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Eur J Cell Biol. Author manuscript; available in PMC 2012 February 1.

Published in final edited form as:

Eur J Cell Biol. 2011 ; 90(2-3): 249–260. doi:10.1016/j.ejcb.2010.06.005.

Up-regulation of Paxillin and Focal Adhesion Signaling follows Dystroglycan Complex deletions and promotes a Hypertensive State of Differentiation

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Abstract

Anchorage to matrix is mediated for many cells not only by integrin-based focal adhesions but also by a parallel assembly of integral and peripheral membrane proteins known as the Dystroglycan Complex. Deficiencies in either dystrophin (*mdx* mice) or γ-sarcoglycan (γSG−/− mice) components of the Dystroglycan Complex lead to upregulation of numerous focal adhesion proteins, and the phosphoprotein paxillin proves to be among the most prominent. In *mdx* muscle, paxillin-Y31 and Y118 are both hyper-phosphorylated as are key sites in focal adhesion kinase (FAK) and the stretch-stimulatable pro-survival MAPK pathway, whereas γSG−/− muscle exhibits more erratic hyper-phosphorylation. In cultured myotubes, cell tension generated by myosin-II appears required for localization of paxillin to adhesions while vinculin appears more stably integrated. Over-expression of wild-type (WT) paxillin has no obvious effect on focal adhesion density or the physical strength of adhesion, but WT and a Y118F mutant promote contractile sarcomere formation whereas a Y31F mutant shows no effect, implicating Y31 in striation. Self-peeling of cells as well as Atomic Force Microscopy (AFM) probing of cells with or without myosin II inhibition indicate an increase in cell tension within paxillin-overexpressing cells. However, prednisolone, a first-line glucocorticoid for muscular dystrophies, decreases cell tension without affecting paxillin at adhesions, suggesting a non-linear relationship between paxillin and cell tension. Hypertension that results from upregulation of integrin adhesions is thus a natural and treatable outcome of dystroglycan complex down-regulation.

Keywords

adhesion; contractility; differentiation; paxillin; muscular dystrophy; hypertensive; glucocorticoid

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INTRODUCTION

Tissue cells not only attach to but also pull on matrix as part of 'tactile' signaling mechanisms (Discher *et al.*, 2005). Myosins invariably provide the pulling force in establishing a cytoskeletal tension, and cell anchorage generally occurs *via* the well-studied integrin-based focal adhesion system but *also* – in many cell types – *via* the dystroglycan complex (DGC). Identified first in myocytes (Campbell, 1995), the DGC is increasingly understood to be used by many cells (Campbell, 1995; Muschler *et al.*, 2002) for anchorage to basal lamina. Integrin↔DGC signaling appears bidirectional (Yoshida *et al.*, 1998), and yet the interplay with cell tension and contractility is unknown, as is any impact on cell differentiation.

The DGC linkage between the cytoskeleton and the extracellular matrix (ECM) is often perturbed or disrupted in muscular dystrophies (MD). Myoblasts are relatively unaffected because the DGC is expressed only in post-fusion, non-dividing myotubes, but tension-induced damage to the mature muscle membrane ultimately causes muscle weakness, massive degeneration, and premature death (Campbell, 1995; Straub and Campbell, 1997; Lim and Campbell, 1998; Cohn and Campbell, 2000). Importantly, in both dystrophin-deficient patients (Duchenne Muscular Dystrophy) as well as in dystrophin-deficient *mdx* mice, the contractile myotubes partially compensate for the lack of an intact DGC by up-regulating integrins, particularly α7β1 (Figure 1A, right sketch) (Hodges *et al.*, 1997). An intermediate level of compensation occurs with deficiency of the DGC component γ-sarcoglycan, leading to what also appears to be a more apoptotic phenotype (Griffin *et al.*, 2005). Intentional over-expression of α7β1 has proven protective (Yoshida *et al.*, 1998; Allikian *et al.*, 2004; Burkin *et al.*, 2005), but whether this is strictly from stabilizing transmembrane force transmission or also from the recruitment of additional cytosolic proteins to the integrin complex has not yet been addressed.

Talin, α-actinin, and perhaps filamin contribute scaffolding roles in integrin-based Focal Adhesions (FA), whereas other components such as paxillin, vinculin, and FAK diffuse in and out as part of a phospho-Tyrosine based signaling nexus (Panetti 2002; Shemesh *et al.*, 2005; Zaidel-Bar *et al.*, 2007; Pasapera *et al.*, 2010). Essential for embryonic development (Furuta *et al.*, 1995; Xu *et al.*, 1998; Hagel *et al.*, 2002; Charlesworth *et al.*, 2006), FA-derived signals promote assembly of cytoskeletal tension structures such as stress fibers and also propagate cell survival signals into the MAPK pathway (Turner, 2000; Hagel *et al.*, 2002; Brown and Turner, 2004) with activation of ERK (Fluck *et al.*, 1999; Turner, 2000; Most *et al.*, 2003; Schaeffer *et al.*, 2003; Lunn and Rozengurt, 2004; Melendez *et al.*, 2004; Mizukami *et al.*, 2004; Subauste *et al.*, 2004; Lin *et al.*, 2005; Palfi *et al.*, 2005; Vittal *et al.*, 2005; Das *et al.*, 2006; Peng *et al.*, 2006; Wei *et al.*, 2006) – which is already known to be enhanced in stretched *mdx* muscle (Kumar *et al.*, 2004) and in $\gamma SG^{-/-}$ muscle (Griffin *et al.*, 2005). In maturing myotubes, FAs are the nucleation sites for myofibrillogenesis (McKenna *et al.*, 1986; Sanger *et al.*, 2002) during which extensive cytoskeletal remodeling ultimately replaces non-muscle myosin-II (NMM-II) mini-filaments with the contractile striations of skeletal muscle myosin-II (Figure 1A). Given the upregulation of integrins in muscular dystrophies, as well as the known enrichment of filamin at the sarcolemma of both *mdx* and $\gamma SG^{-/-}$ mice (Thompson *et al.*, 2000), we hypothesized that additional FA components would also be modulated and would influence downstream outputs ranging from cell tension and myofibrillogenesis to viability. Transcript profiles of *mdx* versus normal muscle indeed hint at increases in paxillin (+15%; see Table S1A) as well as other components, such as vinculin (+35%) and γ-actin, and the latter has recently been shown to be elevated at the protein level (Hanft *et al.*, 2006). However, signaling and phenotype depend on the protein levels, post-translational modifications, and collective interactions with feedback loops in and between signaling networks. Here we demonstrate – as part of compensatory

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mechanisms within mouse dystrophic muscle – a major upregulation of paxillin and adhesive signaling that promotes general contractility, as confirmed by ectopic expression studies. We use micropatterned strips of collagen with finite length that standardize cell shape and permit novel studies of cell adhesion, and then we employ Atomic Force Microscopy to probe cell stiffness – comparing to cells relaxed by a myosin inhibitor – in order to determine an effective cell tension that relates to the adhesion-cytoskeleton state. Changes in signaling that are coupled to structure could suggest new interventions or better understanding of current therapeutic interventions, as illustrated here with initial data on the pro-relaxant activities of a major, clinical glucocorticoid.

RESULTS

Adhesive-contractile signaling is upregulated in muscular dystrophies

Tissue lysates from dystrophic *mdx* and $\gamma SG^{-/-}$ muscle show paxillin to be upregulated compared to normal muscle in Western blots (Figure 1B). α7-integrin was confirmed to be up in both dystrophic cell types (Hodges *et al.*, 1997), but paxillin overexpression appears even higher at about 4-fold in *mdx* and above 3-fold in $\gamma SG^{-/-}$ muscle (+300% and +230%, respectively). Vinculin was also significantly elevated in *mdx* muscle (+84%; see Table S1B) although FAK was unchanged (not shown). Immunostaining of paxillin in normal and *mdx* muscle sections showed paxillin localization at the sarcolemma with perhaps more paxillin in *mdx* cells both at the membrane and throughout the cytosol (Figure 1C).

A number of (P)-Tyr sites on paxillin are known to be modified by cell stretch (Yano *et al.*, 1996; Sokabe *et al.*, 1997), shear stress (Cuvelier *et al.*, 2005), and osmotic shock (Hirakawa *et al.*, 2004). Screening of muscle tissue lysates for 20 phospho-sites within and beyond the paxillin-FAK-MAPK signaling nexus shows that relative levels of phosphorylation in *mdx* versus normal muscle vary from higher to lower (Figure 2), with quantitative changes made explicit in the step plots as red solid lines. Both *mdx* and $\gamma SG^{-/-}$ dystrophic muscle types show significant hyperphosphorylation (beyond +25%) of (P)-ERK1/2, (P)-FAK, and (P)-MAPKAPK2^{T222}. The greatest hyperactivation is found for (P)-ERK1, which is up 2.8-fold in resting *mdx* and 2.3-fold in $\gamma SG^{-/-}$ muscle, while (P)-FAK^{Y577} is up almost as high in both *mdx* and $\gamma SG^{-/-}$ muscle. Some phosphoprotein levels such as (P)-PKC-α ^{S657}, -ε^{S729} change little in both dystrophies whereas others are consistently and significantly decreased (below −25%) in both, especially (P)-MAPKAPK2^{T334} and (P)-Adducin-γ^{S662}. For paxillin, both (P)-paxillin^{Y118} and (P)-paxillin^{Y31} are increased more than 2-fold in *mdx* but unaffected in $\gamma SG^{-/-}$ muscle. Additional signaling molecules such as CREB also show activation in *mdx* tissue (+1.9-fold) but remain unaltered in $\gamma SG^{-/-}$ muscle.

Similarities and differences in relative activation of the paxillin-FAK-MAPK nexus were formally assessed with a simple cross-correlation analysis. Briefly, maximum activation among all proteins in the results of Fig. 2 is set to +1 (i.e. ERK, which is about 4-fold above Normal for *mdx* becomes +1), and the maximum deactivation is set to −1 (i.e. MAPKAPK2). The re-scaled result for each phosphoprotein in a set (eg. mdx_{ERX} = +1) is then multiplied by all of the others within a pairwise set (eg. mdx_i x mdx_j) and this is averaged over the set to obtain the cross-correlation coefficient, φ. If all proteins are up (or down) to the same extent relative to Normal cells, then they are correlated and $\varphi = +1$; anti-correlations ($\varphi = -1$) or null correlations ($\varphi = 0$) might also emerge. For *mdx* muscle, $\varphi =$ +0.35 indicates a general hyperactivation for the selected set of signaling pathways; whereas for $\gamma SG^{-/-}$ muscle, $\varphi = +0.16$ indicates less overall activation for the same set (Table 1). The difference occurs despite similar upregulation of α7-integrin (~2-fold per Figure 1C). The cross-correlation between $\gamma SG^{-/-}$ and *mdx* is small (in absolute value) but negative, indicating a weak anti-correlation. This difference suggests different pathways are involved

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in the increased (P)-ERK1 in γSG$^{-/-}$ mice, while the smaller φ suggests a moderating effect of the residual DGC complex in signaling.

Since the paxillin-FAK-MAPK nexus is implicated in mechanoactivation within numerous cell types, and has the potential for long-term effects in contractile muscle, we assessed late-stage activation after a sustained muscle extension of 10% (Figure S2). In this imposed state of contractile tension, (P)-paxillin shows no sustained changes in either dystrophy whereas ERK and MAPKAP2 appear hyper-activated from 3- to 10-fold after 20 min in normal and dystrophic muscles. Hyper-activation is also seen for (P)-p38MAPK, which is not elevated significantly in unstimulated dystrophic muscle, and (P)-FAK is activated sustainably under mechanical stretch only in *mdx* muscle. Given the lack of acute paxillin response and the noted elevation of paxillin in dystrophy, we conducted model studies in C2C12 myocytes of paxillin distribution and dynamics as well as overexpression contributions to phenotype.

Paxillin localization to Focal Adhesions depends on Contractility

Immunostaining for paxillin and vinculin in myocytes cultured on collagen-coated glass (Figure 3A, B; Figure S3) demonstrates the typical localization to focal adhesions as well as cytosolic staining consistent with the muscle sections (Figure 1C). In culture, 6 days in differentiation medium causes myoblasts to stop dividing and fuse into multinucleated myotubes, but differences in FA density and intensity between myoblasts and definitively fused myotubes were generally not apparent here. Phospho-paxillin, specifically (P)-Y31 and (P)-Y118, preferentially localizes to FA, and treatment of cultured myotubes with blebbistatin, which inhibits non-muscle myosin as well as skeletal muscle myosin (Straight *et al.*, 2003), shows that FA localization of paxillin – but not vinculin – is lost within 20 min (Figure 3B). Recent studies of fibroblasts show that a 60 min treatment with blebbistatin (at lower dose than here) leads to a partial loss of both paxillin and vinculin from adhesions (Pasapera *et al.*, 2010), but the same studies also used FRAP (Fluorescence Recovery After Photobleaching) to show that GFP-paxillin is lost from adhesions more than 3-fold faster than GFP-vinculin. Quantitative intensity analysis here (Figure 3C, D) further shows that most (but not all) of paxillin is lost from FA in non-dividing myotubes, and while 5 min is not enough time, 20 min is sufficient for a saturating effect that is dynamically reversed after the drug is washed out. Formation of FA in non-muscle cells had long been known to depend on the contractile activity of non-muscle myosin, and, at least in T-lymphoblasts, paxillin also reportedly interacts in the cytosol with tubulin (Herreros *et al.*, 2000). However, the putative tubulin depolymerizer drug myoseverin (Rosania *et al.*, 2000), which also dedifferentiates myotubes back into mono-nucleated blasts (see below also), has no obvious effect on FA-localized paxillin or vinculin, consistent with the stated lack of obvious FA differences between myoblasts and myotubes. Nocadozole also has no effect (not shown).

Blebbistatin is expected to decrease cytoskeletal tension (contractility, or 'prestress') in both striated myotubes and also in the more stress-fiber filled cells that adhere to collagen-coated rigid glass; this is because blebbistatin inhibits both the non-muscle and skeletal muscle myosin-II isoforms. To test this effect, myotubes were grown on 20 μm wide strips of collagen-coated rigid glass that vary in length but help to standardize cell geometry (Figure 3E). Phalloidin-stained control cells show actin stress fibers aligned with the patterns, indicative of a tensed myotube. Blebbistatin treatment produces a more anastamosing network of relaxed actomyosin fibers. Functional assessment of the blebbistatin-relaxation of muscle tissue was performed by stretching excised muscles 10% and monitoring the tetanic force in a bath of blebbistatin (with slow permeation) (Figure S4; see Supplemental Methods). Within 45 min, the contractile force had indeed decreased (~60±15%), consistent with known effects of blebbistatin on skeletal muscle myosin (Linari M, 2004). Phospho-

activation of ERK also proved to be about half that of untreated controls (Figure 2, S2). Stretch-activated phospho-signaling thus depends on contractility.

Paxillin overexpression promotes Myofibrillogenesis without affecting Adhesion

Given our original observation of dystrophic upregulation of paxillin, we overexpressed paxillin and phospho-mutants of paxillin in well-controlled cell cultures to study mobility and additional effects. C2C12 myoblasts transfected with GFP-paxillin (~30% transfection efficiency) overexpress total paxillin by well over fivefold at 24 hrs post-transfection by Western blot (not shown). FRAP studies of these cells show high paxillin mobility and full recovery of fluorescence within seconds (Figure S5), consistent with other recent reports for GFP-paxillin (von Wichert *et al.*, 2003; Lele *et al.*, 2006). Paxillin binding within FAs is suggested by the 3-fold slower recovery of paxillin fluorescence within bleached FAs compared to bleached cytosol (Figure S5). Blebbistatin treatment decreases cytosolic mobility, suggesting enhanced interactions in the cytosol when FA are inhibited. This highlights once again a relationship between paxillin and cell tension.

Culture results above for (P)-Y31 and (P)-Y118 in paxillin suggested no difference in FA localization and recent results (Pasapera *et al.*, 2010) suggest no more than a ~10% difference in blebbistatin sensitivity, but these phospho-forms might still have differential roles in other adhesion-directed cell functions. Multinucleated myotubes initiate myofibrillogenesis at their adhesions as they exchange nonmuscle myosin II for skeletal muscle myosin II into a striated organization – a hallmark of striated muscle differentiation (Sanger *et al.*, 2002). The degree of muscle myosin striation depended strongly on the ability of the overexpresed GFP-paxillin to be phosphorylated on Y31 (Figure 4A). In initial studies, we used micropatterned multi-layer cultures in which a first layer of cells is grown on collagen-strips for 1–2 days followed by plating of a second layer of myoblasts (Engler *et al.*, 2004b; Griffin *et al.*, 2004b); about 40% of upper control myotubes striate by day 6 in such cultures. In comparison, when Pax^+ cells constitute the upper layer, nearly 100% of the green fluorescent Pax^+-myotubes appeared striated; this was found for both chicken and mouse wild-type paxillin. We then investigated the effects of inhibiting phosphorylation at different sites on paxillin. While the Y118F mutant behaved similarly to WT, the Y31F mutant did not striate beyond WT and neither did the tetra-mutant Y31/40/118/181F (Figure 4B), which implicates phosphorylation of Y31 in contractility and striation. This result suggests that paxillin, specifically Y31 phospho-paxillin, and muscle tension are part of a feedback loop in which changes in one affects the other.

Myoseverin has also been shown to drastically affect adhesions and cytoskeleton of myotubes, causing muscle to de-differentiate into mononucleated myoblasts (Musa *et al.*, 2003). Indeed, within 24 hrs and even within hours of drug addition, control myotubes treated with myoseverin began to fission and completely reverted to single cells within 48 hrs (Figure 5). Paxillin immunostaining also appears relatively diffuse by 24 hrs at which point 80% of cells are (non-apoptotic) monomyocytes. This process is reportedly reversible (Rosania *et al.*, 2000), as washing out the drug allowed re-formation of myotubes, albeit roughened and imperfect myotubes. In Pax^+ myotubes, structures are better protected from disassembly, and only 20% of Pax^+ cells appear mononucleated. Assuming myoseverin's reported action on microtubules, these results suggest that paxillin delays de-differentiation by maintaining microtubule structure – which we also observe (Figure 5). These de-differentiation results are consistent with a more committed contractile phenotype for Pax^+ cells.

The additional paxillin and contractile tension in Pax^+ cells could in principle drive stronger adhesion, but direct measurements show that neither adhesion structure nor adhesiveness are affected. Vinculin immunostaining in day 6 myotubes attaching to the rigid ECM-coated

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glass indeed shows the same statistical distribution of FA area in Pax^+ and GFP control myotubes (Figure S6A). The strength of adhesion of individual myotubes was determined by a micropipette aspiration method in which individual myotubes are controllably peeled into a large bore micropipette as the fluid shear stress pulls the cell in (Griffin *et al.*, 2004a). The externally applied tension (not the cell-generated internal tension) can be estimated from the fluid mechanics and is visibly sufficient to break the cell adhesion bonds which limit the rate of peeling along the length of the myotube. Importantly, Pax^+ and control cells yield the same cell detachment curve (Figure S6B). A minimum tension T_0 (≈ 6 nN/μm) is always needed to initiate cell peeling, but in agreement with past theory and experiment (Griffin *et al.*, 2004a) the peeling velocity then increases logarithmically with the externally applied tension. Paxillin over-expression thus does not alter – directly or indirectly – the adhesion strength of myotubes.

Hypertension of Paxillin-overexpressing C2C12 cells and primary *mdx* myotubes

Paxillin over-expression has no obvious effects on myoblasts (eg. FA density, spread area, stress fibers), which is consistent with past reports on a vascular smooth muscle cell line (Engler *et al.*, 2004a), but Pax^+ could still promote cytoskeletal contractility since myofibrillogenesis starts at the adhesions (Engler *et al.*, 2004b; Griffin *et al.*, 2004b). The contractile tension stresses the adhesions, but our cell peeling measurements reveal a minimum tension T_0 that a cell would need to generate in order to detach itself. Given the high concentration of FA vinculin and paxillin at the ends of normal myotubes (Griffin *et al.*, 2004a,b; Griffin *et al.*, 2005) as well as the *mdx* myotubes shown here (Figure 6A-inset), these cells are attached more strongly at their ends, and so forced detachment relieves a barrier for self-peeling of these tensed cells (Figure 6A, B). One might consider the cell to be a rubberband that is stretched and glued to a benchtop most strongly at the ends of the rubberband but also lightly in the middle. The tension in the rubberband, like actomyosin contractility, is clear, and can drive some amount of slow self-peeling if bonds are weakened, but then the cell tension would relax and no further 'self-peeling' would occur – unless one forcibly pulled on the rubberband to quickly peel it as done in our micropipette aspirations above. Note that self-peeling is slow and does not involve micropipette aspiration or any externally imposed tension that drives more rapid peeling.

As cells detach and self-peel, they relieve a fraction of their internal tension and exponentially approach a new equilibrium – similar to the blebbistatin-sensitive tetanic force relaxation at constant length (Figure S4). Indeed, blebbistatin-treated cells do not self-peel significantly when compared to controls, but Pax^+ cells detach to a greater extent than controls. The amplitude of final relaxation ($L – L_{\mathrm{Blebb}}$) thus provides an indication of contractility or cell tension since blebbistatin treatment largely eliminates myosin-II based contractility (both non-muscle and skeletal muscle myosin II). Further estimations of cell tension from self-peeling are difficult although the initial cell tension should exceed the T_0 (≈ 6 nN/μm) measured above. Nonetheless, the additional relaxation (and additional cell tension) in Pax^+ cells is most prominent (+60%, Figure 6C) with the striated myotubes in the upper layer (per Figure 4's skeletal muscle myosin II) compared to the lower layer of stress fiber rich cells (+40%). Paxillin overexpression in C2C12 cells thus drives striation (see Y31 requirement above) and thereby generates a more contractile phenotype.

Primary myotubes derived from *mdx* mouse myoblasts and grown in culture for one week are found to relax more than C57-derived control cells in the self-peeling measurements (Figure 6C). As shown here only for comparison, we reported similar results for $\gamma SG^{-/-}$ myotubes (Griffin *et al.*, 2005). The results thus suggest a hypertrophic or hypertensive phenotype for both dystrophic cell types here, although the $\gamma SG^{-/-}$ derived myotubes seem to relax more and thus perhaps possess even greater hypertension. Indeed, a significant fraction of $\gamma SG^{-/-}$ cells in culture – but not *mdx* cells – spontaneously relax and appear

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bulbous in the middle (Figure S7), suggesting that the needed balance between adhesion and contractility is shifted more to contractility.

Another physical measurement made on the various cell types is based on the general relation between cell elasticity and cell tension. In detailed studies by others of smooth muscle cells adhering to deformable gel substrates (Wang *et al.*, 2002) (and expressing smooth muscle myosin-II), cell prestress σdeduced from traction stresses in the gels is found proportional to cell elasticity E as measured by a micro-bead method. Such results imply $\sigma \cong a(E - Eo)$, in which Eo is the tension-free cell elasticity (positive) and a is a proportionality factor of order ~1–10. These two parameters (a, Eo) likely depend on cell-type and details of elasticity measurements. In order to estimate σ here with the skeletal myotubes we assume $a \approx 1$ and that $E\text{o} = E_{Blebb}$. We measured cell elasticity using an atomic force microscope (AFM) in which the force required to indent a cell allows determination of E (Figure 6D) (Rotsch *et al.*, 1999). Representative force-indentation curves for control (CTL), Pax^+, and blebbistatin-treated cells indicate greater tension in CTL cells and Pax^+ cells compared to the drug-relaxed cells ($\sigma \approx 0$). Averaging such data over multiple locations and multiple myotubes we obtain a mean E = 12 kPa for the CTL myotubes, in agreement with past results (Collinsworth *et al.*, 2002), but we obtain 22 kPa for the Pax^+ myotube. We establish a 'stress-free' state of $E\text{o} = E_{Blebb} \approx$ 3–4 kPa for blebbistatin-treated myotube (Figure 6E, acute treatment of 1–2 hrs).

With a baseline Eo ≈ 3–4 kPa established, the various measures of cell elasticity yield an intrinsic cell tension $\sigma_{CTL} \approx 9$ kPa for control cells and $\sigma_{Pax+} \approx 19$ kPa for Pax^+ myotubes. This +110% increase in apparent tension with paxillin overexpression is roughly consistent with the +60% increase in the apparent tension that drives self-peeling (Figure 6B). With primary cells likewise, elasticity measurements of fresh muscle excised from C57 controls and the paxillin-upregulated *mdx* mice (Figure 6F) show the latter is similarly hypertensive and also close to matching the relaxation differences (Figure 6C). A simple analysis of these biophysical results proves remarkably consistent with signaling changes: since the contractile prestress σ cannot exceed the critical peeling tension T_0 without causing spontaneous cell detachment, one can estimate distinct 'attachment length' scales for the different cell types. For Pax^+ myotubes, $T_0/\sigma_{Pax+} \leq 0.3$ μm, and for CTL myotubes, $T_0/\sigma_{CTL} \leq 0.67$ μm. The 2.3-fold smaller length scale for Pax^+ phenotypes certainly reflects the higher contractility and also approximates the higher contractile signaling of Figure 2. Therefore, the various physical measurements here quantify in a very consistent way the hypertensive phenotype of paxillin over-expressing muscle cell lines as well as dystrophic muscle cells.

Prednisolone acts as a relaxant in culture

The glucocorticoid prednisolone (PDN) is currently the most widely used palliative treatment for muscular dystrophy. Mechanisms of PDN action are not well understood, and there are dose-limiting side effects (Lo Cascio *et al.*, 1995), but PDN helps maintain muscle strength and muscle function for a few years (Fenichel *et al.*, 1991). Anti-inflammatory effects might result from down-regulation of CAMs (cell adhesion molecules) on leukocytes and vascular cells (Wehling-Henricks *et al.*, 2004), while further benefits possibly include enhanced myogenesis (Passaquin *et al.*, 1993), stabilization of muscle fiber membranes (Jacobs *et al.*, 1996), reduced necrosis (Takagi *et al.*, 1998) or decrease in intracellular calcium levels (Vandebrouck *et al.*, 1999). Gene expression profiling of PDN-treated *mdx* mice after 1 and 6 wks revealed over-expression of genes relating to metabolism and proteolysis as well as differential expression of genes relating to calcium metabolism (Fisher *et al.*, 2005), which of course could impact contractility.

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PDN effects on cultured myotubes are physically detectable by the methods here: AFM probing after a 12 hr treatment at the typical clinical dose (1 μM PDN) reveals statistically softer cells with $E_{app} \approx 8$ kPa corresponding to a cell tension of ~5 kPa (Figure 7). This is equivalent to relaxing ~50% of myosins when calibrated against full myosin inhibition by blebbistatin. However, a key difference in PDN activity is the fact that a 1 hr treatment has no significant relaxant effect whereas blebbistatin shows full effect well within 1 hr (also see Figure S4), which is more than sufficient time for inhibition of myosin-II's ATPase. On the other hand, PDN had no clear effect on paxillin localization to adhesions or levels of (P)-Y31. All of the results collectively suggest a non-linear, likely multivariate, relationship between total paxillin in the FA's and cell tension (Figure 8A, plot) with ~50% decreases in cell tension having no effect on paxillin whereas larger increases (~100% in Figure 8) produce an increase in cell contractility.

DISCUSSION

Shifting the cell attachment system from dystroglycan to focal adhesions might well be 'mechanically' neutral for the cells, but downstream remodeling and signaling derived from these different complexes appears significantly perturbed. Immunoblots and (P)-protein screens here (Figures 1B, 2) amplify and extend previously published *mdx* muscle transcript profiles (Table S1) which indicate at most a slight paxillin increase (+14%). Dystroglycan and γ-sarcoglycan are also well-known to be lacking at the protein level in *mdx* mice (Table S1) even though decreases in mRNA are similar in magnitude (about −15%). Paxillin is further shown here to signal and promote – in long term culture – myofibril formation and cytoskeletal tension which is synonymous with cell tension, contractility, and 'prestress' (Figure 4, 6). Surprisingly, increased paxillin does not lead to more abundant FA's or stronger adhesion (Figure S6). In the short term, paxillin diffuses in and out of FA in seconds (Figures S4), and its localization closely couples to sustained activation of myosin-II (Figure 3) and perhaps also to ERK (Figure 2), ultimately promoting viability. It should be noted, however, that the phosphorylation states of the MAPK components appear more responsive than paxillin to 20 min of imposed tension (Figure S2), suggesting perhaps that phosphopaxillin contributes to long-term signaling – especially Y31 in the contractile differentiation (Figure 4) – rather than short-term signaling. Consistent with a hypertensive phenotype, the *mdx* transcriptome (Table S1) further indicates upregulation of (*i*) nonmuscle myosin-IIA as well as γ-actin, (*ii*) MAPK (ERK1/2), and of course (*iii*) integrins including α7. Such a profile provides a basis for hypertrophy that is usually attributed to injury-induced regeneration.

While (P)-Y31-paxillin seems to promote striation and contractility, non-muscle myosin IIs almost certainly contribute to cell tension and contractility as well as myofibril formation. RhoA is also found elevated in dystrophic muscle (not shown), but RhoA is *not* known to activate skeletal muscle myosin, and so it seems more likely that in the diseased muscle, skeletal muscle myosin is activated by elevated resting calcium (Law *et al.*, 1994) as is typical of hypertensive states. Membrane stability/permeation as well as anchorage to basement membrane are expected to couple to contractility: an 'A-C-V' triangle of Anchorage-Contractility-Viability is illustrated in Figure 8. Using a signaling circuit appropriate to muscle, emphasis is first on the switch toward α7β1 integrins (Hodges *et al.*, 1997; Allikian *et al.*, 2004; Burkin *et al.*, 2005) and integrin-based ECM signaling and also C-type filamin (Thompson *et al.*, 2000) that occurs in the muscular dystrophies. Upregulation of the nexus-(Paxillin, Vinculin and FAK) drives cytoskeletal remodeling, which includes upregulation of γ-actin (Hanft *et al.*, 2006) as well as vinculin and talin (Law *et al.*, 1994) beyond what transcription profiles might suggest (Table S1). Increased filamins (Thompson *et al.*, 2000) are interesting because they help sustain the tension in a cell and because they are mechanotransducers (Kasza *et al.*, 2009). Hyperactivation of ERK1/2 and

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MAPKAPK2 in screens of both *mdx* and $\gamma SG^{-/-}$ indicate downstream perturbations of common signaling pathways in muscular dystrophy. While ERK1/2 and MAPKAPK2 contribute to the contractile loop, ERK1/2 and FAK likely modulate the apoptosis/survival pathway. The differential regulation of FA proteins such as (P)-paxillin might also regulate the extent of adhesive viability response to prestress. As evidence, the hypertensive $\gamma SG^{-/-}$ cells lack much (P)-paxillin and they also apoptose faster (Griffin *et al.*, 2005).

Additional structures are coupled to signaling. During myofibrillogenesis, sarcomeric organization requires a stable microtubule network for mechanical support (Palazzo *et al.*, 2004; Pizon *et al.*, 2005). Since depletion of paxillin at FA inhibits force development without having any effect on myosin activity (Tang *et al.*, 2002), paxillin might also be involved in stabilizing structural proteins like tubulin and thereby provide myofibrillar stability. In muscular dystrophies, microtubule depolymerization by LIMK1 and Rho GTPases is misregulated (Gorovoy *et al.*, 2005; Hu *et al.*, 2006). A major function of paxillin *in vivo* also seems to be to regulate the p190RhoGAP/p120-Ras-GAP regulatory pathway as well as tubulin polymerization (van Horck *et al.*, 2001; Tsubouchi *et al.*, 2002; Nishiyama *et al.*, 2004); hence, it could be that rapid turnover of paxillin at FA ultimately provides adhesion stability. Although, we observe no significant changes in adhesion, our in vitro measurements on C2C12 cells were performed on rigid glass substrates that promote strong adhesions relative to soft matrices (Engler *et al.*, 2004b). The inability to detect adhesion differences here suggests that adhesion responses may be saturated, and subtle changes in adhesion due to paxillin overexpression are completely over-ridden by the rigid substrate (Pelham, 1997).

Our biophysical measurements of self-peeling cells as well as our AFM measurements of cell tension provide novel evidence of a hypertensive disease state. Dystrophic cells with already weakened sarcolemma are more susceptible to injury from cell-generated forces, with hypertension driven apoptosis evident in $\gamma SG^{-/-}$ cells. Blebbistatin treatment, which relaxes the cell tension, also downregulates ERK activation (Figure S4), thereby highlighting the tension-linked viability signaling in these cells. Elevated levels of spontaneous contractions in *mdx* mice can be brought down to normal levels in the presence of the muscle relaxant relaxin through upregulation of endogenous nitric oxide (Baccari *et al.*, 2005). Here, the first-line gluococorticoid Prednisolone that is used to preserve muscle mass is also shown to relax muscle – perhaps through calcium regulation (Fisher *et al.*, 2005) – and so part of its utility in treating dystrophic patients might lie in relaxing the unappreciated hypertensive phenotype of dystrophic cells. PDN also helps to show that the basic relationship between cell tension and total paxillin at adhesions is likely to be non-linear.

Material and Methods

Tissue lysates and Western Blot Analyses

TA muscles were isolated within 5 min of anesthetizing 8–10 week *mdx*, $\gamma SG^{-/-}$, and normal C57 mice, and the isolated muscles were either snap-frozen immediately in liquid nitrogen or else stretched by 10% for 20 min in Ca^{++} Ringer's per (Griffin *et al.*, 2005). Muscle lysates were made by grinding the tissues to powder, suspending in lysis buffer, and collecting the supernatant after centrifugation at 13,000 rpm at 4 °C. Lysates were stored at −70 °C, and western blotting was performed using the standard protocol with color developed by BNIP substrates. Monoclonal antibodies for paxillin were obtained for total protein (Research Diagnostics, NJ), while polyclonal antibodies were used for (P)-paxillin (BioSource). The phospho-protein screens of lysates were generated and quantitated as described elsewhere (Griffin *et al.*, 2005).

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Cell Cultures, Drug Treatments, and Transfections

In preparation for the experiments, coverslips or fully patterned slides were coated with ECM per (Griffin *et al.*, 2004a) and seeded with roughly 10^4 cells. Primary myoblasts were harvested from 1-day-old mice. After harvesting, ~10^5 cells were plated onto the patterned glass coverslips. Cultures were maintained in DMEM supplemented with 22% horse serum (GIBCO), 8% embryo extract (US Biological, MA), 1% L-glutamine (GIBCO) and 1% penicillin/streptomycin at 10,000 units/ml and 10,000 μg/ml, respectively. Media was changed every other day. Skeletal muscle derived C2C12 mouse cells are maintained in 75 cm^2 cell culture flasks (Corning, NY) in 10 mL DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 20 % fetal bovine serum (GIBCO), 0.5 % chick embryo extract (GIBCO), and 0.5 % penicillin/streptomycin (10,000 units/mL and 10,000 μg/mL, respectively) (GIBCO). Cells are passaged every 2–3 days by detaching with trypsin-EDTA (GIBCO). For differentiation, media is changed to differentiation media (DM) (DMEM supplemented with 10% horse serum (GIBCO) plus 1% penicillin/streptomycin), and replaced every other day. For cell-on-cell studies, a first layer of cells was established on the patterns, and one day later a second layer of C2C12 cells was plated and the media was switched within 24 hr to differentiation media (Griffin *et al.*, 2004a).

Inhibition of myosin by 50 μM blebbistatin (Sigma) or 50 μM BTS (Sigma) used DMSO solvent controls in parallel. For studies with myoseverin (MS; Calbiochem), differentiated myotubes (14 days) were treated at 20 μM in DM. For studying the effect of prednisolone, one week old myotubes were incubated with 1 μM prednisolone for 12 hours before being probed with the AFM. Transfection with GFP-paxillin in C2C12 cells was performed using Lipofectamine-2000 following standard protocols.

Immunofluorescence, Microscopy, and FRAP

Tissue sections of the TA muscle from C57 and *mdx* mice were immunostained using paxillin antibody. Cultured cells were rinsed with phosphate buffered saline (PBS), fixed with 10% formaldehyde solution for 3 minutes, permeabilized with 0.5% Triton X100, and blocked in 5% BSA for 1 h at 37° C. Cells were incubated overnight at 4 °C in one of the following primary antibody solutions in PBS at a dilution of 1:100: mouse anti-myosin (Zymed), mouse anti-tubulin (BioSource), mouse anti-vinculin (Sigma). Secondary FITC anti-mouse IgG (Molecular Probes) was incubated at 1:300 for 1 h at 37 °C. Staining with secondary antibodies alone allowed for background detection. F-actin staining was perfomed with 60 μg/ml TRITC-phalloidin (Sigma), and cell nuclei were labeled with 1:100 Hoechst 33342 (Molecular Probes). Samples were mounted onto slides using Gel-Mount (Biomeda).

Microscopy was done with either a Nikon TE300 inverted microscope or Olympus IX71 microscope, using either a 40× (NA 0.45) or a 60× (NA 1.45) oil objective. Images were recorded under constant, calibrated settings with a Cascade CCD camera (Photometrics, Tucson, AZ), and fluorescence intensity was measured using ImageJ (NIH). For FRAP of GFP-paxillin, a nanosecond pulse laser (MicroPoint Laser, Photonic-Instruments, St. Charles, IL) exciting Coumarin-440 was done with a single pulse so as to limit bleaching of the mobile pool. Recovery curves, normalized to total signal to account for photobleaching, were computed for a circular area of maximum bleach; d_{bleach} ~ 1μm.

Single cell peeling, Self-Relaxation, and AFM measures of Cell tension

Myotubes were forcibly peeled from the substrate by a micropipette peeling technique described elsewhere (Griffin *et al.*, 2004a). Briefly, a large-bore micropipette was attached to a syringe pump and the flow rate was set to 10 μl/min. After one end of the cell was mechanically detached, the syringe pump was used to initiate a flow and cells were peeled from the substrate using a motorized stage. Because of the large size of the micropipette

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compared with the dimensions of the cell, the cell peeling velocity (V_{peel}) is driven by the peeling tension (T_{peel}), which is simply the integral of the shear stress over the length of cell inside the pipette.

For self-relaxation experiments per (Griffin *et al.*, 2004b), briefly, one end of a patterned cell was mechanically detached with a glass micropipette incubated in BSA. As the cell relaxed, the stage was repositioned in order to image the cell.

For AFM measures of cell tension, samples were placed on the stage of an Asylum 1-D AFM (Asylum Research, Santa Barbara) and indented perpendicular to the myotube axis by a pyramid-tipped probe (Veeco) The spring constant was determined with the thermal calibration method and was within 10% of the nominal value. Force-indentation profiles were obtained for multiple myotubes (cultured or freshly isolated) at multiple locations with a total count of 100 measurements. Each profile was fit with a modified Hertz model for a cone (Rotsch *et al.*, 1999) to determine their elastic modulus. Histograms of the determined elastic moduli and the intrinsic tension were plotted, and their mean magnitudes reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support from the NIH, MDA, and NSF is very gratefully acknowledged. Mouse GFP-Paxillin was generously provided Dr. J.F. Mushinski (NIH).

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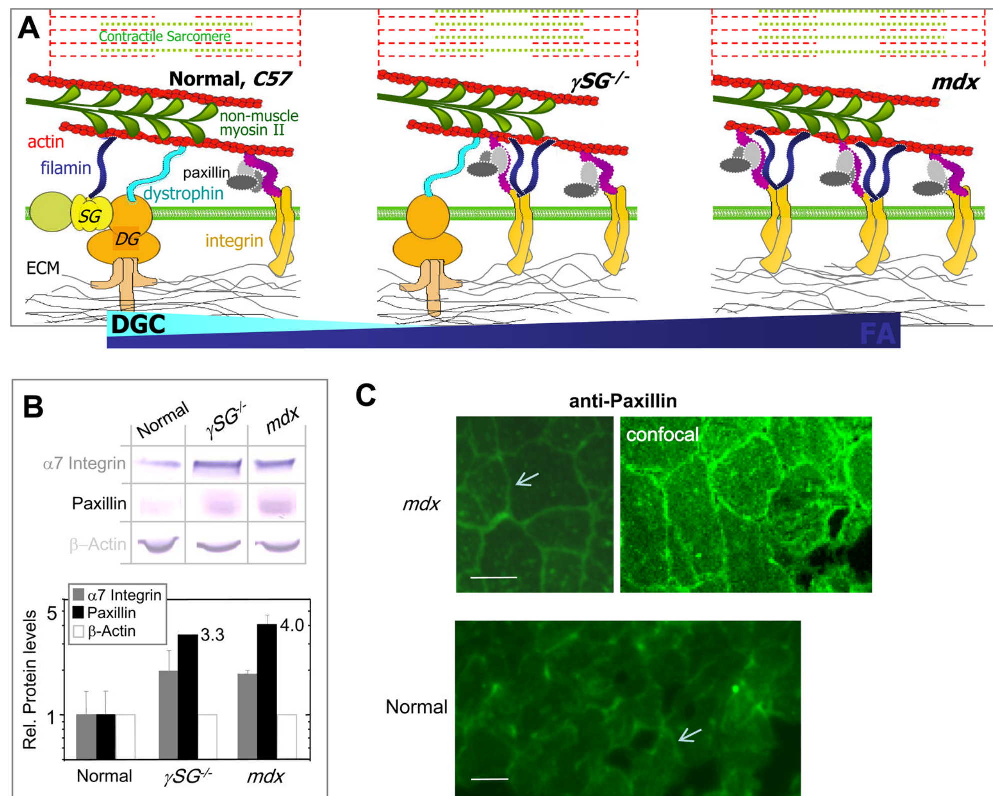

Figure 1. Upregulated adhesive complex components in muscular dystrophies

(**A**) Sarcolemma scaffolding in normal (C57), γSG$^{-/-}$, and *mdx* muscle cells illustrates the shift in cell attachment mediated by Dystroglycan Complex (DGC) to increasingly integrin-based Focal Adhesions (FA).

(**B**) Western blot analysis of C57, *mdx* and γSG$^{-/-}$ muscle lysates shows a two-fold upregulation of α7 integrins in dystrophic muscle together with 4-5 fold increases in paxillin (n=3). β-actin is used to normalize, and in so doing the integrin result appears consistent with past reports (Hodges *et al.*, 1997).

(**C**) Muscle sections immunostained with monoclonal anti-paxillin plus secondary and imaged under identical conditions by conventional fluorescence microscopy and confocal (*mdx*). Secondary alone shows no labeling. Paxillin intensity in normal muscle appears dim relative to *mdx*, and only the latter showed sufficient intensity to be imaged by laser scanning confocal, suggesting paxillin localization to sarcolemma (arrows) as well as cytosol. Scalebars ~20 μm.

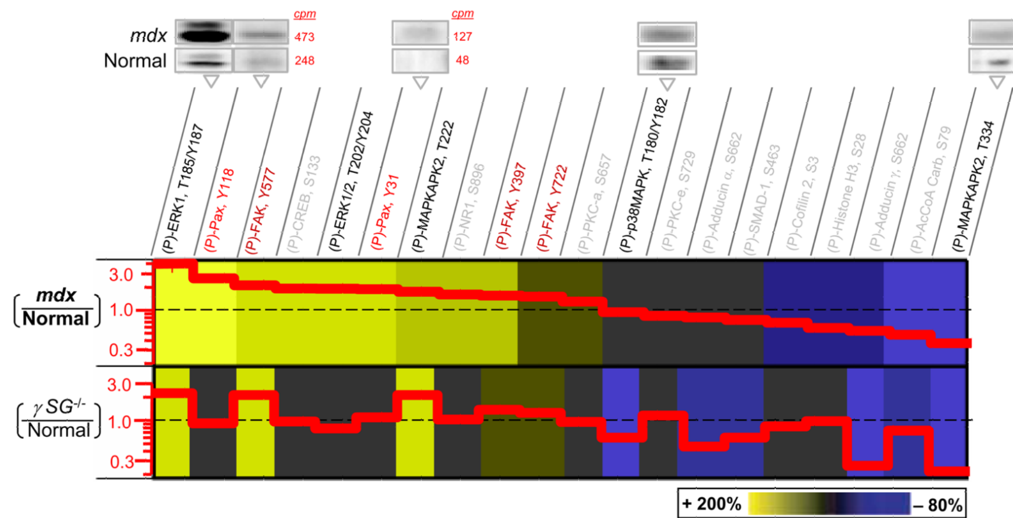

Figure 2.

Hyperphosphorylated MAPKs, FAK, and Paxillin in muscular dystrophies. Phosphoprotein immunoblots of normal (C57) and dystrophic (*mdx*) muscle tissue were quantified (with radio-labeled secondary) and normalized for direct comparison to results of γ$SG^{-/-}$ (Griffin *et al.*, 2005). Representative blot images are shown with cpm's indicated for phosphopaxillins to illustrate the methods. Greater phosphorylation of paxillin is found in *mdx*. Increased levels of phospho-paxillin (Fluck *et al.*, 1999; Melendez *et al.*, 2004; Subauste *et al.*, 2004), ERK (Most *et al.*, 2003; Schaeffer *et al.*, 2003; Mizukami *et al.*, 2004; Palfi *et al.*, 2005; Vittal *et al.*, 2005; Das *et al.*, 2006; Wei *et al.*, 2006), and FAK (Lunn and Rozengurt, 2004; Lin *et al.*, 2005; Peng *et al.*, 2006) are associated with either survival or apoptosis, but only γ$SG^{-/-}$ cells have been reported to be apoptotic (Griffin *et al.*, 2005). Representative error bars for phospho-ERK are <20% (S.D./Avg.; *n*=3).

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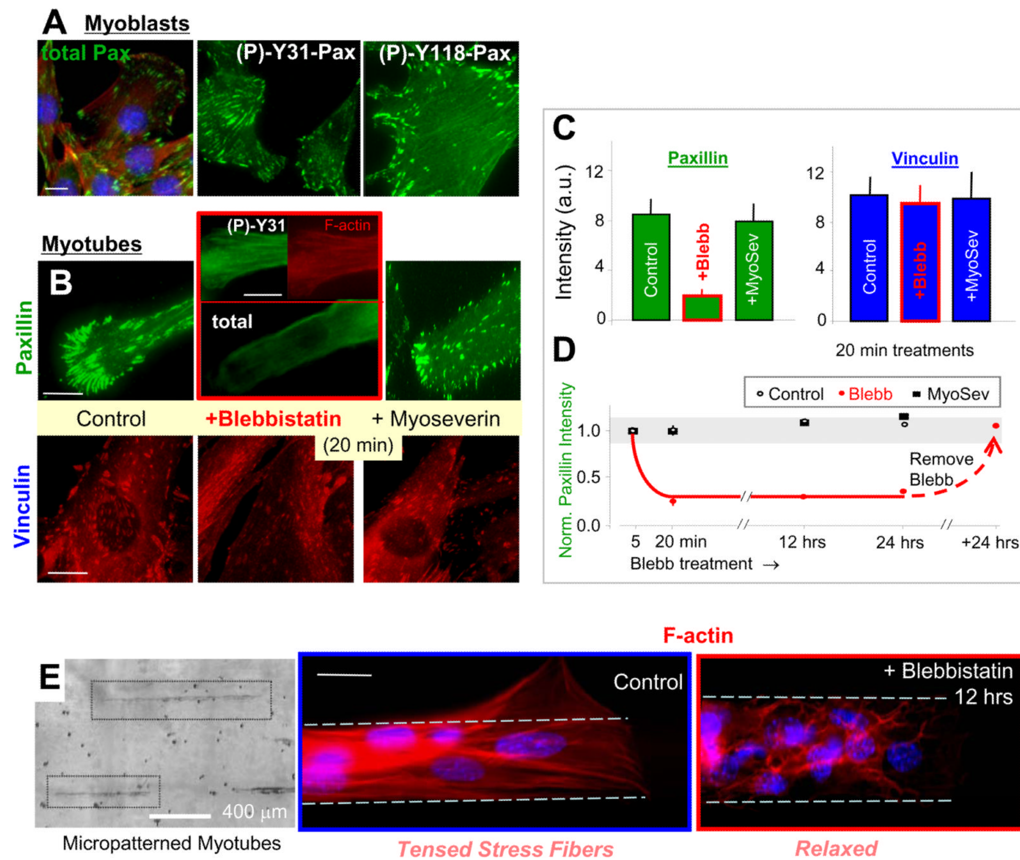

Figure 3. Paxillin localization to focal adhesions is contractility dependent

(**A**) Immunostaining of paxillin and its phosphorylated forms, (P)-Y31 and (P)-Y118, at focal adhesions and in the cytosol of myoblasts.

(**B–C**) Myotubes treated with blebbistatin, which inhibits non-muscle myosin-II and skeletal muscle myosin, show loss of paxillin but not vinculin from adhesions within 20 min. Note that the vinculin stained cells were within differentiated myotubes cultures and no distinctions with residual myoblasts were obvious. Myoseverin, which depolymerizes tubulin, has no such effect (total paxillin shown). Quantitative intensity analysis of 20 arbitrary myotubes (Avg. ± S.D.).

(**D**) Kinetics of paxillin loss and re-localization at focal adhesions after blebbistatin treatment and washout.

(**E**) Nascent myotubes attached to micropatterned collagen strips on glass exhibit stress fibers, whereas hours of blebbistatin treatment leads to relaxation of these structures into an anastamosing network of bundles. Scalebars ~10 µm.

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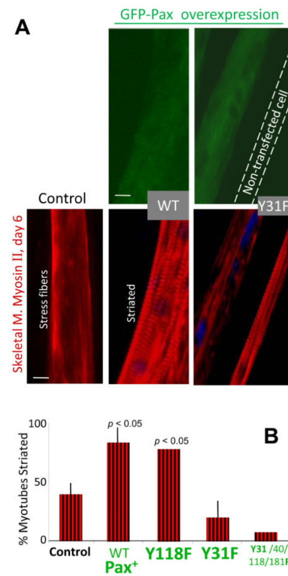

Figure 4. Paxillin overexpression promotes striation-differentiation and depends on Y31

(A) Cell cultures immunostained for skeletal muscle myosin show contractile sarcomeric striations in nearly 100% of Pax^+ myotubes (green). Most control cells show only stress fibers at day 6, with about 40% exhibiting striations. Similar results were found for chicken and mouse WT paxillin. Paxillin mutant Y118F gives a similar phenotype to WT, but mutant Y31F shows no effect.

(B) Quantitation for 30 cells per expt.; S.D. shown for $n \geq 3$ transfections. Scalebars ~10 μm.

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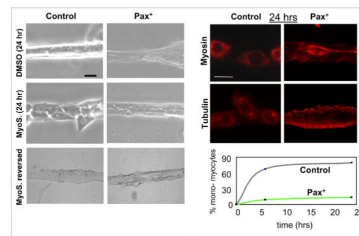

Figure 5.

Paxillin overexpression protects against de-differentiation. Phase contrast images of myotubes treated with DMSO solvent show no perturbation, but myoseverin treatment for 24 hrs (and as short as 6 hrs) leads to disassembly of control myotubes and microtubules. Pax^+ protects myotubes against this de-differentiation and microtubules against depolymerization at 24 hrs. Scalebars ~20 μm.

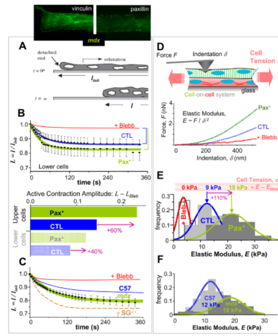

Figure 6. Hypertension of paxillin-overexpressing and *mdx* myotubes

(**A**) Self-peeling is initiated by detaching the firmly attached end of the myotube at $t = 0$. Upper insets show cells on micropatterned strips with a typical distribution of FA proteins – here in *mdx* myotubes. Note the end-concentrated FA's, which must be disrupted for the cells to peel under their own tension.

(**B**) Exponential relaxation of control, Pax^+, and blebbistatin-treated myotubes for both single layer and cell-on-cell arrangements. The asymptotic relaxation amplitudes are used to obtain the relaxation attributable to myotube contractility (L - L_{bleb}). Pax^+ myotubes are more contractile than controls: 40% moreso for the bottom layer and 60% moreso for the top layer of striated cells (8–10 cells each).

(**C**) Self-relaxation of 6-day old *mdx* myotubes (12 cells) suggests contractility between that of C57 and $\gamma SG^{-/-}$ myotubes (Griffin *et al.*, 2005). After subtraction of the blebbistatin results, *mdx* myotubes relax 16% *versus* 11% for C57 control cells and ~23% for $\gamma SG^{-/-}$ myotubes.

(**D**) AFM probing of cell-on-cell arrangements yields the apparent myotube stiffness E_{app}, which relates to the structural organization and also the intrinsic stress σ or contractility of the myotubes. Representative force-indentation curves are shown for control (CTL), Pax^+ and blebbistatin treated myotubes.

(**E**) Normal myotubes have an elasticity of E_{app} = 12 kPa, but Pax^+ myotubes are significantly stiffer at 22 kPa. Since blebbistatin suppresses contractility, E_{Blebb} = 3 kPa is subtracted from the two other cell systems to yield an intrinsic tension of 9 kPa for control myotubes and 19 kPa for Pax^+ myotubes.

(**F**) EDL muscle was dissected from normal and *mdx* mice and also probed in buffer using AFM. *mdx* muscle have an E_{app} of 18 kPa and appear 1.5 times stiffer than C57 muscle with a mean stiffness of 12 kPa. The relative frequency is normalized to 100 contacts on at least 10 myotubes.

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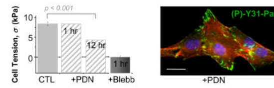

Figure 7. Prednisolone is a mild relaxant but Paxillin is unaffected

Prednisolone (PDN, 1 μM) treatment for 12 hr, softens C2C12 myotubes to ~50% of that achieved with Blebbistatin. AFM measurements were converted to cell tensions per Figure 8D. Immunofluorescence and immunoblotting show that Y31-Paxillin is unaffected by PDN. Scalebar ~10 μm.

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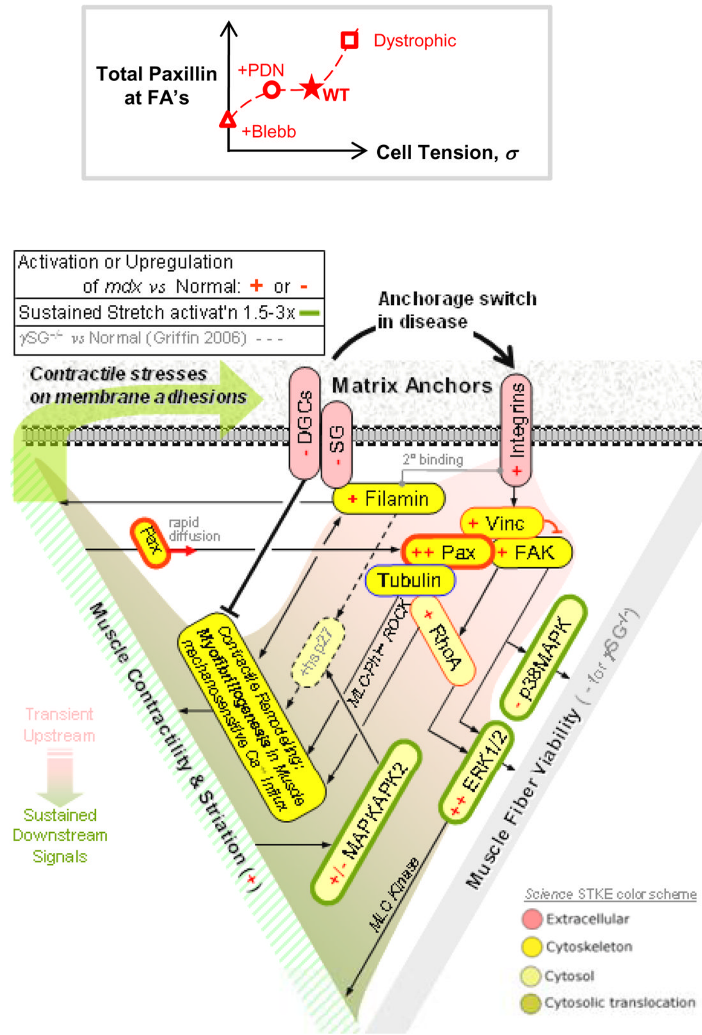

Figure 8. Non-linearities in paxillin vs tension and a Signaling Circuit for Anchorage-dependent Contractility and Viability

(A) Non-linear relationship between total paxillin and cell tension, summarizing the various findings.

(B) The underlying Signaling circuit for anchorage dependent contractility and viability. Dystroglycan-related defects in the muscular dystrophies leads to upregulation of integrins and associated proteins. Upregulation of FA proteins such as paxillin, vinculin and FAK helps to preserve muscle integrity by maintaining a balance between adhesion and contractility. A role for MAPK proteins in sustained stretch-mediated mechanosignaling emerges as a common pathway in both dystrophies studied.

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Table 1

Cross-correlation within Pax-FAK-MAPK nexus

Correlation Coefficient	***mdx*/Norm**	**$\gamma SG^{-/-}$/Norm**
mdx/Norm	0.35	−0.039
$\gamma SG^{-/-}$/Norm	−0.039	0.16