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DISCOVERY

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FRZB and melusin, overexpressed in LGMD2A, regulate integrin β1D isoform replacement altering myoblast fusion and the integrin-signalling pathway

OIHANE JAKA[1,2,3]†, LEIRE CASAS-FRAILE[2,3]†, MARGARITA AZPITARTE[2], ANA AIASTUI[2,3], ADOLFO LÓPEZ DE MUNAIN[2,3,4,5], AMETS SÁENZ[2,3]*

[1]*Centre of Human and Aerospace Physiological Sciences, King's College London, London, UK,* [2]*Neurosciences Area, Biodonostia Health Research Institute, San Sebastián, Spain,* [3]*Center for Networked Biomedical Research on Neurodegenerative Diseases (CIBERNED), Carlos III Health Institute, Spanish Ministry of Economy and Competitiveness, Madrid, Spain,* [4]*Department of Neurology, Donostia University Hospital, San Sebastián, Spain, and* [5]*Department of Neurosciences, University of the Basque Country, San Sebastián, Spain*

Limb-girdle muscular dystrophy type 2A (LGMD2A) is characterised by muscle wasting and progressive degeneration of proximal muscles because of mutations in the *CAPN3* gene. However, the underlying pathophysiological mechanisms of muscle degeneration are still not well understood. The objective of this study was to assess the relevance of genes with differential expression in the muscle of LGMD2A patients. For this purpose, we analysed their *in vitro* expression in primary cultures of human myoblasts and myotubes. Abnormal fusion was observed in the myotubes of these patients, which may be explained by the lack of physiological replacement of integrin β1D. Owing to this observation, we focused on deregulated genes coding proteins that directly interact with integrin, *ITGB1BP2* and *CD9*, as well as *FRZB* gene, because of its *in vitro* upregulation in myotubes. Silencing studies established that these genes are closely regulated, *CD9* and *FRZB* being positive regulators of the expression of *ITGB1BP2*, and in turn, this gene being a negative regulator of the expression of *FRZB*. Interestingly, we observed that *FRZB* regulates integrin β1D expression, its silencing increasing integrin β1D expression to levels similar to those in controls. Finally, the administration of LiCl, an enhancer of the Wnt-signalling pathway showed similar experimentally beneficial effects, suggesting *FRZB* silencing or LiCl administration as potential therapeutic targets, though further studies are required.

Introduction

Limb-girdle muscular dystrophy type 2A (LGMD2A) is one of the most common subtypes of this form of muscular dystrophy, accounting for 20–40% of the families in the different populations studied (Refs 1, 2, 3). This condition is caused by mutations in the calpain 3 gene (Ref. 4). The first clinical signs and symptoms of this type of dystrophy tend to occur in the second decade of life and progressively worsen, 80% of patients becoming wheelchair dependent by 20 years of age (Refs 5, 6), although cases with a milder course have been reported (Ref. 7).

As well as the main alteration in expression of the defective gene, abnormalities in the expression of other genes have been observed in the muscle of LGMD2A patients (Ref. 8). Concerning such changes in expression, one of the greatest challenges is to establish which changes are relevant to the process of muscle degeneration in these patients. In this study, we analysed the expression of some of these genes in different cell types namely myoblasts and myotubes in control and LGMD2A patients.

We show that the genes coding for melusin, FRZB and CD9 are concomitantly overexpressed in LGMD2A and that their expression is reciprocally regulated *in vitro*. Our findings suggest that melusin and FRZB are involved in the process of replacement of the β1A isoform of integrin by β1D in the myotubes of LGMD2A patients. Further, the decrease in FRZB expression in myotubes of these patients led to a decrease in melusin expression, as well as an increase in the level of the β1D isoform, reaching that observed in the myotubes of controls. These results indicate that FRZB is not only an inhibitor of the Wnt-signalling pathway as has been described previously, but also that it is involved in the regulation of the integrin pathway, being a potential link between the two pathways that control the synthesis and distribution of sarcolemmal and costamere proteins. Since our results attest to regulation driving expression and phosphorylation levels of various proteins towards adequate levels in LGMD2A

†These authors contributed equally to the work.

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patients, the regulation of FRZB expression could be a potentially useful therapeutic approach.

Material and methods

Muscle biopsy samples

All participants gave informed consent, using forms approved by the Ethics Committee on the Use of Human Subjects in Research at Donostia University Hospital. Muscle biopsy specimens were obtained from adult patients with LGMD2A, in whom the diagnosis had been confirmed genetically by the identification of both mutations in the calpain 3 gene, and from healthy adult controls. These controls were otherwise healthy individuals that underwent surgery for bone fractures and the muscle biopsies were obtained during this surgery. Samples were obtained from proximal limb muscles (biceps, deltoids, triceps and quadriceps). We used muscle samples from two sets of patients. One of the sets was used for obtaining proteins and included five patients and six controls. However, given the small size of the samples, we used a second set of samples from a further four LGMD2A patients and four controls for obtaining myoblasts and myotubes (Table 1).

Human myoblasts obtained from distal muscles (tibialis anterior) were kindly provided by Dr Schneiderat from the Muscle Tissue Culture Collection, Munich (Germany) (Table 1).

Primary human skeletal muscle culture

Human proximal muscle biopsies were minced and cultured in a monolayer according to the method described by Askanas (Ref. 9). To obtain highly purified myoblasts, primary cultures were sorted by immunomagnetic selection based on the presence of the early cell surface marker CD56 (separator and reagents from Miltenyi Biotec). CD56-positive cells were seeded at 2500–3000 cells/cm^2 in culture medium for the myoblast stage. When the myoblasts started to fuse, the medium was replaced by one containing 2% of fetal bovine serum (FBS) and without growth factors to obtain myotubes. Myoblasts obtained from distal muscles were also cultured using the aforementioned method.

C2C12 cell line culture

C2C12 mice muscle cells were used to confirm the results obtained in human primary myoblast/myotubes. The C2C12 mouse cell line (ATCC-CRL-1772) was purchased from ATCC. Cells were seeded at 10 000 cells/cm^2 in proliferating medium containing 10% FBS. Once confluence was achieved, proliferating

TABLE 1.
ANALYSED, MUSCLE AND MYOBLAST SAMPLES; ORIGIN TISSUE IS DETAILED FOR EACH CASE BETWEEN PARENTHESIS

Biopsy number	Status	Gender	Sample (origin tissue)	Age	Ambulation	*CAPN3* mutations	
						Mutation 1	Mutation 2
Proximal muscles – Set 1							
EXP-01	LGMD2A	M	Quadriceps	34	Ambulant	p.(Gly222Arg)	p.(Arg748Gln)
EXP-02	LGMD2A	F	Deltoid	33	Ambulant	c.946–1G>A	p.(Gln660Arg)
EXP-03	LGMD2A	M	Quadriceps	37	Unknown	p.(Met248Arg)	p.(Arg769Gln)
EXP-05	LGMD2A	M	Deltoid	13	Asymptomatic	p.(Arg788SerfsX14)	p.(Arg788SerfsX14)
EXP-35	LGMD2A	M	Deltoid	48	Wheelchair bound	p.(Gln142X)	p.(Gln142X)
EXP-25	Control	F	Deltoid	57	–	–	–
EXP-27	Control	M	Quadriceps	50	–	–	–
EXP-29	Control	F	Quadriceps	73	–	–	–
EXP-33	Control	M	Deltoid	51	–	–	–
EXP-38	Control	M	Quadriceps	31	–	–	–
EXP-39	Control	M	Quadriceps	41	–	–	–
Proximal muscles – Set 2							
09–21	LGMD2A	M	Myoblasts (Biceps)	19	Ambulant	p.(His690ArgfsX9)	p.(His690ArgfsX9)
09–24	LGMD2A	F	Myoblasts (Deltoid)	47	Wheelchair bound	p.(Arg788SerfsX14)	p.(Lys595ValfsX70)
09–25	LGMD2A	M	Myoblasts (Deltoid)	28	Ambulant	p.(Lys254Glu)	p.(Pro637HisfsX25)
10–39	LGMD2A	M	Myoblasts (Deltoid)	29	Wheelchair bound	p.(Lys254del)	p.(X822Leuext62X)
09–23	Control	M	Myoblasts (Triceps)	26	–	–	–
10–36	Control	M	Myoblasts (Biceps)	23	–	–	–
13–05	Control	M	Myoblasts (Quadriceps)	14	–	–	–
13–07	Control	F	Myoblasts (Biceps)	36	–	–	–
Distal muscles							
2009CAL1	LGMD2A	M	Myoblasts (Tibialis anterior)	27	Ambulant	p.(Thr184Argfs*36)	p.(Thr184Argfs*36)
2009CAL3	LGMD2A	F	Myoblasts (Tibialis anterior)	21	Ambulant	p.(Thr184Argfs*36)	p.(Arg490Trp)
2009CAL4	LGMD2A	M	Myoblasts (Tibialis anterior)	12	Ambulant	c.1992+1G>T	p.(Thr679Serfs*20)
08–08	Control	M	Myoblasts (Tibialis anterior)	34	–	–	–

F, female. M, male.

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medium was switched to medium containing 1% of horse serum (Thermo Fisher Scientific).

RNA extraction from myoblast/myotubes and muscle biopsies

RNA extraction from primary myoblast/myotubes was performed with an RNeasy Mini Kit (Qiagen). In the case of the muscle biopsies, which were snap frozen and stored at −80°C until use, total RNA was obtained using an RNA-Plus Kit (QBiogene).

Quantitative real-time PCR

The isolated RNA was reverse-transcribed to first-strand complementary DNA (cDNA) in a final volume of 50 µl using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. To investigate the levels of expression of the differentially expressed genes in these tissues, TaqMan quantitative RT–PCR assays were performed, using the 7900 HT Fast Real-Time PCR System (Applied Biosystems).

Custom-designed TaqMan Low-Density Arrays (TLDA) (Applied Biosystems) were used to test a series of 63 genes, having selected candidate genes a priori on the basis of an earlier expression profiling study performed using microarrays. This selection was made based on the validation of some of the 74 genes that were found to be deregulated in the muscle of LGMD2A patients (Ref. 8). This selection included genes that showed deregulation in various different biological processes. Genes coding for collagens and fibronectin (which interacts with integrins) were analysed because they are markers of fibrosis. As they are overexpressed in the muscle of LGMD2A patients (Ref. 8), we wanted to establish whether the treatment with si*FRZB* reduced their expression.

The TLDAs were used following the protocol recommended by the manufacturer and the expression of all transcripts was determined relative to the internal housekeeping gene in the TLDAs, *GAPDH*, for which no alterations in expression were detected.

In order to identify genes differentially expressed in LGMD2A patients and healthy controls, we conducted geometric fold-change analysis. The threshold was set at a twofold change in intensity. The *P*-values obtained when comparing the unaffected control data with that from LGMD2A patients were adjusted by the Benjamini–Hochberg method using StatMiner software (Integromics).

For the analysis of gene expression in myotubes, TaqMan probes for Melusin gene *ITGB1BP2*-OMIM: 300332- (Hs00183746_m1), *CD9*-OMIM: 143030- (Hs00233521_m1), *FRZB*-OMIM: 605083- (Hs00173503_m1), *KAL1*-OMIM: 300836- (Hs01085107_m1), *COL1A1*-OMIM:120150- (Hs00164004_m1), *COL5A1*-OMIM: 120215- (Hs00609088_m1), *FOS*-OMIM: 164810- (Hs99999140_m1), *VLDLR*-OMIM: 192977- (Hs01045922_m1), *FN1*-OMIM: 135600- (Hs00365052_m1), *GAPDH*-OMIM: 138400- (Hs99999905_ m1) and *TBP*-OMIM: 600075- (Hs00427620_m1) were purchased from Life Technologies. *TBP* was used as the endogenous control and *GAPDH* as an unrelated control gene to verify the specificity of the siRNAs.

RNA interference knockdown

The siRNAs for *CD9* (s2598), *ITGB1BP2* (s25536) and *FRZB* (s5369) knockdown (in our work identified as si*CD9*, si*ITGB1BP2* and si*FRZB*) were purchased from Life Technologies. A scrambled siRNA was used as a negative control (AM4611, Life Technologies). Cells plated at 24 000 cells/cm^2 were transfected with the siRNA at a concentration of 5 nM using RiboCellin transfection reagent (Eurobio) following the manufacturer's instructions. After 8 days of differentiation, human primary myotubes were incubated with the corresponding siRNA and the transfection agent. Finally, the RNA obtained from these cultures was analysed 48 h post-transfection by quantitative real-time PCR. Likewise, proteins from these cultures were analysed 72 h post-transfection.

After 4 days of differentiation, C2C12 myotubes were incubated only with the *FRZB* siRNA and the transfection agent. Cells were fixed 72 h after silencing.

LiCl administration

LiCl was administered at a 10 mM concentration to the myotubes at day 8 of differentiation. At 48 h after administration of the drug, RNA and proteins were extracted.

Muscle tissue and cell preparation for Western blot analysis

Briefly, muscle samples were weighed and homogenised in a Tissue-Lyser mixer-mill disruptor (Qiagen) in treatment buffer, 19:1 w/v [0.125 mol/L Tris, 4% sodium dodecyl sulphate (SDS), 10% glycerol, 0.1 mol/L ethylenediaminetetraacetic acid and 5% β-mercaptoethanol]. Proteins from cultured cells were extracted using the same treatment buffer. Homogenised samples were loaded onto an SDS–polyacrylamide gel. Western blots were performed as described previously (Ref. 10) with minor modifications and probed with the following antibodies: melusin (Abcam), CD9 (R&D Systems), FRP-3 (anti-FRZB), GAPDH and ERK1/2 (Santa Cruz Biotechnology Inc), MyHC (Developmental Studies Hybridoma Bank), Integrin β1A and Integrin β1D (Millipore), Akt, P-Akt (Ser473), P-ERK1/2 (Thr202, Tyr204), GSK3β and P-GSK3β (Ser9) (Cell Signaling Technology).

Immunoreactive bands were visualised with the enhanced chemiluminescence reagent ECL-Plus (Amersham Pharmacia) and analysed with the Chemi-Doc XRS imaging system (Bio-Rad). The Student's t-test was applied to compare the mean values of different groups. Bars in graphs represent standard deviations.

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Immunofluorescence

Cells grown on coverslips were fixed with 4% paraformaldehyde, pH 7.4, for 10 min. Then, they were washed in PBS, and permeabilised by addition of 0.2% of Triton-X (Sigma-Aldrich) in PBS with 1% bovine serum albumin (BSA) (Biowest) and NaN_3 (0.01%) for 10 min, and then blocked in a solution containing 1% BSA. For the immunostaining, fixed cells were incubated with the primary antibody overnight at room temperature. After several washes with PBS, they were incubated with the corresponding secondary antibody for 1 h at room temperature. The solutions for primary and secondary antibodies contained 1% BSA in PBS. Cells were further washed with PBS and coverslips mounted on glass slides in a drop of ProLong mounting medium with DAPI (Life Technologies). The primary antibodies used were monoclonal mouse anti-MyHC at a dilution of 1:50 (Developmental Studies Hybridoma Bank) and mouse monoclonal anti-active-β-catenin at a dilution of 1:150 (Millipore), while the secondary antibodies used were goat anti-mouse conjugated to Alexa-Fluor 488 at a dilution of 1:300 and goat anti-mouse conjugated to Alexa-Fluor 555 at a dilution of 1:300 (Life Technologies). Cells were examined using a Nikon 80i microscope and the NIS-Element software. For myonuclei, the same myotubes were double stained with DAPI to visualize the nuclei and anti-MyHC antibody to outline the shape of the myotube. Nuclei located inside the fibre were counted as myonuclei. The distribution of myotubes with different numbers of nuclei in control and LGMD2A cultures was assessed by counting the number of each in 12 randomly chosen areas in each culture. The myotubes were classified depending on the number of myonuclei (Ref. 11). The fusion index was calculated as the percentage ratio of the number of nuclei inside MyHC+ myotubes (defined by the presence of at least two nuclei within a continuous cell membrane) to the total number of nuclei at day 10 of myogenic differentiation. The number of nuclei was estimated by calculating the average number of nuclei counted in 20 (control) and 18 (LGMD2A) independent and randomly chosen microscope fields of view.

Results

Myoblast cell culture and the subsequent differentiation to myotubes gave rise to the observation of different shapes in the myotubes of LGMD2A patients. The abnormal morphology of the patients' myotubes indicated that nuclei were clustered, and the myotubes with nuclear clusters were also more rounded. A more detailed study revealed that the distribution of the number of nuclei was different in patients and controls. Oversized myotubes containing more than 50 nuclei were found in LGMD2A cultures. Such myotubes were absent in control cultures (Fig. 1a). The observation of an anomalous distribution of myonuclei suggested an abnormal process of fusion of myoblasts in LGMD2A. Therefore, the fusion index was also measured and it was found to be significantly higher in myotubes from LGMD2A patients (44.15 ± 10.60%) than those from controls (31.69% ± 10.68) (Fig. 1a).

Integrin β1A and β1D isoform replacement is altered in LGMD2A patients' myotubes

Integrins are transmembrane glycoprotein receptors that are essential for myoblast fusion (Ref. 12). Under normal physiological conditions, the β1A isoform is replaced by the β1D isoform in muscle fibre maturation (Ref. 13), and this replacement has been shown to be altered in myotubes of calpain 3-knockout (C3KO) mice, which show a similar anomalous distribution of myonuclei as our LGMD2A myotubes (Ref. 11). Therefore, we next studied the distribution of the β1A and β1D isoforms of integrin in LGMD2A patients and controls.

We analysed the presence of these integrins in myoblasts and myotubes at different stages (days) of differentiation. Regarding expression of the β1A isoform, we observed hardly any differences in the differentiation process between patients and controls. Further, we observed less of the β1D isoform in myotubes of patients than controls, and the level of this isoform progressively increased in controls as the differentiation process advanced (Fig. 1b).

From the analysis performed on day 16 of myotube differentiation, we found evidence that while β1A integrin was slightly upregulated, the β1D isoform of integrin was underexpressed in myotubes from LGMD2A patients (Fig. 1c). This underexpression indicates that the replacement of the integrin isoforms does not occur correctly in these patients. In muscle samples, the variability in β1A and β1D integrin protein levels was too high (including among healthy controls) to obtain conclusive results.

In order to direct our study to the analysis of genes/proteins that may have relevance in the pathophysiology of LGMD2A, we focused on melusin and CD9 specifically, since both proteins interact with integrins (specifically melusin with β1A, β1B and β1D) (Refs 12, 14, 15, 16) and both are overexpressed in patients' muscle according to our previous study (Ref. 8).

Moreover, when we analysed the expression of genes from our previous microarray study (Ref. 8) in patient's cultured cells, some gene expression differences were observed in the myoblast/myotubes of LGMD2A patients. Regarding myoblasts, most of the altered genes were downregulated in patients, while in the case of myotubes, few genes showed an altered expression pattern and, compared with muscle, only *FRZB* was concordantly upregulated (fold-change value: 3.35) (Supplementary Material Table S1). This is the reason why we also focused on *FRZB* gene, since it was the only gene included in our analysis that was upregulated *in vitro* in the same way as in LGMD2A muscle.

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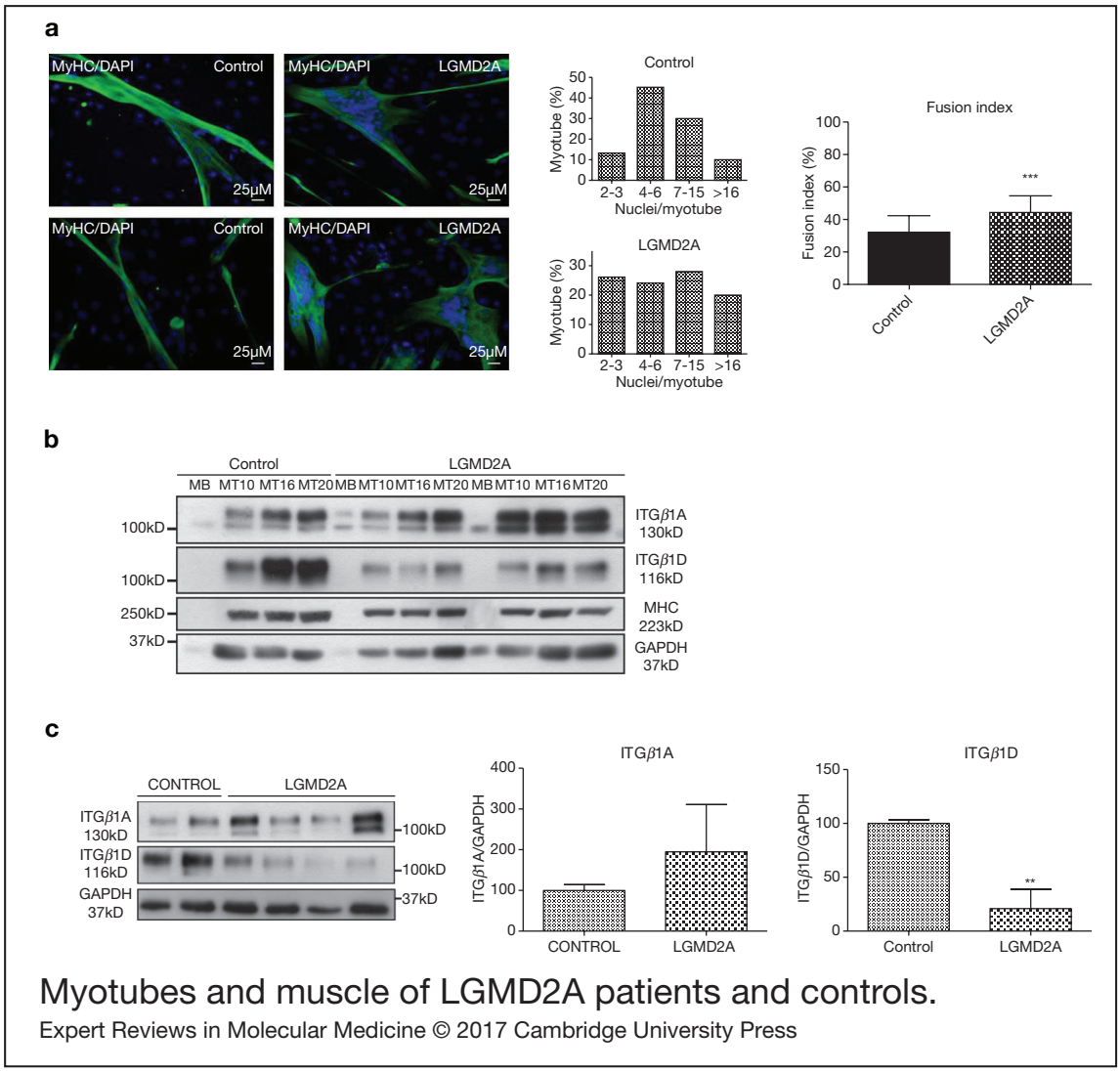

FIGURE 1.

(a) Myotubes on day 10 of differentiation in LGMD2A patients and controls. Green: myosin heavy chain. Blue: DAPI. The graphs indicate the distribution of the number of nuclei in patients and controls. Fusion index calculated from 18–20 fields of view in LGMD2A and control myotubes at day 10 of differentiation ($P = 0.0009$). Bars represent mean + standard deviation. (b) Western blot analysis of integrin isoforms in myoblasts and myotubes at days 10, 16 and 20 of differentiation. Expression of the β1A isoform increased as the differentiation process advanced in one control and in two LGMD2A patients, and expression of the β1D isoform increased in controls, but its level in LGMD2A patients remained low throughout the differentiation process. (c) Integrin isoforms β1A and β1D in myotubes at day 16 of differentiation (2 controls and 4 patients) ($P = 0.0042$). (d) Western blot and densitometry analysis of CD9 ($P = 0.0148$), melusin ($P = 0.0018$) and FRZB ($P = 0.0168$) in samples from muscles of LGMD2A patients and controls. (e) Overexpression of FRZB ($P = 0.0277$) in myotubes of LGMD2A patients.

Melusin, CD9 and FRZB protein expression is upregulated in LGMD2A muscle

Owing to a putative role of these genes in LGMD2A, we focused our study on the proteins coded by the deregulated genes, *ITGB1BP2, CD9* and *FRZB*, in order to identify whether they play a role in the pathophysiology of the LGMD2A muscle. The protein expression of these genes was further analysed in muscle samples, myoblasts and myotubes.

Western blot confirmed significant overexpression of melusin, CD9 and FRZB proteins in muscle from LGMD2A patients compared with levels in muscle from controls (Fig. 1d). In myotubes, significant overexpression of the FRZB protein was confirmed in Western blot of myotubes from LGMD2A patients compared with levels in myotubes from controls (Fig. 1e).

CD9 *and* FRZB *are positive regulators of* ITGB1BP2 *gene expression and* ITGB1BP2 *is a negative regulator of* FRZB *gene expression in myotubes*

As melusin and CD9 interact with integrin β1 (Refs 12, 14, 15, 16), and the expression of *ITGB1BP2, CD9* and *FRZB* genes is upregulated in calpain 3-deficient patients, siRNA experiments were carried out in myotubes, to find out whether there was any coordinated regulation of these genes. At 48 h after treatment with *CD9* siRNA, cultures showed markedly less *CD9* mRNA (90%) than in those treated with scrambled

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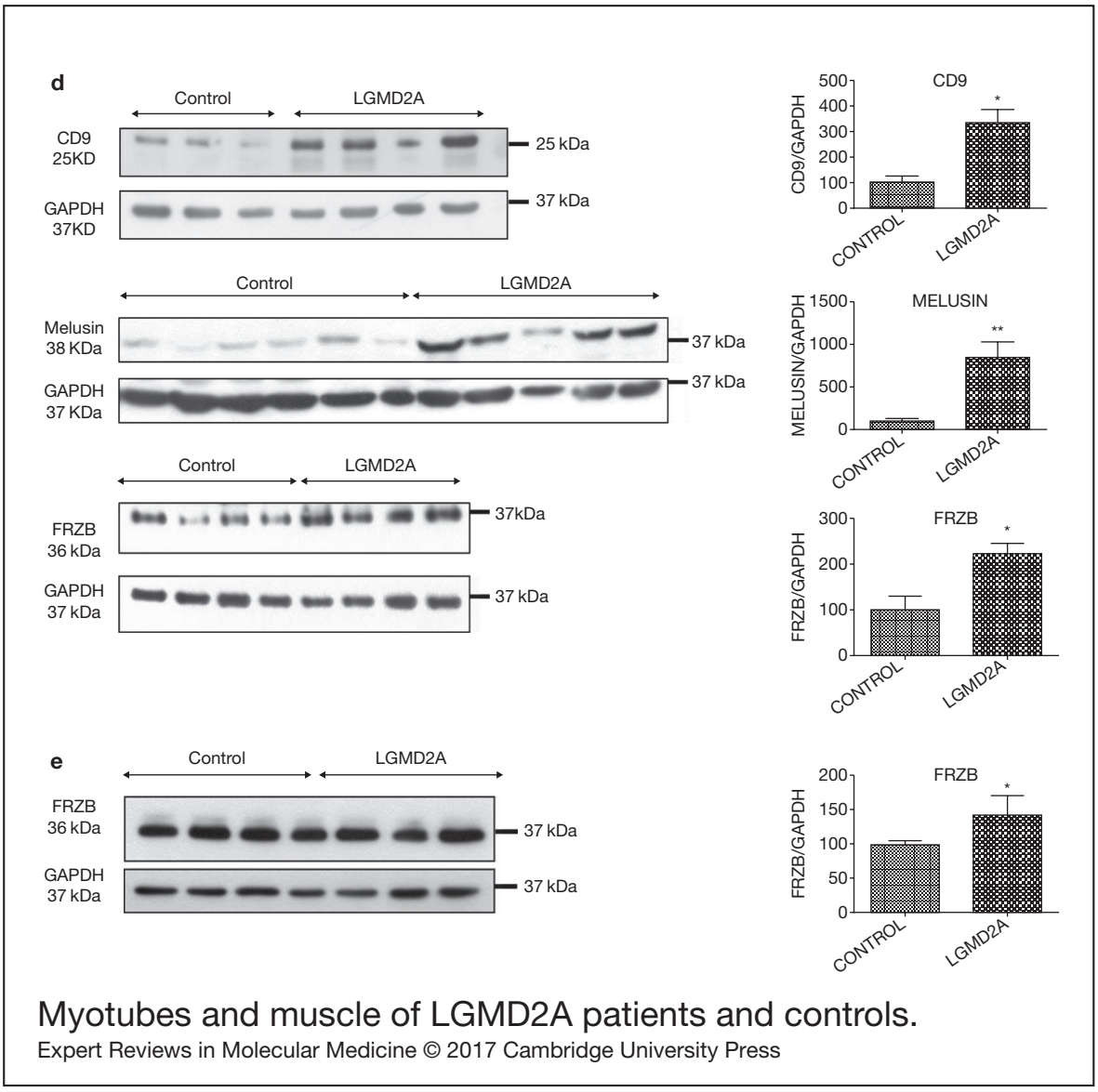

FIGURE 1.
(continued)

siRNA (Fig. 2a). Interestingly, we observed a parallel 40% reduction in *ITGB1BP2* in these cultures. *ITGB1BP2* gene silencing (90%) did not alter *CD9* expression, but it did upregulate *FRZB* expression. Lastly, *FRZB* silencing produced a slight reduction in *ITGB1BP2* expression (Fig. 2a). The same results were observed in myotubes obtained from distal muscles (tibialis anterior); that is, *ITGB1BP2* was downregulated when *CD9* and *FRZB* were silenced and, on the other hand, *FRZB* expression was upregulated when the *ITGB1BP2* gene was silenced (Supplementary Material, Fig. S1).

The efficiency and the effects of the silencing experiments were further confirmed at the protein level (Fig. 2b). In the case of *FRZB*, its silencing reduced melusin protein levels, and *ITGB1BP2* silencing increased FRZB protein levels (Fig. 2c and d). These results demonstrate an interaction between the expression of deregulated genes in LGMD2A patients, *CD9* and *FRZB* being positive regulators of *ITGB1BP2* gene expression and melusin a negative regulator of *FRZB* gene expression.

Integrin β1A–β1D isoform replacement is controlled by melusin and FRZB expression in myotubes

Since β1A–β1D isoform replacement seemed to be defective in LGMD2A myotubes and melusin binds directly to β1 integrins, we next investigated whether silencing of melusin could affect β1 integrin levels in myotubes. Melusin gene silencing produced an increase (not significant) in the β1A isoform, while we observed a trend toward a decrease in the β1D isoform in the myotubes of both patients and controls (Fig. 3a).

As silencing of the melusin gene affected *FRZB* expression and *vice versa*, we also analysed the effect

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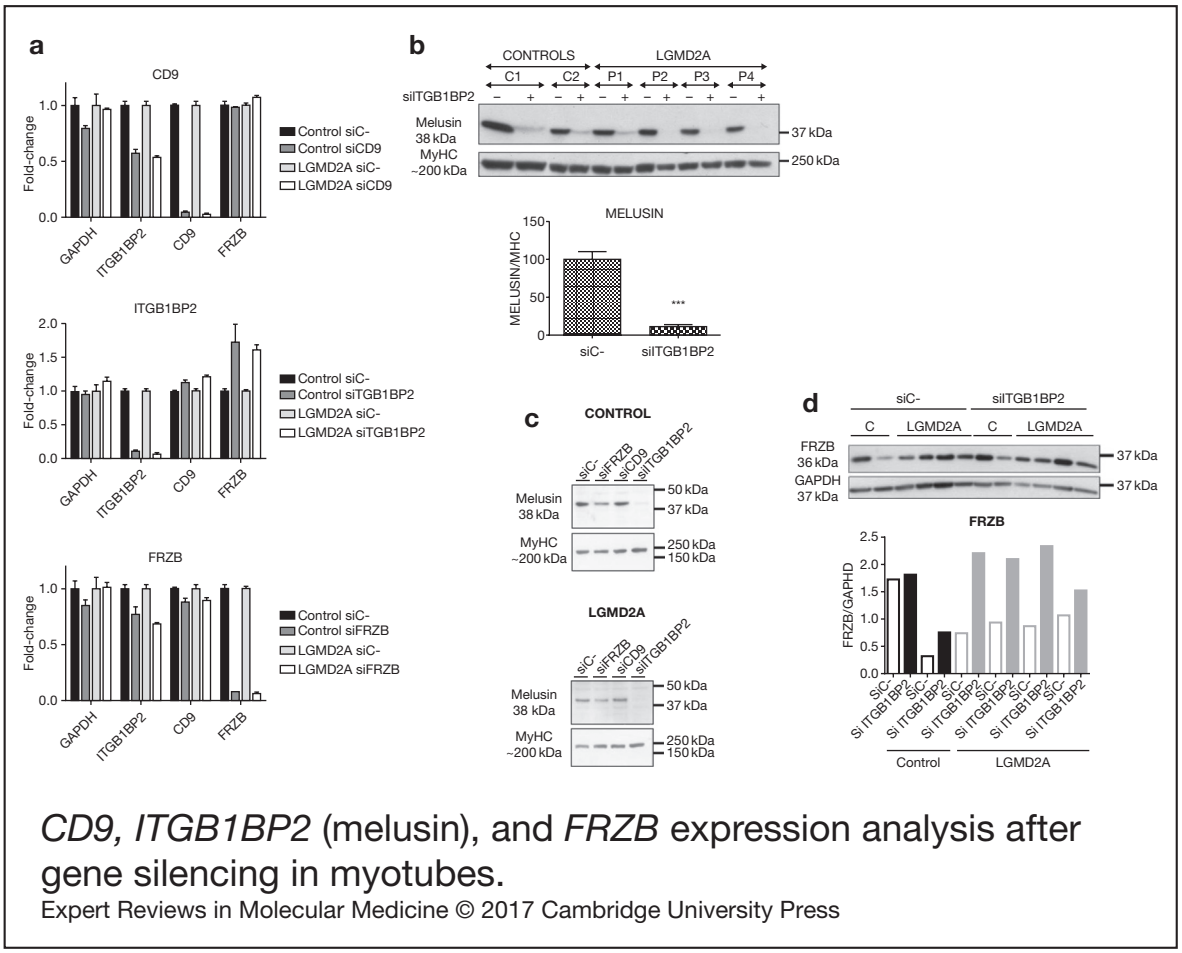

CD9, ITGB1BP2 (melusin), and *FRZB* expression analysis after gene silencing in myotubes.

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FIGURE 2.

CD9, ITGB1BP2 (melusin), and *FRZB* expression analysis after gene silencing in myotubes. (a) Quantification of the expression of the *CD9, ITGB1BP2* (melusin), *FRZB* and *GAPDH* genes after silencing using si*CD9,* si*ITGB1BP2*, and si*FRZB* in control and LGMD2A myotubes. siC- (control siRNA, scramble RNA). *GAPDH* was used to assess whether silencing was specific. *TBP* was used as the endogenous control. (b) Effect of si*ITGB1BP2* on melusin protein levels in controls and patients ($P = 0.0001$). (c) Effect of silencing using si*ITGB1BP2*, si*CD9* and si*FRZB* on the expression of melusin at the protein level in control and LGMD2A myotubes. MyHC: loading control. (d) Effect of silencing using si*ITGB1BP2* on the expression of FRZB at the protein level. GAPDH: loading control. Bars represent mean + standard deviation.

of *FRZB* silencing on β1 integrins. This silencing led to a decrease in levels of the β1A isoform only in LGMD2A patients, while this decrease was less clear in controls (Fig. 3b). In the case of the β1D isoform, *FRZB* silencing induced an increase in its protein levels in the myotubes of both controls and patients, though variability was observed ($P = 0.0546$). In LGMD2A patients, after *FRZB* silencing, we even observed a level of the β1D isoform that was similar to that in controls, without any additional treatment (Fig. 3b).

FRZB regulates phosphorylation of several signalling pathways

To elucidate the potential effect of the reduction in integrin β1D on the signal transduction proteins, we analysed the expression of Akt and P-Akt, which act downstream of the integrin pathway. Further, we analysed the phosphorylation status of other kinases, since melusin (the expression of which is reduced through *FRZB* silencing) phosphorylates ERK1/2 (Ref. 17), as well as Akt kinase (which in turn phosphorylates the glycogen synthase kinase 3).

The total amount of Akt was not different after silencing the *FRZB* gene, but the signal corresponding to phosphorylated Akt was lower. In addition, the P-Akt/Akt ratio was significantly lower after si*FRZB* treatment, indicating a reduction in Akt activity. In the case of GSK3β, we observed a lower level of phosphorylation, and a lower P-GSK3β/GSK3β ratio, which suggests an increase in the activity of this kinase.

Finally, the ERK phosphorylation levels also decreased after si*FRZB* treatment, showing a significantly lower P-ERK/ERK ratio than in nonsilenced myotubes and therefore a reduction in the activity of the protein (Fig. 4).

FRZB regulates β-catenin localisation in human and C2C12 mice myotubes

FRZB is an antagonist of Wnt1 and Wnt8 involved in the Wnt/β-catenin pathway (Refs 18, 19, 20) but its

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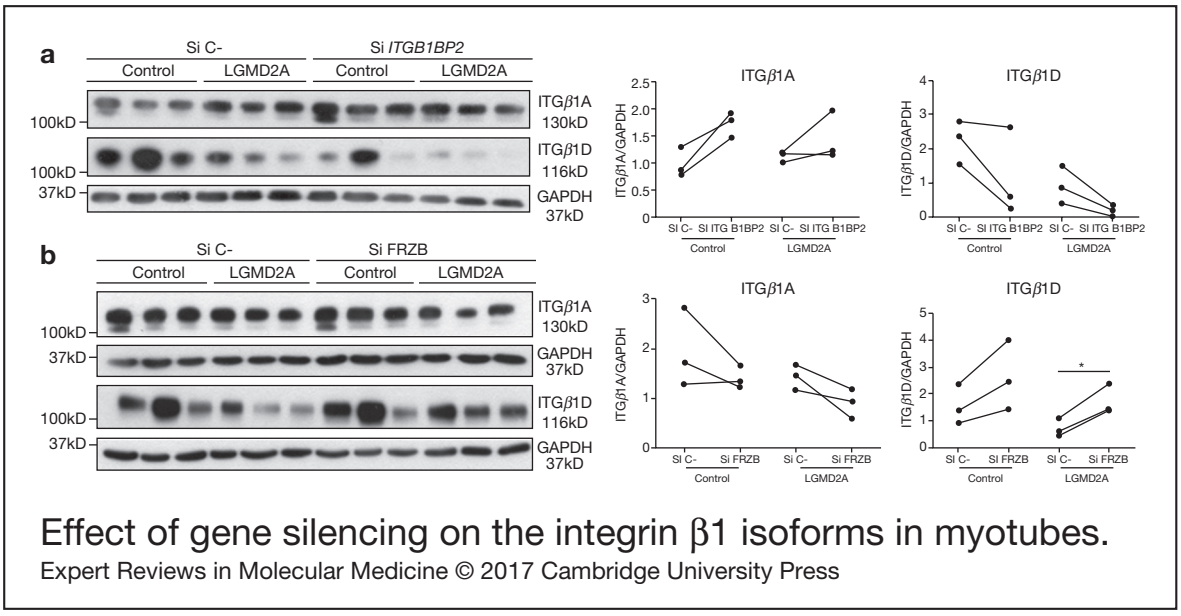

Effect of gene silencing on the integrin β1 isoforms in myotubes.

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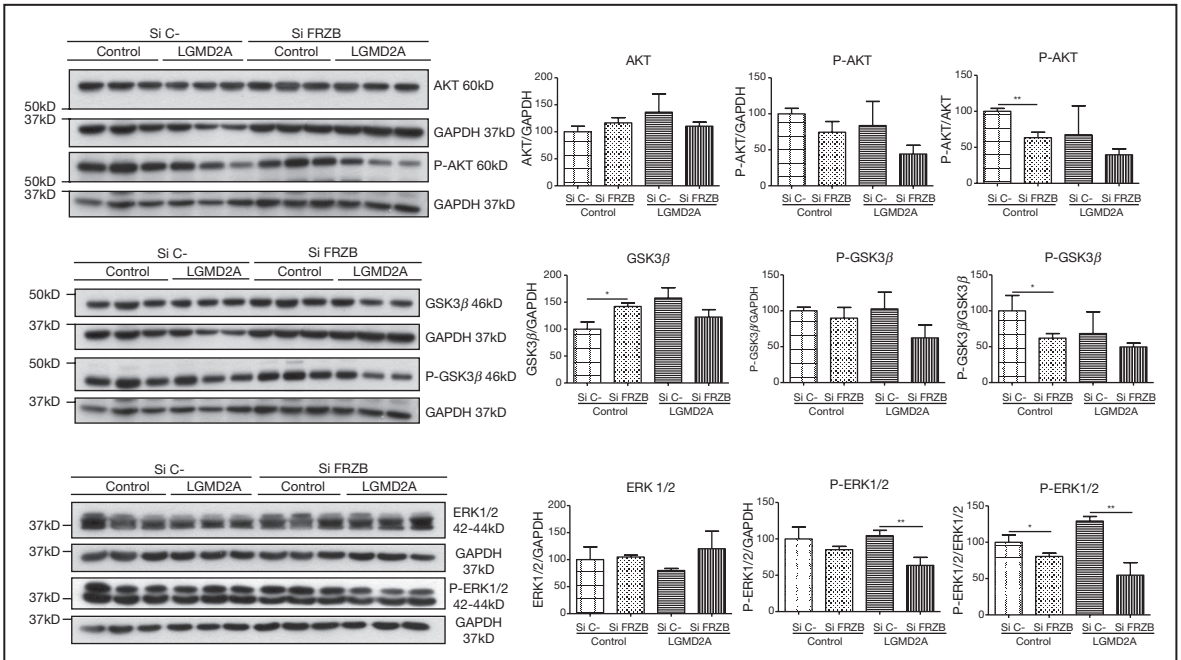
FIGURE 3.

Effect of silencing of (a) the melusin gene (si*ITGB1BP2*) (three controls and three patients) and (b) the *FRZB* gene (si*FRZB*) (three controls and three patients) on the integrin β1 isoforms in myotubes. ITGβ1D in LGMD2A ($P = 0.0546$). siC- (control siRNA, scramble RNA). GAPDH: loading control.



Akt, GSK3β and ERK1/2 phosphorylation analysis after FRZB gene silencing in control and LGMD2A myotubes.

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FIGURE 4.

Akt, GSK3β and ERK1/2 phosphorylation analysis by Western blot after *FRZB* gene silencing in control and LGMD2A myotubes. P-Akt/Akt in controls, $P = 0.0018$; GSK3β in controls, $P = 0.0466$; P-GSK3β/GSK3β in controls, $P = 0.0414$; P-ERK1/2 in LGMD2A, $P = 0.0058$; P-ERK1/2/ERK1/2 in controls, $P = 0.0359$ and in LGMD2A, $P = 0.0021$. siC- (control siRNA, scramble RNA). GAPDH: loading control. Bars represent mean + standard deviation.

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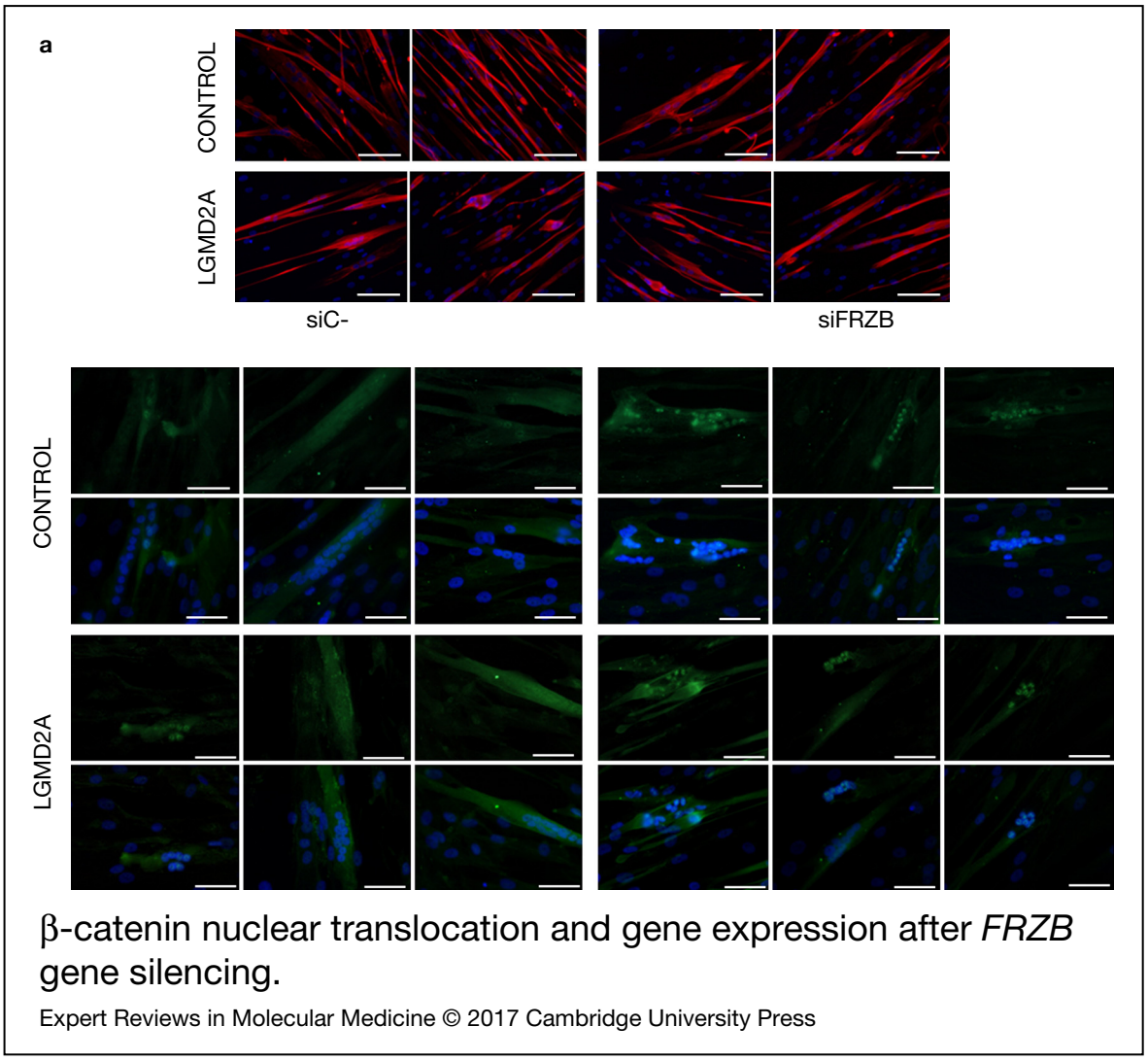

FIGURE 5.

β-catenin nuclear translocation and gene expression after *FRZB* gene silencing in (a) human myotubes (control and LGMD2A patients) and (b) mouse C2C12 myotubes. Red: myosin heavy chain. Green: active β-catenin. Blue: DAPI. Scale bar in human myotubes: 100 μm (upper panel), and 50 μm (lower panel), and in C2C12: 100 μm. (c) Gene expression analysis of *COL1α1, COL5α1, FN1, VLDLR, KAL1* and *FOS* in control (C1–C2–C3) and LGMD2A patients (P1–P2) after si*FRZB* treatment. C- (control siRNA, scramble RNA). Bars represent mean + standard deviation.

role in human muscle is not well defined. Given that we found overexpression of FRZB in LGMD2A muscle and myotubes, we analysed Wnt activation by assessing the localisation of β-catenin. The silencing of FRZB had an effect on the Wnt/β-catenin pathway: we observed nuclear translocation of β-catenin in human myotubes (both in patients and controls), and this was further confirmed in mouse C2C12 myotubes (Fig. 5a and b).

FRZB controls the expression of genes coding for ECM proteins and proteins participating in Wnt and integrin-signalling pathways in myotubes

The expression of some genes previously reported to be deregulated in the muscle of LGMD2A patients (Ref. 8) was analysed to establish whether FRZB expression was involved in their regulation. Interestingly, some of the ECM coding genes, involved in fibrosis, such as *COL1A1* (OMIM: 120150), *COL5A1* (OMIM: 120215) and *FN1* (OMIM: 135600) showed a trend to upregulation after *FRZB* silencing, although great variability was observed between the samples. Additionally, genes coding for FOS (OMIM: 164810) (a transcription factor), anosmin-1 *(KAL1* gene, OMIM: 300836) (which is part of the integrin β1 complex interacting with fibronectin) (Refs 21, 22), and very low-density lipoprotein receptor (*VLDLR*, OMIM: 192977) (which binds to LRP6, a transmembrane protein involved in the Wnt pathway), all deregulated in LGMD2A muscle (Ref. 8), showed a trend to upregulation once *FRZB* had been silenced (Fig. 5c).

In silico analysis (AliBaba v.2.1) of the promoter of *KAL1* gene (coding for anosmin-1) indicated the presence of various binding sequences for transcription factors such as *c-FOS* (upregulated after FRZB silencing) (Fig. 5c), *c-MYC* and *c-JUN*, which are regulated

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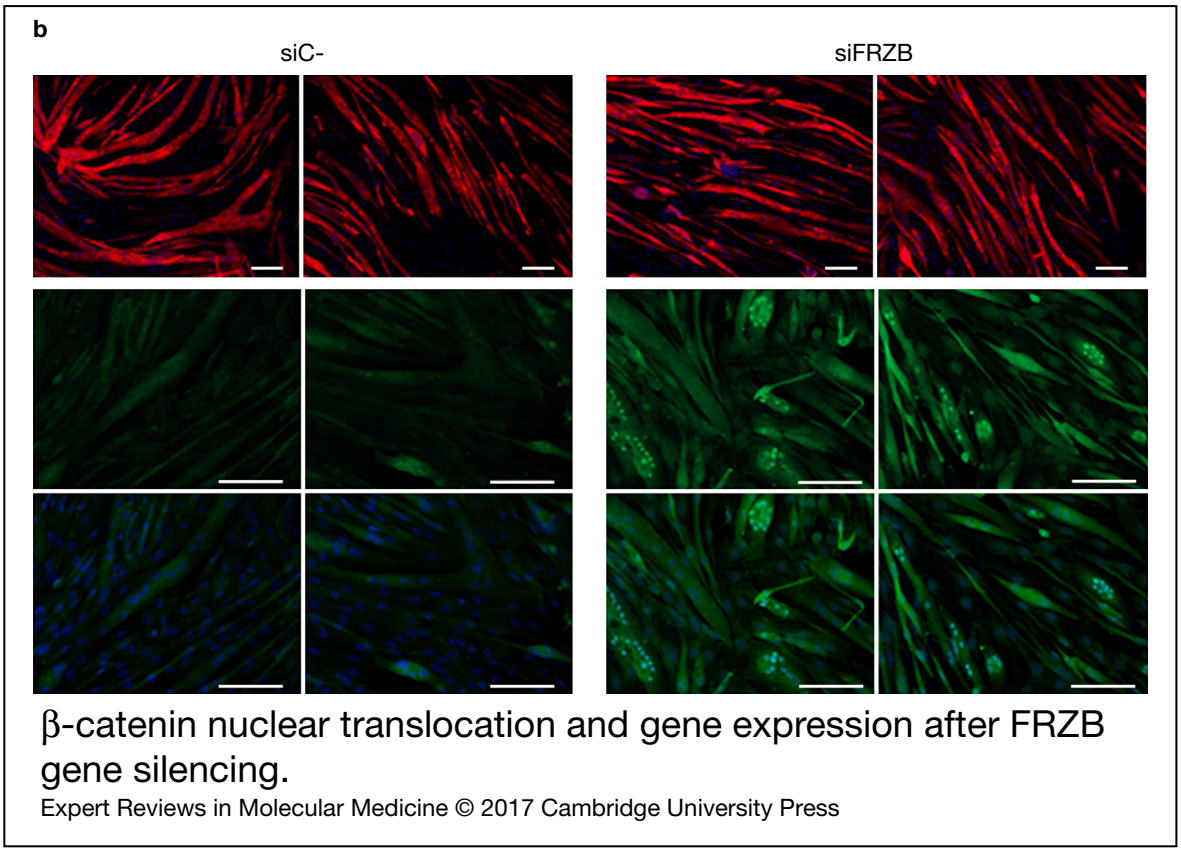

FIGURE 5.
(continued)

by β-catenin (Refs 23, 24), and which were previously shown to be downregulated in LGMD2A muscle (Ref. 8).

*Wnt signalling is enhanced by LiCl mimicking most of the effects of si*FRZB

As Hiyama and colleagues (Ref. 25) previously reported that LiCl increased Wnt1 expression, we assessed the effect of LiCl on myotube cultures at 8 days of differentiation, as a positive control for the activation of the Wnt pathway. The treatment with LiCl, an enhancer of the Wnt pathway, produced similar results to those obtained in the analysis of si*FRZB*. On the one hand, we observed lower levels of expression of the *FRZB* gene, and on the other, a higher level of expression of the *FOS, KAL-1* and *VLDLR* genes, although there was variability between different samples (Fig. 6a). Finally, with regards to protein expression, we found that LiCl treatment was also associated with higher levels of the β1D adult isoform of the integrin in primary myotubes (Fig. 6b).

There are, however, some differences in the effect of the treatments for activating the Wnt pathway. Specifically, the expression of melusin was lower after *FRZB* silencing but not after LiCl treatment (data not shown). Further, we observed less phosphorylation of Ser9 of GSK3β after *FRZB* silencing, unlike with the treatment with LiCl, this being associated with an increase in the phosphorylation of Ser9, and in turn an inhibitory effect on GSK3β (Fig. 6c). Given that the administration of LiCl also increases the expression of β1D integrin, we assessed downstream phosphorylation in the integrin pathway. We found that there was a trend to a decrease in the phosphorylation of Akt, though there was great variability between samples. Finally, LiCl administration was associated with less ERK phosphorylation (Fig. 6c).

Discussion

As there are physiological differences between what happens in culture and *in vivo* in muscle, results obtained from culture studies need to be interpreted with caution. Nevertheless, human myology research is mostly based on knowledge obtained from culture systems. Specifically, this approach can help to shed light on the poorly understood muscle pathophysiology caused by calpain 3 deficiency. It remains to be determined how calpain 3 and its related proteins interact with each other in cells and tissues. Calpain 3 activity may proteolyse certain parts of different molecules, modifying their functions post-translationally. It could be speculated that proteins interacting with calpain 3 may transduce signalling pathways or alter the fusion of the myoblasts required for correct muscle regeneration, a process that is impaired in LGMD2A patients.

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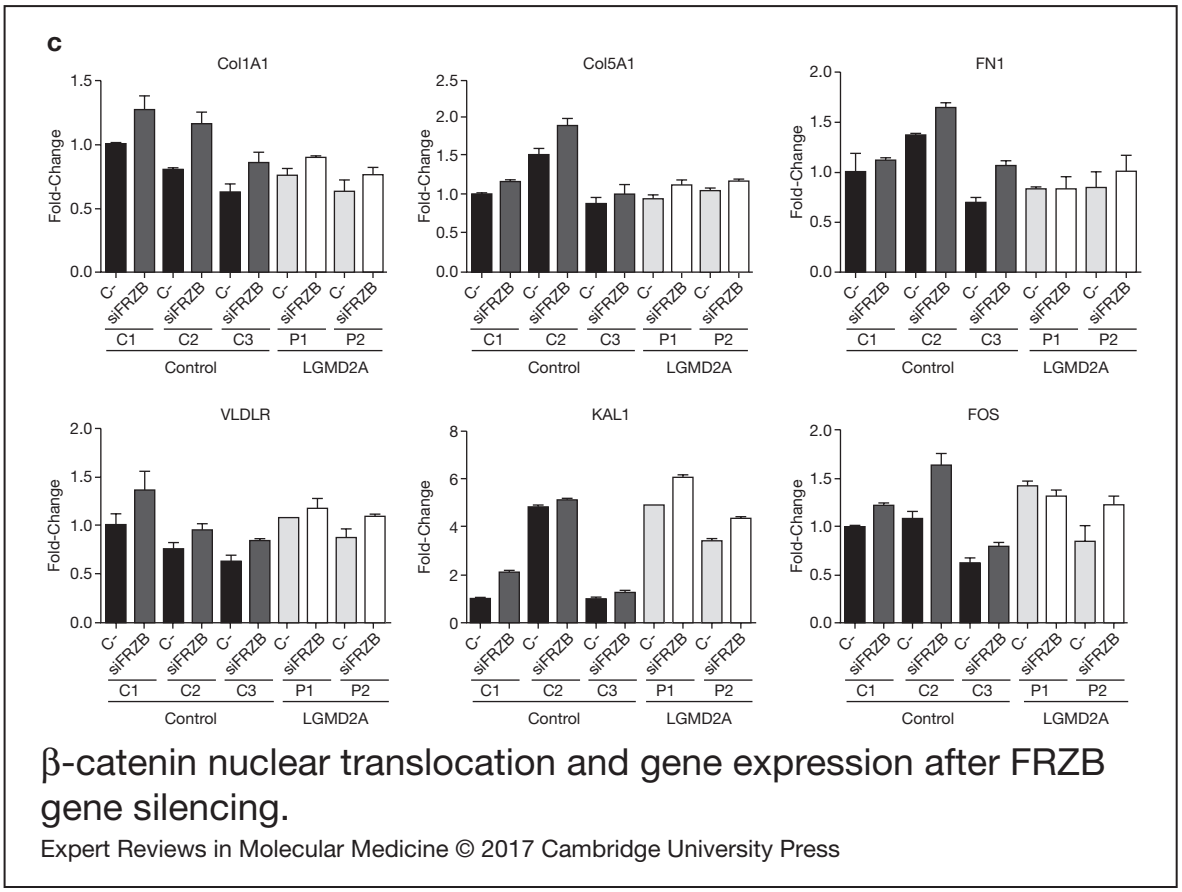

FIGURE 5.
(continued)

In this study, we have shown that genes coding for melusin, CD9 and FRZB are upregulated in the muscles of LGMD2A patients. CD9 upregulation may explain the abnormal distribution of the nuclei observed in LGMD2A myotubes given that normal muscle regeneration requires a tight control of myoblast fusion by the tetraspanins CD9 and CD81 (Ref. 26).

Additionally, although a direct interaction of melusin, CD9 and FRZB has not previously been described, we have observed that at least *in vitro*, they are related at the level of expression control. As siRNA experiments to silence *CD9* and *FRZB* expression have produced a parallel downregulation of melusin, it could be suggested that CD9 and FRZB act upstream on the regulation of the *ITGB1BP2* gene (Fig. 7a). Similarly, the fact that *ITGB1BP2* gene silencing upregulates FRZB expression suggests that these proteins are involved in a common regulatory pathway that is impaired in LGMD2A dystrophy. Further studies are required to determine the exact mechanism that controls this apparent contradictory regulation, but it could be suggested that there is a negative feedback mechanism for the activation or control of both pathways.

The fact that the proteins of these genes are all directly or indirectly involved in the integrin pathway demonstrates the importance of an appropriate coordination in the expression of proteins that interact with or are components of the costamere. So far, mechanisms underlying common expression of different genes are not well known. However, it is likely that genes whose products function together are under a common regulatory system such that they are expressed in a coordinated manner (Refs 27, 28).

Gene silencing experiments have provided evidence of the importance of FRZB expression regulation for proper muscle fibre growth and/or maturation. The deregulation of the FRZB protein in LGMD2A patients seems to affect the composition of cell membrane-forming proteins and hence to defects in the associated signalling pathways. Specifically, FRZB overexpression seems to have a negative impact on the cell as silencing this gene caused the β1D integrin isoform level to return to normal levels in myotubes from LGMD2A patients (Fig. 3b).

Regarding previous knowledge, the incorrect replacement of the β1A isoform by the β1D isoform has also been observed in the myotubes of C3KO mice (Ref. 11). Other studies have also shown the involvement of FRZB in the integrin pathway, for instance, it has been reported that KO $Frzb^{-/-}$ mice show an overrepresentation of upregulated genes

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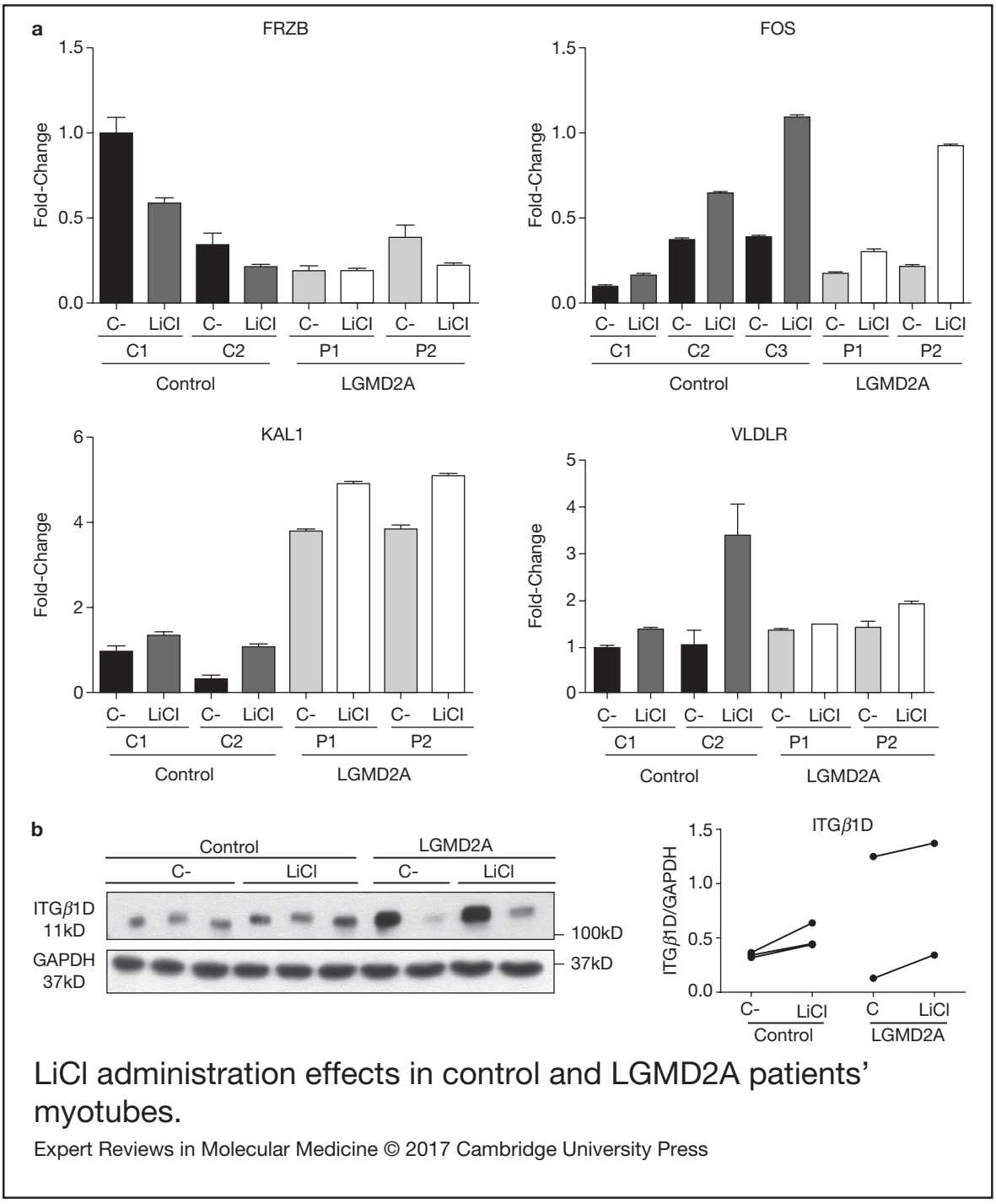

FIGURE 6.

LiCl administration effects in control and LGMD2A patients' myotubes. (a) Gene expression of *FRZB, FOS, KAL1* and *VLDLR* after LiCl treatment in control (C1–C2) and LGMD2A (P1–P2) myotubes. (b) ITGβ1D protein expression increase after LiCl administration in control and LGMD2A patients' myotubes and (c) Akt, GSK3β and ERK1/2 phosphorylation analysis by Western blot after LiCl administration. GSK3β, control versus LGMD2A without treatment, $P = 0.0377$; P-GSK3β in controls, $P = 0.0093$; P-GSK3β/GSK3β in controls, $P = 0.0173$; P-ERK1/2/ERK1/2 in controls, $P = 0.0432$. C- (control siRNA, scramble RNA). GAPDH: loading control. Bars represent mean + standard deviation.

related to this pathway (Ref. 29). As observed in a gastric cancer cell line model (Ref. 30), we found that FRZB regulates the localisation of β-catenin downstream of the Wnt pathway in primary human myotubes (Fig. 5a), and this suggests that FRZB may play a role in the crosstalk between integrin and Wnt-signalling pathways. The link between these pathways may involve the nuclear translocation of β-catenin that would activate transcription factors such as *FOS* that have binding sites for the promoter of *KAL1* gene, which codes for anosmin-1, a protein that also interacts with β1D integrins (Ref. 21) (Fig. 7b). *KAL1* gene was

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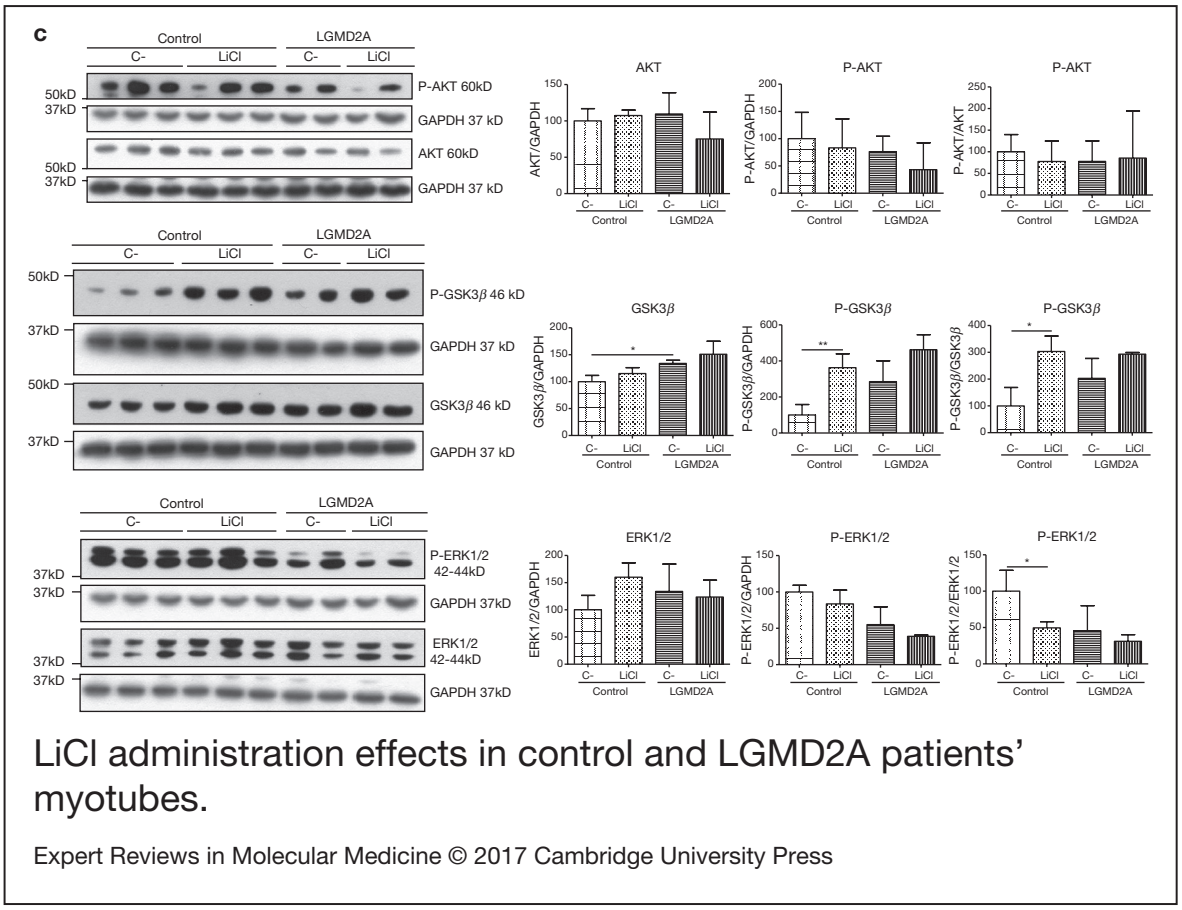

FIGURE 6.
(continued)

underexpressed in the muscle of LGMD2A patients with eosinophylic infiltrates (significantly lower) and the level of expression was also notably lower in other LGMD2A patients (two-fold lower, though the difference did not reach statistical significance; Ref. 8 and unpublished data). Expression of this protein is elevated after si*FRZB* or LiCl treatment, and this could facilitate an increase in the β1D isoform of the integrin. These findings suggest that there is a relationship between the regulation of the two pathways and that anosmin-1 (coded by *KAL1*) may be involved in the organisation of the integrin complex, to ensure its correct replacement in the maturation process of muscle fibres.

On the other hand, melusin upregulation and its silencing are both associated with a decrease in the integrin β1D isoform. Based on these results, we suggest that this decrease occurs both when too much melusin is present (potentially because of steric problems) but also when there is too little melusin (because of lack of an anchoring protein). These data therefore would indicate that the quantity of melusin needs to be finely tuned for proper formation of the protein complex. There are similar biological systems responsible for reverse effects in the cell, such as the one reported by Bernick and colleagues in 2010 (Ref. 31), in which loss or overexpression of unc-45b leads to defective myofibril organisation; that is, unc-45b expression must be precisely regulated to ensure normal myofibril organisation. And there are other examples, for instance, abnormally high or low levels of *IQGAP1* and *SRPK1* expression reduce activation of MEK and ERK or promote cancer, respectively (Refs 32, 33).

The nuclear translocation of β-catenin may also act as a regulator of the Wnt/β-catenin pathway, given that we observed elevated expression of the *VLDLR* gene after si*FRZB* treatment. This gene has binding sites to transcription factors modulated by β-catenin and the resulting protein forms heterodimers, VLDLR-LRP6, that accelerate the turnover of LRP6 (a transmembrane protein that binds to Frizzled and that is involved in the canonical Wnt pathway) (Fig. 7b); this represents a potential new mechanism for the regulation of the Wnt/β-catenin pathway (Ref. 34) which could serve to limit the duration or intensity of a Wnt-initiated signal.

We have used two methods for activating the Wnt pathway, the first, genetic silencing (si*FRZB*) and the second, a chemical-based method (LiCl), with similar results. Despite some differences, both approaches produced an increase in β1D integrin levels. It would be expected that by increasing levels of β1D integrin, we would observe an increase in P-Akt (Ref. 35).

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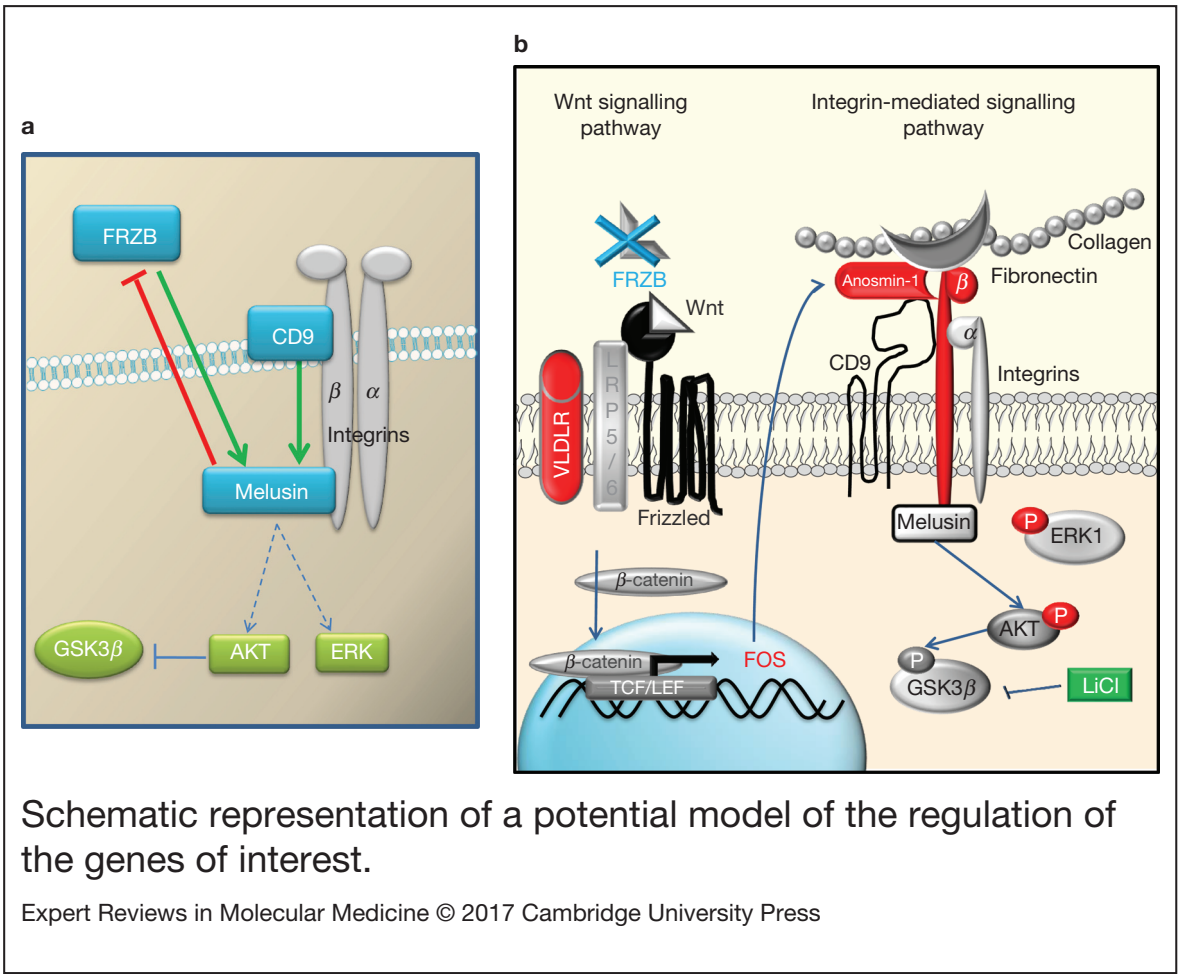

FIGURE 7.

Schematic representation of a potential model of the regulation of the genes of interest. (a) CD9 and FRZB are positive regulators of melusin gene expression (green arrow) and melusin is a negative regulator of *FRZB* gene expression (red line). (b) FRZB and LiCl regulation effect in sarcolemmal, costamere and transduction signalling proteins. Once *FRZB* gene silencing occurs, the Wnt-signalling pathway, that activates its downstream effector β-catenin, is activated. β-catenin in turn activates various transcription factors, including *FOS*. The gene coding for anosmin-1 (*KAL1*) has regions in its promoter that could be targets for *FOS*. Anosmin-1 interacts with integrin β, and it could be suggested that it regulates the organisation of the integrin complex and that the increase in the expression of this gene, after FRZB silencing, would lead to an increase in β1D integrin in the costamere. Red indicates upregulated proteins or an increase in phosphorylation after FRZB silencing or LiCl administration.

However, FRZB silencing seemed to be more closely associated with a reduction in the phosphorylation of all the kinases analysed, and this may be mainly because of a reduction in the expression of melusin, given that melusin is known to phosphorylate ERK1/2 and Akt (Ref. 17). In the case of LiCl, despite no reduction in melusin, we also observed a trend to decrease in the phosphorylation of kinases Akt and ERK, suggesting that the phosphorylation of these kinases is not regulated by melusin in these circumstances. Even though LiCl is widely used experimentally, the molecular mechanisms by which LiCl treatment regulates ERK phosphorylation have not yet been elucidated, various different responses having been obtained depending on the type of cell studied (Ref. 36).

We have no evidence of a direct interaction between calpain 3 and integrin. It could be suggested that there is an intermolecular interaction, as reported for titin, but at present there is no evidence of that. On the other hand, the possibility of integrin being a direct calpain 3 substrate has already been ruled out by Kramerova and colleagues (Ref. 11), who showed that neither β1A nor β1D integrin are digested by calpain 3, suggesting that the changes in integrin isoform levels are because of an indirect effect.

With regard to a potential direct interaction between calpain 3 and integrin regulating transcription factors, it could be supposed that a decrease in *FOS* (as observed in LGMD2A patients) (Ref. 8) would also reduce the expression of integrin because integrin has FOS and AP-1 binding sequences in its promoter. However, we did not observe a reduction in integrin RNA levels in patients muscle biopsies (unpublished data). Since the *ITGB1* gene encodes both integrin isoforms, β1D and β1A, we suspect that the alteration in the amount of integrin β1D is because of post-translational events and the substitution of the isoform might be mediated by the increase in anosmin-1.

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In LGMD2A patients, myogenesis is impaired, because of the lack of replacement of the integrin isoforms required for appropriate costamere assembly, as well as for the fusion of myotubes. The regulation of FRZB expression can be proposed as a potential therapeutic target, given that *in vitro* studies support the idea that it may be possible to bring expression and phosphorylation of various proteins back towards appropriate levels in LGMD2A patients.

Further studies are required to assess whether FRZB expression is abnormal in other types of muscular dystrophy, to establish whether it is also involved in their pathophysiology. Similarly, further research should investigate the mechanisms of action of FRZB, since controlling an inhibitor of the Wnt pathway may be useful for the treatment of other conditions, as already described for osteoarthritis (Refs 37, 38) and it might also be applicable to ischemic cardiopathy, in which low levels of β1D integrin are observed (Ref. 35).

To conclude, in line with what we have found in this study, and given unexpected difficulties that have arisen in the application of cell therapies, we should consider using pharmacological treatments to regulate the Wnt pathway, at least, until the development of more specific therapies, and until we have a better understanding of the mechanisms secondary to the regulation of gene expression.

Supplementary Material

The supplementary material for this article can be found at https://doi.org/10.1017/erm.2017.3

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*Corresponding author:
Amets Sáenz
Neurosciences Area, Biodonostia Health Research Institute, San Sebastián, Spain and Center for Networked Biomedical Research on Neurodegenerative Diseases (CIBERNED), Carlos III Health Institute, Spanish Ministry of Economy and Competitiveness, Madrid, Spain.
E-mail: amets.saenzpena@osakidetza.eus

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