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ZZ DOMAIN OF DYSTROPHIN AND UTROPHIN: TOPOLOGY AND MAPPING OF A β -DYSTROGLYCAN INTERACTION SITE

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Short title: ZZ domain binds dystroglycan

Synopsis

Dystrophin forms part of a vital link between actin cytoskeleton and extracellular matrix via the transmembrane adhesion receptor dystroglycan. Dystrophin and its autosomal homologue utrophin, interact with β -dystroglycan via their highly conserved carboxy-terminal cysteine-rich regions, comprising WW, EF hand and ZZ domains. The EF hand region stabilises the WW domain providing the main interaction site between dystrophin or utrophin and dystroglycan. The ZZ domain, containing a predicted zinc finger motif, stabilises the WW and EF hand domains and strengthens the overall interaction between dystrophin or utrophin and β -dystroglycan. Using bacterially expressed ZZ domain we demonstrate a conformational effect of zinc binding to the ZZ domain, and identify two zinc binding regions within the ZZ domain by SPOTs overlay assays. Epitope-mapping of the dystrophin ZZ domain was carried out with new monoclonal antibodies by ELISA, overlay assay and immunohistochemistry. One monoclonal antibody defined a discrete region of the ZZ domain that interacts with β -dystroglycan. The epitope was localised to the conformationally sensitive second zinc binding site in the ZZ domain. Our results suggest that residues 3326-3332 of dystrophin, forms a crucial part of the contact region between dystrophin and β -dystroglycan and provides new insight into ZZ domain organisation and function.

Keywords: cysteine-rich region, Duchenne muscular dystrophy, dystroglycan, dystrophin, utrophin, zinc binding,

Abbreviations:

AAS	atomic absorption spectroscopy
BCA	bicinchoninic acid
β -DG	β -dystroglycan
BMD	Becker muscular dystrophy
CBP	c-AMP response element binding protein
DMD	Duchenne muscular dystrophy
Dys	dystrophin
F-actin	filamentous actin
KLH	keyhole limpet haemocyanin
LIM	Lin-11, Isl-1, Mec-3
Utr	utrophin

EF, WW and ZZ are not abbreviations but the actual names of the domains.

INTRODUCTION

Dystrophin loss leads to the progressive muscle wasting disease Duchenne muscular dystrophy (DMD). Following the identification of the DMD gene by Kunkel and colleagues by positional cloning in the late 1980's [1] it was realised that the protein product dystrophin was a large cytoskeletal protein with homology to the spectrin family of proteins [2-4]. Like spectrin and α -actinin, dystrophin and utrophin can provide a link between the F-actin cytoskeleton and transmembrane proteins, and in the specific case of dystrophin through the transmembrane adhesion receptor protein dystroglycan, and so to the extracellular matrix [5]. The much rarer and often milder allelic form of DMD known as Becker muscular dystrophy (BMD) allows the expression of a variable amount of altered dystrophin protein [6]. Analysis of the genotype/phenotype relationship for deletion mutations in these patients has revealed valuable information about the functional importance of different regions of the dystrophin protein [7]. Dystrophin and its autosomal homologue utrophin [8] comprise 4 main regions. From N to C terminus: an actin-binding domain, a series of spectrin-like repeats, a cysteine-rich region, and a dimeric coiled coil region. The actin-binding domain and cysteine-rich region show highest overall sequence similarity between the two proteins, and the mutational spectrum from analysis of BMD patients points to a greater functional importance for these regions in dystrophin. Mutations in the actin binding region give rise to severe BMD, whilst despite the presence of some dystrophin protein product, mutations in the cysteine-rich region give rise to an DMD phenotype [7]. These findings from human subjects were recapitulated in an elegant series of experiments using transgenic mice expressing dystrophin constructs containing a similar spectrum of deletion and mutations, reviewed in [9]. The human and mouse data demonstrate that from a functional point of view the physical links between dystrophin and the actin cytoskeleton via the actin-binding domain, and the link between cysteine-rich region and dystroglycan are crucial to dystrophin function. The spectrin repeats are to a large extent redundant, and only mild phenotypes are associated with loss of the C-terminal coiled coil repeats. Within the cysteine-rich region, arguably the most important region of dystrophin, are three distinct domains that contribute to the interaction between dystrophin and the c terminus of dystroglycan: the WW, EF-hand and ZZ domains [10-12]. The WW domain is the primary site of interaction between dystrophin (or utrophin) and the last c-terminal 15 amino acids of β -dystroglycan [13,14]. The stability of the WW domain and its affinity for β -dystroglycan is greatly increased by the EF

hand region which cradles the WW domain [15]. The overall complex is then further stabilised and supported by interactions between dystroglycan and the ZZ domain [13,16]. Whilst the interactions between β -dystroglycan and the WW and WW-EF regions of dystrophin and utrophin have been studied in some detail, less attention has been paid to the ZZ domain. Here we describe features of the ZZ domain with respect to its zinc binding properties, and using a panel of new monoclonal antisera to the ZZ domain, define a binding site for dystroglycan on the ZZ domain.

MATERIALS AND METHODS

Peptides and constructs - All peptides used were obtained as previously described [17]. Overlapping 7 residue synthetic peptides were produced to span the dystrophin or utrophin ZZ domains, D3311-3342 and U3068-3099 respectively as represented in Fig. 1. Dystrophin and utrophin differ by only two isoleucines in this region I3322 and I3336 (dystrophin numbering) replaced by two valine residues in utrophin sequences (V3079 and V3093). A ZZ domain construct comprising residues 3049-3114 of human utrophin was generated by PCR including Nde1 and Sal1 restriction sites and cloned into the modified pET vector pSJW1 [18]. Point mutations in cysteine residues were generated by overlap extension mutagenesis. The non-fusion protein was expressed in *E. coli* BL21(DE3) and purified from inclusion bodies using CM Sephadex and Gel filtration chromatography in the presence of 6M urea. Urea was removed by step-wise dialysis into 10mM PIPES pH 7.0, 1mM ascorbate with either 1mM ZnCl₂ or 1mM phenanthroline. The cytoplasmic domain only of mouse β -dystroglycan, residues 775-895, was expressed in *E. coli* BL21(DE3) and purified as described previously [19].

Antibodies - The dystrophin ZZ domain sequence (3311-3342) was linked to keyhole limpet haemocyanin (KLH) and injected into mice according to a previously described protocol [20]. Spleen cells were fused to myeloma to obtain specific hybridomas and antibodies were selected by ELISA using the antigenic peptide. The dystrophin and utrophin C-terminal polyclonal antibodies (H4 and K7 respectively) were raised in New Zealand rabbits by repeated intradermal injections. Peptides of the last 16 and 11 C-terminal amino acids of dystrophin or utrophin respectively were conjugated via a cysteine residue to KLH and used as antigen. Antibodies were purified and characterised as previously described [21].

Tissues - All tissues; muscle, lung and sciatic nerve, were dissected immediately after death and rapidly frozen in 2-methylbutane, cooled in liquid nitrogen and stored at -80°C until use. Crude muscle membrane homogenate was prepared using freshly dissected muscle according to a previous protocol [22].

ELISA - ZZ domain sequence synthetic peptide (3311-3342) was coated on micro-titre plates at 0.1 mg/ml, and incubated at 4°C overnight in PBS. After washes, the plates were blocked with 0.05% Tween 20 in PBS buffer containing 1% BSA for 20 min at 37°C, then incubated with each monoclonal antibody for 2h at 37°C. Reaction was revealed by adding alkaline phosphatase labelled anti-mouse IgG for 30 min at 37°C and signal was detected at 405 nm using p-nitrophenylphosphatase (Sigma) in diethanolamine buffer, pH 9.8. ELISA assay was performed to test epitope mapping for each monoclonal antibody using the 7 residue synthetic peptides corresponding to the overlapping sequences spanning the D3311-3342 or U3068-3099 ZZ domain. Freshly prepared peptide, 7.7 mg/ml in 0.05% Tween 20 PBS buffer, was coated onto micro-titre plates in triplicate then incubated with each monoclonal antibody to test their reactivity. For competitive ELISA experiments micro-titre plates were coated in triplicate with peptide 19. After blocking as above, plates were incubated at 4°C overnight with either crude muscle membrane homogenate (0.1 mg/ml), purified β -dystroglycan (9 μ g/ml) or with blocking buffer. Monoclonal antibody (13D2) and the commercial β -dystroglycan antibody (1:500, 43DAG/8D5 Novocastra) were added successively. Reactions were revealed as described above.

Immunofluorescence - Cryostat sections (10 μ m) of unfixed muscle (Rabbit and *Torpedo marmorata*) were labelled with the antibodies described above. Immunoreactivity was detected with Cy3-conjugated sheep anti-mouse IgG (Euromedex).

Western blotting - Fresh extracts were prepared from 0.01 g of muscle tissue homogenised in 150 μ l of 5% SDS buffer (50 mM Tris/HCl pH 8.0, 10 mM EDTA) supplemented with 1% of trypsin inhibitor and 1% saponin. After centrifugation (10 min at 13000 g), supernatant protein concentrations were estimated using the BCA protein assay (Pierce). Samples were separated in duplicate by SDS-PAGE (3-10% or 5-15%), transferred to nitrocellulose and developed with appropriate antibody essentially as described previously [13] All monoclonal antibodies were tested in competition with an excess of corresponding synthetic peptides (1mg of peptide per μ g/ml of monoclonal antibody [23]) on both cryostat sections or western blots. All monoclonal antibodies were completely blocked by their specific peptides (Fig 4).

Far western blotting overlay - Blotted nitrocellulose sheets (0.2 μm) containing total muscle protein extract were blocked for 1 h at room temperature with 10 mM triethanolamine, pH 7.6, 140 mM NaCl, 1mM CaCl₂, and 1mM MgCl₂ containing 5% BSA and then pre-incubated with peptide 19 (0.1 mg/ml) in 0.5 % BSA containing 1mM dithiothreitol or 0.5% BSA alone (control) for 24 hours. Crude membrane homogenates were applied to the nitrocellulose membrane overnight at 4°C with gentle agitation. Membranes were blocked in Tris buffer containing 0.1% Tween 20, 3% BSA (w/v). After washing, proteins were revealed with dystrophin C-terminal antibody (H4).

ZZ domain zinc binding - Circular dichroism (CD) was carried out on a Jobin Yvon CD6 spectrophotometer using 0.6 ml cuvettes. ZZ domain was analysed in the presence of 1mM ZnCl₂ or 1mM 1,10-phenanthroline. Aliquots of similar samples were also subjected to SDS-PAGE in the absence or presence of zinc. Atomic absorption spectroscopy (AAS) was performed on a Unicam Solaar 929 spectrometer using ZnCl₂ solutions prepared in the same buffer as the ZZ domain as standards at a wavelength of 213.9 nm with a bandpass of 0.5 nm and taking the average of 3 readings at 1 sec intervals. For metal ion competition assays, 10 μg of purified ZZ was spotted onto nitrocellulose and allowed to air dry. Membranes were probed with ⁶⁵ZnCl₂ as described previously [24] using 200 μM ⁶⁵ZnCl₂ (20 $\mu\text{Ci/ml}$) and an excess of the indicated counter ions. Autoradiographs were quantified by densitometry and data expressed relative to ⁶⁵ZnCl₂ alone = 1.0. A SPOTs peptide array comprising 46 peptides of 20 residues each differing by 1 amino acid covering the complete utrophin ZZ domain (3049-3114) was synthesised as described previously [13]. ⁶⁵ZnCl₂ binding was performed as described above; a separate control experiment was performed in the presence of excess 10mM ZnCl₂.

RESULTS

ZZ domain zinc binding: Like other proteins that bind divalent cations, purified bacterially expressed utrophin ZZ domain showed a small upward mobility-shift on SDS-PAGE when the protein was prepared in the presence of zinc, whereas ZZ domain prepared in the presence of the chelator 1,10-phenanthroline ran slightly faster (Fig 2A inset). This is indicative of a structural change induced by zinc binding altering the SDS-PAGE mobility, a phenomenon commonly seen in cation binding proteins, such as calmodulin when subjected to SDS-PAGE in the presence or absence of calcium, for example see [25]. A conformational change upon zinc binding was also substantiated by CD. The CD spectrum of ZZ domain in the presence of zinc showed a slightly higher propensity toward helical structure compared to ZZ domain with phenanthroline (Fig 2A). Determination of divalent cation specificity for the ZZ domain by ^{65}Zn -overlay revealed most competition for zinc binding by excess zinc or copper (Fig 2B) which is in keeping with the complexing ability of divalent cations of the transition metals which generally follow the Irving-Williams series $\text{Mn} < \text{Fe} < \text{Co} < \text{Ni} < \text{Cu} > \text{Zn}$ which in turn are all greater than the alkaline earth metals, Ca^{2+} , Mg^{2+} etc [26]. This trend is also evident on quantification of this and two other independent experiments (Fig 2C). These data suggest the ZZ domain is a bona fide zinc binding protein. The original description of the ZZ domain highlighted 4 conserved cysteine residues in the cysteine rich regions of dystrophin and utrophin [12] leading to the suggestion that the ZZ domain might adopt a structure equivalent to half of a LIM domain [27]. ZZ domains in most other proteins however, contain 6 conserved cysteine residues in positions that could coordinate zinc with additional histidine and other potential liganding residues (Fig 1B) [12].

^{65}Zn -overlay assays: We therefore mutated each of the five cysteine residues in the utrophin ZZ domain and performed ^{65}Zn -overlay assays. No qualitative difference was observed in zinc binding as determined by autoradiography suggesting that the ZZ domain did not adopt a half LIM domain-like conformation, as removal of any one cysteine should prevent zinc coordination. For the constructs where enough soluble material could be produced, we further examined the ZZ domain zinc binding using AAS. The wild type purified protein, refolded from urea, contained sub-stoichiometric quantities of zinc, 0.65 ± 0.03 mol Zn/mol ZZ suggesting that the protein was not all correctly folded. However the zinc content of the utrophin ZZ domain mutant C3071A

appeared stoichiometric at 2.14 ± 0.1 mol Zn/mol ZZ: thus two rather than one zinc ion bound to the ZZ domain. We hypothesise that the mutation of C3071 to alanine removed a 'free' uncoordinated cysteine residue that led to inappropriate zinc coordination, possible disulphide formation and aggregation of the domain leading to insolubility. To confirm the stoichiometry of zinc binding determined by AAS, we used a peptide SPOTs array of 20 amino acid peptides each differing from the previous by one amino acid spanning the 66 residues of the ZZ domain to examine ^{65}Zn binding. As shown in Figure 3A, two distinct regions of the utrophin ZZ domain bound zinc, in part corroborating the AAS data. Zinc is typically coordinated by Cys and His, but Asp, Glu are also able to act as ligands with water contributing in catalytic situations [28]. In the first zinc-binding region, peptides 6-16, contained a glutamic acid, a histidine, two of the conserved cysteines and a further cysteine unique to dystrophin and utrophin. Each potential liganding residue was separated by two residues which could theoretically accommodate the coordination of a zinc ion (Fig 3A). The other region, peptides 30-37, contained the second pair of conserved cysteines, a histidine and an aspartic acid (Fig 3A). Rather than a zinc finger, the ZZ domain may therefore contain two zinc 'knuckles' as depicted in Figure 3B, though other conformations are possible. The binding of zinc to the peptides regions described above must rely on some local sequence specificity and not the simple presence of potential coordinating ligands, as peptides 43-46, which all contain a pair of histidines and a pair of cysteines in a configuration one might expect to be able bind zinc, showed no labelling with ^{65}Zn (Fig 3A). The synthesis of SPOTs peptides is not quantitative, therefore strength of binding interaction cannot necessarily be inferred from the intensity in any one SPOT as the quantities of peptide in each SPOT may differ. However, the presence of radioactivity of broadly similar intensity across several SPOTs is likely to indicate a similar affinity for the ligand over those SPOTs.

Monoclonal antibody selection and epitope mapping: Monoclonal antibodies directed towards the ZZ domain of dystrophin (3311-3342) were collected as hybrid supernatants, selected by ELISA using recombinant ZZ domain and named 13D2, 12D7, 14A4 and 4G3. Specificity and epitope mapping was performed for the four selected supernatants by ELISA against the series of 7 residue peptides from the dystrophin ZZ domain (Fig. 1A) including those common to dystrophin and utrophin. A specific response is obtained with peptide 19 (D3326-3332 / U3081-3089) for 13D2 supernatant, with peptide 1 (D3315-3331 / U3072-3072) for 12D7 supernatant and peptide 33 (D3335-3341) for 14A4 supernatant whilst no specific response was obtained

with supernatant 4G3 (Fig. 4A). Peptide 33 is unique to dystrophin as it contains one of the isoleucines that is substituted by valine in the utrophin ZZ domain. We therefore re-screened serum 14A4 hybrid supernatant against all peptides that spanned the equivalent region in dystrophin and utrophin, but as shown in Figure 4B, supernatant 14A4 was specific for peptide 33 and did not recognise the equivalent peptide form utrophin with an Ileu to Val substitution (peptide 34). The respective epitopes of the selected antibodies are presented schematically in Fig. 4C taking into account the topological information derived from Figure 3.

Immunodetection of the dystrophin ZZ domain: The four monoclonal antibodies were tested by immunofluorescence detection using both *Torpedo marmorata* and rabbit muscle (Fig. 5AB). Serum 13D2 shows no specific sarcolemmal labelling whereas both 12D7 and 14A4 antibodies produced the expected sarcolemmal labelling typical of dystrophin staining in skeletal muscle. Specific staining was blocked by preincubation of the hybrid supernatant with the respective peptide. The 4G3 antibody, which did not detect any specific dystrophin or utrophin ZZ domain peptide, produced a labelling pattern only in the cytoplasmic compartment of muscle fibres. These antibodies were further tested by western blot on total protein homogenates from rabbit skeletal muscle (Fig. 5C) or *T. marmorata* (not shown). In keeping with the immunofluorescence detection pattern in muscle sections, no protein band was detected using the 13D2 antibody while a 400 kDa protein band, corresponding to the expected molecular mass of dystrophin, was obtained by 12D7 and 14A4 antibodies (Fig. 5). Specific staining was again blocked by preincubation of the serum with the respective peptide. Using protein extract from dystrophin deficient mdx mouse muscle (Fig. 5D) 12D7 and 14A4 antibody fail to give a 400 kDa protein band indicating that these antibodies recognize specifically the dystrophin ZZ domain sequence. The 4G3 monoclonal antibody revealed an unknown protein band with molecular mass of about 170 kDa. Monoclonal supernatant 13D2 specifically recognised peptide 19 common to the dystrophin/utrophin ZZ domain (Fig 4A). It is surprising therefore that it did not recognise dystrophin in tissue sections or on western blots (Fig 5AB). This lack of reactivity might imply that the epitope for 13D2 is masked in tissue sections or is dependent on conformation, the latter might also explain its lack of reactivity in western blots.

ELISA competition assays: In order to further investigate the reasons behind this lack of reactivity we performed competitive ELISA experiments in micro-titre plates coated with peptide 19. In two separate experiments we examined the competition between purified β -dystroglycan or

a skeletal muscle membrane fraction enriched for β -dystroglycan, and the 13D2 ZZ domain antibody or 43DAG/8D5, a monoclonal hybrid supernatant against β -dystroglycan. All experiments, including controls were carried out in the presence of 1% BSA. The two experiments produced qualitatively similar results (Fig. 6AB). 13D2 incubation produced a consistent high signal across all wells (Fig. 6AB), consistent with the specificity of this monoclonal antibody for peptide 19 (Fig 4). The monoclonal antibody 43DAG/8D5 against β -dystroglycan however, gave a consistent low signal across all wells, demonstrating that, like the related antiserum MANDAG2, this hybridoma supernatant does not recognise peptide 19 and is specific for the WW domain interaction sequence and PPPYVP epitope at the C-terminus of β -dystroglycan (Supplementary Fig 1.) and as reported previously [13,29]. Increasing concentrations of either purified recombinant β -dystroglycan (Fig. 6A) or dystroglycan-containing membrane fraction (Fig. 6B), competed for 13D2 binding to peptide 19 reducing the signal from successive wells. Conversely the detection of β -dystroglycan with 43DAG/8D5 revealed an increase in β -dystroglycan binding to peptide 19 with increasing β -dystroglycan or dystroglycan-containing membrane fraction competition across successive wells. Thus peptide 19 appears to be able to bind β -dystroglycan which can compete specifically for the 13D2 monoclonal antibody suggesting that the region of the dystrophin and utrophin ZZ domains, corresponding to peptide 19, is an additional binding site for β -dystroglycan on dystrophin and utrophin. Furthermore, the ability of 43DAG/8D5 to still recognise β -dystroglycan bound to peptide 19, suggests that the two binding events are independent in this context.

Far-western blotting of β -dystroglycan with Dystrophin and utrophin ZZ domains: Total mouse muscle extract transferred to nitrocellulose was overlaid with a dystrophin-enriched crude membrane homogenate of mouse skeletal muscle, or with a utrophin-enriched crude membrane homogenate of *mdx* mouse skeletal muscle or sciatic nerve. The dystrophin in the enriched dystrophin homogenate bound to β -dystroglycan on the nitrocellulose membrane as revealed by blotting for the dystrophin c terminus (Fig 7, panel A3; Pep19-). When the blot was pre-incubated with peptide 19 however, the binding of dystrophin in the membrane homogenate to dystroglycan on the blot was strongly reduced (Fig 7, panel A3; Pep19+). This result demonstrates that the peptide 19 can effectively block dystrophin binding to intact β -dystroglycan again highlighting the importance of the ZZ domain for the dystrophin-dystroglycan interaction. In a similar experiment overlaying an enriched utrophin homogenate from

dystrophin-deficient *mdx* muscle or sciatic nerve, utrophin detection using the C-terminal utrophin antibody (K7) showed that the fraction of utrophin that bound β -dystroglycan was less compared to that observed with dystrophin, suggesting that utrophin has a lower affinity for β -dystroglycan (Fig 7, panel B3; Pep19-). However, when peptide 19 was applied there was still a slight reduction in utrophin binding (Fig.7 panel B3; Pep19+). The apparent difference in inhibition of binding to β -dystroglycan is not likely to be due to the slightly lower dystrophin+utrophin content seen in the *mdx* mice samples, compare quantifications in panels A4 and B4. The difference in competition for β -dystroglycan binding probably reflects a real difference in the mode of binding between the dystrophin or utrophin ZZ domain, and β -dystroglycan. This may be due to other DGC components assembly differently in the *mdx* mouse in the presence of utrophin which contributes to a slightly different mode of interaction with dystroglycan.

DISCUSSION

The ZZ domain: ZZ domains are a highly conserved and widespread zinc binding motif first identified in dystrophin/utrophin and the transcriptional co-activator CBP/p300 [12] (Figure 8). In dystrophin, the first half of the carboxy-terminal domain and ZZ domain-containing cysteine-rich region has been implicated in binding to β -dystroglycan, and when mutated gives rise to a severe muscle-wasting phenotype [2,30,31]. This region contains several modular protein domains: a WW domain, two incomplete but putative calcium-binding sites (EF hands) [32] and a ZZ domain (zinc finger domain) [12]. An examination of the spectrum of point mutations in the human dystrophin gene in the ZZ domain region (Table 1), reveals that 4 of these mutations, where a full-length protein product may be produced, are in zinc liganding residues implicated in our studies. The missense mutation C3313F produced a DMD phenotype, though no information is available as to whether this individual had any dystrophin protein expression [33]. D3335H on the other hand was identified in an individual with a normal level of correctly localised full length dystrophin *and* dystrophin associated proteins, but yet a severe DMD phenotype [34]. From our analysis of ^{65}Zn peptide SPOTs overlay, D3335 is a potential liganding residue (Fig 3), but mutation of this residue to histidine in biochemical experiments had no effect on β -dystroglycan binding [35]. Although Asp to His is not normally considered a conservative substitution, in this context His could replace Asp and still coordinate zinc, thus preserving protein conformation, and as suggested from the patient phenotype, presumably some function too. It may be that D3335 has another important functional role that so far has not been elucidated and that is not supported by a His substitution, but it is difficult to predict what that might be. Aspartic acid in this position is conserved in the majority of ZZ domain sequences (Figure 8), suggesting a functional importance [12].

Zinc binding: An NMR structure of the ZZ domain from CBP (CRE Binding Protein) revealed a cross-brace zinc finger motif [36], where the individual zinc ions are coordinated by non-linear sequence elements, with the protein backbone crossing back on itself to form a zinc binding site (Figure 8B). The SPOTs analysis carried out in Figure 3 could not reveal coordination of zinc through such a structure as the individual peptides were only 20 amino acids long, and from the CBP ZZ domain structure the liganding residues are 21 and 26 residues apart in the linear

sequence [36]. Due to insertions in the dystrophin/utrophin sequences this corresponds to 27 and 28 residues apart, too long to provide an unambiguous interpretation in the peptide array. But as highlighted in Figure 3, it does not appear that just any combination of 4 residues that *could* coordinate zinc, *will* bind zinc. The two pairs of cysteine and histidine residues in peptides 43-46 of the SPOTs are appropriately spaced to bind zinc (Fig 3A) but do not, suggesting a degree of specificity to the other sequences identified by this method that do bind zinc. Moreover, unlike almost all other ZZ domain sequences in the databases, vertebrate dystrophin and utrophin lack three of the conserved residues that form the second zinc binding site in the CBP ZZ domain: the central pair of cysteines and one of the c-terminal histidines (Figure 8B) [12,36]. This would suggest that dystrophin and utrophin bind zinc in a different conformation and this conformation may be specific to the ability of these proteins to interact with dystroglycan through multiple modes of interaction as discussed below. Interestingly the vertebrate dystrobrevins and invertebrate dystrophins and dystrobrevins, do have cysteine residues in these positions, suggesting some functional divergence with the emergence of distinct dystrophin and utrophin genes in vertebrates.

Dystroglycan binding, role of the WW domain: We and others have independently mapped the site for dystrophin and utrophin binding to dystroglycan to the last 15 amino acids at the c-terminus of β -dystroglycan [13,14,16,37-39]. The major determinant within these 15 residues being the sequence PPPY a consensus for type I WW domain interactions [13,16,37,39]. Despite a necessary requirement for the PPPY motif in dystroglycan for dystrophin/utrophin binding via their WW domains [29], this is not sufficient. Where this requirement has been analysed in more detail, it has been found that constructs corresponding to the WW domain of dystrophin or utrophin alone cannot bind β -dystroglycan, the addition of the EF hand region allows some binding, but full binding potential is not realised until the ZZ domain is included [13,14,16,31,35,39,40]. Unlike most other WW domains which exhibit autonomous binding activity toward their cognate peptide ligands [29], the WW domains of dystrophin and utrophin as a minimum require the support of the EF hand region. The reasons for this became clear on elucidation of a crystal structure of the dystrophin WW-EF region in complex with a β -dystroglycan peptide [15]. This revealed that the WW domain was supported extensively by the EF hand region including a contribution to the β -dystroglycan binding interface and confirming earlier elegant biochemical studies by this group [16].

Dystroglycan binding, role of the ZZ domain: Detailed biochemical analyses have pointed to a role for the ZZ domain in further supporting and contributing to this binding interface [16,35]. Moreover Rentschler et al. even go so far as to suggest that there is an additional binding site between dystroglycan and non-WW domain regions in dystrophin [16], possibly the ZZ domain. Transgenic *mdx* mice expressing various dystrophin gene constructs showed that the dystrophic change in *mdx* muscle can be rescued [41,42]. Because the binding of dystrophin to β -dystroglycan is required to prevent the dystrophic phenotype, deletion models can be considered to exhibit effective and physiological binding activity in these mice. In this context, the full-length cDNA with deletion of exons 68-70 (ZZ domain) failed to rescue the phenotype [41] despite the presence of the WW-EF region. Another model with deletion of exons 64-67 (EF hands) also failed to rescue the phenotype [42]. This model, which includes the WW domain encoded by exons 62-63 but lacks the subsequent EF1 and EF2, suggests as in the biochemical experiments, that the WW domain alone is not sufficient for effective binding to β -dystroglycan. On the other hand, a mouse with deletion of exons 71-78 could rescue the phenotype, suggesting that the C-terminal region encoded by these exons is not essential for the binding [42]. All these results are compatible with the fact that the C-terminal region of dystrophin spanning 3311-3342 - the ZZ domain, is crucial for binding to β -dystroglycan. Our results suggest that we could limit the direct binding activity of this crucial region to the residues D3326-3332 or U3083-3089 of dystrophin or utrophin respectively (peptide 19). Taken together these results are consistent with there being a second dystroglycan-binding site between dystrophin/utrophin ZZ domain and dystroglycan in addition to the WW domain-mediated interaction with the extreme c-terminus of β -dystroglycan. As shown in figure 6, this region is likely to be distinct from the well characterised PPPYVP site due to the inability of 43DAG/8D5 to compete for peptide 19 binding to purified β -dystroglycan. This additional binding site further stabilises the β -dystroglycan - WW domain interaction, which itself is supported by the EF hand region, and explains why the complete WW-EF-ZZ region appears to be required for full binding activity between β -dystroglycan and dystrophin or utrophin.

ZZ domain mutations in humans: The first missense mutation reported in the C-terminal region was C3340Y from a patient with DMD and mental retardation, but with ~20% of normal levels of dystrophin immunoreactivity and some β -dystroglycan staining at the sarcolemma [43]. From our analyses, C3340 would be predicted to be essential for the formation of the second zinc

knuckle (Fig 3), and mutation to tyrosine would ablate the zinc binding site. Biochemical analysis of this mutation, and also an engineered C3340S substitution, in the context of the interaction between dystrophin and β -dystroglycan also demonstrates a loss of function [35] in support of the patient data and our own analysis of the zinc binding site. From the biochemical analysis of mutations of all the cysteines in the dystrophin ZZ domain, only C3340Y prevents dystroglycan binding. Mutations C3313Y, C3316Y and C3319Y still allow dystroglycan binding [35], suggesting some plasticity in zinc binding in the first zinc knuckle. This is evident from the SPOTs overlay in Figure 3A, where there are five potential liganding residues, but only four would be required to coordinate zinc. The situation for utrophin appears different however. Mutation of any of the 3 cysteine residues in the first zinc knuckle did not support dystroglycan binding, whereas mutation of either cysteine in the second zinc knuckle did support dystroglycan binding [35]. This observation is supported in part by our own data, where antibody 13D2 directed against the second zinc knuckle completely blocked dystroglycan binding to dystrophin but had a reduced ability to block utrophin binding (Fig 7) suggesting that the second zinc knuckle is less important for the utrophin-dystroglycan interaction.

Summary: The above data provides a conceptual advance on previously reported findings [16,35] and defines the ZZ domain in concert with the WW domain as being a crucial structural and functional part of the dystrophin/utrophin – β -dystroglycan interaction that is involved in anchoring these proteins to the cell membrane. The identification of the short dystrophin peptide YRSLKHF defines a second β -dystroglycan binding site in dystrophin and utrophin and raises the possibility of identifying further specific antibodies that could be used to distinguish between dystrophin and utrophin in this highly similar region. Finally biochemical approaches, epitope mapping, ELISA and overlay, provide new evidence for the zinc binding properties and β -dystroglycan interaction site on the ZZ domain, and highlights distinct differences in the mode of interaction between β -dystroglycan and the dystrophin or utrophin ZZ domains.

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CONFLICT OF INTEREST

The authors declare they have no competing interests.

REFERENCES

- 1 Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C. and Kunkel, L. M. (1987) Complete cloning of the Duchenne muscular-dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* **50**, 509-517
- 2 Hoffman, E. P., Brown, R. H., Jr. and Kunkel, L. M. (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* **51**, 919-928
- 3 Koenig, M., Monaco, A. P. and Kunkel, L. M. (1988) The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* **53**, 219-226
- 4 Davison, M. D. and Critchley, D. R. (1988) Alpha-actinins and the DMD protein contain spectrin-like repeats. *Cell* **52**, 159-160
- 5 Ervasti, J. M. and Campbell, K. P. (1993) A role for the dystrophin glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell. Biol.* **112**, 809-823
- 6 Koenig, M., Beggs, A. H., Moyer, M., Scherpf, S., Heindrich, K., Bettecken, T., Meng, G., Muller, C. R., Lindlof, M., Kaariainen, H., et al. (1989) The molecular-basis for Duchenne versus Becker muscular-dystrophy - correlation of severity with type of deletion. *Am. J. Hum. Genet.* **45**, 498-506
- 7 Beggs, A. H., Hoffman, E. P., Snyder, J. R., Arahata, K., Specht, L., Shapiro, F., Angelini, C., Sugita, H. and Kunkel, L. M. (1991) Exploring the Molecular-Basis For Variability Among Patients With Becker Muscular-Dystrophy - Dystrophin Gene and Protein Studies. *Am. J. Hum. Genet.* **49**, 54-67
- 8 Tinsley, J. M., Blake, D. J., Roche, A., Fairbrother, U., Riss, J., Byth, B. C., Knight, A. E., Kendrick-Jones, J., Suthers, G. K., Love, D. R., et al. (1992) Primary structure of dystrophin-related protein. *Nature* **360**, 591-593
- 9 Chamberlain, J. (2006) The structure and function of dystrophin. In *Molecular mechanisms of muscular dystrophies* (Winder, S. J., ed.), pp. 14-34, Landes Bioscience, Georgetown
- 10 Andre, B. and Springael, J. Y. (1994) WWP, a new amino acid motif present in single or multiple copies in various proteins including dystrophin and the SH3-binding Yes-associated protein YAP65. *Biochem. Biophys. Res. Commun.* **205**, 1201-1205
- 11 Bork, P. and Sudol, M. (1994) The WW domain: a signalling site in dystrophin? *Trends Biochem. Sci.* **19**, 531-533
- 12 Ponting, C. P., Blake, D. J., Davies, K. E., Kendrick-Jones, J. and Winder, S. J. (1996) ZZ and TAZ: new putative zinc fingers in dystrophin and other proteins. *TIBS* **21**, 11-13
- 13 James, M., Nuttall, A., Ilsley, J. L., Ottersbach, K., Tinsley, J. N., Sudol, M. and Winder, S. J. (2000) Adhesion-dependent tyrosine phosphorylation of β -dystroglycan regulates its interaction with utrophin. *J. Cell Sci.* **113**, 1717-1726
- 14 Jung, D., Yang, B., Meyer, J., Chamberlain, J. S. and Campbell, K. P. (1995) Identification and characterization of the dystrophin anchoring site on β -dystroglycan. *J. Biol. Chem.* **270**, 27305-27310
- 15 Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M. and Eck, M. J. (2000) Structure of a WW domain containing fragment of dystrophin in complex with β -dystroglycan. *Nature Struct. Biol.* **7**, 634-638

- 16 Rentschler, S., Linn, H., Deininger, K., Bedford, M. T., Espanel, X. and Sudol, M. (1999) The WW domain of dystrophin requires EF-hands region to interact with beta-dystroglycan. *Biol. Chem.* **380**, 431-442
- 17 Pons, F., Robert, A., Fabbriozio, E., Hugon, G., Califano, J.-C., Fehrentz, J. A., Martinez, J. and Mornet, D. (1994) Utrophin localisation in normal and dystrophin-deficient heart. *Circulation* **90**, 369-374
- 18 Winder, S. J. and Kendrick-Jones, J. (1995) Protein production in 3 different expression vectors from a single PCR product. [published erratum in *Anal. Biochem.* Vol 236, p190, 1996]. *Anal. Biochem.* **231**, 271-273
- 19 Chen, Y.-J., Spence, H. J., Cameron, J. M., Jess, T., Ilsley, J. L. and Winder, S. J. (2003) Direct interaction of β -dystroglycan with F-actin. *Biochem. J.* **375**, 329-337
- 20 Fabbriozio, E., Leger, J., Anoa, M., Leger, J. J. and Mornet, D. (1993) Monoclonal antibodies targetted against the C-terminal domain of dystrophin or utrophin. *FEBS Letters* **322**, 10-14
- 21 Chazalette, D., Hnia, K., Rivier, F., Hugon, G. and Mornet, D. (2005) $\alpha 7$ B Integrin changes in mdx mouse muscles after l-arginine administration. *FEBS Lett.* **579**, 1079
- 22 Fabbriozio, E., Latouche, J., Rivier, F., Hugon, G. and Mornet, D. (1995) Re-evaluation of the distributions of dystrophin and utrophin in sciatic nerve. *Biochem J* **312 (Pt 1)**, 309-314
- 23 Khurana, T. S., Watkins, S. C. and Kunkel, L. M. (1992) The subcellular distribution of chromosome 6-encoded dystrophin-related protein in the brain. *J. Cell Biol.* **119**, 357-366
- 24 Schiff, L. A., Nibert, M. L. and Fields, B. N. (1988) Characterization of a Zinc Blotting Technique: Evidence that a Retroviral Gag Protein Binds Zinc. *PNAS* **85**, 4195-4199
- 25 Chantler, P. D. (1985) Calcium-dependent association of a protein complex with the lymphocyte plasma membrane: probable identity with calmodulin-calceinurin. *J. Cell Biol.* **101**, 207-216
- 26 Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1986) *Data for Biochemical Research*. Clarendon Press, Oxford
- 27 Winder, S. J. (1996) Structure-function relationships in dystrophin and utrophin. *Biochem. Soc. Trans.* **24**, 497-501
- 28 Vallee, B. L. and Auld, D. S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* **29**, 5647-5659
- 29 Ilsley, J. L., Sudol, M. and Winder, S. J. (2002) The WW domain: linking cell signalling to the membrane cytoskeleton. *Cell. Signal.* **14**, 183-189
- 30 Campbell, K. P. and Kahl, S. D. (1989) Association of dystrophin and an integral membrane glycoprotein. *Nature* **338**, 259-262
- 31 Suzuki, A., Yoshida, M., Yamamoto, H. and Ozawa, E. (1992) Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain. *FEBS Lett.* **308**, 154-160
- 32 Chung, W. and Campanelli, J. T. (1999) WW and EF hand domains of dystrophin-family proteins mediate dystroglycan binding. *Mol Cell Biol Res Commun* **2**, 162-171
- 33 Flanigan, K., Niederhausern, A. v., Dunn, D., Alder, J., Mendell, J. and Weiss, R. (2003) Rapid direct sequence analysis of the dystrophin gene. *Am. J. Hum. Genet.* **72**, 931-919
- 34 Goldberg, L. R., Hausmanowa-Petrusewicz, I., Fidzianska, A., Duggan, D. J., Steinberg, L. S. and Hoffman, E. P. (1998) A dystrophin missense mutation showing persistence of dystrophin and dystrophin-associated proteins yet a severe phenotype. *Ann Neurol* **44**, 971-976

- 35 Ishikawa-Sakurai, M., Yoshida, M., Imamura, M., Davies, K. E. and Ozawa, E. (2004) ZZ domain is essentially required for the physiological binding of dystrophin and utrophin to beta-dystroglycan. *Hum. Mol. Genet.* **13**, 693-702
- 36 Legge, G. B., Martinez-Yamout, M. A., Hambly, D. M., Trinh, T., Lee, B. M., Dyson, H. J. and Wright, P. E. (2004) ZZ Domain of CBP: an Unusual Zinc Finger Fold in a Protein Interaction Module. *Journal of Molecular Biology* **343**, 1081
- 37 Ilsley, J. L., Sudol, M. and Winder, S. J. (2001) The interaction of dystrophin with β -dystroglycan is regulated by tyrosine phosphorylation. *Cell. Signal.* **13**, 625-632
- 38 Rosa, G., Ceccarini, M., Cavaldesi, M., Zini, M. and Petrucci, T. C. (1996) Localisation of the dystrophin binding site at the carboxyl terminus of β -dystroglycan. *Biochem. Biophys. Res. Commun.* **223**, 272-277
- 39 Tommasi di Vignano, A., Di Zenzo, G., Sudol, M., Cesareni, G. and Dente, L. (2000) Contribution of the different modules in the utrophin carboxy-terminal region to the formation and regulation of the DAP complex. *FEBS Lett* **471**, 229-234
- 40 Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y. and Ozawa, E. (1994) Molecular organisation at the glycoprotein-complex-binding site of dystrophin: three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. *Eur. J. Biochem.* **220**, 283-292
- 41 Rafael, J. A., Cox, G. A., Corrado, K., Jung, D., Campbell, K. P. and Chamberlain, J. S. (1996) Forced expression of dystrophin deletion constructs reveals structure-function correlations. *J. Cell Biol.* **134**, 93-102
- 42 Crawford, G. E., Faulkner, J. A., Crosbie, R. H., Campbell, K. P., Froehner, S. C. and Chamberlain, J. S. (2000) Assembly of the Dystrophin-associated Protein Complex Does Not Require the Dystrophin COOH-terminal Domain. *J. Cell Biol.* **150**, 1399-1410
- 43 Lenk, U., Oxele, K., Voit, T., Ancker, U., Hellner, K.-A., Speer, A. and Hubner, C. (1996) A cysteine 3340 substitution in the dystroglycan-binding domain of dystrophin associated with Duchenne muscular dystrophy, mental retardation and absence of the ERG b-wave. *Hum. Mol. Genet.* **5**, 973-975
- 44 Winder, S. J. (1997) The membrane-cytoskeleton interface: the role of dystrophin and utrophin. *J. Muscle Res. Cell Motil.* **18**, 617-629
- 45 <http://www.dmd.nl/> Leiden Muscular Dystrophy Pages(c) DMD sequence variants. 13-14
- 46 Hofstra, R. M. W., Mulder, I. M., Vossen, R., Koning-Gans, P. A. M. d., Kraak, M., Ginjaar, I. B., Hout, A. H. v. d., Bakker, E., Buys, C. H. C. M., Ommen, G.-J. B. v., et al. (2004) DGGE-based whole-gene mutation scanning of the dystrophin gene in Duchenne and Becker muscular dystrophy patients. *Hum. Mutat.* **23**, 57-66
- 47 Becker, K., Robb, S. A., Hatton, Z., Yau, S. C., Abbs, S. and Roberts, R. G. (2003) Loss of a single amino acid from dystrophin resulting in Duchenne muscular dystrophy with retention of dystrophin protein. *Hum. Mutat.* **21**, 651-656

FIGURE LEGENDS

Figure 1: Amino acid alignment of the ZZ domains of dystrophin (Dys) and utrophin (Utr) indicating identical residues (|) and highly conserved residues (:). Above and below these sequences are the sequences of the series of overlapping 7 residue peptides used in this study, all are numbered except the first four which could not be synthesised to high enough purity. Peptides above the alignment are to dystrophin including those which are underlined which are common between dystrophin and utrophin, those below the alignment are unique to utrophin.

Figure 2: Zinc binding to the utrophin ZZ domain. A. CD spectrum of the utrophin ZZ domain in the presence of 1mM ZnCl₂ (dashed line) or absence of zinc (presence of phenanthroline, solid line) indicating a modest structural change on zinc binding. This is reflected in the slightly altered mobility of the ZZ domain on SDS-PAGE in the presence of ZnCl₂ (inset). B. Autoradiographs of individual ⁶⁵ZnCl₂ overlays of purified ZZ domain in the absence (top left square) or presence of an excess of the indicated competing divalent cation. ⁶⁵Zn binding is competed in keeping with the Irving-Williams series Mn<Fe<Co<Ni<Cu>Zn>>Ca/Mg. A subset of the data in part B were published previously in brief [44]. C. Densitometric analysis of the data in B and two additional independent experiments, with ⁶⁵ZnCl₂ binding in the presence of the indicated competing divalent cations represented relative to ⁶⁵ZnCl₂ alone = 1. Mean ± SEM n=3.

Figure 3: Utrophin ZZ domain Peptide SPOTs ⁶⁵Zn overlay. A. A 46 peptide, 20 residue SPOTs peptide array overlapping by one amino acid and covering the utrophin ZZ domain was overlaid with ⁶⁵ZnCl₂ and subjected to autoradiography. Individual peptides are numbered 1-46 with the corresponding sequence shown. Positions 47-50 in the array were blank. Two regions of ⁶⁵Zn binding were observed spanning peptides 6-16 and 30-37 respectively, potential residues involved in the coordination of zinc are highlighted in grey and bold underline. Another region with the potential to bind ⁶⁵Zn (peptides 43-46: potential coordinating residues highlighted in grey) showed no ⁶⁵Zn binding by this approach. The two zinc binding regions are represented schematically in B.

Figure 4: ZZ domain monoclonal antibody epitope mapping. A. Epitope determination of monoclonal antibodies specificity by ELISA assay using synthetic peptides (number on x axis; see Fig 1). Results are represented as histograms; 13D2, 12D7 and 14A4 antibodies all recognised a single specific dystrophin peptide, whereas antibody 4G3 did not recognise any peptide. 14A4 was reprobated against a series of overlapping dystrophin and utrophin peptides (panel B). Again the 14A4 antibody appeared specific for the dystrophin sequence D3335-3341 sequence, and did not recognise the when I3336 substitution to V3039 in the utrophin sequence (peptide 34). The epitopes are represented on the topological map of the ZZ domain derived from Figure 3, individual epitopes are shaded grey and the non-conserved isoleucine residues (valine in utrophin) are indicated by asterisks.

Figure 5: Immunodetection of dystrophin in muscle. Serial cryostat sections of *Torpedo marmorata* (A) or rabbit muscle (B) were labelled using the four monoclonal antibodies. Antibodies 12D7 and 14A4 both produced a clear sarcolemmal staining pattern consistent with dystrophin staining (A2/B2,A3/B3), which in both cases was competed by an excess of the specific peptide to which the antibody was raised (A2'/B2',A3'/B3'). Antibody 4G3, which did not detect a specific peptide by ELISA exhibited extensive background staining in both *T. marmorata* and rabbit muscle (A4/B4). Surprisingly antibody 13D2 produced only a very weak background staining pattern that was not further reduced in the presence of excess peptide, with no clear sarcolemmal staining in either muscle type (A1/A1', B1/B1'). Bar =50 μm . In keeping with the immunohistological observation antibodies 12D7 and 14A4 recognised an $\sim 400\text{kDa}$ band in western blots of rabbit muscle extracts, again consistent with dystrophin staining, which was competed away with the specific peptide, whereas antibody 13D2 did not detect anything (C). Antibody 4G3, which did not detect a specific peptide or label the sarcolemma, recognised an unknown 170kDa protein in western blots. The specificity of antibodies 12D7 and 14A4 was further tested on muscle extracts from *mdx* mice that lack dystrophin (D). A previously characterised antibody specific for the carboxy-termini of dystrophin (H4, far left lane) confirmed the absence of dystrophin, whereas the presence of utrophin was revealed by antibody K7 (near left lane). Antibodies 12D7 and 14A4 detected no bands on western blots of *mdx* skeletal muscle confirming their specificity for dystrophin (right lanes). The protein bands detected are indicated according molecular mass markers (Biorad).

Figure 6: Competitive ELISA binding for peptide 19. Microtitre plates coated with or without peptide 19 were detected with antibody 13D2 (raised against peptide 19) or 43DAG/8D5 (43DAG; raised against the β -dystroglycan cytoplasmic domain). In panel A, peptide 19 was detected by 13D2 at similar levels in all wells (open squares) and not detected by 43DAG (closed squares). On incubation with increasing concentrations of purified β -dystroglycan cytoplasmic domain (β -DG; 0.5-9 μ g/ml) 13D2 detection of peptide 19 was specifically reduced (open diamonds) whereas incubation of empty or peptide 19 coated wells with β -DG and detection with 43DAG showed a specific increase in signal. These results demonstrate a β -DG specific competition for 13D2 binding to peptide 19, confirming peptide 19 as a β -dystroglycan binding site. Qualitatively similar results were obtained in competitive ELISA binding for peptide 19 using a dystroglycan-enriched fraction of skeletal muscle (Ht; 1-10 μ g/ml panel B). Increasing concentrations of homogenate competed for 13D2 detection of peptide 19 (open triangle), whereas the homogenate had no effect on the detection of β -DG coated wells by 43DAG (closed circles), other controls were as panel A.

Figure 7: Overlay experiments using electroblotted total crude muscle membrane homogenate (Ht). Panels (A1 and A2) correspond to the native nitrocellulose with total electrotransferred Ht revealed by LG5 and H4 polyclonal antibodies (anti- β -dystroglycan and anti-dystrophin respectively). Membranes were overlaid with enriched dystrophin homogenate comprising dystrophin and its associated proteins. Detection was performed with a dystrophin C-terminal antibody (H4). When peptide 19 was pre-incubated before overlay with the dystrophin enriched homogenate only a 400 kDa band was detected with H4 polyclonal antibody suggesting that the binding site for β -dystroglycan was blocked (A3). Panel B represents the same overlay experiment performed with utrophin enriched homogenate from *mdx* mouse comprising utrophin and its associated proteins detected by the C-terminal β -dystroglycan and utrophin antibodies (panel B1 and panel B2 respectively). Panel B3 showed that the utrophin binding properties for β -dystroglycan are slightly different from that of dystrophin and were not reduced to the same extent when peptide 19 was applied. Panels A4 and B4 represent densitometric quantification of the amounts of dystrophin (DYS) and utrophin (UTR) present in the two fractions.

Figure 8. Alignment of ZZ domain sequences from dystrophin family proteins and other unrelated proteins from different phyla, A. Gene name, organism and residue numbers for the Z domain used in the alignment are shown. The alignment was generated using ClustalW and the 60% consensus sequence derived from the SMART database entry for the ZZ domain is shown at the bottom. All cysteines are coloured yellow, single amino acids that fit the consensus and conserved aromatic and basic residues that form the core of the ZZ domain are also coloured. Capitalised letters represent conserved amino acids (in the full alignment), and the lowercase letters and symbols denote conserved features; positive, +; aromatic, a; small, s; polar, p; big, b and hydrophobic, h. A more extensive alignment of 47 ZZ domain sequences can be found at <http://www.expasy.org/cgi-bin/aligner?psa=PS01357&color=1&maxinsert=10&linelen=0>

B. A scheme to demonstrate the positions of Zn coordinating residues as proposed for the utrophin ZZ domain and as elucidated structurally for the CBP ZZ domain [36]. Numbers beneath each sequence refer to the positions of the liganding residues coordinating each individual zinc ion. For utrophin these are proposed to be consecutive in the linear sequence, whereas CBP adopts a cross-braced organisation.

Table 1.

Phenotype	Dystrophin ZZ domain	Source
	3311 AKCNICKECPIIGFRYRSLKHFNYDICQSCFF 3343	
DMD	AK <u>F</u> NICKECPIIGFRYRSLKHFNYDICQSCFF	[33]
BMD	AKCNICKE <u>R</u> PIIGFRYRSLKHFNYDICQSCFF	[45]
DMD	AKCNICKECPIIGFRYRSLKHFNY <u>H</u> ICQSCFF	[34]
DMD	AKCNICKECPIIGFRYRSLKHFNYDI <u>Y</u> QSCFF	[43]
DMD ^a	AKCNICKECPIIGFRYRSLKHFNYDIC <u>L</u> SCFF	[45]
DMD ^b	AKCNICKECPIIGFRYRSLKHFNYDIC <u>F</u> SCFF	[46]

Table 1. Missense mutations in the ZZ domain of dystrophin. ^{a,b}These mutations produced frame-shifting stop codons 34^a and 8^b codons respectively downstream of the point mutation. Consequently the DMD phenotypes could be due to the frame-shift and or truncation rather than any specific point mutation. A rare single amino acid deletion of a conserved glutamate 3367 just c-terminal of the ZZ domain resulted in a DMD phenotype despite normal correctly localised protein [47].

Figure 1. Hnia et al.

6ECPIIGF 19YRSLKHE 31YDICQSC
4KECPIIG 18RYRSLKH 29NYDICQS
2CKECPPII 17FRYRSLK 27FNYDICQ
1ICKECPI 16GFRYRSL 25HFNYDIC
NICKECP 14IGFRYRS 23KHFNYDI
CNICKEC 12IIGFRYR 22LKHFNYD
KCNICKE 10PIIGFRY 21SLKHFNY 35ICQSCFF
AKCNICK 8CPIIGFR 20RSLKHFN 33DICQSCF
Dys 3311...AKCNICKECPIIGFRYRSLKHFNYDICQSCFF...3342
| | | | | | | | | | : | | | | | | | | | | : | | | | |
Utr 3068...AKCNICKECPIVGFYRSLKHFNYDVCQSCFF...3099
3CKECPV 24KHFNYDV
5KECPVIG 26HFNYDVC
7ECPIVGF 28FNYDVCQ
9CPIVGF 30NYDVCQS
11PIVGFY 32YDVCQSC
13IVGFYR 34DVCQSCF
15VGFYRS 36VCQSCFF

Figure 2. Hnia et al.

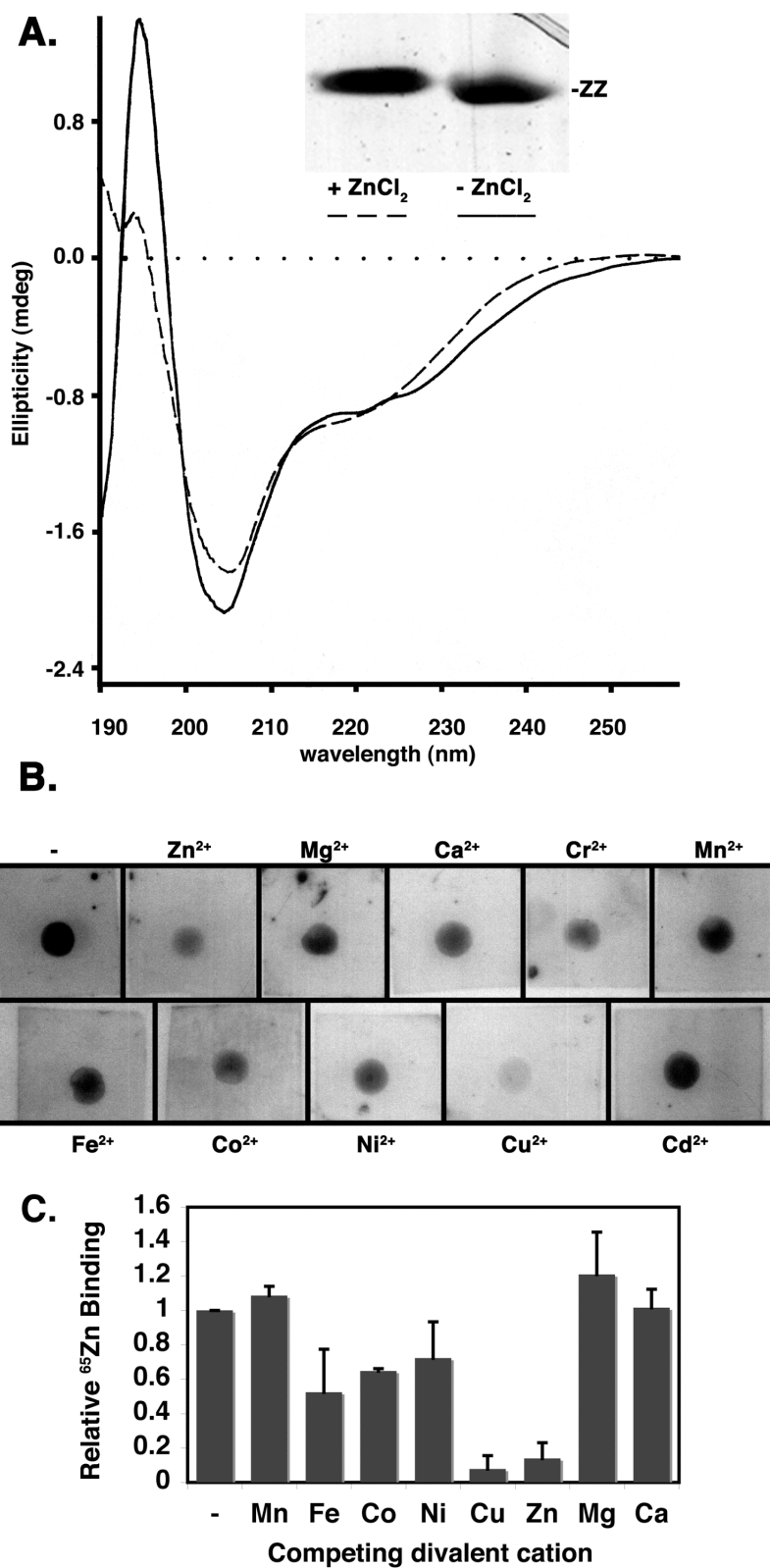
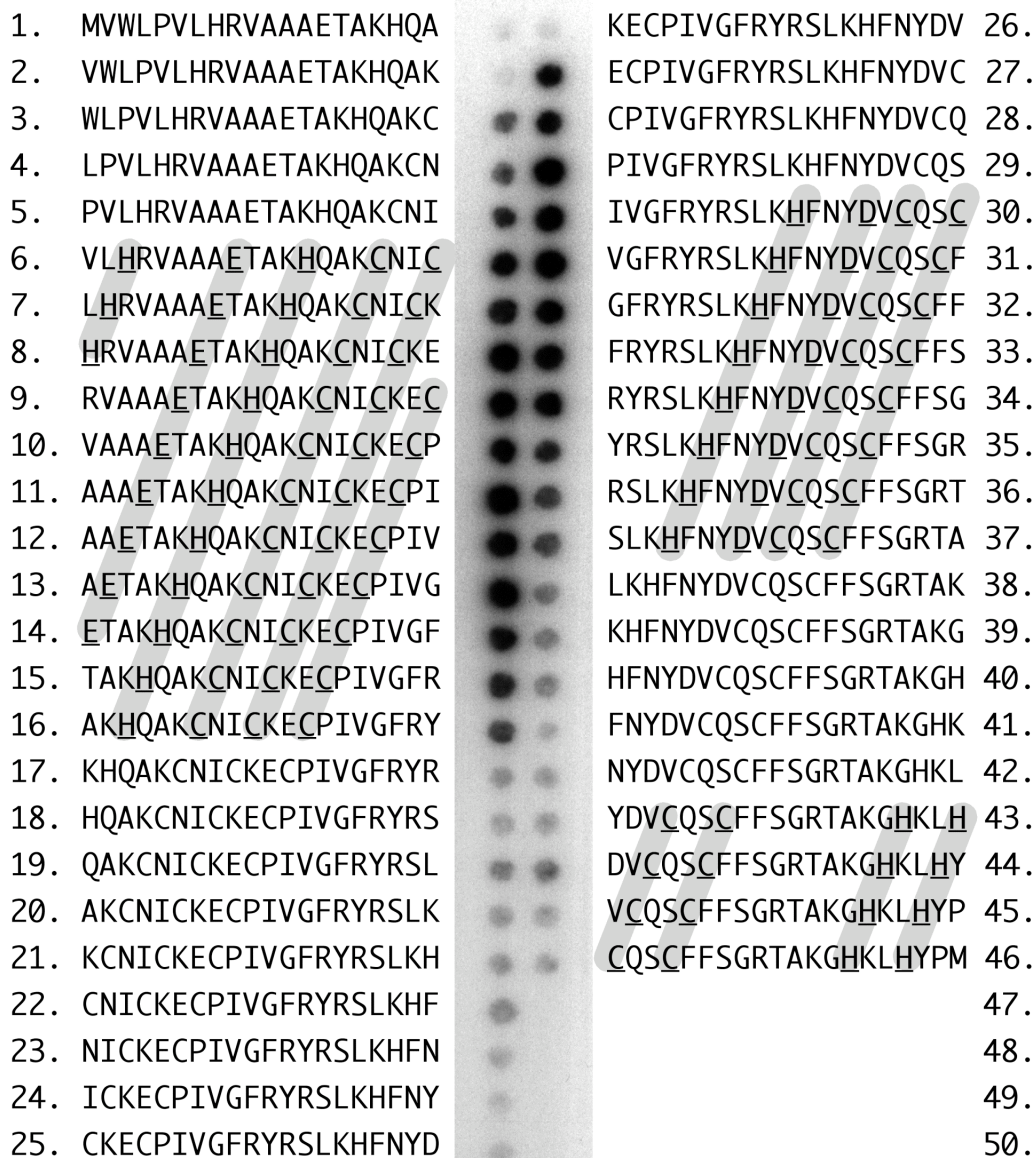


Figure 3. Hnia et al.

A.



B.

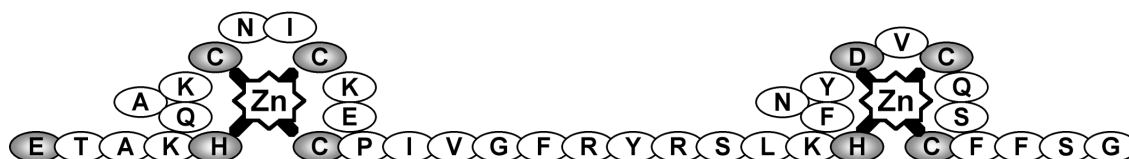


Figure 4. Hnia et al.

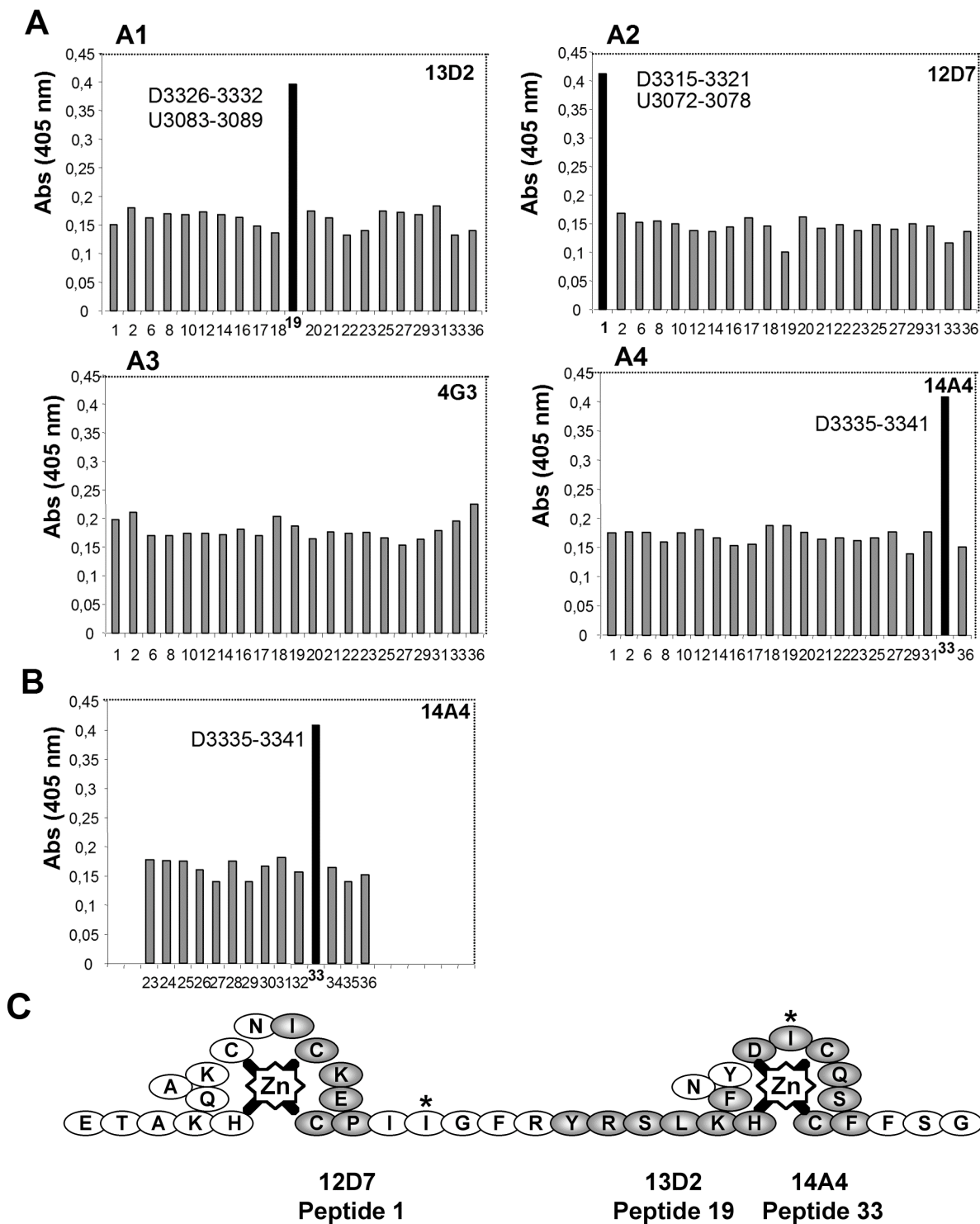


Figure 5AB. Hnia et al.

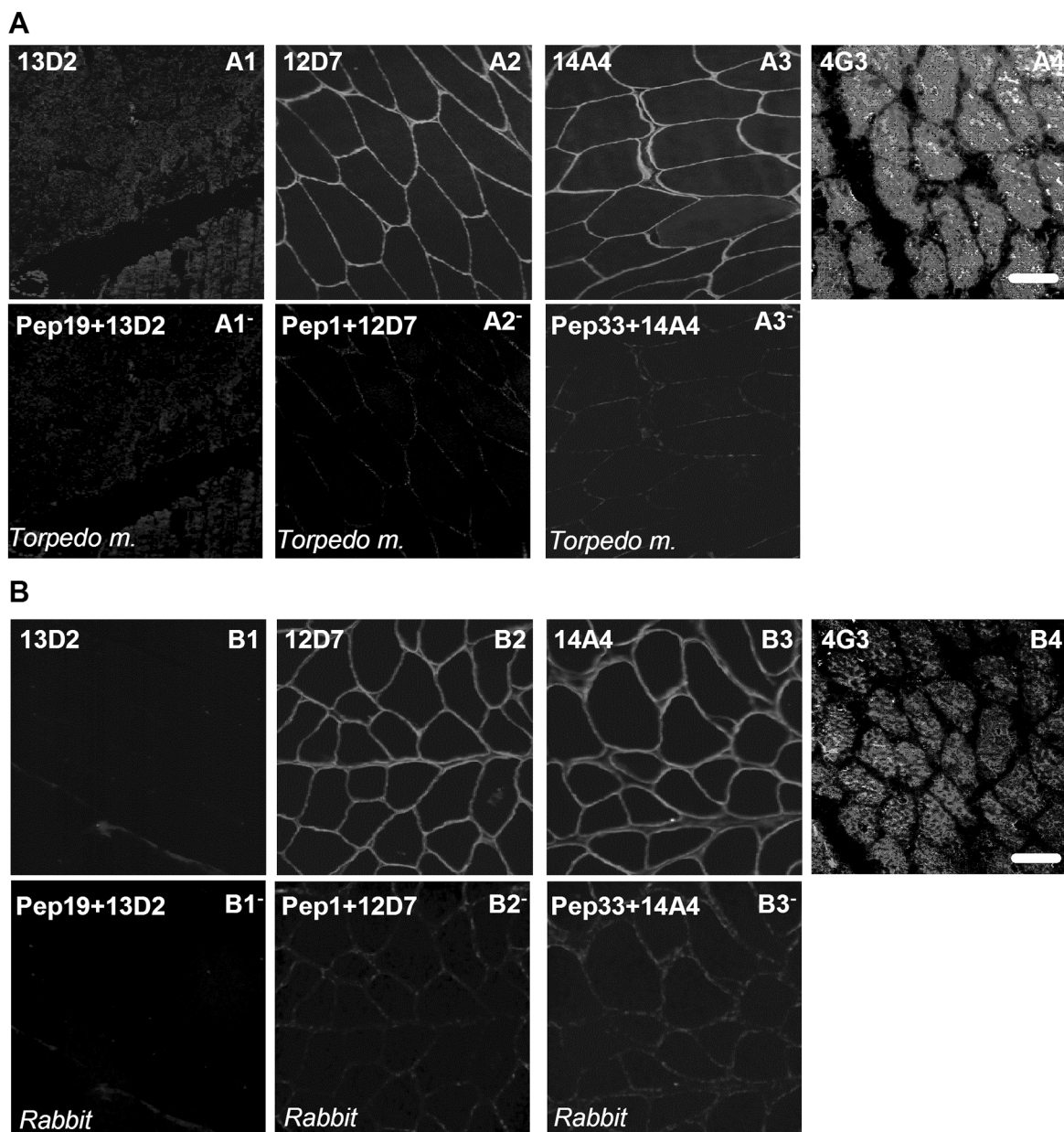
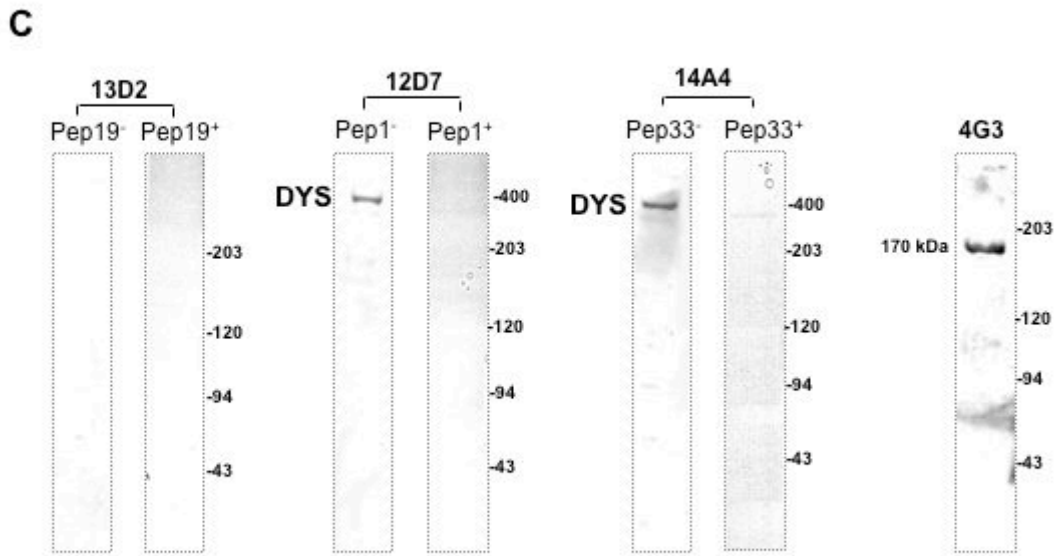
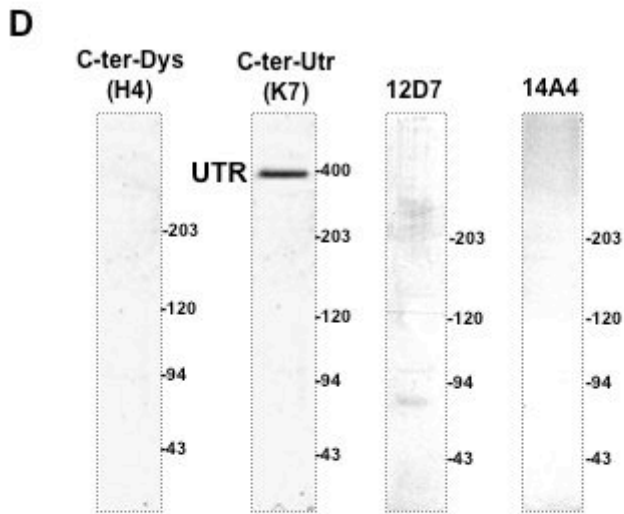


Figure 5CD. Hnia et al.



Rabbit skeletal muscle



mdx mouse skeletal muscle

Figure 6. Hnia et al.

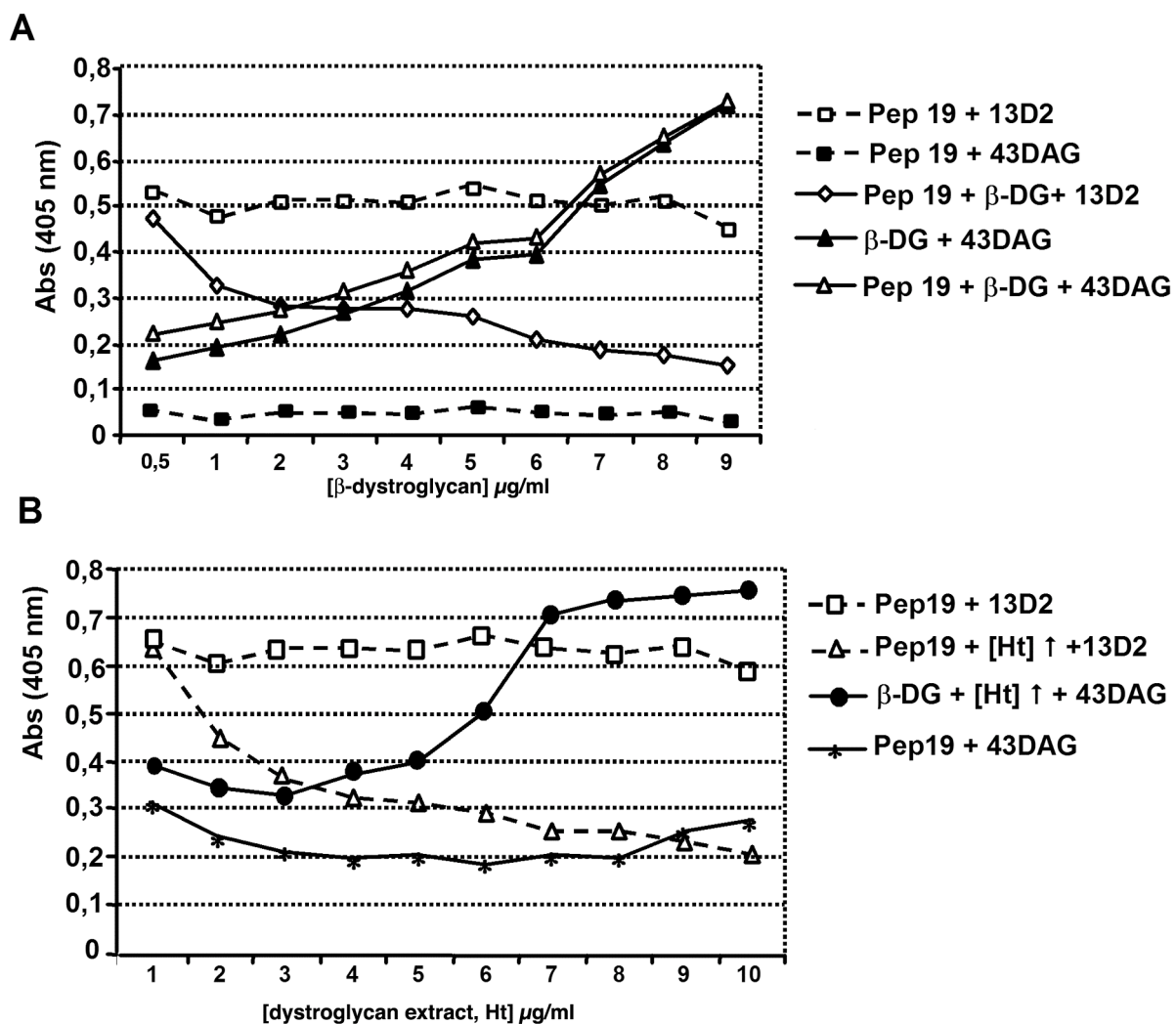


Figure 7. Hnia et al.

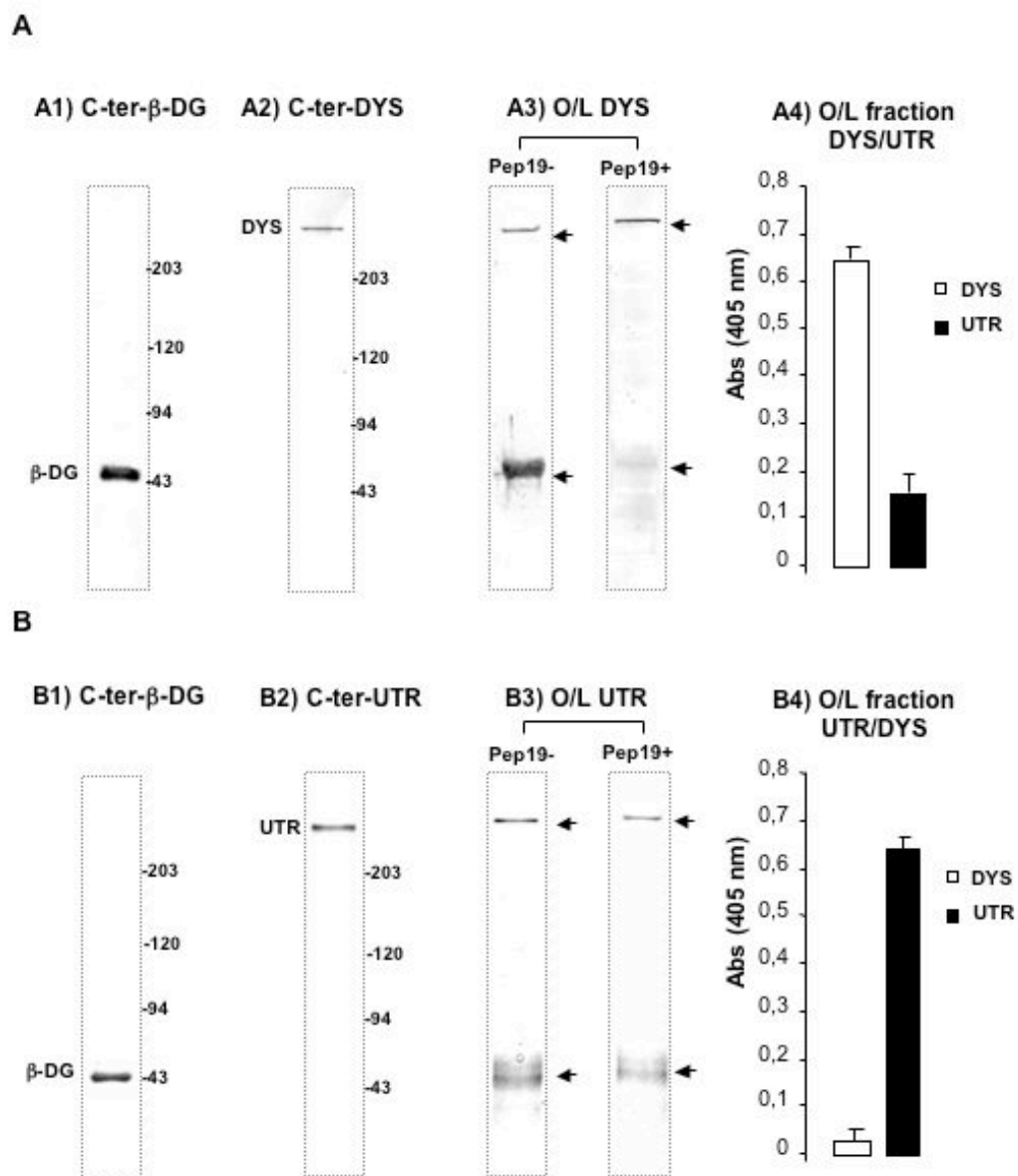


Figure 8. Hnia et al.

A

UTRO_HUMAN/3064-3109	AKHQAKCNI	CKECPIVGF	FRYRSLKHF	---	NYDVCQSC	FFSGRTAKGHKL
DMD_HUMAN/3307-3354	AKHQAKCNI	CKECPIIG	FRYRSLKHF	---	NYDICQSC	FFSGRVAKGHKL
DTNB_HUMAN/237-282	VFHPVECSY	CRCESMMGF	FRYRCQCH	---	NYQLCQNC	FWRGHAGGPHSN
CBP_human/1701-1744	DRFVYTCNE	CKHH--	VETRWHTVCE	---	DYDLCINCYNT	--KSHAHKM
MIB_DROME/173-218	KHEGTMCDT	CRQPIFG	IRWKCAEC	---	INYDLC	SIYHGDKHHLRHRF
REF2P_DROME/121-165	IHDAVECDG	CGLAPLIG	FRYKCVQCS	---	NYDLCQKCELA	-HKHPEHLM
YOY6_CAEEL/8-52	THEGVS	CDGCAFTAFAG	NRYKCLRCS	---	DYDLCFSC	FTT-KNYGDQOT
CBP1_CAEEL/1493-1532	KGMEYTCNK	CS-----	PAVWHCQSCD	---	DFDLCDGCKPT	--TQHPHEM
ADA2_YEAST/1-46	MSNKFHCDV	CSADCTN	RVVSCAICP	---	EYDLCVPC	FSSQ--GSYTGKH
RSC8_YEAST/254-298	IHKVYICHT	CGN-ESIN	VRYHNLRAR	---	DTNLC	SRCFQE--GHFGANF
SMCL_ARATH/304-350	PAVEYHCNS	CSAD-CSR	KRYHCPKQA	---	DFDLC	TECFNS--GKFSSDM
PRT1_ARATH/2005-255	VHFGAGCD	SGVYPIIG	DRYRCKD	CKEEIGYDLC	CKDCYE	-TPSKVPGRF
SMART Consensus/60%	spppsp	CssC.p.	.bsGhRa+ChbC.		DaDLCps	Cbsp...bphpH.

B

UTRO_HUMAN/3064-3109	AKHQAKCNI	CKECPIVGF	FRYRSLKHF	---	NYDVCQSC	FFSGRTAKGHKL
DYS/UTR Zn binding	1	1	1	1	2	2 2 2
CBP_human/1701-1744	DRFVYTCNE	CKHH--	VETRWHTVCE	---	DYDLCINCYNT	--KSHAHKM
CBP Zn binding	1	1	2	2	1	1 2 2