



# Band 4.1 proteins regulate integrin-dependent cell spreading

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## ABSTRACT

Integrins link the extracellular matrix (ECM) to the cytoskeleton to control cell behaviors including adhesion, spreading and migration. Band 4.1 proteins contain 4.1, ezrin, radixin, moesin (FERM) domains that likely mediate signaling events and cytoskeletal reorganization via integrins. However, the mechanisms by which Band 4.1 proteins and integrins are functionally interconnected remain enigmatic. Here we have investigated roles for Band 4.1 proteins in integrin-mediated cell spreading using primary astrocytes as a model system. We demonstrate that Proteins 4.1B and 4.1G show dynamic patterns of sub-cellular localization in astrocytes spreading on fibronectin. During early stages of cell spreading Proteins 4.1B and 4.1G are enriched in ECM adhesion sites but become more diffusely localized at later stages of spreading. Combinatorial inactivation of Protein 4.1B and 4.1G expression leads to impaired astrocyte spreading. Furthermore, in exogenous expression systems we show that the isolated Protein 4.1 FERM domain significantly enhances integrin-mediated cell spreading. Protein 4.1B is dispensable for reactive astrogliosis in experimental models of cortical injury, likely due to functional compensation by related Protein 4.1 family members. Collectively, these findings reveal that Band 4.1 proteins are important intracellular components for integrin-mediated cell spreading.

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## 1. Introduction

The Protein 4.1 superfamily is comprised of a diverse group of proteins that share common FERM domains [1]. Members of this superfamily include unconventional myosins [2], talins [3], kindlins [4], focal adhesion kinase [5] and the proto-typical Band 4.1 proteins [6]. The five members of the Band 4.1 family show overlapping expression in various tissues where they interact with cytoplasmic signaling effectors and link transmembrane proteins to the actin cytoskeleton [7–10].

Various studies have revealed important roles for Protein 4.1B in tumor cell growth and migration. For example, Protein 4.1B gene expression is down regulated in cancers of the brain [11,12] and lung [13]. Protein 4.1B has also been identified as a suppressor of metastasis in mouse models of sarcoma and prostate cancer [14,15]. However, mice genetically null for Protein 4.1B do not develop spontaneous malignancies, suggesting that loss of Protein 4.1B in combination with other tumor suppressors and/or activating mutations in oncogenes drive tumorigenesis. Alternatively, other related Band 4.1 family members may compensate for loss of Protein 4.1B expression in knockout mice

[16]. Indeed, in various Band 4.1 knockout mouse models functional compensation by different family members has been suggested [17,18].

Protein 4.1B contains an N-terminal FERM domain that likely interacts with cell surface receptors including integrins. Here, we demonstrate that Protein 4.1B and  $\beta 1$  integrins associate in ECM adhesion sites in primary astrocytes spreading on fibronectin, with the Protein 4.1B FERM domain promoting  $\beta 1$  integrin-mediated cell spreading. Collectively, these data reveal important functional links between Protein 4.1B and  $\beta 1$  integrins in regulating astrocyte spreading.

## 2. Materials and methods

### 2.1. Experimental mice and primary cell culture systems

4.1B<sup>+/−</sup> mice were interbred to generate wild type and 4.1B<sup>−/−</sup> littermates as previously described [16]. Genotypes were determined using PCR-based methods as described previously [19]. Primary wild type and 4.1B<sup>−/−</sup> astrocytes were isolated from P1–P3 as described previously [20,21]. Wild type and 4.1B<sup>−/−</sup> cells were transduced with retroviruses expressing E6/E7 oncogenes, and cells were selected in growth media containing 1  $\mu$ g/ml puromycin for five days as described previously [20]. SMART Pool siRNAs targeting murine Band 4.1G gene sequences were purchased from Dharmacon, Inc.

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## 2.2. Immunoblotting, immunofluorescence and immunoprecipitation

Generation of the anti-Protein 4.1B rabbit polyclonal antibody has been described previously [22]. The following antibodies are from commercial sources: anti-4.1G and anti-4.1N (Protein Express); anti-actin and anti-talin (Sigma), anti- $\alpha$ -actinin (Abcam), anti-paxillin (BD Biosciences), anti-myc (Invitrogen); anti- $\beta$ 1 and anti- $\alpha$ 8 (Santa Cruz Biotech); anti- $\beta$ 1, anti- $\alpha$ 2, anti- $\alpha$ 3, anti- $\alpha$ 4, anti- $\alpha$ 6, anti- $\beta$ 1, and anti-GFAP (Millipore); anti- $\alpha$ 2 and anti- $\alpha$ 5 mAbs (Emfret Analytics). The anti- $\alpha$ v and anti- $\beta$ 8 integrin antibodies have been described elsewhere [23,24]. Secondary fluorescent antibodies are from Molecular Probes.

Cells on ECM-coated glass cover slips were fixed with 4% PFA/PBS and then permeabilized with PBS containing 0.5% NP40. Samples were blocked with PBS containing 10% goat serum, incubated with appropriate primary and secondary antibodies. E11.5 wild embryos were dissected, embedded in Tissue-Tek OCT (Sakura Finetek) and coronal sections were prepared. To analyze interactions between myc-tagged Protein 4.1B and  $\beta$ 1 integrin, transfected cells were plated on fibronectin for 30 min. Detergent-soluble lysates were immunoprecipitated using anti-myc mAbs (Invitrogen) and immunoblotted with anti- $\beta$ 1 integrin pAbs (Millipore).

## 2.3. Biotinylation and immunoprecipitation

Primary astrocytes growing on laminin were labeled with 0.1 mg/ml Sulfo-NHS-biotin (Thermo Scientific) for 30 min at 37 °C. After briefly washing cells with PBS and TBS, cells were lysed in NP40 buffer. Lysates were pre-cleared with goat anti-rabbit-agarose (Sigma), rabbit anti-goat IgG-agarose (Research Diagnostics), or goat anti-rat IgG-agarose (Sigma) prior to immunoprecipitation with anti-integrin antibodies.

## 2.4. Cell adhesion assays

Glass coverslips were coated with 3  $\mu$ g/ml fibronectin (Sigma) for 16 h at 4 °C. Primary astrocytes were allowed to adhere and spread for varying times before fixation and permeabilization. Adherent cells were stained with crystal violet, and absorbance at 560 nm was detected. COS7 cells were transfected with pcDNA3.1 vectors expressing 4.1B FERM-myc, full-length 4.1B (FL)-myc, and lacZ-myc fusion proteins using Effectene transfection reagents (Qiagen). Cells were fixed with 4% PFA/PBS, permeabilized with PBS containing 0.5% NP40 and immunofluorescently labeled with anti-myc antibodies. Areas encompassed by myc-expressing cells were quantified using ImageJ software (NIH).

## 2.5. In vivo brain injury models

To induce cortical injury in control and 4.1B $^{-/-}$  littermates a single incision was made from the anterior pole of the skull to the posterior ridge. A sterile scalpel blade was lowered into the brain 3 mm below the pial surface and a 3–5 mm wound was generated posterior to the initial stereotactic point of entry (1.5 mm rostral, 1.5 mm anterior). Three or seven days after the surgery control and 4.1B $^{-/-}$  groups ( $n = 3$  mice per genotype and time point) were sacrificed and brain sections were analyzed.

## 2.6. Statistical analyses

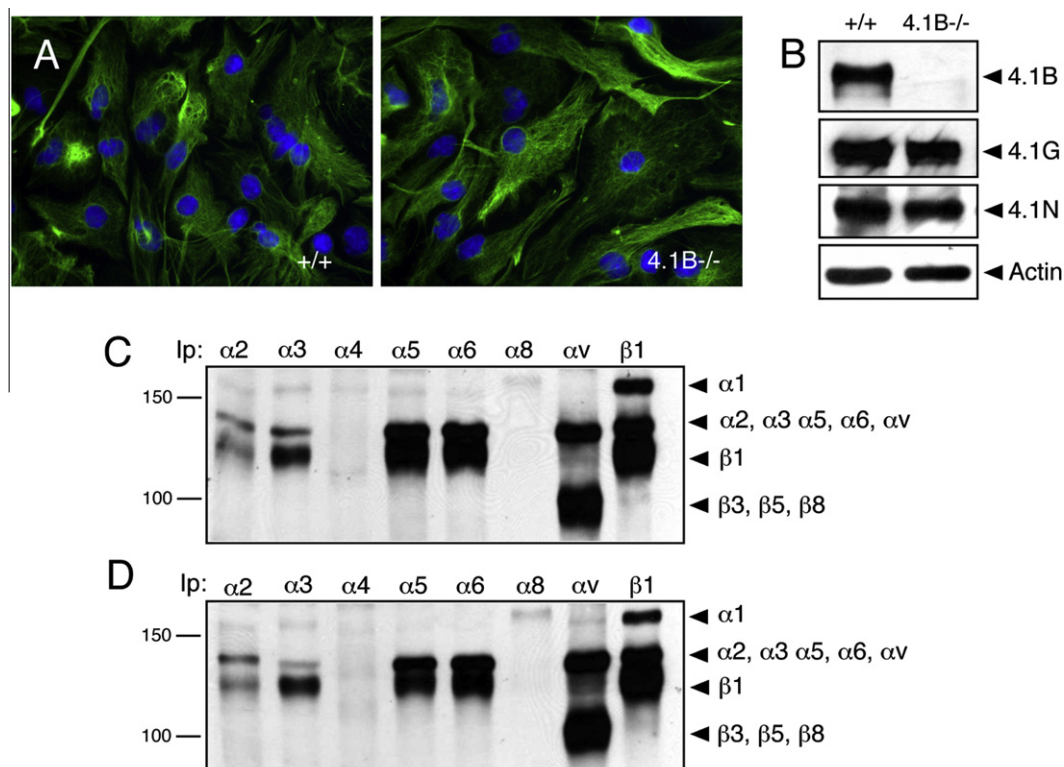
All data were analyzed using student's *t*-test to determine statistically significant differences.

## 3. Results

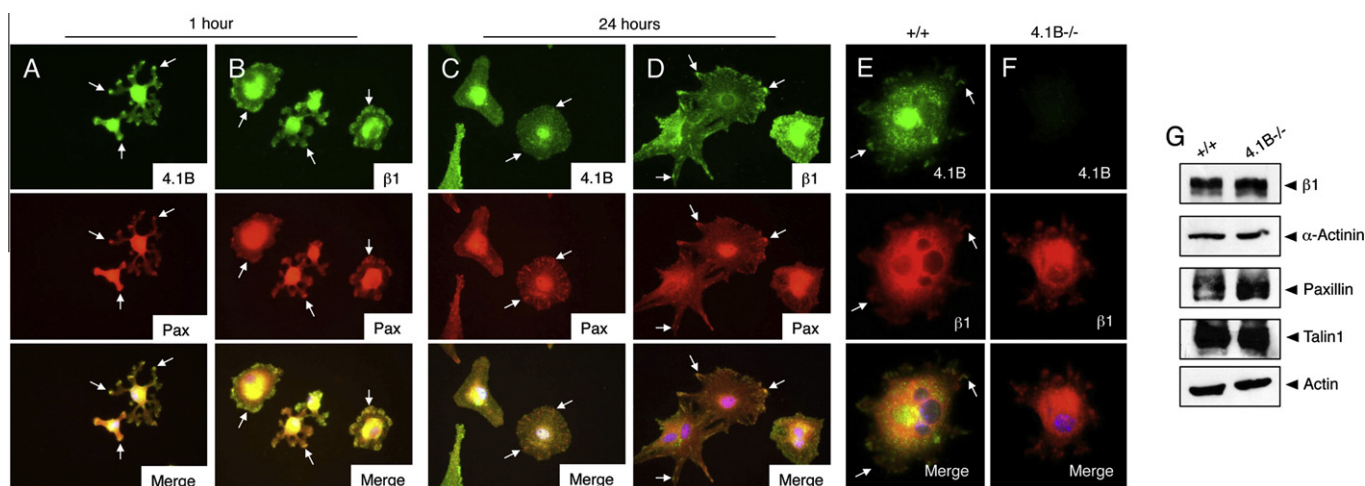
To characterize functions for Protein 4.1B and other related Band 4.1 family members in cell adhesion and spreading [25], we cultured primary brain astrocytes from wild type (control) and 4.1B $^{-/-}$  (mutant) newborn mice. Astrocytes from control and 4.1B $^{-/-}$  mice expressed robust levels of the intermediate filament proteins glial fibrillary acidic protein and nestin, and did not display obvious morphological differences (Fig. 1A). Detergent-soluble lysates from control and 4.1B $^{-/-}$  astrocytes were immunoblotted with anti-4.1B, anti-4.1G, anti-4.1N, and anti-4.1R rabbit polyclonal antibodies. As shown in Fig. 1B, in wild type cells the anti-4.1B antibody was immunoreactive with a band of approximately 145 kDa. Robust levels of protein 4.1G and 4.1N bands were also detected in wild type and 4.1B $^{-/-}$  astrocytes with approximate molecular weights of 150 kDa and 100 kDa, respectively (Fig. 1B). No significant differences in 4.1G and 4.1N protein levels were observed between wild type and 4.1B $^{-/-}$  cells. Similar levels of Proteins 4.1B, 4.1G and 4.1N were detected in lysates prepared from wild type and 4.1B $^{-/-}$  cerebral cortices (Supplementary Fig. 1). Band 4.1 proteins contain N-terminal FERM domains that are reported to interact with cell surface receptors [6], and a recent report has shown interactions between the Protein 4.1R FERM domain and  $\beta$ 1 integrin [26]. We analyzed surface expression of various integrins in wild type and 4.1B $^{-/-}$  astrocytes by labeling cells with amine-reactive biotin. As shown in Fig. 1C, in wild type astrocytes we detected robust levels of  $\alpha$ 1 $\beta$ 1,  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1,  $\alpha$ 5 $\beta$ 1,  $\alpha$ 6 $\beta$ 1, and  $\alpha$ v-containing integrins. In addition, 4.1B $^{-/-}$  astrocytes displayed comparable levels of integrin cell surface expression (Fig. 1D).

Sub-cellular localization of Protein 4.1B and  $\beta$ 1 integrin was next analyzed in astrocytes spreading on fibronectin. Cells adhering for 1 or 24 h were immunolabeled with anti-4.1B, anti- $\beta$ 1, or anti-paxillin antibodies. At 1 h post-adhesion both Protein 4.1B (Fig. 2A) and  $\beta$ 1 integrin protein (Fig. 2B) were detected in ECM contact sites as determined by co-localization with paxillin. However, after 24 h when cells were well spread, Protein 4.1B was more diffusely localized (Fig. 2C), whereas  $\beta$ 1 integrin showed continued enrichment in paxillin-positive adhesion sites (Fig. 2D). We next analyzed Protein 4.1B-dependent cell adhesion and spreading on fibronectin. Wild type and 4.1B $^{-/-}$  astrocytes plated on fibronectin were labeled with anti-4.1B polyclonal antibodies and anti- $\beta$ 1 integrin mAbs. Protein 4.1B and  $\beta$ 1 integrin co-localized in spreading cells (Fig. 2E), although 4.1B $^{-/-}$  astrocytes did not display altered  $\beta$ 1 integrin protein enrichment in cell-ECM contact sites (Fig. 2F). Furthermore, Protein 4.1B-dependent differences in expression of common focal contact proteins such as talin, paxillin,  $\alpha$ -actinin and  $\beta$ 1 integrin were not detected (Fig. 2G). To quantify 4.1B-dependent adhesion on fibronectin, wild type and 4.1B $^{-/-}$  astrocytes were plated for 60 min and cell adhesion was measured by crystal violet staining. Morphologies of adherent wild type and 4.1B $^{-/-}$  astrocytes on fibronectin revealed similar actin cytoskeleton architecture as revealed by phalloidin staining (Supplementary Fig. 2).

Given that Protein 4.1G and 4.1N are expressed in wild type and 4.1B $^{-/-}$  astrocytes (Fig. 1), we next analyze spatial patterns of expression of these proteins in spreading astrocytes. Wild type and 4.1B $^{-/-}$  astrocytes were allowed to adhere to fibronectin for varying times, and cells were immunostained with anti-4.1B, anti-4.1G, and anti-4.1N antibodies in combination with anti-paxillin to visualize ECM adhesion sites. Protein 4.1B and 4.1G, but not Protein 4.1N, were expressed in cell-ECM contact sites (Fig. 3A–C). Like wild type cells, 4.1B $^{-/-}$  astrocytes were adherent to fibronectin and contained paxillin-positive ECM adhesion sites, although Protein 4.1B protein did not co-localize with paxillin (Fig. 3A)



**Fig. 1.** Multiple Band 4.1 and integrin proteins are expressed in primary brain astrocytes. (A) Astrocytes cultured from wild type (left panel) or 4.1B<sup>-/-</sup> (right panel) neonatal brains were immunolabeled with anti-GFAP antibodies. Note that control and 4.1B mutant cells display similar cytoarchitecture. (B) Lysates from wild type and 4.1B<sup>-/-</sup> astrocytes were immunoblotted with anti-4.1B, anti-4.1G, and anti-4.1N rabbit polyclonal antibodies. (C and D) Primary astrocytes from wild type (C) and 4.1B<sup>-/-</sup> mice (D) were labeled with amine-reactive biotin and detergent-soluble lysates were immunoprecipitated with various anti-integrin antibodies.



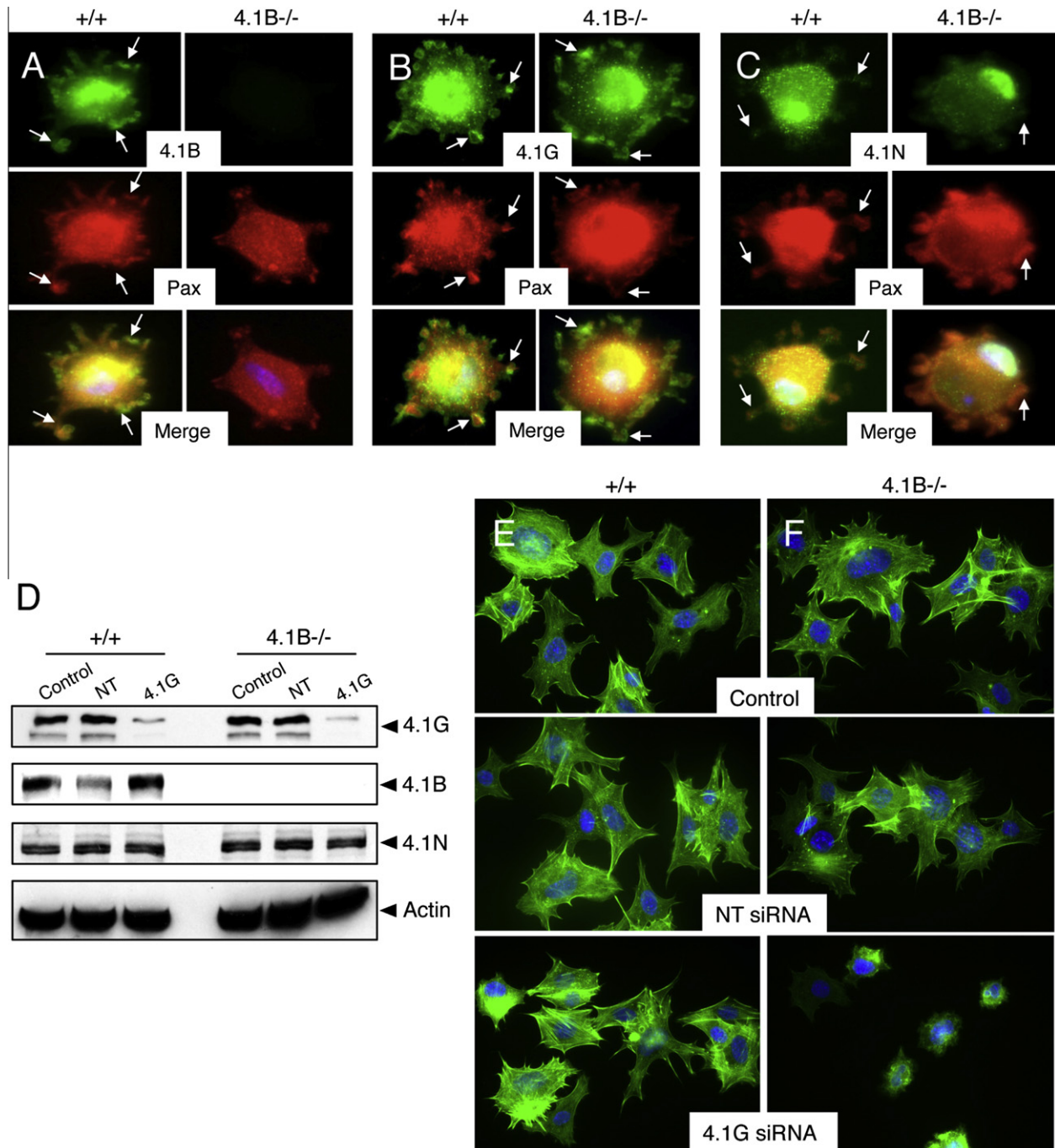
**Fig. 2.** Protein 4.1B displays dynamic sub-cellular localization in spreading astrocytes. (A–D) Wild type astrocytes were plated on fibronectin-coated dishes for 60 min (A and B) or 24 h (C and D). Spreading cells were then labeled with anti-4.1B (A and C) or anti-β1 integrin (B and D) rabbit polyclonal antibodies in combination with anti-paxillin mAbs. (E and F) Wild type (E) and 4.1B<sup>-/-</sup> (F) astrocytes were plated on fibronectin for 1 h and then immunolabeled with anti-4.1B polyclonal antibodies and anti-β1 integrin mAbs. Protein 4.1B and β1 integrin co-localize at 1 h post-adhesion on fibronectin (arrows in E). Images are shown at 400 $\times$ . (G) No differences in expression of common focal adhesion proteins in wild type and 4.1B<sup>-/-</sup> astrocytes.

owing to gene deletion. Obvious differences in localization of Proteins 4.1G and 4.1N were not detected in 4.1B<sup>-/-</sup> cells (Fig. 3B and C). Analysis of Protein 4.1B, 4.1G, and 4.1N sub-cellular localization in wild type and 4.1B<sup>-/-</sup> primary astrocytes at 24 h post-adhesion to fibronectin showed diffuse, cytoplasmic expression (Supplementary Fig. 3). In confluent cultures of adherent astrocytes Proteins 4.1B and 4.1G were enriched at sites of direct cell–cell contact, although Protein 4.1N remained diffusely expressed in

confluent astrocyte cultures (Supplementary Fig. 4). In scratch-wound migration assays we did not detect Band 4.1 proteins enriched at the leading edge of migrating astrocytes (Supplementary Fig. 5A).

We hypothesized that 4.1G may have overlapping functions or functionally compensate in the absence of 4.1B. Therefore, we used siRNAs to silence murine Band 4.1G gene expression in wild type and 4.1B<sup>-/-</sup> cells. Cultured primary astrocytes display limited





**Fig. 3.** Proteins 4.1B and 4.1G, but not 4.1N, are enriched in ECM adhesion sites in spreading astrocytes. (A–C) Wild type and 4.1B<sup>-/-</sup> astrocytes were plated on fibronectin for 60 min and cells were labeled with anti-4.1B (A), anti-4.1G (B), or anti-4.1N (C) rabbit polyclonal antibodies in combination with anti-paxillin mAbs. Note that Proteins 4.1B and 4.1G co-localize with paxillin in cell-ECM adhesion sites (arrows). Images are shown at 400 $\times$ . (D) Wild type and 4.1B<sup>-/-</sup> astrocytes were mock transfected or transfected with non-targeting (NT) siRNAs or siRNAs targeting Protein 4.1G. Cell lysates were immunoblotted with anti-Band 4.1G, 4.1B or 4.1N antibodies. (E and F) Wild type and 4.1B<sup>-/-</sup> astrocytes that were mock transfected or transfected with non-targeting siRNAs or siRNAs targeting 4.1G were plated on fibronectin for 60 min and cells were stained with phalloidin to visualize the actin cytoskeleton. Images are shown at 400 $\times$ .

growth properties and are not amenable for siRNA transfection (data not shown); therefore, astrocytes were immortalized with retroviruses expressing E6/E7 oncogenes as we have described previously [20]. Immortalized astrocytes displayed high transfection efficiency as determined using siRNAs targeting murine cyclophilin (>90% reduction in protein expression, data not shown). As shown in Fig. 3D, we detected a significant reduction in Protein 4.1G levels in wild type and 4.1B<sup>-/-</sup> cells following siRNA transfection. Protein 4.1B and 4.1N levels were not altered following 4.1G gene

silencing (Fig. 3D), and we did not detect changes in the cell surface expression of  $\beta 1$  integrins in control and 4.1B<sup>-/-</sup> cells (data not shown). Wild type or 4.1B<sup>-/-</sup> astrocytes that were mock transfected or transfected with non-targeting siRNAs did not display differences in spreading on fibronectin. In addition, spreading differences were not detected in wild type astrocytes transfected with siRNAs targeting 4.1G (Fig. 3E), likely due to expression of Protein 4.1B. However, upon silencing 4.1G gene expression in 4.1B<sup>-/-</sup> astrocytes obvious defects in cell spreading and actin

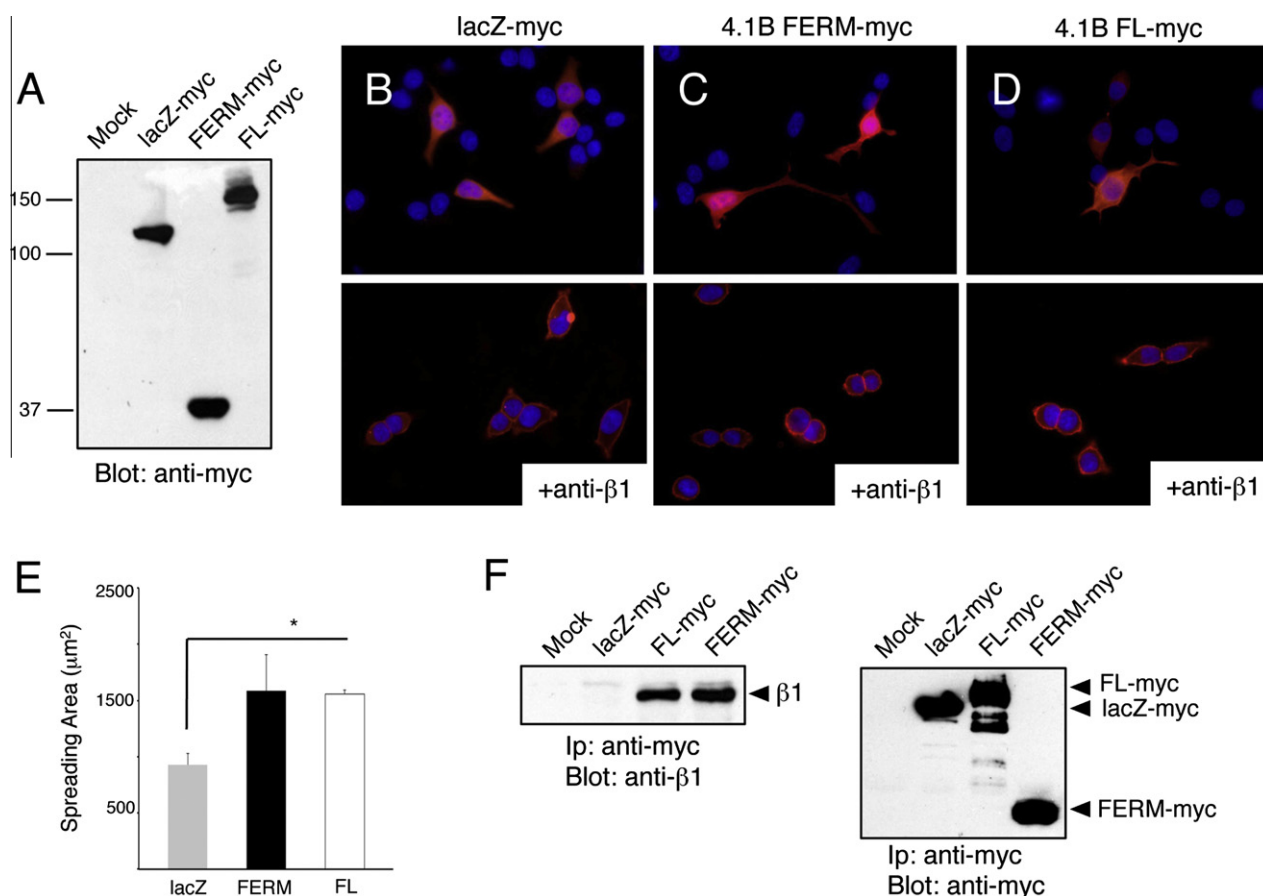
cytoskeleton organization were apparent (Fig. 3F). Collectively, these data reveal that Protein 4.1B and 4.1G both regulate astrocyte spreading on fibronectin, and their combined inhibition leads to spreading defects.

We next used exogenous expression approaches to analyze 4.1B domains involved in cell spreading. COS7 cells, which express low levels of endogenous Protein 4.1B and 4.1G, were transfected with pcDNA3.1 plasmids expressing lacZ-myc, myc-tagged full length Protein 4.1B, or the myc-tagged Protein 4.1B FERM domain (Fig. 4A). After 48 h cells were plated on fibronectin for 1 h and then stained with anti-myc mAbs. Unlike COS7 cells transfected with lacZ-myc (Fig. 4B, upper panel), cells expressing 4.1B FERM-myc and full-length 4.1B-myc displayed increased spreading on fibronectin (Fig. 4C and D upper panels). ImageJ software was used to quantify myc-positive cell surface areas, revealing increased spreading in cells expressing Protein 4.1B or the isolated FERM domain (Fig. 4E). Cells transfected with the 4.1B FERM-myc, full-length 4.1B-myc, and lacZ-myc constructs were also treated with anti- $\beta$ 1 integrin neutralizing antibodies prior to plating on fibronectin. Protein 4.1B-mediated enhancement of cell spreading was inhibited by anti- $\beta$ 1 integrin blocking antibodies (Fig. 4B–D, lower panels). We next analyzed associations between Protein 4.1B and  $\beta$ 1 integrin in COS7 cells spreading on fibronectin by co-immunoprecipitation strategies. As shown in Fig. 4F, in COS7 cells we

detect interactions between endogenous  $\beta$ 1 integrin and transfected myc-tagged full-length Protein 4.1B and the isolated FERM domain. We performed similar experiments with HEK-293 cells, which express low levels of endogenous Protein 4.1B, and found similar 4.1B FERM domain-dependent increases in spreading on fibronectin (data not shown).

Next, co-localization of Protein 4.1B and  $\beta$ 1 integrin was analyzed by labeling brain sections with anti-Protein 4.1B rabbit polyclonal antibodies and anti- $\beta$ 1 integrin mAbs. Within the developing cerebral cortex Protein 4.1B and  $\beta$ 1 integrin co-localized in neuroepithelial cells (Supplementary Fig. 6), which give rise to most astrocytes and neurons in the post-natal brain [27]. In addition, robust expression of  $\beta$ 1 integrin protein, but not Protein 4.1B, was detected in cerebral blood vessels (arrows in Supplementary Fig. 6).

To analyze functions for Protein 4.1B in astrocytes in vivo we induced cortical stab-wounds [28]. Upon brain injury astrocytes develop ‘reactive’ morphologies and upregulate expression of the intermediate filament proteins nestin and GFAP. Over several days reactive astrocytes polarize and invade through the brain microenvironment to form a ‘glial scar’ surrounding the wound [29,30]. We induced stab wounds in the cerebral cortices of P60 wild type and 4.1B $^{-/-}$  mice ( $n=6$  mice per genotype). Three and 7 days after injury mice ( $n=3$  per genotype per time point)



**Fig. 4.** The Protein 4.1B FERM domain enhances cell spreading and associates with  $\beta$ 1 integrin. (A) Detergent-soluble lysates from mock-transfected COS7 cells or cells overexpressing lacZ-myc, 4.1B FERM-myc, or full-length 4.1B-myc fusion proteins were immunoblotted with anti-myc antibodies. Note the similar levels of myc fusion protein expression. (B–D) COS7 cells were transiently transfected with plasmids expressing lacZ-myc (B), 4.1B FERM-myc (C), or full-length 4.1B-myc (D) fusion proteins. After 48 h cells were plated on fibronectin for 30 min and then immunolabeled with an anti-myc antibodies. Alternatively, cells were pre-incubated with anti- $\beta$ 1 integrin blocking mAbs (B–D, lower panels) prior to adhesion to fibronectin. Note that  $\beta$ 1 integrin inhibition blocks 4.1B-mediated cell spreading. Images are shown at 400 $\times$ . (E) Surface areas of transfected COS7 cells, as visualized by anti-myc immunofluorescence, were quantified using ImageJ software, revealing that cells expressing 4.1B FERM-myc or full length 4.1B-myc proteins showed statistically significant increases in spreading in comparison to lacZ-myc controls,  $*p < 0.01$ . (F) COS7 cells overexpressing lacZ-myc, 4.1B FERM-myc, or full-length 4.1B-myc proteins were plated on fibronectin for 30 min. Lysates were immunoprecipitated with anti-myc antibodies and immunoblotted with anti- $\beta$ 1 integrin (left panel) or anti-myc polyclonal antibodies (right panel).

were sacrificed and brain sections were labeled with anti-GFAP antibodies. As shown in [Supplementary Fig. 7A and B](#), in the non-injured cerebral cortex we detected GFAP expression in perivascular astrocytes of wild type and 4.1B<sup>-/-</sup> mice. Following cortical injury astrocytes displayed reactive morphologies and expressed elevated levels of GFAP; however, in wild type and 4.1B<sup>-/-</sup> mice no apparent differences in numbers of reactive GFAP-expressing astrocytes were detected at three days post-injury ([Supplementary Fig. 7C and D](#)). In both control and 4.1B<sup>-/-</sup> mice astrocytes formed a well-defined glial scar around the wound region. Similar patterns of GFAP-expressing astrocytes were detected at 14 days post-injury in wild type and 4.1B<sup>-/-</sup> brains ([Supplementary Fig. 8](#)). Consistent with the lack of Protein 4.1B-dependent astrocyte migration defects in vivo, 4.1B<sup>-/-</sup> astrocytes also showed apparently normal migration behaviors in scratch-wound assays ([Supplementary Fig. 5B and C](#)). These data reveal that Protein 4.1B is dispensable for reactive astrogliosis following experimental-induced brain injury.

#### 4. Discussion

Protein 4.1B localization is highly dynamic in spreading cells, showing enrichment in cell-ECM adhesions during early stages of spreading and displaying more diffuse localization patterns during later stages of spreading. Similar to Protein 4.1B,  $\beta$ 1 integrin localizes to paxillin-containing ECM adhesions during initial stages of spreading; however, unlike Protein 4.1B,  $\beta$ 1 integrin protein remains in focal adhesions beyond early stages of spreading. These data suggest that Proteins 4.1B and 4.1G may regulate focal adhesion assembly. Alternatively, Protein 4.1B may link  $\beta$ 1 integrins to the actin cytoskeleton or serve as a binding intermediate between  $\beta$ 1 integrin and other protein components of focal adhesions. Indeed, Protein 4.1R has been shown to interact directly with the intracellular signaling effectors  $\beta$ -catenin [31] and IQGAP1 [32] to promote cell adhesion and spreading.

We propose functions for Protein 4.1 family members that are partially distinct from other FERM domain-containing proteins such as talin, which activates integrins [33], but is dispensable for focal adhesion assembly [34]. It is possible that Protein 4.1B and related members are important for promoting focal adhesion formation prior to talin stabilizing these sub-cellular structures. Indeed, while this manuscript was in preparation An and colleagues reported associations between the Protein 4.1R FERM domain and  $\beta$ 1 integrins [26]. Protein 4.1R was shown to modulate  $\beta$ 1 integrin protein levels in primary mouse keratinocytes, with 4.1R<sup>-/-</sup> cells showing decreased integrin cell surface expression. In vitro scratch-wound assays and in vivo wound models also revealed essential roles for Protein 4.1R in keratinocyte adhesion and migration. The data we report here in for Protein 4.1B in astrocytes differs in some ways from the Protein 4.1R study. First, integrin surface expression levels are not altered in 4.1B<sup>-/-</sup> astrocytes ([Fig. 1](#)). Second, unlike 4.1R<sup>-/-</sup> keratinocytes, 4.1B<sup>-/-</sup> astrocytes do not display migration defects in scratch-wound assays ([Supplementary Fig. 5](#)) and Protein 4.1B in astrocytes appears to be dispensable for reactive astrogliosis in vivo ([Supplementary Figs. 7 and 8](#)). Therefore, characterization of in vivo functional overlap between Protein 4.1B and related family members, particularly Protein 4.1G, may require combinatorial gene deletion or protein inhibition in astrocytes.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2012.08.129>.

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