain is made up of 3 subdomains (F1, F2, F3) connected by short linkers. Recently, the crystal structure was determined of a part of the chicken talin FERM domain containing the F2 (residues 209-304) and F3 (residues 311-400) subdomains [35] (Fig. 3). The talin rod comprises approximately 60 alanine-rich repeats [87]. It was found to contain a significant proportion of the  $\alpha$ -helical structure [91]. The rod is very long (219 kDa) and is ended by a highly conserved C-terminal, the best characterized actin-binding site (residues 2345-2541) ABS3. This is one of the three actin-binding domains located in talin [53]. One of the remaining actin-binding sites is exposed in the talin rod (residues 951-1327, ABS2) and the other within the talin FERM domain (residues 102-497, ABS1) [72]. Both the F2 and F3 FERM subdomains contribute to F-actin binding. Actin binding is correlated with the binding and activation of integrin. Subdomain F3 of the FERM domain contains overlapping binding sites for integrin cytoplasmic domains and for the type 1 gamma isoform of PIP-kinase

(phosphatidylinositol 4-phosphate 5-kinase) [8]. The interaction of PIP-kinase with talin in the presynaptic compartments determines the mechanism for PIP<sub>2</sub> synthesis, actin dynamics, endocytosis, and functional connections between actin and clathrin-mediated endocytosis [85]. The large C-terminal domain contains the second integrin-binding site [72]. The 219 kDa rod fragment of talin contains three sites known to have vinculin-binding activity: residues 498-656, 852-950 and 1929-2029 [9, 53] (Fig. 3).

Talin is structurally and functionally regulated by interactions with the membrane key regulatory lipid - phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) [39, 80]. The binding of its N-terminal domain, enriched in basic amino acids [106], with the negatively charged head groups of PIP<sub>2</sub> is thought to play a significant role in the regulation of adhesion dynamics [89].

Two small segments of the 47 kDa head part of the talin homodimer, named S19 and H17, respectively containing 21-39 and 385-406 amino acid residues, were found to mediate the interaction with the lipid membrane [118]. These peptides, synthesized separately, can penetrate the POPC/POPG and POPC monolayers. Amino acid residues 385-406 (H17) may represent a potential membrane-anchoring domain. The helical wheel projection shows its amphipatic character. Amino acid residues 21-39 (S19) could provide an additional (hydrophobic and electrostatic) contribution of talin anchorage in the lipid membrane [118]. The strong amphipatic nature of talin peptide and the hydrophobicity gradient along its sequence suggest a partial penetration of the lipid bilayer that can destabilize it and lead to inverted micelle formation, which triggers membrane fusion as observed by cryo-electron microscopy [62].

### **The PTPH family**

Protein tyrosine phosphatases belong to larger family of PTPs containing FERM domain, but till now the PTPH protein family consisted of only two well-defined members: PTPH1 and PTPMEG. These proteins are constructed predominantly from the N-terminal FERM domain and a C-terminal (260 residues) protein tyrosine phosphatase (PTP) catalytic domain [45]. The other proteins, such as PTP36/PTPD2/Pez or PTPD1/PTP-RL10, or the largest known cytoplasmic PTP, the 250 kDa PTPL1, are predominantly localized in the apical region of the plasma membrane and in the dorsal microvilli of HeLa cells [13]. As in the other members of FERM family, there are two potential PIP<sub>2</sub>-binding motifs within the FERM domain of PTPL1; these are probably responsible for the intracellular targeting of PTPL1 [13].

**PTPH1.** PTPH1 (protein tyrosine phosphatase Hela1, UniProtKB/Swiss-Prot: P26045) was isolated as a putative intracellular PTP from a HeLa cell cDNA library. Its

open reading frame encodes a protein of 913 amino acid residues with  $M_r = 104030$  Da [143]. Along with N- and C-terminal domains, it contains a central segment bearing putative phosphorylation sites for protein-serine/threonine kinases (Fig. 3). PTPH1 may act as a linker between the plasma membrane and the cytoskeleton, *e.g.* in focal adhesions - specialized regions of the plasma membrane through which cells adhere to the external substrate, anchoring actin stress fibers [17]. PTPH1 was reported to regulate cell cycle progression by dephosphorylating valosin-containing protein [144] and is capable of interacting with and dephosphorylating the T-cell receptor  $\zeta$  subunit [123]. Through binding of the PDZ domain of PTPH1 to the C-terminal part of TACE, PTPH1 can also interact with tumor necrosis factor alpha-convertase (TACE), a metalloprotease involved in the ectodomain shedding of several proteins and critical for proper murine development. This interaction leads to the inhibition of TACE. This is the first evidence of the regulation of TACE by a cytoplasmic protein [145].

**PTPMEG.** PTPMEG (protein tyrosine phosphatase, megakaryocyte) was isolated from the human MEG-01 cell line. It has a predicted  $M_r = 105780$  Da with 926 amino acid residues (gi:4506295 for human protein tyrosine phosphatase, non-receptor type 4, UniProtKB/TrEMBL:Q9WU22 for mouse gene Ptpn4 product). It has two functional domains: a protein 4.1 homology domain and a tyrosine-phosphatase domain, separated by 292 amino acid residues unrelated to any previously sequenced protein [45]. Within the intermediate domain, multiple sites of serine and threonine phosphorylation were found (between amino acids 386 and 503). This region also contains two PEST sequences (residues 436-461 and 490-503) and two proline-rich motifs (residues 389-397 and 462-468) that may confer the ability to bind to Src homology 3 domains. In the C-terminal part of this domain, a DHF motif was found (residues 518-599) (Fig. 3) [43]. The function of this fragment remains unknown; it could presumably play a role in the interaction with some other molecules or control the activity of the phosphatase domain [43]. In the COS-7 cell lines, PTPMEG can inhibit cell proliferation, reduce saturation density, and block the ability of these cells to grow without adhering to a solid matrix [44]. PTPMEG associates directly with the mouse GluRdelta2 (glutamate receptor delta2 subunit, selectively expressed in cerebellar Purkinje cells) and plays a role in the downstream signaling of the GluRs and/or in the regulation of their activities through tyrosine dephosphorylation [55]. The homology between the N-terminal domain of PTPMEG and protein 4.1 suggests that this protein can attach to a glycoporphin-like transmembrane molecule and could have a cytoskeletal association with an additional PTPase activity that may influence the cytoskeletal rearrangement,

organization and regulation of cytoskeletal events [43, 44]. This family may become bigger: there are three cDNA clones, NBL1, NBL2 and NBL3, with high sequence homology to PTPH1/PTPMEG [128].

### The NBL4 family

The last two clones (NBL4 and NBL5) mentioned by Takeuchi [128] form the NBL4 protein family. These proteins are not very closely related to any of the known members of the protein 4.1 superfamily. There are several isoforms of NBL4: human (hNBL4, UniProtKB/Swiss-Prot: Q9HCS5) with 598 residues and  $M_r = 69375$  Da, murine (mNBL4, UniProtKB/Swiss-Prot: P52963) [120] with 554 residues and  $M_r = 64082$  Da, and zebrafish NBL4 (UniProtKB/Swiss-Prot: O57457) [67] with 619 residues and  $M_r = 70709$  Da. There is no clear data on the appearance or role of NBL5. A comparison of the predicted amino acid sequences of these proteins indicated a high degree of similarity of the overall structures (87% and 71%, respectively) [63]. The deduced overall structures of hNBL4 and mNBL4 indicate the presence of two FERM subdomains (amino acid residues 70-102 and 181-210) separated by a small tyrosine kinase phosphorylation domain [63] (Fig. 3). The examination of various types of human cancer showed that NBL4 is an important component of the  $\beta$ -catenin/Tcf signaling pathway, and therefore is probably involved in establishing the cell polarity or proliferation [63]. NBL4 may be also involved in adhesion, in cell motility and/or in cell-to-cell communication [63].

### New members of protein 4.1 superfamily

Despite the variety and diversity of the protein 4.1 isoforms discovered in the eighties and nineties, new members of protein 4.1 superfamily are still being recognized. One of the examples could be the *urp1* gene, found during the identification of biomarkers of human lung and colon carcinomas [138]. URP1 reveals a strong sequence homology to the previously characterized *C. elegans* gene, UNC-112 [105]. URP1 is also closely related to other novel genes, URP2 and the previously discovered human mitogen-inducible (MIG-2) gene. The genomic structure for all three genes is nearly identical, all being encoded by 15 exons, although the URP1 gene localized to chromosome 20p13, URP2 to 11q12, and MIG-2 to 14q22. This conserved exon structure suggests that all three members probably arose by gene duplication from one ancestral gene. The predicted URP1 polypeptide is 677 residues long an expected molecular mass of 77408 Da (GenBank accession number: AF443278). URP1, URP2, MIG-2, and two genes from *Drosophila* (CG7729 and CG14991) share a unique domain structure consisting of two FERM domains that surround a PH domain and appear to form a novel membrane-asso-

ciated FERM and PH domain-containing protein family (Fig. 3). The presence of multiple FERM domains characteristic to many plasma membrane cytoskeleton linkers and a PH domain typical of membrane-anchored proteins involved in signal transduction suggests an important role for URP1 in tumorigenesis [138].

Another member is a new FERM domain-containing protein called willin [47], the result of the yeast two-hybrid screening of the transmembrane L1 family member neurofascin that identified a novel FERM-containing cDNA. This cDNA clone from human uterine leiomyosarcoma tissue was sequenced and found to contain 614 amino acid residues (about 71 kDa), with the FERM domain within its N-terminus (residues 14-322) and a localization and length similar to the ERM proteins [47] (Fig. 3). Immunohistochemical studies indicated that willin can have different intracellular distribution. In the oral mucosa squamous cell carcinoma it is localized predominantly in the cytoplasm, but was also found along the plasma membrane. In the plasma membrane, willin can co-localize with actin, which is commonly observed for the protein 4.1 superfamily members. The chimeric GST-willin showed the same phospholipid-binding profile as purified GST-moesin [47].

Apart from these, there are many other proteins containing the FERM domain that could in the future be classified as new members of the protein 4.1 or FERM family. One of these could be a focal adhesion kinase (FAK), which is a non-receptor tyrosine kinase that localizes to focal adhesions in adherent cells [19]. The N-terminal region of FAK contains a fragment that shares the sequence homology with band 4.1 and ERM proteins, a FERM domain. Three subdomains (F1, F2, and F3) form a tertiary fold, similar to those of known FERM structures despite the low sequence conservation (only 12-15% sequence identity with ERM family members). The difference concerns the sequence and relative orientation of the F3 subdomain that alters the nature of the interdomain interface. A putative protein interaction site on the F3 lobe is masked by the proximal region of the linker. The phosphoinositide-binding site found in ERM family proteins is absent in the FERM domain of FAK. The adjacent Src SH3 and SH2 binding sites in the linker of FAK can associate with the surfaces of the F3 and F1 lobes. These structural features suggest the possibility that interactions of the FAK FERM domain can be regulated by binding of Src kinases to the linker segment [19]. Deletion analyses suggest that the FERM domain of FAK can inhibit enzymatic activity and repress FAK signaling [29]. The purified FERM domain of FAK can interact with full-length FAK *in vitro*, and mutation of this sequence disrupts the interaction.

In an inactive state of FAK, the FERM domain (especially the F2 subdomain) is believed to interact with the catalytic domain of FAK to repress its activity. This

interaction may be disrupted upon activation, and the enzyme can expose its kinase activity [29].

### Protein 4.1 evolution

The variety of protein 4.1 superfamily members suggests the involvement of these proteins in many cellular events. The comparative analyses of the amino acid sequences of the N-terminal domains of these proteins allowed the estimation of the genetic distances and possible evolutionary relationships between the FERM family members [128]. Based upon this data, the presumable phylogenetic tree was constructed using the unweighed pair-group method [33]. Using this interpretation, talin and the primordial form of the other members of the protein 4.1 family were generated first. The subsequent differentiation of the primordial form of the FERM family members led to band 4.1 "prefamily" and ERM "prefamily" proteins, and then the band 4.1 "prefamily" differentiated into the band 4.1, NBL4/5 and PTPH1 families. The ERM "prefamily" differentiated into the ERM family, merlin and the NBL6/7 family, respectively [128].

Recently, more precise studies concerning the evolution of merlin in comparison to the other ERM family proteins were based on phylogenetic analysis of 50 proteins of the ERM family which were identified from 30 different taxa [42]. Using the neighbor-joining method [68] combined with the molecular evolutionary genetics analysis program MEGA2 [69], the phylogenetic tree of the ERM family of proteins was constructed; the entire ERM family could be subdivided into the ERM branch and the merlin branch. While both branches have a strongly supported monophyletic origin, the merlin branch can be separated from the ERM branch and divided into 3 groups according to the order of derivation: worms, insects and Chordata, with the earliest separated genus, *Ciona*, in the last taxonomic unit [42]. The lack of a merlin-like sequence in many species of Platyhelminthes and the absence of merlin-like sequences in the complete genomes of protozoa, fungi and plants suggests that the derivation of merlin occurred in the early metazoa after their separation from flatworms. The ERM-like proteins found in parasites can be grouped together and form a branch separate from the rest of the ERM proteins. In vertebrates, the ERM-like proteins evidently expand, which occurred after separation from Urochordata (*Ciona intestinalis*), and led to the appearance of three related groups of proteins: ezrin (the most ancient), radixin and moesin, and the increasing number of ERM members paralleled the evolutionary complexity of the organism. Amino acid sequence alignment reveals the absence of an actin-binding site in the C-terminal region of all merlin proteins from various species but the presence of a conserved internal binding site in the N-terminal domain of the merlin and ERM proteins. A more conserved pattern of seven amino acid residues

(<sup>170</sup>YQMTPEM<sup>177</sup>) was found in the N-terminal domain of *Drosophila* merlin, called the "Blue Box"; these seven are identical between human and *Drosophila* merlins [70], although some amino acid substitutions in this region exist in the merlin sequences of worms, fish, and *Ciona* [42].

Secondary structure prediction reveals the presence of a conserved  $\alpha$ -helical domain in the central to C-terminal region of the merlin proteins of various species. The conserved residues and structures identified correspond to the important sites highlighted by the available crystal structures of the merlin and ERM proteins. Analysis of the merlin gene structures from various organisms shows the increase in gene length during evolution due to the expansion of the introns. However, a reduction in intron number and length appears to occur in the merlin gene of the insect group [42]. The overall similarity among the primary and secondary structures of all the merlin proteins and the conservation of several functionally important residues suggest a universal role for merlin in a wide range of metazoa.

### Concluding remarks

Although a lot has been done to understand the structure and role of protein 4.1 and the other FERM family members, many details still remain unknown. To better understand the protein 4.1 superfamily and its large diversity, it is necessary to explain in which cellular events the proteins from particular protein 4.1 subfamilies are involved, and to examine how these proteins evolved and how their functions diversified. In particular the FERM domain seems to play specific roles in regulation of activity of FERM proteins and determination of their function. Recent data indicate a novel sequence domain, the FERM-adjacent (FA) region detected in a subset of many proteins containing FERM domain [6]. The presence of conservative motifs that are potential substrates for kinases in this region suggests a possible regulatory adaptation in this subset including substantial amount of proteins: true 4.1 proteins, some tyrosine phosphatases, rho-GEF proteins, type II transmembrane proteins and many uncharacterised FERM proteins [6].

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