Quantitative changes in focal adhesion kinase and its inhibitor, FRNK, drive load-dependent expression of costamere components

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Running title: Costameres during load-dependent muscle plasticity
Abstract

Costameres are mechno-sensory sites of focal adhesion in the sarcolemma that reinforce the muscle-fiber composite and provide an anchor for myofibrillogenesis. We hypothesized that elevated content of the integrin-associated regulator of costamere turnover in culture, focal adhesion kinase (FAK), drives changes in costamere component content in anti-gravity muscle in a load-dependent way in correspondence with altered muscle weight.

The content of FAK in soleus muscle being phosphorylated at auto-regulatory tyrosine 397 (FAK-pY397) was increased after 20 seconds of stretch. FAK-pY397 content remained elevated after 24 hours of stretch-overload due to up-regulated FAK content. Overexpression of FAK in soleus muscle fibers by means of gene electro-transfer increased the beta 1 integrin (+56%) and meta-vinculin (+88%) content. Alpha 7 integrin (p=0.46) and gamma-vinculin (p=0.18) content was not altered after FAK overexpression. Co-overexpression of the FAK inhibitor FRNK reduced FAK-pY397 content by 33% and increased the percentage of fast type fibers that arose in connection with hybrid fibers with gene transfer. Transplantation experiments confirmed the association of FRNK expression with slow-to-fast fiber transformation.

7 days of unloading blunted the elevation of FAK-pY397, beta 1 integrin and meta-vinculin content with FAK-overexpression and this was reversed by one day of reloading.

The results highlight that the expression of components for costameric attachment sites of myofibrils is under load- and fiber type-related control via FAK and its inhibitor FRNK.

Keywords: FAK, muscle, vinculin, focal adhesion, hypertrophy, atrophy
Introduction

Sites of focal adhesion in the sarcolemma of muscle fibers, costameres, provide physical links between Z-disks in the fiber interior and the extracellular matrix on the outside (13, 23, 42). Costameres assemble through the clustering of cytoskeletal and signaling proteins at the intracellular portion of integrin- and/or dystrophin/sarcoglycan-type extracellular matrix receptors (3, 13, 23, 38, 47). They integrate contraction between neighboring muscle fibers and are essential for muscle fiber stability (reviewed in 18, 33). In analogy to focal adhesions of non-contractile cells, costameres are thought to mediate the conversion of physical forces into biochemical signaling which modulates cell morphology, gene and protein expression (13, 23, 42) (4, 27, 30, 32). In culture, this process of mechano-transduction is initiated within seconds through a conformational change in integrins (26). It is transduced via sequential phosphorylation of associated phosphotransfer enzymes (kinases), i.e. focal adhesion kinase (FAK), mitogen-activated protein kinases (MAPK), c-jun N-terminal kinase (JNK) and c-src (13, 26, 32, 53). Subsequent modifications in associated cytoskeletal proteins (51) rapidly alter the composition and adhesive strength of focal adhesions before changes in downstream gene expression are apparent (2, 22).

Costamere formation is coordinated with the assembly of myofibrils during muscle cell growth in culture (45). Costamere component content is also modified with repeated and/or chronic alterations in muscle loading that affect muscle (fiber) size (1, 5, 6, 21). For instance the content of focal adhesion components, paxillin and FAK, in anti-gravity muscle of rats is increased after 1 and 8 days of stretch overload by tenotomy along with augmented muscle weight (21). Conversely, expression of the gamma- and meta-isoform of vinculin, talin and beta 1-integrin is compromised beyond 3 weeks of unloading in human and rodent muscle when muscle fiber atrophy installs (1, 5, 6) and this is reversed with resistance types of exercise (5). The observations argue for a role of costamere remodeling in the regulation of fiber size in fully developed muscle, and highlight that this is subject to control by mechanical factors. The mechano-induced chemical signal mechanism assisting activity/load-dependent costamere organization has not been exposed (1).
The integrin-associated tyrosine kinase, FAK is a potential key regulator of load-regulated costamere content (14, 21, 39) and FAK is instrumental for coupling costamere formation with myofibril assembly during muscle fiber growth in culture (7, 27, 45, 48). FAK assists the hetero-dimerization of integrins and the recruitment of focal adhesion components upon the impact of mechanical stress to integrins (22, 55).

Trans-phosphorylation of FAK at tyrosine residue 397 (Y397) is of central importance for FAK activation (50). It is favored through a conformational change in its N-terminus through a non-identified mechanism (50). Phosphorylated Y397 (pY397) creates high affinity binding sites for c-src that enables FAK to develop its full catalytic activity and phosphorylate auxiliary sites (i.e. Tyr 576/577). This facilitates the interaction of FAK with further binding partners (49) that mediate the clustering of cytoskeletal and signaling proteins to integrins (38). This role is emphasized by modified focal adhesion turnover with overexpression of the FAK inhibitor FRNK (FAK-related non-kinase), derived from the C-terminal portion of FAK (11). Increased FAK-pY397 content is understood to serve as readout for the initiation of chemical mechano-transduction (28, 32, 53). This is highlighted by the increased phosphorylation of FAK at Y397 and auxiliary phosphorylation sites in human and rodent muscle with increased loading and a concomitant drop in tyrosine phosphorylated FAK content with unloading (8, 9, 12, 21, 27, 54). In culture, this is a rapid process with FAK-pY397 content being 3-fold increased within 2 minutes of stretch and being decreased within the next ten minutes without changes in soluble FAK protein (32). By contrast, with chronic muscle overload the increased content of FAK-pY397 is explained by elevated FAK protein expression (16, 17, 21). This view is supported by an elevated content of N-terminally immunoreactive FAK, which is indicative of activated FAK, at the sarcolemma in frequently recruited muscle fiber types (12, 16). Equally it is supported by differences in costamere content between slow and fast type muscle fibers, which demonstrate varying levels of recruitment (3, 6). The time course and mechanism underlying the possibly fiber type specific regulation of costameres by mechanical factors is not understood.

We set out to expose the involvement of increased FAK content to the load-dependent regulation of FAK-Y397 and costamere component content in anti-gravity muscle and its relation to muscle weight and slow-to-fast fiber transformation. Towards this end we characterized the time course of changes in FAK-pY397 and FAK protein content
with overload of the slow soleus muscle in rats and studied the effect of FAK overexpression in soleus muscle fibers by gene electro-transfer on changes for protein levels of the costamere markers, meta- and gamma-vinculin, beta 1-integrin, and its major sarcolemmal binding partner in skeletal muscle, alpha 7 integrin (40) with unloading and reloading. We hypothesized that elevated FAK content in m. soleus increases the content of costamere markers in a load-dependent way compared to empty-transfected contralateral controls and which would correspond to alterations in muscle weight. Co-overexpression of the inhibitor of FAK-mediated focal adhesion turnover, FRNK, was employed to dissect the role of FAK and its Y397 phosphorylation for costamere turnover.

**Materials and Methods**

**Experimental design** - Anti-gravity muscle of female Wistar rats, m. soleus, was subjected to physiological interventions reducing or increasing muscle loading. This comprised overload by bilateral tenotomy, unloading by hindlimb suspension or reloading after hindlimb suspension. To expose the load-dependent role of FAK we combined hindlimb suspension-reloading with somatic transfection of soleus muscle with a cytomegalovirus-driven expression construct for the chicken homologue of FAK (gift of Thomas Parsons, University of Virginia, Charlottesville, USA) or an empty construct, alone, and in combination with a construct for the inhibitor of FAK, FRNK. Muscle were stored at -80°C until being processed for the detection of costameric proteins (alpha 7 integrin, beta 1 integrin, meta-vinculin, gamma-vinculin, FAK, FRNK) and the phosphorylation status of FAK at pY397. The analysis combines muscle samples from a previous investigation into the effects of hindlimb suspension (12) with samples from new experiments on tenotomy and cage controls performed at the Insel-Hospital in Berne (Switzerland). Transport of samples between laboratories was carried out on dry ice in insulated boxes. Plasmids were propagated endotoxin-free by Plasmidfactory (Bielefeld, Germany, www.plasmidfactory.de). The experiments were conducted under approval of the Animal Protection Commission of the Canton Bern, Switzerland. Transplanted soleus muscle stem from previous experiments performed on male rats (strain Zur:SIV; Institute of Laboratory Animal Science, University of Zürich,
Tenotomy - Rats were anaesthetized with 5% isoflurane inhalation and were maintained in 3% isoflurane during the surgical procedure with the help of a commercial tube system (Provet AG, Lyssach b. Burgdorf Switzerland). An approximately 4 mm long incision was made at the dorsal side of the lower leg of the left hindlimb towards the tendinous end of the gastrocnemius muscle. The Achilles tendon being connected to the tendinous end of the gastrocnemius muscle (i.e. the medial and lateral part) was exposed and lifted with the help of a pair of sharp forceps and capped with a scalpel. The skin was closed with 3 sutures. Tenotomy was repeated for the Achilles tendon of the right leg. The intervention per leg was complete within 3-5 minutes. Subsequently animals were released from the anesthesia and allowed to wake up under normal oxygenation in a Macrolon type III cage. After an average of 10 minutes, animals would regain consciousness and began to explore the cage. Muscle activity during the first hour of resuming consciousness was stimulated in regular intervals (i.e. 1 min, 3 min, 5 min, 10 min and 30 min). After 1, 6 and 24 hours of cage activity under tenotomy rats would be anesthetized again with isoflurane to collect soleus muscle from both legs. On average this added an extra 10 minutes of anesthesia and operation time to the interventions. In addition mock tenotomy experiments only exposing the Achilles tendon (but not capping it) where muscle activity after anesthesia was stimulated in the same manner as described above were conducted and soleus muscles harvested 1 and 24 hours after the rats gained consciousness after the mock intervention. Non-operated, age-matched control rats were kept in standard cages for the same duration.

Hindlimb suspension-reloading of rats: Unloading of the soleus muscles by 7 days of hindlimb suspension and subsequent 1 or 5 days of reloading was carried out as described (12). At the end of the respective protocol, m. solei of both hindlimbs were harvested a described below.

Somatic transfection of anti-gravity rat muscle - Solei muscles of anaesthetized rats (3% isoflurane) were transfected using gene electrotransfer in a paired design to assess the effects of FAK overexpression vs. contralateral controls essentially as
described (12). 35 µg of construct for FAK, pCMV-FAK, in 35 µl was injected into
the belly portion of the right muscle. The equivalent amount of empty pCMV-plasmid
was injected into the contralateral left muscle.
Animal were kept as cage controls for 9 days or after 2 days of normal cage activity
subjected to 7 days of hindlimb suspension, or 7 days of hindlimb suspension
followed by 1 or 5 days of reloading to alter m. soleus muscle loading. M. solei of
both hindlimbs were harvested as described below. Additionally, right soleus muscle
was transfected with a mix of pCMV-FAK (25 µg) and pCMV-FRNK (35 µg) in a
final volume of 70 µL. The same relative amounts of pCMV-FAK (25 µg) / pCMV
(45 µg) plasmid were injected in the contralateral, left muscle.

Harvesting of soleus muscle – Rats were anesthetized in 3% isoflurane, weighed and a
circular incision was drawn around the heel just above the calcaneus followed by a
long medial cut from the heel towards the knee. The musculature of the lower leg was
exposed by stripping the skin towards the knee. The two tendons that insert distally
into the lateral and medial head of the gastrocnemius muscle were clamped with a pair
of forceps and then capped with a scalpel. Subsequently, the triceps group was turned
over the knee heel with the forceps; exposing the reddish soleus muscle lying on top
of the triceps group as a single compartment. Finally the proximal tendinous end of
the soleus muscle under the knee heel was capped, and the muscle frozen within 20
seconds in liquid nitrogen-cooled isopentane while hanging down on a pair of forceps.
Euthanasia of the anesthetized animals was carried out by dislocation of the cervical
vertebrae and rapid exsanguination.

Stretch of soleus muscle in vivo- Soleus muscles was exposed as described for muscle
harvesting while the proximal tendon was left intact. Using a pair of forceps the
muscle was pulled once for 2 seconds via its distal tendon to approximately 1.2-times
its resting length. The proximal tendon of the soleus was capped and the muscle
frozen within 20 seconds in nitrogen-cooled isopentane.

Immunodetection of FAK – Staining of FAK was carried out histochemically on 12-
micrometer cryosections with FAK N-terminal antibody A-17 (Santa Cruz
Biotechnology) and horseradish peroxidase-coupled secondary antibody against rabbit
from goat (ICN Biochemicals), essentially as described by Fluck et al (2002). Signal was recorded digitally at 10x magnification on an Axioskop 2 microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). The percentage of muscle fibers with immunoreactivity against the FAK N-terminus was examined as described (12). Co-staining of FAK and vinculin was carried out using FAK N-terminal antibody A-17 and a monoclonal vinculin antibody (14) flowed by secondary, Alexa-Fluor 488 and Alexa-Fluor 555 labeled antibodies (12, 27). Fluorescent signal was recorded in separate channels using a TCS SP5 confocal microscope operated by Leica Application Suite software (LAS; Leica Microsystem CMS, Milton Keynes, UK).

Fiber typing – Cross-sections of soleus muscle were stained for type I and type II myosin heavy chain, respectively, using immunohistochemistry and evaluated for the distribution of type I, type II and hybrid type (i.e. type I/type II positive) muscle fibers as described (12).

Analysis of protein content – Proteins were analyzed essentially as described (12, 14, 27). In brief, 20 microgram protein from the homogenization of cryosections in modified RIPA buffer was separated using SDS-PAGE, western blotted onto nitrocellulose membrane (Schleicher & Schuell) and subjected to immunodetection with specific antibodies against gamma-vinculin and meta-vinculin (gift of Dr. M. A. Glukhova, Paris, FRANCE), the FAK C-terminus (gift of Prof. Andrew Ziemiecki, University of Berne, Switzerland), alpha 7 integrin (40) or beta 1 integrin (AB1952, Millipore). Protein transfer was verified by staining the membrane with Ponceau S. Content of FAK-pY397 was assessed in the supernatant from 1-milligram total protein homogenate using immunoprecipitation as described (27). For the analysis of transplanted muscles a deoxycholate buffer (50 mM Tris-HCl pH 7.8, 2% deoxycholate, 2 mM EDTA, 5 mM N-ethyl maleimide, 2 mM phenyl-methyl-sulfonyl fluoride) was used to extract FRNK from cryosections as described (15). Signal detection was carried out with enhanced chemoluminescence using a Geldoc system that was operated using Quantity One 1-D analysis software 4.6.1 (Bio-Rad Laboratories Ltd, Hemel Hempstead, United Kingdom). The molecular weight of the detected proteins was compared against molecular weight markers. Signal intensity of the protein bands was determined using the rectangle density mode and background
from an empty sample lane of equal size was subtracted. The pixel resolution was 4096 levels of gray per pixel of 0.316 x 0.316 mm² in size. Samples were loaded in a specific design to reduce variability when pooling data from separate experiments. For this purpose samples from control interventions (i.e. mock controls for tenotomy, empty plasmid transfection controls from the contralateral leg) were analyzed on each immunoblot. Background-subtracted signal intensities for the protein band of interest on a given immunoblot were normalized to the mean signal for the respective control intervention. Subsequently normalized signal intensities (being proportional to relative protein content) from the different immunoblots were pooled and subjected to statistical analysis.

**Statistics:** Statistical significance was assessed using Statistica 9 (Statsoft Inc, Tulsa, USA). An ANOVA design with post-hoc test of Fisher was employed to assess the statistical significance of differences. The effect of FAK overexpression on soleus-to-body weight and protein content was assessed with a two-way ANOVA for the factor ‘loading condition’ (cage control, unloading, 1 and 5 days of reloading) and the repeated factor of plasmid transfection (‘pCMV’, ‘pCMV-FAK’). A one-way ANOVA was employed to test the effect of ‘loading condition’ on percentage changes in protein content between ‘pCMV’ and ‘pCMV-FAK’ transfected muscles. The effect of FRNK co-expression was assessed with a one-way ANOVA for the repeated factor of plasmid transfection between muscle pairs (‘CMV-FRNK / pCMV-FAK’, ‘CMV / pCMV-FAK’). A one-way ANOVA was employed to test the effect of ‘loading condition’, or tenotomy duration, on the content of FAK, FAK-pY397, and costamere component content, respectively, or to test the effect of transplantation on fast fiber type percentage. Linear relationships were assessed based on Pearson correlations. Differences were called significant at a p-value < 0.05.

**Results**

*Time course of FAK and FAK-pY397 regulation by stretch overload* – We assessed the temporal response of FAK-Y397 phosphorylation and protein content to muscle stretch. FAK-pY397 content per total muscle protein was 1.9-fold increased after 20
seconds of muscle stretch (Fig. 1B). FAK-pY397 content was elevated after 1 and 24 hours of stretch overload by bilateral tenotomy (Fig. 1C/E). The early increase in FAK-pY397 content was explained by a specifically elevated phosphorylation per FAK protein. By contrast the increase after 24 hours of tenotomy was due to a progressive up-regulation of FAK protein content (Fig. 1D-F). FAK-pY397 content was reduced after 1 hour of mock tenotomy and did not recover until 24 hours after the mock intervention (Fig. 1F). This response differed significantly to the response with tenotomy (p=0.04). Table 1 shows the soleus-to-body weight ratio with tenotomy.

Studies in the hindlimb suspension-reloading model demonstrated that muscle loading regulated FAK protein levels in an on/off manner. FAK concentration was 60% reduced after 14 days of unloading by hindlimb suspension and increased 144% within the first day of reloading (Fig. 2B). The content of both vinculin isoforms was down-regulated with unloading; one day of reloading established the normal content of gamma-vinculin (Fig. 2C).

**FAK overexpression enhances costamere protein levels in skeletal muscle** - We assessed the consequences of increased FAK content on costamere protein content. Using somatic transfection we introduced the expression plasmid pCMV-FAK for the native chicken FAK homologue in soleus muscle. An average of 12% of soleus muscle fibers demonstrated FAK overexpression (Fig. 3A) in line with previous observations (12). FAK signal primarily localized at the sarcolemma (Fig. 3B). FAK content in soleus muscle of rats kept under normal cage conditions was 62%-increased 7 days after transfection with the pCMV-FAK plasmid compared to empty pCMV plasmid-transfected contralateral soleus muscle (Fig. 3C/D). FAK overexpression elevated the content of the costamere components beta 1 integrin (+56%) and meta-vinculin (+88%) compared to empty plasmid transfection controls (Fig. 3E/G). Alpha 7 integrin (p=0.46) and gamma-vinculin levels (p=0.18) were not altered in rats housed under normal cage conditions (Fig. 3F,H).

Table 2 summarizes the effect of FAK overexpression and muscle unloading and reloading on soleus-to-body weight ratio. Soleus-to-body weight ratio was 12% higher in pCMV-FAK compared to pCMV transfected soleus muscle (p<0.05) and was correlated to levels of gamma-vinculin (r=0.76, p=0.01).
**Inhibition of focal adhesion turnover by FRNK promotes slow-to-fast fiber transformation** - We assessed the implication of focal adhesion turnover to the changes in costamere component expression with FAK overexpression. Co-transfecting of pCMV-FAK with the plasmid for the inhibitor of focal adhesion turnover, pCMV-FRNK, elevated FRNK protein from virtually non-detectable levels in control-transfected soleus muscle (Fig. 4A/E). FAK-pY397 content was reduced by FRNK overexpression (Fig. 4G) and the percentage of fibers showing immune reactivity against the FAK N-terminus was increased (Fig. 4F). The assessed costamere components were not significantly affected by FRNK co-expression (Fig. 4E, H-J).

We have reported that FAK overexpression reduces the percentage of hybrid slow/fast type fibers in slow soleus muscle that arise after gene electro-transfer (Fig. 5;(12). FRNK co-expression did increase the percentage of fast type fibers compared with FAK overexpression alone and reduced the percentage of hybrid fibers compared to the transfection with empty plasmid (Fig. 5A). In untreated rats, expression of both FRNK and meta-vinculin was readily detectable in the fast type muscle *extensor digitorum longus* (EDL) but not the slow type soleus muscle of rats (Fig. 5B,C). Slow-to-fast fiber transformation of soleus muscle induced by its transplantation into the bed of the EDL elevated FRNK and meta-vinculin levels (Fig. 5D).

**FAK mediated costamere component expression is load dependent** – 7 days of unloading and subsequent reloading reduced and increased, respectively, FAK-pY397 content and N-terminal FAK immune reactivity at the sarcolemma in soleus muscle which overexpressed FAK protein as reported (12). Load regulated changes in FAK-pY397 in soleus muscle were paralleled by changes in beta 1 integrin content (Fig. 6A). Similarly, the content of meta-vinculin and gamma-vinculin was reduced with unloading in FAK overexpressing muscle and this down-regulation faded with continued reloading (Fig. 6B/C). The mean fold changes in the content of pY397 phosphorylated FAK over all loading conditions and transfected muscles were correlated to the fold changes in meta- (r=0.96) and gamma-vinculin content (r=0.97), respectively. Equally, gamma-vinculin levels co-related to soleus-to-body weight over all sample points (r=0.62, p<0.001; Fig. 6D).
Skeletal muscle size demonstrates a pronounced dependence on muscle loading (20, 34) and this probably involves costameres as these anchor myofibrils and initiate mechano-transduction through post-translational activation of phospho-transferases (45, 47). It is however not known through which mechanism load-modulated muscle atrophy and hypertrophy is associated with the regulation of costamere components in intact skeletal muscle. Specifically it is not understood how acute post-translational regulation of focal adhesion kinase (FAK) on tyrosine 397 - which serves as readout for integrin-associated mechano-transduction (28, 32, 53) - relates to the reported changes in FAK content with prolonged changes in muscle loading (14, 17, 21); and how this may come into play in the regulation of costamere component expression. To this end, we measured the relationship between post-translational regulation and expression of FAK in the tenotomy model of rat soleus muscle overload. FAK and the FAK inhibitor, FRNK, as specificity control were overexpressed in soleus muscle and muscles were analyzed for subsequent changes in costamere components content in the hindlimb unloading-reloading model. Indicated relationships between FAK / FRNK and fiber type transformation were verified in transplantation experiments.

We identify that regulation of the costamere markers, beta 1 integrin and meta-vinculin, and FAK itself, by muscle unloading and overloading is reversible in rat soleus muscle (Fig. 2) and is amplified by increases in FAK content imposed by somatic gene transfer (Fig. 3 and 6). Thereby the effects of FAK overexpression on meta-vinculin content were correlated to those of the proxy of costamere turnover, FAK-pY397, and modified by the inhibitor of FAK mediated focal adhesion turnover, FRNK. As well we noted that meta-vinculin content demonstrated a trend for a 48%-increase (p=0.07) with FRNK co-overexpression (Fig. 4H) and found that increased FRNK and meta-vinculin levels relate to slow-to-fast transformation in transplanted muscle (Fig. 5). Microscopic analysis resolved that the effect of FRNK co-overexpression was related to an increased percentage of fast fiber types.

An interesting point of our observation was that FAK-driven elevation of the beta 1 subunit of integrins was not matched by level alterations by alpha 7 integrin (compare Fig. 3E/F). Alpha 7 integrin is considered to represent the major binding partner of
beta 1 integrin at the sarcolemma (reviewed in 37, 40). This is highlighted by the finding that alpha 7 is strongly up-regulated with differentiation of myoblasts towards a syncytial myotube when the other binding partner of beta 1, the integrin alpha 5 subunit, is down-regulated (19). A possible explanation for the unaffected levels of integrin alpha 7 in our experiments therefore may be that this reflects the primary localization of overexpressed FAK proteins to the sarcoplasma of muscle fibers (Fig. 3A) and not satellite cells that correspond to the myoblast pool.

Analysis of the time course of Y397 phosphorylation of FAK in rat soleus muscle after stretch overload (Fig. 1) shows that regulation of FAK by mechanical stress has two phases: an early increase in specific Y397 phosphorylation detectable after seconds and one hour, but not 6 hours, of mechanical stress (Fig. 1B/G); and a later increase in FAK-pY397 content driven by elevated FAK content (Fig. 1E/H). These observations point out that FAK is indeed activated early after mechanical stimulation of skeletal muscle (44). The increase in FAK-pY397 content distinguished to its down-regulation with the controlled mock intervention (Fig. 1D/I). We have not identified the mechanism underlying this effect. Possibly this reflects an interaction between the minimally invasive surgery and anesthesia with the associated tissue ischemia known to reduce FAK phosphorylation and content in a later phase (56). By contrast the early responsiveness of FAK-pY397 content relates to the recently reported stimulation of FAK phosphorylation at Y576/Y577 with massage therapy (8); thus emphasizing that early post-translational regulation of FAK in intact skeletal muscle has a low threshold. The observations imply a new paradigm of how feed forward regulation of FAK-pY397 content by mechanical loading is achieved.

Y397 phosphorylation of FAK has been postulated to relate to a conformational change in FAK’s N-terminus that enables this non-receptor tyrosine kinase to interact with binding partners (41, 49). We now show that the co-expression of the inhibitor of FAK’s binding to integrins, FRNK (24), reduces FAK-pY397 content in relation to an increased count of fibers showing N-terminal immune reactivity for FAK (Fig. 4F vs. G). This observation is of interest in regard to the trend for an accentuated increase in the content of meta-vinculin (+48%, p=0.07) with FRNK and FAK co-overexpression (Fig. 4H-J). FRNK expression increases focal adhesions in culture with (24) and FAK-mediated interaction with vinculin regulates focal adhesion...
 assembly (reviewed in 29, 31, 43, 55). We interpret our findings to suggest that
FRNK decouples the conformation change in FAK’s N-terminus and Y397
phosphorylation, thereby possibly slowing down its turnover and prolonging the half-
life time of focal adhesions.

The role of FRNK in control of meta-vinculin levels was corroborated by a
correspondence in their protein content in non-transfected muscles (Fig. 5B-E). As
pointed out from the comparison between the slow soleus with the fast extensor
digitorum longus muscle and transplantation experiments, this relationship was found
to reflect the content of fast type fibers. Gene electro-transfer produces a cycle of
regeneration of muscle fibers that increases fast type myosin expression (12, 46) and
which likely involves increased turnover of focal adhesion due to remodeling of the
extracellular matrix (25). An association between meta-vinculin with fast muscle fiber
has been reported before (6). The influence of FRNK on features of the fast fiber type
content has, however, to our knowledge not been reported. The present novel
observation thus points out a new molecular paradigm whereby FAK and the
endogenous gene product, FRNK work in opposition to control aspects of the
contractile muscle phenotype. The mechanism by which this connects to the
suggested regulation of costamere turnover and attached myofibrils remains to be
addressed.

It is now understood that FAK is part of the physiological pathway governing load-
dependent regulation of the hypertrophic phenotype in a number of musculoskeletal
tissues (10, 35, 52). Our study emphasizes that load-dependent regulation of muscle
weight is paralleled by adjustment in the content of FAK and the costamere
component gamma-vinculin (Fig. 2). This is underscored through an interaction effect
of FAK overexpression and muscle (un)loading on gamma-vinculin content (Fig. 6C)
and a correlation between gamma-vinculin content and body weight-related soleus
muscle weight (Fig. 6D). We see these observations to reflect the force-sensitive
maturation process of cell adhesion that has been reported to involve the load-
dependent recruitment of vinculin to focal adhesions in culture (22). Our observations
imply that FAK content controls this phenomenon. We identify that FAK-mediated
regulation of costamere components involves expression control of beta 1-integrin
and meta-vinculin content under normal loading conditions (Fig. 3) and interacts with
the loading state (Fig. 6). The time course during which this occurred is compatible with a role of mechanical stress in regulation of the lifetime of focal adhesions (22). Together with the relationship between the content of FAK-pY397 and costamere components this implies that the load-modulated content of FAK-pY397 acts as a force-sensitive switch for costamenergenesis in muscle fibers.

**Conclusions:** Content and Y397 phosphorylation of the integrin-associated FAK and its inhibitor FRNK mediate load- and fiber type dependent expression of costameres components.

**Perspectives and Significance**

Using a systematic approach, we provide evidence that FAK Y397 phosphorylation is a force-sensitive switch corresponding to changes in costamere component expression and muscle mass. The rapid and chronic nature of FAK regulation and its fiber type-related control by the inhibitor FRNK emphasize its implication in mechano-regulation of the contractile muscle phenotype. This highlights the possibility that load-regulated control of costamere content signifies the tuning and coordination of myofibril attachment during situations of altered muscle use.

**Author’s contribution**

Conception and design of research: MF, ACD; Performed experiments: MF, SK, RL, SR, ACD; Analyzed data: MF; Interpreted results of experiments: MF, SK; Prepared figures: MF; Drafted manuscript: MF; Edited and revised manuscript: MF; Approved final version of manuscript: MF, SK, RL, ACD, SR.

**Acknowledgements**

We are grateful for the proficient help of Aurélien Frobert, Céline Ferrié and members of the Department of Cardiovascular Surgery, University Hospital of Berne, Switzerland during the animal experiments, Professor Markus Müntener for access to
muscles samples from transplantation experiments, and Hans Degens for the
 provision of rat muscle sample in relaxing buffer. We thank Prof Ulrike Mayer for the
 provision of antibody against alpha 7 integrin. This study was financially supported
 by a grant from the Région Rhône-Alpes (France). The authors acknowledge financial
 support through the Swiss National Science Foundation (grant number 112139).
 We declare a conflict of interest in relation to patent application “Regulation of
 Muscle repair” (US2009/0280103 A1).

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Figure captions
Figure 1: Mechano-regulation of FAK tyrosine phosphorylation in rat soleus muscle.

A) Example of the detection of FAK-pY397 content in immunoprecipitates from 500 microgram protein in RIPA extract of a soleus muscle of a control rat without (lane 1) and with first antibody against FAK-pY397 (lane 2, CTL) as detected with immunoblots using FAK primary antibody. The respective position of the detected pY397-phosphorylated FAK protein (FAK-pY397, top) and immunoglobulin (IgG bottom) is indicated. B) Example of FAK-pY397 content in two rat soleus muscles harvested without stretch (CTL; lanes 1 and 2) or 20 seconds after stretch (S; lanes 3, 4). C) Example of the change in FAK-pY397 content in soleus muscle vs. control during the first 24 hrs of tenotomy. D) Changes in FAK-pY397 content in soleus muscle after 1, 6 and 24 hours of mock tenotomy vs. control (CTL). E, F) Example of the detection of FAK content in rat soleus muscle during the first 24 hrs of tenotomy (E) and its mock control (F). Images stem from two immunoblots and are separated by a line. G-J) Mean and s.e.m. of FAK-pY397 content per total protein (G, H) and FAK content per total protein (I, J) at different time points during the first 24 hours after bilateral tenotomy (G, I) or mock tenotomy (H, J) compared to controls (CTL). * and ** indicate p< 0.05, and p < 0.01 vs. CTL. $$ indicates p<0.005 vs. ‘+24 h tenotomy’ (one-way ANOVA with post-hoc test of Fisher, n=4-8).

Figure 2: Regulation of costamere protein component levels by muscle unloading and subsequent reloading. A,B) Representative immunoblots visualizing FAK protein, and vinculin isoform content as a function of muscle loading. Two biological replicas per condition are shown. MW, molecular weight marker. The positions of 100 and 150-kDa being indicated. C-E) Mean and s.e.m. of FAK (C), gamma-vinculin (D) and meta-vinculin (E) per total protein in soleus muscle of cage control (CTL), 14 days suspended (HU14) and 1 day reloaded (HUR1) rats. Asterisk and $ indicate p < 0.05 vs. CTL and HU14, respectively (two-tailed T-test, n=4-6).

Figure 3: Effect of FAK overexpression on costamere protein levels in cage controls. A,B) Confocal image of C-terminal FAK (red) and vinculin (yellow) staining in a rat soleus muscle 7 days after gene electro-transfer with pCMV-FAK expression construct. Arrows point to examples of fibers demonstrating FAK overexpression. Bar designates 50 micrometer. C) Representative immunoblots visualizing the detected FAK, gamma- and meta-vinculin and beta 1 integrin in 20 microgram total protein of
a muscle pair transfected with pCMV-FAK (right muscle) or empty pCMV construct
(left muscle) of a same rat. D-H) Mean and s.e.m. of the protein levels of FAK (D),
beta 1 integrin (E), the light chain of the processed alpha 7 integrin variant (F), meta-
vinculin (G) and gamma-vinculin (H) per total protein in pCMV-FAK- and pCMV-
transfected contralateral muscle pairs in cage controls. Asterisks indicate p < 0.05 vs.
pCMV-transfected muscle (one-way ANOVA with post-hoc test of Fisher, n=6).

Figure 4: FRNK co-overexpression enhances costamere protein levels. A-C)
Representative immunoblots visualizing the effect of double transfection of soleus
muscle with expression construct for FRNK (pCMV-FRNK) together with pCMV-
FAK compared to co-transfection with empty pCMV and pCMV-FAK on
FAK/FRNK content (A), vinculin and integrin (B), FAK-pY397 content and IgG (C).
D) Loading control showing the Ponceau S stained membrane. E-J) Mean and s.e.m.
of the level of FRNK and FAK (E), N-terminal immunoreactivity of FAK (F), FAK-
pY397 content (G), meta- and gamma-vinculin (H), beta 1 integrin (I) and alpha 7
integrin (J) in soleus muscle after pCMV-FRNK or pCMV co-transfection in pCMV-
FAK-transfected soleus muscle pairs. Grey and black bars, respectively, denote ‘pCMV-FAK + pCMV’ and ‘pCMV-FAK + pCMV-FRNK’ transfected muscles,
respectively. Asterisks and + denote p<0.05 and 0.05 ≤ p< 0.10, respectively, for
differences between soleus muscle pairs (repeated two-way ANOVA with post-hoc

Figure 5: FRNK expression promotes fast muscle phenotype. A) Bar graph visualizing
the mean + s.e.m. of type I, type II and hybrid fiber type percentage in differently
transfected soleus muscles. B,C) Vinculin isoform (B) and FAK/FRNK (C)
expression in soleus (SOL), extensor digitorum longus (EDL) and transplanted soleus
(T-SOL). Respective loading controls visualizing the position of sarcomeric alpha
actin on the Ponceau S stained membrane are included at the bottom of each panel.
D,E) Mean + s.e.m. of relative meta-vinculin (D) and FRNK content (E) in the
respective muscle types. N=3-6. * denotes p < 0.05 for the indicated comparisons
(one-way ANOVA with post-hoc test of Fisher).

Figure 6: Loading modifies the effect of FAK overexpression. A-C) Mean and s.e.m.
of the difference in protein levels of beta 1 integrin (A), meta-vinculin (B) and
gamma-vinculin (C) per total protein in pCMV-FAK- vs. pCMV-transfected contralateral muscle pairs (i.e. FAK overexpression) in cage controls (while bar), 7 days unloaded (black bar), 1 day reloaded (light gray) and 5 days reloaded soleus muscle (dark gray). Asterisks and + denote p<0.05 and 0.05 ≤ p< 0.10, respectively, for the changes between pCMV-FAK vs. pCMV transfected muscle (repeated two-way ANOVA with post-hoc test of Fisher). N=4-6. † denotes p < 0.05 in changes ‘pCMV-FAK vs. pCMV’ between the indicated loading states (one-way ANOVA with post-hoc test of Fisher). D) Graph visualizing the correlation between soleus-to-body weight and gamma-vinculin over all muscles examined. Diamonds indicate the values form cage controls demonstrating an r-value of 0.76.
E

Tenotomy

CTL  +1h  +6h  +1h  +24h

FAK  actin

F

Mock tenotomy

CTL  +6h  +24h

FAK  actin
**A**

[Image: Immunofluorescence microscopy of cells showing red and yellow fluorescence with white arrows indicating specific regions.]

**B**

[Image: Another fluorescence microscopy image with red fluorescence and white arrows pointing to specific areas.]
Figure 3

**D** FAK

**E** beta 1 integrin

**F** alpha 7 integrin

**G** meta-vinculin

**H** gamma-vinculin
A

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4
**E**

Protein content per total protein

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<td>80%</td>
<td>100%</td>
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- pCMV-FAK + pCMV
- pCMV-FAK + pCMV-FRNK

**F**

Immunoreactivity of FAK N-terminus [% fibres]

- FAK-pY397

**G**

FAK-pY397 [% fibres]

- FAK+pCMV [pCMV=1]

**H**

Protein [per total protein]

<table>
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<th>gamma-</th>
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<td>200%</td>
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- Meta-vinculin
- Gamma-vinculin

**I**

Protein [per total protein]

<table>
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**J**

Protein [per total protein]

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A. **beta 1 integrin**

![Bar chart showing % change of beta 1 integrin](image)

B. **meta-vinculin**

![Bar chart showing % change of meta-vinculin](image)

C. **gamma-vinculin**

![Bar chart showing % change of gamma-vinculin](image)

D. **Soleus-to-body weight**

![Scatter plot showing correlation between gamma-vinculin and soleus-to-body weight](image)

- *: p<0.05
- †: p<0.01

**Beta 1 integrin**

- pCMV-FAK vs. pCMV:
  - 40% increase
  - 30% increase
  - 20% increase
  - 10% increase
  - 0% increase
  - 10% decrease
  - 20% decrease
  - 30% decrease

**Meta-vinculin**

- pCMV-FAK vs. pCMV:
  - 100% increase
  - 50% increase
  - 0% increase
  - 50% decrease
  - 100% decrease

**Gamma-vinculin**

- *: p<0.05
- †: p<0.01

**Soleus-to-body weight**

- r=0.62, p<0.001
Table 1: Soleus-to-body weight ratio with tenotomy.

Mean ± s.e.m. and p-value of differences are given for each loading condition. * indicates p< 0.05 vs. CTL. (Two-way ANOVA with post-hoc test of Fisher, n=4-8).

<table>
<thead>
<tr>
<th>Soleus-body [mg/g]</th>
<th>CTL</th>
<th>+1 h</th>
<th>+6 h</th>
<th>+24 h</th>
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<tr>
<td>mock</td>
<td>0.49 ± 0.01</td>
<td>0.51 ± 0.02</td>
<td>0.53 ± 0.03</td>
<td>0.58 ± 0.01 *</td>
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<tr>
<td>tenotomy</td>
<td>0.50 ± 0.02</td>
<td>0.54 ± 0.04</td>
<td>0.57 ± 0.03 *</td>
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Table 2: Effect of FAK overexpression on soleus-to-body weight ratio.

Mean + s.e.m. are represented. * indicates p< 0.05 vs. pCMV of the respective loading condition. $ indicates p < 0.05 vs. CTL of the respective transfection condition. p < 0.001 for the interaction effect of ‘loading condition’ x ‘pCMV/pCMV-FAK transfection’. Two-way ANOVA with repeated factor of pCMV/pCMV-FAK transfection and post-hoc test of Fisher, n=4-8.

<table>
<thead>
<tr>
<th>Soleus-body [mg/g]</th>
<th>CTL</th>
<th>HU7</th>
<th>HU7R1</th>
<th>HU7R5</th>
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<tr>
<td>pCMV</td>
<td>0.41 ± 0.03</td>
<td>0.30 ± 0.02 $</td>
<td>0.34 ± 0.02 $</td>
<td>0.47 ± 0.02</td>
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<tr>
<td>pCMV-FAK</td>
<td>0.48 ± 0.02 *</td>
<td>0.31 ± 0.03 $</td>
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<td>p-value</td>
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