Analysis of the dystrophin interactome

Dissertation

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Dedicated to

My mother, Joy Thorley

My father, David Thorley

My sister, Alexandra Thorley

My fiancée, Vera Sakhno-Cortesi

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Abstract

The aim of this project was to systematically identify new interaction partners of the dystrophin protein within differentiated human skeletal muscle cells in order to uncover new roles in which dystrophin is involved, and to better understand how the global interactome is affected by the absence of dystrophin. A more complete understanding of the interactors and roles of dystrophin is vital for increasing the understanding of pathology in patients of Duchenne and Becker muscular dystrophy and other diseases in which dystrophin or dystrophin associated proteins are affected, and will assist in the development and research of treatments aimed at re-establishing the important interactions and pathways that have been lost or disrupted.

Initially, a review of existing literature and bioinformatics resources was carried out and used as the basis of an online interactome of dystrophin's physical interactors. A SILAC-based proteomics approach was then used to identify new interaction partners that were added to this bioinformatics resource. To obtain high protein quantities to maximize sensitivity we required a large number of cells, only feasible though the use of immortalized cell lines. hTERT/cdk4 immortalized myogenic human cell lines represent an important tool for skeletal muscle research however, disruption of the cell cycle has the potential to affect many other cellular processes to which it also linked. We carried out a transcriptome-wide analysis of healthy and diseased lines comparing immortalized lines with their parent primary populations in both differentiated and undifferentiated states in order to test their myogenic character by comparison with non-myogenic cells. We found that immortalization has no measurable effect on the myogenic cascade or on any other cellular processes, and that it was protective against the senescence process - a process observed at higher division counts of primary cells. Selected lines were not aneuploid and did not present any chromosomal rearrangements. In this context the human muscle cell lines are a good in vitro model to study the dystrophin interactome.

Having validated our cell lines, we investigated dystrophin's interactors using a high-sensitivity proteomics approach incorporating (1) isotopic amino acid labelling (SILAC), (2) knockdown of dystrophin expression, (3) immunoprecipitation and (4) mass spectrometry, allowing for the confident identification of dystrophin interactors, eliminating the multitude of false positive proteins pulled down through unspecific binding to both the beads and the antibody. We identified 18 new physical interactors of dystrophin which displayed a high proportion of vesicle transport related proteins and adhesion proteins, strengthening the link between dystrophin and these roles. The proteins determined through previously published data together with the newly identified

interactors were incorporated into a web-based data exploration tool: sys-myo.rhcloud.com/dystrophin-interactome, intended to provide an easily accessible and informative view of dystrophins interactions in skeletal muscle. This tool will benefit future understanding and interpretation of the interaction data, by placing these newly identified physically interacting proteins within the mechanistic context of the wider dystrophin interactome.

Résumé

Le but de ce projet était d'identifier de manière méthodique et standardisée les partenaires interagissant avec la protéine dystrophine dans les cellules musculaires squelettiques humaines différenciées. Cette stratégie devrait nous permettre de découvrir de nouveaux rôles de la dystrophine, et de mieux comprendre comment ce réseau d'interacteurs (interactome) est affectée par l'absence d'expression de la dystrophine. L'identification et la compréhension de cet interactome devrait nous permettre de mieux appréhender les dystrophinopathies (maladie de Duchenne et de Becker) mais également d'autres pathologies musculaires ou les niveaux d'expression de la dystrophine ou de ses partenaires sont affectés. L'identification de cet interactome egalement devrait contribuer à l'élaboration de stratégies thérapeutiques visant à rétablir les interactions et les voies de signalisations importantes qui ont été perdues ou perturbées.

Dans un premier temps, nous avons construit un premier interactome de la dystrophine en utilisant les données de la littérature et les bases de données bio-informatiques. Nous avons ensuite utilisé une approche protéomique SILAC pour identifier de nouveaux partenaires, partenaires qui ont alors été ajoutés à ce premier interactome de la dystrophine.

L'approche proteomique SILAC nécessite des quantités importantes de protéines, et exige donc l'utilisation d'un grand nombre de cellules. Dans ce contexte, nous avons utilisé des lignées de cellules musculaires humaines immortalisées. L'immortalisation a été obtenue en sur-exprimant de manière stable hTERT / CDK4. Dans un premier temps, nous avons vérifié que l'étape d'immortalisation ne perturbait pas divers processus cellulaires, dont le programme myogenique. Pour cela, nous avons réalisé une analyse transcriptomique comparant des lignées immortalisées avec leurs populations primaires correspondantes, à l'état de prolifération et de différentiation. Nous avons inclus dans cette analyse, des cellules non musculaires comme contrôles négatifs. Nous avons constaté que l'immortalisation n'a pas d'effet mesurable sur le programme myogénique ou sur tout autre processus cellulaires, et qu'elle avait un effet protecteur contre le processus de sénescence, processus observés à partir d'un nombre élevé de division des cellules primaires. De plus, les lignes sélectionnées ne présentaient pas d'aneuploïdie ni de réarrangements chromosomiques. Les lignées de cellules musculaires humaines constituent donc de bon model in vitro pour l'étude de l'interactome de la dystrophine.

Dans un second temps, nous avons déterminé l'interactome de la dystrophine en utilisant une approche proteomique hautement sensible combinant (1) l'utilisation d'isotopes stables d'acides aminés (SILAC), (2) le knockdown de l'expression de la dystrophine, (3) l'immuno- precipitation de la dystrophine et (4) la spectrométrie de masse. Cette stratégie nous a permis d'identifier de manière sure les partenaires de la dystrophine et d'éliminer une multitude de faux positifs, faux positifs étant des protéines immunoprécipitées de manière non spécifique par l'anticorps ou les billes. Nous avons identifié 18 nouveaux partenaires directs de la dystrophine, partenaires étant impliqués dans le transport vésiculaire ou étant des protéines d'adhésion. Ces résultats renforcent les données précédemment publiées suggérant un lien entre la dystrophine et le trafic vésiculaire, ainsi que dystrophine et adhesion cellulaire. Ces nouveaux partenaires ont été ajoutés à l'interactome de la dystrophine, interactome accessible sur le Web: sys-myo.rhcloud.com/dystrophin-interactome. Ce site web est dédié à être un outil facile d'utilisation permettant d'explorer et de visualiser l'interactome de la dystrophine du muscle squelettique. Cet outil permet au chercheur de comprendre et d'interpréter l'interactome de la dystrophine, en plaçant ces interacteurs nouvellement identifiés dans leur contexte mécanistique.

Abstrakt

Ziel dieses Projektes war die systematische Aufdeckung neuer Interaktionspartner des Dystrophin-Proteins in differenzierten Skelettmuskelzellen, um neue Funktionen von Dystrophin zu identifizieren und ein eingehenderes Verständnis darüber zu erlangen, welche Auswirkungen das Fehlen von Dystrophin auf das Interaktom insgesamt hat. Eine umfassendere Kenntnis der Wechselspieler und Funktionen von Dystrophin ist entscheidend für ein besseres Verständnis des Krankheitsgeschehens bei Patienten mit Muskeldystrophie vom Typ Duchenne oder Becker und anderer Krankheiten, bei denen Dystrophin oder Dystrophin-assoziierte Proteine beeinträchtigt sind, und wird zur Entwicklung und Erforschung von Therapien beitragen, die auf eine Wiederherstellung fehlender bzw. gestörter fundamentaler Interaktionen und Signalwege abzielen.

Zunächst wurde eine Recherche in der vorhandenen Fachliteratur und in Bioinformatik-Ressourcen durchgeführt und als Grundlage für ein Online-Interaktom der physischen Wechselspieler von Dystrophin verwendet. Anschließend wurden mithilfe der in der Proteomik gängigen SILAC-Technik neue Interaktionspartner identifiziert und dieser Bioinformatik-Ressource hinzugefügt. Um im Sinne einer Maximierung der Empfindlichkeit hohe Proteinmengen zu erhalten, benötigten wir eine große Anzahl von Zellen, was nur durch Verwendung immortalisierter Zelllinien möglich war. Immortalisierte myogene Humanzelllinien vom Typ hTERT/cdk4 sind ein wichtiges Hilfsmittel in der Skelettmuskelforschung, allerdings könnten durch die Unterbrechung des Zellzyklus zahlreiche andere zelluläre Prozesse beeinflusst sein, mit denen der Zellzyklus verknüpft ist. Wir führten eine Transkriptom-weite Analyse gesunder Zelllinien und krankheitsspezifischer Zelllinien durch, indem wir immortalisierte Zelllinien mit ihren Primärzell-Elternpopulationen sowohl im differenzierten als auch im undifferenzierten Zustand verglichen, um ihren myogenen Charakter durch Vergleich mit nicht-myogenen Zellen zu prüfen. Bestimmte Zelllinien wurden außerdem im Hinblick auf Aneuploidie und chromosomale Rearrangements überprüft. Wir stellten fest, dass Immortalisierung keine messbaren Auswirkungen auf die myogene Signalkaskase oder auf andere zelluläre Prozesse hatte und dass sie vor den systemischen Auswirkungen von Seneszenz, die nach einer großen Zahl von Teilungen von Primärzellen zu beobachten sind, schützte.

Nachdem wir unsere Zelllinien validiert hatten, untersuchten wir Dystrophin-Wechselspieler im Rahmen eines hochempfindlichen Proteomik-Ansatzes mit Knockdown, Isotopenmarkierung von Aminosäuren (SILAC), Knockdown der Dystrophin-Expression, Immunpräzipitation und Massenspektrometrie. Dies ermöglichte eine zuverlässige Identifizierung von Dystrophin-

Wechselspielern unter Eliminierung der Präzipitation der zahlreichen falschpositiven Proteine infolge einer unspezifischen Bindung an die Beads und den Antikörper. Wir identifizierten 18 neue physische Wechselspieler von Dystrophin, darunter anteilsmäßig viele Proteine, die am Vesikeltransport beteiligt sind, sowie Adhäsionsproteine, was die Verknüpfung zwischen Dystrophin und diesen Funktionen untermauert. Die aus publizierten Daten ermittelten Proteine sowie die neu identifizierten Wechselspieler wurden in ein internetbasiertes Datenexplorationstool (sys-myo.rhcloud.com/dystrophin-interactome) eingegeben, um eine übersichtliche und informative Darstellung der Dystrophin-Interaktionen im Skelettmuskel zu erhalten. Dieses Tool wird künftig dem Verständnis und der Interpretation der Interaktionsdaten dienlich sein, indem es diese neu identifizierten physisch interagierenden Proteine in den Kontext des größeren Dystrophin-Interaktoms einbindet.

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Abbreviations

2OMePS – 2'-O-methyl phosphorothioate
AAV – Adeno-associated virus
ABS – Actin-binding site
BMD – Becker muscular dystrophy
Cas9 – CRISPR associated protein 9
Cdk4 – Cyclin-dependent kinase 4
CH domain – Calponin homology domain
CR – Cysteine rich
CRISPR – Clustered, regularly interspaced, short, palindromic repeats
C-terminal – Carboxylic terminal
DAPC – Dystrophin-associated protein complex
DMD – Duchenne muscular dystrophy
Dp – Dystrophin protein
DRP2 – Dystrophin-related protein 2
DTNA – Alpha-dystrobrevin
EHD2 – EH domain-containing protein 2
FDA – Food and drug administration
FDR – False discovery rate
FSHD – Facioscapulohumeral muscular dystrophy
GEO – Gene Expression Omnibus
GO – Gene Ontology
GSEA – Gene set enrichment analysis
hTERT - Human telomerase reverse transcriptase
ITR – Inverted terminal repeats
kb – Kilobases
kDa – Kilodalton
LAMP1 – Lysosome-associated protein 1
LGMD – Limb-girdle muscular dystrophy
Lis1 – Lissencephaly 1
MCK promoter – Muscle creatine kinase promoter
MURC – Muscle-related coiled-coil protein / cavin-4
MYADM – Myeloid-associated differentiation marker
Nde1 – Nuclear distribution protein nude homolog 1
NMDA receptors – N-methyl-D-aspartate receptors
nNOS – Neuronal nitric oxide synthase
N-terminal – Amino terminal
Par1b – Polarity-regulating kinase partitioning-defective 1b
PCA – Principal component analysis
PH domain – Pleckstrin homology domain
PMO – Morpholino
PSICQUIC – Proteomics Standard Initiative Common QUery InterfaCe
PSI-MI format – Proteomics Standard Initiative Molecular Interaction format
PTRF – Polymerase I and transcript release factor / cavin-1
QUICK – Quantitative immunoprecipitation combined with knockdown
R[X] – Spectrin repeat [X]
SH domain – Src homology domain
SILAC – Stable isotope labelling with amino acids in cell culture

SNTA/B1/B2 – Syntrophin (alpha/beta-1/beta-2)
TALEN – Transcription activator-like effector nucleases
TNS1 – Tensin-1
TRPC – Transient receptor potential channels
UGC – Utrophin–glycoprotein complex
ZFN – Zinc finger nucleases
ZZ domain – ZZ-type zinc finger domain

Chapter 1 – Introduction

Muscle can be categorised into three groups; skeletal muscle, smooth muscle and cardiac muscle, skeletal muscle being responsible for voluntary movements. The constituent cells of muscles are called myoblasts. These myoblasts, upon myogensis, differentiate and fuse together to form elongated multinucleated myotubes. These myotubes initially have centrally located nuclei, but upon further maturity the nuclei migrate to the periphery of the cell and are referred to as muscle fibres. Muscles are made up of hundreds or thousands of aligned muscle fibres. Myoblasts can also fuse to previously differentiated myotubes

Upon myoblast differentiation the cell will alter protein expression as it moves from the proliferating myoblast to the muscle-functioning myotube. Muscle cells also express proteins specific to muscle. The differing protein expressions between myoblasts, myotubes, and cells of other tissue types allows for easy determination of cells types. Such common used markers include desmin (myoblasts and myotubes) (Kaufman & Foster, 1988; Lawson-Smith & McGeachie, 1998; Lazarides & Hubbard, 1976), myosin heavy chain (myotubes) (Bandman, Matsuda, Micou-Eastwood, & Strohman, 1981; Roy, Sreter, & Sarkar, 1979; Rushbrook & Stracher, 1979; Sréter, Bálint, & Gergely, 1975) and CD56 (myoblasts) (Belles-Isles et al., 1993; Illa, Leon-Monzon, & Dalakas, 1992; Schubert, Zimmermann, Cramer, & Starzinski-Powitz, 1989) to determine myogenicity. CD56 is a cell surface protein, making it useful for determining cell type and selecting cells while the cells are still alive (a property which is taken advantage of during the cell immortalisation process)

Muscles undergo constant degeneration and regeneration. In order for muscle to maintain its mass the two processes must occur at the same rate. Muscle atrophy occurs when the rate of degeneration exceeds the rate of regeneration. Skeletal muscle, being involved in voluntary movements, experiences external forces during animal movement. If the muscle is weakened and its ability to cope with the additional strain is diminished, degeneration will increase, which is the case during dystrophin absence from the cell.

Dystrophin is a vital protein within muscle cells encoded by one of the largest human genes at 2.4 megabases, comprising 79 exons which transcribes a 3,685 amino acid full length protein in humans (Figure 1).

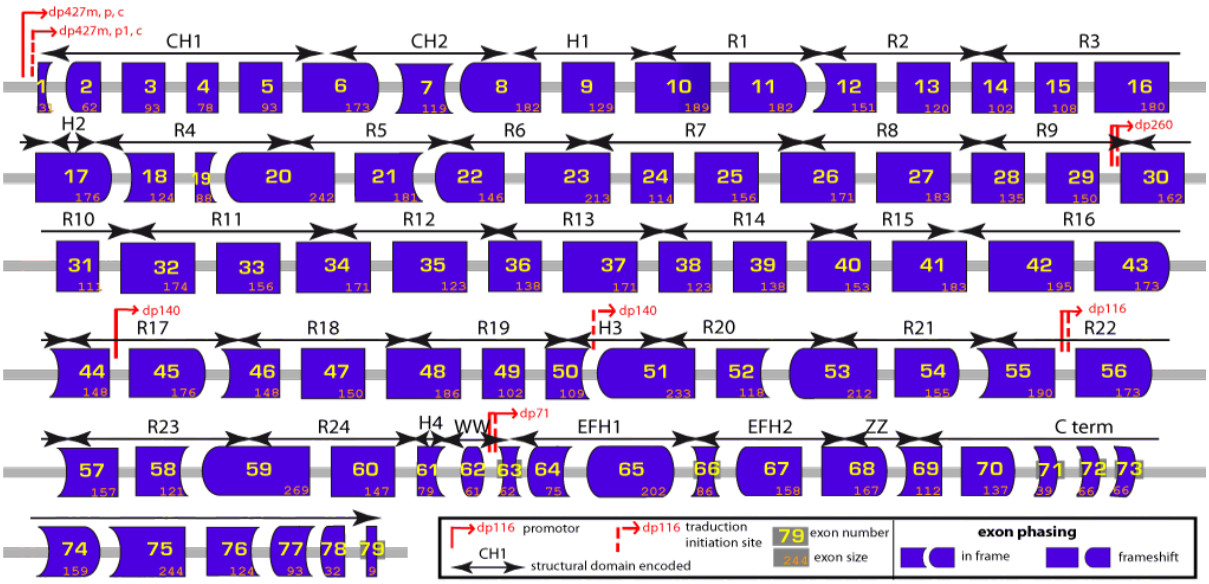


Figure 1. Representation of the domains encoded by the exons within the dystrophin gene. Figure from Nicolas *et al.* 2012 (Nicolas et al., 2012). Deletions of exons which result in-frame deletions result in a truncated dystrophin to be expressed whereas exon deletions resulting in a frameshift of the reading frame will result in an unstable protein.

Mutations in the gene can result in Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD). DMD is generally caused by a mutation that results in an out-of-frame deletion (the absence of exon 51 for example, Figure 1) or a nonsense mutation leading to no expression of functional dystrophin. Substitution of a single nucleotide is responsible for approximately half of reported cases, followed by small deletions, exon duplications, exon deletions, small duplications, small duplications and small insertions (Buzin et al., 2005; Hofstra et al., 2004; Tuffery-Giraud et al., 2004). BMD is generally caused by an in-frame mutation which results in a partially functional, shortened dystrophin being expressed (the absence of exon 49 for example). An example of the effects of exon deletions on the dystrophin protein resulting in BMD or DMD is shown in Figure 2.

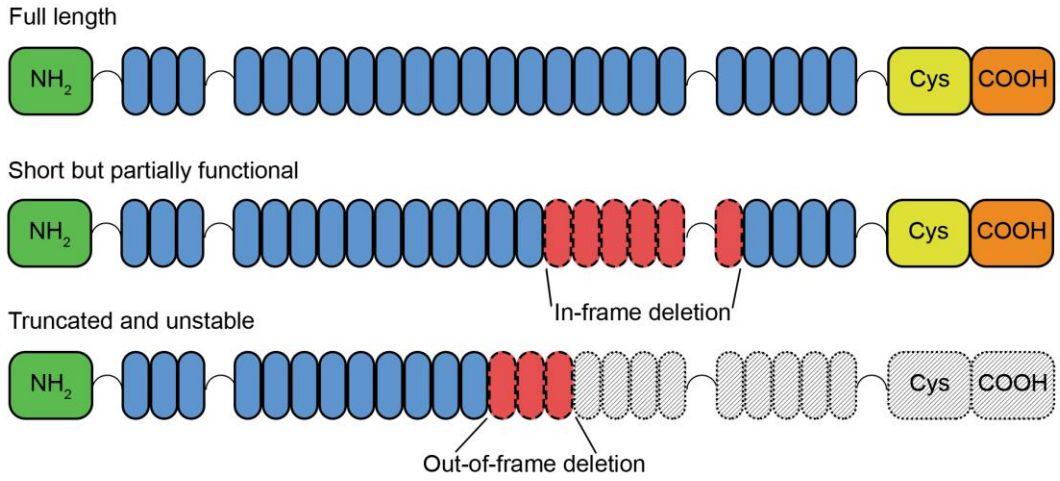


Figure 2. Representation of the effects of in-frame and out-of-frame deletions on the resulting dystrophin proteins. Spectrin repeats expressed in blue, gene deletions of spectrin in red and subsequently disrupted out-of-frame domains in black and white stripes. Figure adapted from Muntoni *et al.* 2003 (Muntoni, Torelli, & Ferlini, 2003). In-frame deletions result in a truncated protein in which domains further along the gene are still represented in the protein (deletion of exons 36-41 represented in this example) predicting a BMD phenotype. Out-of-frame deletions result in disruption of the reading frame causing domains downstream of the deletion to be disrupted and resulting in an unstable protein (deletion of exons 41-52 in this example) predicting a DMD phenotype.

DMD is the more severe of the two phenotypes and many efforts to treat the disease attempt to convert the DMD phenotype to the less severe BMD phenotype. Each in-frame mutation pattern has a different effect on the coding sequence, resulting in truncated dystrophin proteins of different lengths and structural composition. In addition, mutations may have varying effects on the expression level and stability of the resulting protein. This results in differing degrees of severity of the disease among those affected by BMD. In some cases, in-frame mutations can result in DMD phenotypes usually resulting from a large deletion in the spectrin repeats whereas some mutations allow BMD patients to go through their whole life needing only a walking stick to aid walking (Nevo et al., 2003), and others are entirely asymptomatic (Christophe Béroud et al., 2007). About two-thirds of cases are thought to be inherited, while the remainder result from spontaneous mutations (T. Lee et al., 2014). DMD affects approximately 1 in 5000 males (Mendell et al., 2012), and is characterized by severe muscle wasting with loss of the ability to walk during childhood and a life expectancy now of approximately 25 years with the use of corticosteroids and non-invasive ventilation (Beytía, Vry, & Kirschner, 2012) .

The DMD pathology usually becomes apparent between the ages of two and seven years of age when subjects experience their first difficulty in walking. Deterioration in other muscle functions become increasingly evident as the disease progresses. Muscle wasting occurs due to the inability to repair or recruit new muscle cells to sufficiently replace those lost by necrosis.

Some mutations affect the expression of shorter isoforms driven by alternative promoters within the gene. In particular, cognitive function may be impaired (Doorenweerd et al., 2014). The majority of patients with DMD and BMD will ultimately die as a result of respiratory failure or cardiac complications including dilated cardiomyopathy and irregular heartbeats (T. Matsumura, Saito, Fujimura, Shinno, & Sakoda, 2011; Stromberg, Darin, Kroksmark, & Tulinius, 2012), resulting from progressive loss of the muscles of the thoracic cavity, including the heart and diaphragm.

Such is the severity and high frequency of DMD among muscular dystrophies, numerous attempts have been made to treat the disease. Approaches include gene therapy and exon skipping (Abdul-Razak, Malerba, & Dickson, 2016; Popplewell et al., 2013; Yue et al., 2015), the latter having currently progressed beyond phase II clinical trials. Many of the treatments attempt to convert the Duchenne phenotype to a less severe BMD phenotype. Attempts to treat the disease through gene editing have recently gained traction (Long et al., 2015; Nelson et al., 2016; Tabebordbar et al., 2016). An alternative method involving overexpression of the dystrophin-related-protein utrophin to substitute for the absent dystrophin have also been explored with some preclinical success (Moorwood et al., 2011).

A great deal of research has gone into investigating dystrophin, its roles within the cell, and the pathology of dystrophinopathy since the discovery of the DMD gene in 1986 (Monaco et al., 1986) and subsequent discovery of the protein in 1987 (Hoffman, Brown, & Kunkel, 1987). During this time many proteins have been found to interact with dystrophin, many of which form the dystrophin associated protein complex (DAPC), a group of proteins that have a prominent role in maintaining cell structure by providing a link between the extracellular matrix and the cytoskeleton (Ehmsen, Poon, & Davies, 2002). Animal and cell models of the DMD pathology (such as the dystrophin-deficient mdx mice (Bulfield, Siller, Wight, & Moore, 1984)) have been used to research the effects of dystrophin and loss and potential treatments.

Gene therapy approaches involving dystrophin gene transfection using adeno-associated virus (AAV) have been extensively researched (Hollinger & Chamberlain, 2015; Jarmin, Kymalainen, Popplewell, & Dickson, 2014; Ramos & Chamberlain, 2015) and successfully completed phase 1 clinical trials to exhibit AAV safety (Bowles et al., 2012). Owing to the unusually large size of the dystrophin coding sequence, a limitation of the gene therapy approach arises from the physical capacity of the AAV capsid, which can hold around 4.5 kilobases (kb) of DNA. Genes encoding truncated dystrophin proteins (also known as

microdystrophin constructs) have been designed in an effort to produce a dystrophin that will reduce the DMD phenotype as much as possible within the AAV size constraints. This requires judgement on which are the most important dystrophin regions to retain, and which can be deleted with minimal loss of function.

To be able to fully understand the effects of dystrophin absence or the loss of specific fragments and to design the most effective exon-skipping and micro-dystrophin approaches, it is important to know which other proteins dystrophin is interacting with. The identification of all important binding partners is particularly pertinent to the gene therapy approach where there is some freedom to select which regions of dystrophin to include into the truncated product.

Chapter 2 – Literature review

2A. Dystrophin domains

2A1. Introduction

The 79 exons of dystrophin can be split into four distinct regions. The N-terminal/actin binding domain encompasses amino acids 14-240 (exons 1 to 8), forming two calponin homology domains (CH1 and CH2) (Koenig, Monaco, & Kunkel, 1988). The x-ray structure of this domain has been resolved (Figure 3A). The rod domain encompasses amino acids 253-3040 (exons 8 to 61), made up of 24 triple helical spectrin like repeat units and four short 'hinge' regions (x-ray structure Figure 3B). The rod domain comprises 75 percent of the entire dystrophin protein. Amino acids 3080-3360 (exons 62-69) make up the cysteine-rich domain. This includes the end of a WW domain (Figure 3C), two EF hands and a zinc finger domain (ZZ). Finally, there is the C-terminal domain of amino acids 3361- 3685 encoded by exons 70 to 79 containing a coiled-coil region. The cysteine rich and C-terminal domains contain a large proportion of the binding sites for proteins that make up the dystrophin-associated protein complex. A full crystal structure of dystrophin has not been obtained due to its large size.

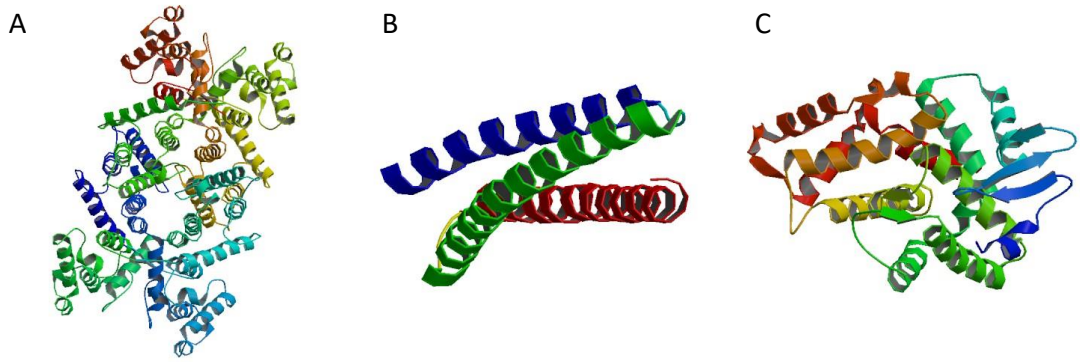


Figure 3. Crystal structures of dystrophin's N-terminal actin binding domain (A), first spectrin repeat (B) and WW domain fragment (C), taken from RCSB with PDB IDs of 1DXX, 3UUN and 1EG3 respectively. A complete crystal structure of dystrophin has yet to be obtained.

2A2. Isoforms and expression

Many dystrophin isoforms exist with expression specific to different tissues. Dystrophin isoform nomenclature follows the structure Dp (for the dystrophin protein), the proteins weight in kilodaltons (kDa), and a letter to represent either tissue expression or splicing. Full length dystrophin is represented as Dp427m (Dp for dystrophin, 427 kDa length, m for expression in

muscle tissue). Other important isoforms of dystrophin include Dp427c in the brain (cortical) and Dp71, which is the most abundant outside of muscle, particularly in the brain.

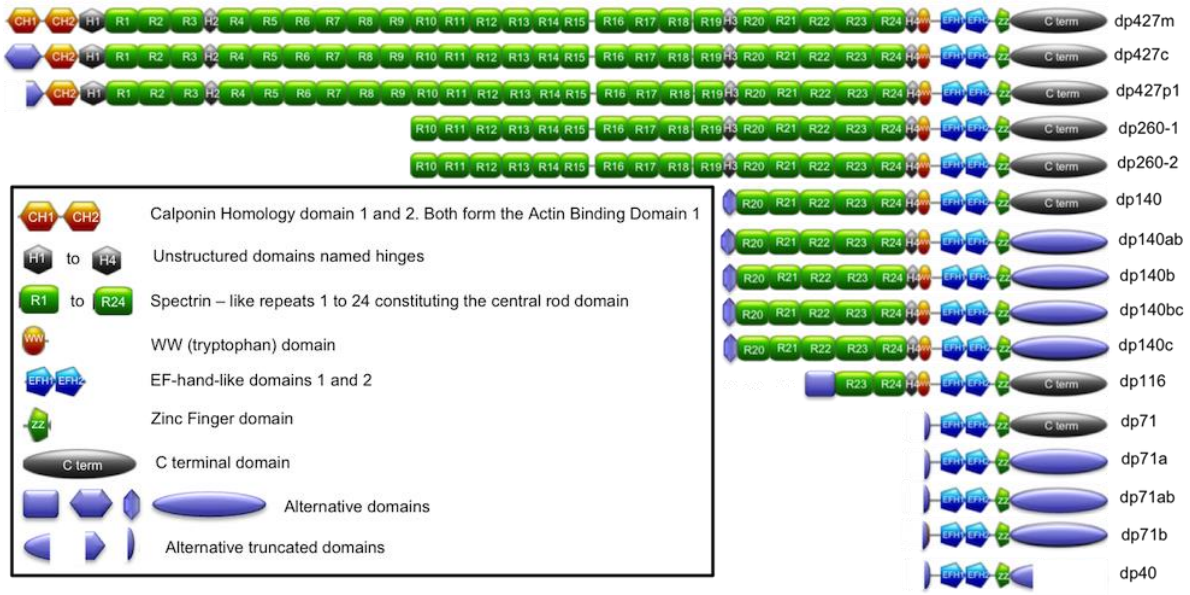


Figure 4. The domains present in each of the dystrophin isoforms. Image from Nicolas *et al.* 2012 (Nicolas et al., 2012). Skeletal muscle dystrophin isoform Dp427m contains all domains that are encoded in the dystrophin gene and often referred to as full length dystrophin. Domains WW, EF hand 1, EF hand 2 and ZZ are together known as the cysteine rich domain.

The different dystrophin isoforms (Figure 4) result from alternative promoter regions or splicing, or from an alternative poly-A addition site in the specific case of Dp40 (apo-dystrophin-3) of the DMD gene. Dp427 encodes the full length dystrophin with all the domains present. Tissue-specific promoters create a large diversity of dystrophin domains present in the different isoforms. Promoters within the gene drive expression of numerous isoforms lacking the full-length N-terminal regions. For example, Dp260, Dp140, and Dp116, lack the CH domains and part of the rod domain up to spectrin repeats 9, 18, and 21, respectively, while Dp71 lacks the CH domains and the entire rod domain. In total, dystrophin has 7 unique promoters. Additional variations through splicing can also occur. Dp40, for example, encodes only the glycoprotein binding site of dystrophin.

Multiple dystrophin isoforms can be expressed within the same cell, with differing expression patterns in both developmental time points and in protein localisation (Bolaños-Jiménez et al., 2001), indicating that dystrophin isoforms have unique roles within the cell. Skeletal muscle expresses the Dp427 isoform as the actin binding CH domains are required to combat muscle contraction-induced damage (Banks, Gregorevic, Allen, Finn, & Chamberlain, 2007). It is also

the prominent isoform in cardiac muscle. Full-length dystrophin is expressed only upon differentiation.

Dp71 is expressed across a wide range of tissues, being most abundant in brain and has been reported in myoblasts (de León et al., 2005). Some variations of the Dp71 isoform have been shown to localise within the nucleus where it anchors an alternative version of the DAPC (Fuentes-Mera et al., 2006). Its expression is subsequently downregulated during differentiation when the full length isoform becomes prominent. No other isoforms have been detected in healthy skeletal muscle.

In myoblasts, YY1 protein was found to downregulate the promoter region of the full length dystrophin (Galvagni, 1998). The protease m-calpain was found to be upregulated and subsequently degrade the YY1 protein during skeletal muscle cell differentiation, allowing Dp427 to be expressed. Conversely, the Sp1 and Sp3 proteins were found to promote Dp71 expression in myoblast, while these proteins were absent upon differentiation (de León et al., 2005).

2A3. Dystrophin phosphorylation

Various phosphorylation sites of dystrophin have been identified, some of which have been found to regulate the interactions of dystrophin. These phosphorylations are performed by a large number of kinases such as cAMP-dependent/cGMP-dependent protein kinase (DGC-PK), CaM kinase, casein kinase II, protein kinase C, p44 MAP kinase and p34cdc2 protein kinase.

Phosphorylation at S3066 enhances the dystrophin-dystroglycan interaction (Swiderski et al., 2014). Phosphorylation within dystrophin's rod domain is able to regulate actin binding, with PKA phosphorylation increasing dystrophin's actin binding properties and phosphorylation by CK-II or PKC inhibiting actin binding (Senter, Ceoldo, Petrusa, & Salviati, 1995). Phosphorylation within the dystrophin C-terminal domain can inhibit syntrophin binding (Madhavan & Jarrett, 1999).

The majority of the currently known dystrophin phosphorylation sites are located at the C-terminal, a region responsible for binding many of the DAPC members. Several other members of the DAPC have been found to be phosphorylated and implicated in their function, such as

syntrophin phosphorylation initiating Rac1 signalling (Y. W. Zhou, Thomason, Gullberg, & Jarrett, 2006).

Dystroglycan phosphorylation has been implicated in signalling for proteasomal degradation of the DAPC. Preventing beta-dystroglycan phosphorylation at Y890 in mdx mice (mdx mice being a commonly used mouse model lacking dystrophin expression (Bulfield et al., 1984; Sicinski et al., 1989), discussed in further detail in chapter 2.F2) was found to ease the dystrophic phenotype by reinstating the correct localisation of the members of DAPC (excluding dystrophin) and increasing the resistance to contraction-induced damage (Miller et al., 2012). It has been suggested that kinase inhibitors could be used in therapeutic approaches to alleviate dystrophic symptoms (Lipscomb, Piggott, Emmerson, & Winder, 2016).

2A4. Calponin homology domain, CH1 and CH2

There are three types of calponin domains, type 1, 2 and 3 (CH1, CH2 and CH3). Type 1 and 2 are found in tandem, typically in cytoskeleton proteins, and bind F-actin. Type three is involved in regulatory and signalling roles. Dystrophin contains tandem CH1 and CH2 domains (Figure 5). They are located at the N-terminal of dystrophin at amino acids 14-240, encoded by exons 2 to 8.

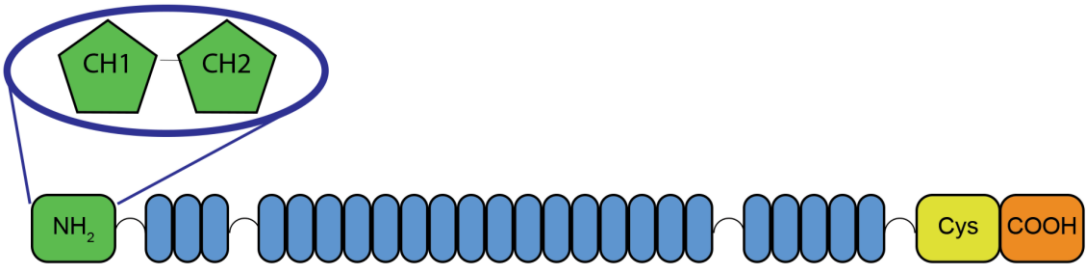


Figure 5. The dystrophin domains with the calponin homology domains highlighted. Dystrophin contains two calponin homology domains, CH1 and CH2, located at its N-terminal, which act together to bind actin. Two CH domains are present in other actin-binding proteins such as spectrins and filamins.

Each CH domain is made up of a four alpha-helix core (Norwood, Sutherland-Smith, Keep, & Kendrick-Jones, 2000). The two CH domains are connected by a short linker alpha-helix region, and are able to bind one F-actin monomer between them. Individually, the N-terminal CH domain is able to bind F-actin with an affinity comparable to the two CH domains in parallel while the second CH domain is significantly weaker at binding actin and is predominantly required for stabilisation of the tandem domains (Singh, Bandi, & Mallela,

2015). The two CH domains have three distinct actin binding sites between them, located at amino acids 18-27 (actin-binding site 1, ABS1), 88-116 (ABS2) and 131-147 (ABS3) in exons 2, 5 and 6 respectively (Norwood et al., 2000). The shorter dystrophin isoforms are all without the calponin homology domains, since these domains are located at the N-terminal, and all other smaller dystrophin isoforms have their promoter regions located further downstream.

Microdystrophins lacking the calponin domains were found to be highly susceptible to contraction-induced injury when compared to microdystrophins incorporating the same domains and the additional calponin domains (Banks et al., 2007), likely due to the disrupted binding of dystrophin to actin and subsequent decrease in dystrophin-mediated linkage of the cytoskeleton to the extracellular matrix. Additionally, microdystrophin constructs lacking this N-terminal region have been found to have reduced stability and expression (Corrado et al., 1996). As a result, microdystrophin constructs are usually designed to include the two CH domains intact.

2A5. Central Rod Domain/Spectrin Repeats

Dystrophin's central rod domain contains 24 spectrin repeat units, with two hinge units flanking the entire domain, and a further two hinge units located between spectrin repeats R3/R4 and R19/R20 (Figure 6A). Each spectrin repeat unit is comprised of a three alpha-helix bundle (Muthu et al., 2012) forming triple-helical coiled-coil structures (Figure 6C).

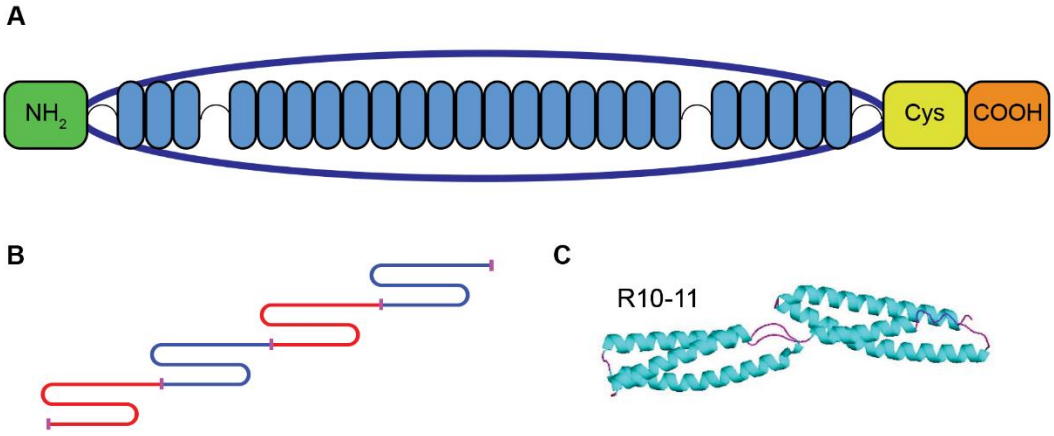


Figure 6. A. The dystrophin domains with the spectrin repeat domains highlighted. Spectrin repeats (coloured in light blue) form an elongated rod structure with hinge domains open half circle) located between repeats 3 to 4 and 19 to 20, with hinge domains also separating repeats 1 and 24 from the calponin domains at the N terminus and the cysteine rich domain near the C terminus respectively. The 24 spectrin repeats of dystrophin are not identical. B. A diagram representing how the individual spectrin repeats link to each other. Alternating blue and red represent separate spectrin repeat units. Image from Grum *et al.* 1999 (Grum, Li, MacDonald, &

Mondragón, 1999). C. Predicted structure of the dystrophin spectrin repeats 10 and 11 from Legrand *et al.* 2011 (Legrand, Giudice, Nicolas, Delalande, & Le Rumeur, 2011).

The spectrin repeats are homologous to the repeats found in other proteins including spectrin, utrophin and alpha-actinin (Winder, Gibson, & Kendrick-Jones, 1995). Each spectrin repeat is made up of approximately 110 amino acids, although the individual spectrin repeats in dystrophin vary in length. Consecutive spectrin repeats form longer rod structures (Figure 6B and 6C) (Grum et al., 1999).

The hinge units are proline-rich small amino acid sequences that may add flexibility or specific conformation to the general form of the spectrin rod structure (Legrand et al., 2011). The hinges are important to dystrophin function and deletions which alter the neighbouring spectrin domains of the hinges can have effects on disease severity in BMD (Banks, Judge, Allen, & Chamberlain, 2010; Carsana et al., 2005).

One role of the rod domain may be to elongate dystrophin to increase the distance between the actin-binding N-terminal and the DAPC-implicated C-terminal. The spectrin repeats have also been implicated in the binding of neuronal nitric oxide synthase (nNOS) (Lai et al., 2009), actin (Kurt J Amann, Renley, & Ervasti, 1998), and membrane lipids (DeWolf et al., 1997).

The rod domain consists of amino acids 253-3040 from exons 8 to 61, making up the majority of the entire dystrophin molecule. Spanning 54 of dystrophins 79 exons, many dystrophin mutations capable of causing DMD and BMD phenotypes have been reported to occur within the spectrin repeats (X. Li et al., 2015). In-frame mutations in the spectrin repeat region generally cause less severe phenotypes than those affecting the N-terminal, cysteine rich domain, and C-terminal (C Béroud, Collod-Béroud, Boileau, Soussi, & Junien, 2000; Tuffery-Giraud et al., 2009). Out-of-frame deletions around exons 45 to 52 are particularly common in patients with DMD (Barzegar, Habibi, Bonyady, Topchizadeh, & Shiva, 2015; X. Li et al., 2015), likely due to the relatively long introns adjacent to these exons.

Microdystrophin designs remove large portions of this domain in order to reduce the size of dystrophins sufficiently for AAV transfections. Much of the functionality of dystrophin appears to be retained in the absence of some of these spectrin repeats (Harper et al., 2002). The shorter dystrophin isoforms, whose roles differ from those of the full length dystrophin in skeletal muscle, are without some (Dp260, Dp140, Dp116) or all (Dp71, Dp40) of the spectrin repeats.

2A6. Cysteine rich region (CR)

The cysteine rich region, as the name suggests, is enriched in cysteine residues. In dystrophin it is encoded by exons 63 to 69 and makes up amino acids 3080-3360. Located within this region are the WW domain, EF hands and ZZ domain (Figure 7).

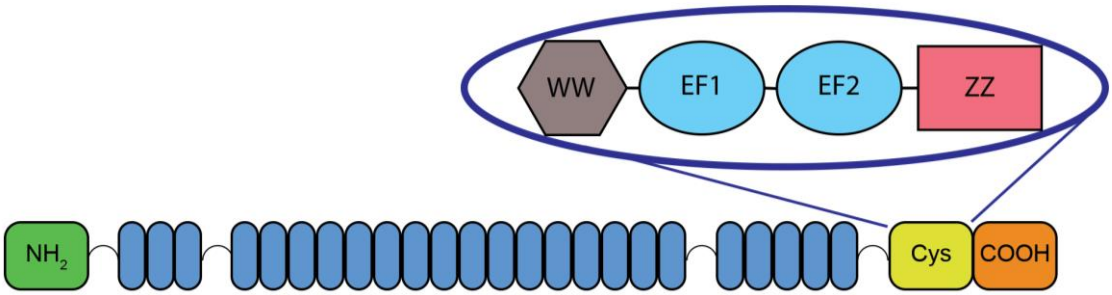


Figure 7. The dystrophin domains with the WW, EF hand 1, EF hand 2 and ZZ domains of the cysteine rich domain highlighted. The cysteine rich domain is important for dystrophin with EF1, EF2 and ZZ domains present in all dystrophin isoforms.

Dystrophin interactors mapped to the cysteine rich region include beta-dystroglycan (Hnia et al., 2007; Rentschler et al., 1999), synemin (Bhosle, Michele, Campbell, Li, & Robson, 2006), plectin (Rezniczek et al., 2007) and ankyrin 2 and 3 (Ayalon, Davis, Scotland, & Bennett, 2008), all of which make use of the three different domains within the cysteine rich domain for their binding.

The region is highly important to dystrophin function. Deletions in this region have not been reported in BMD patients and microdystrophin constructs without this region disrupt the DAPC and cause severe pathologies (Rafael et al., 1996; Suminaga, Takeshima, Wada, Yagi, & Matsuo, 2004). It is the only region that is present in all dystrophin isoforms.

2A7. WW domains

WW domains are triple stranded beta-sheets that contain two tryptophan residues (though which the domain gets its name) approximately 20-22 amino acids apart, in addition to a conserved proline. The WW domain recognises proteins with a proline rich sequence followed by a PPXP (H. I. Chen & Sudol, 1995; Macias, Wiesner, & Sudol, 2002) or PPXY (A. Yatsenko et al., 2009) motif. It is also able to recognise phosphoserine and phosphothreonine containing motifs. The domain has diverse functional roles but is most commonly involved in signalling or regulation. Dystrophin's WW domain is located at amino acids 3056-3092, encoded by

exons 62 and 63. It has been found to have binding affinities for beta-dystroglycan at two regions (A. Yatsenko et al., 2009).

2A8. EF hands

The EF hand domains are made up of two alpha-helices linked by a loop region of 12 amino acids (Lewit-Bentley & Réty, 2000) and often found in pairs. The domain is commonly found to bind calcium however this is not thought to be the case in dystrophin as they do not contain the calcium-binding residues. No calcium ions were observed binding during crystal structure determination and the ion was not found to affect the affinity of the dystrophin-beta-dystroglycan-binding (Huang et al., 2000). In addition to calcium recognition, the domain can also recognise magnesium ions (Malmendal, Linse, Evenäs, Forsén, & Drakenberg, 1999).

It is commonly involved in either regulatory or structural roles. When involved in regulatory processes, binding of calcium to the EF hands will induce conformational changes. When involved in structural roles the EF hand will not undergo conformational changes. As dystrophin does not bind calcium it does not undergo conformational changes, and the EF and WW domains were found to be rigid. Binding of the EF domains to dystroglycan in conjunction with the WW and ZZ domains of dystrophin also produced no conformational changes. The domain is therefore thought to be involved in a structural role. The EF hands on dystrophin are located at amino acids 3130-3157 encoded by exon 65 and 3178-3206 exons 65 and 66, between the WW and ZZ domains.

2A9. ZZ domains

The ZZ domain, also known as the ZZ-type zinc finger domain, contains two distinct CXXC motifs that commonly bind to zinc ions. It is located at amino acids 3307-3354 encoded by exons 68 and 69. The domain has been shown to strengthen the interaction of dystrophin and beta-dystroglycan which primarily occurs through the WW domain. The distinct binding sites of the ZZ domain with beta-dystroglycan have been reported (Hnia et al., 2007; Ishikawa-Sakurai, Yoshida, Imamura, Davies, & Ozawa, 2004).

2A10. C-terminal domain

Dystrophin's C-terminal (labelled COOH in figures 5, 6A and 7) is unique to itself and utrophin, an evolutionarily closely related protein. The C-terminal comprises amino acids

3361-3685 encoded by exons 70 to 79. The domain is made up of leucine heptad repeating units consisting of two right-handed α-helices that form a coiled-coil motif. It contains the dystrophin binding sites of syntrophins and dystrobrevins. Dystrophin's coiled-coil site interacts with a coiled-coil site on dystrobrevin. This interaction was found to be specific as the coiled-coil domains do not homodimerise (Sadoulet-Puccio, Rajala, & Kunkel, 1997).

Forms of dystrophin with C-terminal deletions have been shown to be functional, retaining the localization of dystrophin, dystrobrevin, and syntrophin, at the sarcolemma (Crawford et al., 2000), through binding of other DAPC proteins. As a result, many microdystrophin constructs have been designed with truncated or absent C-terminal regions and have shown promising results (Yue, Liu, & Duan, 2006).

2B. Dystrophin Interactors

2B1. Dystrophin Associated Protein Complex

Introduction

To fully understand the roles of a protein, it is necessary to know its binding partners. Additionally, protein interactions help to explain the fundamental causes of pathologies. This is of particular relevance to dystrophin due to its involvement in many muscular dystrophies, including DMD and BMD, but also other DAPC-related pathologies such as the sarcoglycanopathies. The specific interaction sites of a protein are of particular use in understanding diseases resulting from proteins expressed with delete or mutated domains such as in BMD. The therapies being developed to treat DMD are also able to make use of specific binding sites during therapeutic design.

The dystrophin associated protein complex is comprised of proteins that strongly associate with dystrophin at its C-terminal at the sarcolemma. The complexes main role is structural, linking the extracellular matrix with the actin cytoskeleton in order to distribute contractile induced stress and stabilise the sarcomere. It has also been strongly implicated in signalling. The proteins usually classed as being part of the DAPC are dystrobrevins, syntrophins, dystroglycans, sarcoglycans, sarcospan and nNOS (Figure 8).

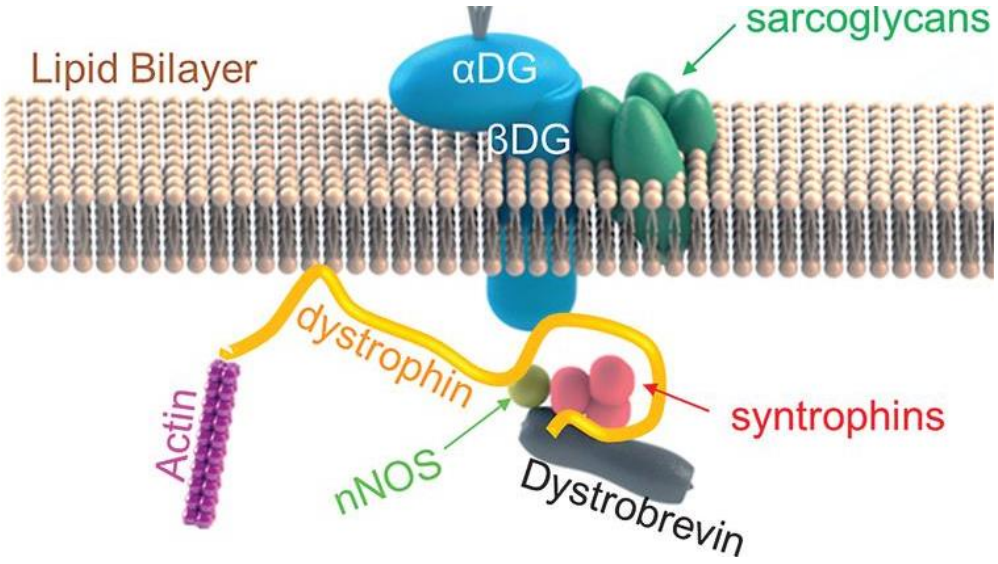


Figure 8. Typical schematic of the dystrophin-associated protein complex. The important interaction of dystrophin with the actin cytoskeleton is also included. Image derived from Rodrigues et al. (2016) (Rodrigues, Echigoya, Fukada, & Yokota, 2016). The dystrophin-associated protein complex includes the transmembrane proteins beta-dystroglycan and several members of the sarcoglycan family. Its most well studied role is in linking the actin cytoskeleton to the extracellular matrix however it is also involved in several other roles. As well as dystrophin's protein interactions its rod domain also associates with the lipid membrane.

The DAPC can itself be separated into three distinct complexes; the dystroglycan complex (alpha-dystroglycan and beta-dystroglycan), the sarcoglycan-sarcospan complex (alpha-, beta-, delta- and gamma-sarcoglycan and sarcospan) and the dystrophin/dystrobrevin/syntrophin complex (dystrophin, alpha-dystrobrevin and alpha-, beta-1- and beta-2-syntrophin). Diseases resulting from mutations in proteins of the DAPC often have similarities in their pathology (Table 1).

Table 1. Dystrophin associated protein complex proteins and their related diseases. The absence of many dystrophin related proteins result in distinct diseases, although not all of the proteins have been linked to a disease.

Protein name	Related disease code	Full disease name	Notes
Dystrophin	DMD BMD	Duchenne Muscular Dystrophy Becker Muscular Dystrophy	
Alpha-sarcoglycan	LGMD-2D	Limb-Girdle Muscular Dystrophy type 2D	
Beta-sarcoglycan	LGMD-2E	Limb-Girdle Muscular Dystrophy type 2E	
Delta-sarcoglycan	LGMD-2F	Limb-Girdle Muscular Dystrophy type 2F	
Gamma-sarcoglycan	LGMD-2C	Limb-Girdle Muscular Dystrophy type 2C	
Alpha-dystrobrevin	- LVNC1	Meniere's disease Left ventricular noncompaction 1	Ear only Cardiac only
Alpha-dystroglycan	LGMD-2P	Limb-Girdle Muscular Dystrophy 2P	Dystroglycanopathy
Beta-dystroglycan	Non	-	
Alpha-syntrophin	LQT12	Long QT syndrome 12	Cardiac only

Beta-1-syntrophin	Non	-	
Beta-2-syntrophin	Non	-	
Sarcospan	Non	-	
nNOS	-	Achalasia type 3	Striated/Smooth muscle (esophagus)
Other gene mutations of note			
Genes responsible for Alpha-dystroglycan including POMT1 / POMT2 / ISPD / FKTN / FKRP / LARGE / POMGNT1	WWS / MEB / FCMD / MDC1C / MDC1D / LGMD-2I/L/N	Walker–Warburg syndrome / Muscle–Eye–Brain disease / Fukuyama-type muscular dystrophy / Congenital muscular dystrophy type 1C/D / Limb girdle muscular dystrophy type 2I/L/N	Alpha-dystroglycan aberrant glycosylation related dystroglycanopathies (Bouchet-Seraphin C, 2015)
Caveolin-3	LGMD-1C	Limb-Girdle Muscular Dystrophy 1C	
Alpha-Laminin-2	MDC1A	Merosin-Deficient Congenital Muscular Dystrophy type 1A	

Dystrobrevins

The dystrobrevin protein family is comprised of alpha- and beta-dystrobrevin isoforms in human. Dystrobrevins are closely related to dystrophin. The dystrobrevin proteins have a close homology to dystrophin at their C-terminals, both having coiled coil motifs containing EF domains. Dystrobrevins are significantly shorter than dystrophin with the alpha and beta human isoforms having length 743 and 627 amino acids respectively.

Dystrobrevin is able to bind to dystrophin (Sadoulet-Puccio et al., 1997), syntrophins (Newey, Benson, Ponting, Davies, & Blake, 2000) and sarcoglycans (M. Yoshida et al., 2000) within the DAPC. Other important interactions include the intermediate filaments syncoilin (Newey et al., 2001) and synemin (Mizuno et al., 2001). Synemins are also able to bind to dystrophin (Bhosle et al., 2006). The intermediate filament interactions link the DAPC, providing additional mechanical support.

Although both alpha- and beta-dystrobrevin are highly expressed across a wide range of tissues, alpha-dystrobrevin is the isoform prominently expressed in skeletal muscle. It directly binds dystrophin's coiled coil domain through its own coiled-coil domain (Sadoulet-Puccio et al., 1997). The coiled coil domains of both proteins are in close proximity to syntrophin-binding domains also present in both proteins, and located in the C-terminus of both. Specifically, Dystrophin's WW domain binds to the WW domain of dystrobrevin, with EF hands of

dystrobrevin stabilising the interaction. The crystal structure of this interaction has been solved (Huang et al., 2000).

While dystrophin is known to be the central protein in formation of the dystrophin associated protein complex in muscle, experiments on mice lacking all dystrophin isoforms suggested that dystrobrevins may be essential for DAPC assembly outside of muscle (Loh, Newey, Davies, & Blake, 2000). In DMD skeletal muscle, the DAPC is disrupted, resulting in the loss of alpha-dystrobrevin localisation at the sarcolemma (D. Li, Long, Yue, & Duan, 2009; Metzinger, 1997).

Alpha-dystrobrevin deficient mice exhibited skeletal and cardio myopathy (Grady et al., 1999). Dystrobrevin plays a role in stabilisation of the sarcolemma, but the DAPC forms intact in its absence. The signalling protein nNOS has a decreased localisation at the membrane as a result of dystrobrevin knockdown despite dystrophin localisation being normal. This suggests a role in signalling for alpha-dystrobrevin. Alpha-dystrobrevin's direct interaction with alpha-syntrophin is likely to mediate the association of dystrobrevin with nNOS. Affecting only the inner ear, a mutation resulting in skipping of alpha-dystrobrevin's exon 21 resulted in Meniere's disease with symptoms of vertigo, tinnitus and permanent hearing loss, possibly due to the disruption of DAPC roles in the inner ear (Requena et al., 2015).

Syntrophins

Syntrophins are scaffolding adapter protein, linking the DAPC to signalling proteins. Humans express five isoforms of syntrophin: alpha, beta-1, beta-2, gamma-1 and gamma-2, with all except gamma-1 expressed in skeletal muscle. Gamma-2-syntrophin has distinct functions and localises to the endoplasmic reticulum rather than being present in the DAPC (Alessi et al., 2006). As such, it is only alpha-, beta-1- and beta-2-syntrophins that interact with dystrophin *in vivo*. Beta-1-syntrophin is predominantly located at the sarcolemma, while beta-2-syntrophin is mostly found at the neuromuscular junction. Alpha-syntrophin is significantly located in both regions. All subsequent discussions on syntrophins will be solely referring to the alpha and beta isoforms.

The syntrophin proteins range from 505 to 540 amino acids in length, and all feature two tandem pleckstrin homology (PH) domains, a PDZ domain and a C-terminal syntrophin unique domain. The C-terminal of dystrophin is able to bind to the syntrophin unique domains of

alpha-, beta-1- and beta-2-syntrophins in skeletal muscle, binding at exons 73-74 (Ahn & Kunkel, 1995; Yang, Jung, Rafael, Chamberlain, & Campbell, 1995). Dystrophin is thought to be capable of binding up to two syntrophins simultaneously (Newey et al., 2000). Alpha-dystrobrevin contains three sites that bind to this syntrophin unique domain (Böhm, Constantinou, Tan, Jin, & Roberts, 2009). Exons relating to syntrophin binding in both dystrophin and dystrobrevin are subject to alternate splicing allowing for regulation of syntrophin stoichiometry within the DAPC.

This leaves PH and PDZ domains free to bind a variety of signalling proteins. The PH and PDZ domains can work together in order to recruit nNOS and acetylcholine receptors to the neuromuscular junction in alpha-syntrophin (M E Adams, Mueller, & Froehner, 2001; Marvin E Adams, Anderson, & Froehner, 2010). Sodium Ion Channels have been shown to bind to the PDZ domains of the three syntrophins (Gee, Madhavan, et al., 1998). Dystrophin deficiency, which results in syntrophin mislocalisation, results in sodium channel misregulation, increased sodium ion concentration in cells, promoting cell death (Hirn, Shapovalov, Petermann, Roulet, & Ruegg, 2008).

Interestingly, mutations of alpha-syntrophin showed no adverse effects on phenotype, despite a resulting mislocalisation of nNOS (Kameya et al., 1999). No disease has been related to mutations of syntrophins.

Dystroglycans

Alpha- and beta-dystroglycan are located in the membrane of the sarcolemma. Alpha-dystroglycan and beta-dystroglycan bind together and are referred to as the dystroglycan complex. This transmembrane complex binds laminin (and other extracellular proteins) through alpha-dystroglycan and dystrophin through beta-dystroglycan, creating a structural link between dystrophin (and the intracellular cytoskeleton) and laminin (part of the extracellular matrix). The dystroglycan complex has also been implicated in signalling (Y. W. Zhou et al., 2006). It is able to form complexes with proteins other than dystrophin. Some of its interactions, such as signalling interactions with growth factor receptor 2 (Russo et al., 2000), plectin (Rezniczek et al., 2007) and caveolin-3 (Sotgia et al., 2000) are inhibited by dystrophin binding. Caveolin-3 is thought to negatively downregulate dystrophin localisation at the sarcolemma. Actin has been shown to bind to beta-dystroglycan (Y.-J. Chen et al., 2003) but has not been confirmed as part of the DAPC. Ankyrin-G has been shown to bind to

dystroglycan at a different location from the dystrophin binding site (Ayalon et al., 2008). Dystroglycan has been shown to play a role in cell polarity which is independent of dystrophin binding (A. S. Yatsenko et al., 2007).

Beta-dystroglycan binds dystrophin at amino acids 3054-3271 (Jung, Yang, Meyer, Chamberlain, & Campbell, 1995) located at dystrophin's cysteine rich domain, interacting with dystrophin's WW domain, EF hands and ZZ domain (Huang et al., 2000; Ishikawa-Sakurai et al., 2004; Rentschler et al., 1999).

In the dystrophin-deficient mdx mice (tibialis anterior muscle), dystroglycan localisation at the sarcolemma is lost (Ayalon et al., 2008). Dystrophin is still able to localise to the sarcolemma without the presence of the dystroglycans (Côté, Moukhles, & Carbonetto, 2002) along with syntrophins and nNOS. Sarcoglycans, caveolin-3 and integrins were found to be dependent on dystroglycan for their localisation, each of which is located in the membrane. Alpha- and beta-dystroglycan deficiencies result in dystroglycanopathies (Martin et al., 2008; Salih et al., 1996), which are forms of muscular dystrophy. Alpha-dystroglycan requires glycosylation in order to bind to, and provide a mechanical link to, the extracellular matrix (Tran et al., 2012). The loss of glycosylation results in impaired binding between the DAPC and the extracellular matrix leading to contraction-induced membrane damage resulting in dystrophy.

Sarcoglycans

Alpha-, beta-, delta- and gamma-sarcoglycan are transmembrane proteins that interact to form the sarcoglycan complex in skeletal muscle (Weixing Shi et al., 2004). A fifth sarcoglycan, zeta-sarcoglycan, has been reported to localise with DAPC (Wheeler, Zarnegar, & McNally, 2002) although this has not been widely investigated. Epsilon-sarcoglycan is able to localise with the DAPC at lower levels to the rest of the complex and performing a similar role to alpha-sarcoglycan (Imamura, Mochizuki, Engvall, & Takeda, 2005).

Each sarcoglycan has a short intracellular region and a larger extracellular region and is encoded by a distinct gene. Alpha- and epsilon-sarcoglycan are both type 1 transmembrane proteins and are closely related to each other. Delta-, gamma- and zeta-sarcoglycan are type 2 transmembrane proteins and closely related. Beta-sarcoglycan is also a type 2 transmembrane protein though less closely related. Despite similarities, sarcoglycans vary in tissue expression. Alpha- and gamma-sarcoglycan are predominantly expressed in striated muscle, although they

have also been detected in smooth muscle (Giuseppe Anastasi et al., 2005). The other sarcoglycans have a broader range of expression but have all also been detected in striated muscle.

Beta- and delta-sarcoglycan are particularly important for localisation of the sarcoglycan complex. Beta-sarcoglycan is the first to localise to the membrane and subsequently associates with delta-sarcoglycan. Gamma-sarcoglycan is next recruited to the complex, which finally associate with alpha-sarcoglycan (Weixing Shi et al., 2004). Alpha-sarcoglycan does not associate with the sarcoglycan complex as closely as the other subunits. Each of the sarcoglycans are glycosylated at their extracellular regions. Beta-sarcoglycan glycosylation has been found to be essential for correct localisation of the complex.

Epsilon-sarcoglycan replaces alpha-sarcoglycan in the smooth muscle sarcoglycan complex (Straub et al., 1999) and zeta-sarcoglycan is suspected to replace gamma sarcoglycan (Shiga et al., 2006). It has also been found that epsilon-sarcoglycan is capable of replacing alpha-sarcoglycan in striated muscles with alpha-sarcoglycan deficiency when overexpressed (Imamura et al., 2005). Alpha-sarcoglycan deficiency (limb-girdle muscular dystrophy type 2D, LGMD2D) results in mild or no cardiomyopathy, with epsilon levels appearing to be sufficient to substitute for the missing alpha-sarcoglycan (Lancioni et al., 2011).

The sarcoglycan complex does not directly interact with dystrophin. It has been found to bind to alpha-dystrobrevin (M. Yoshida et al., 2000), through which it is associated to dystrophin and forms part of the DAPC. It is also suspected to bind to beta-dystroglycan, strengthening the alpha-dystroglycan-beta-dystroglycan binding. Biglycan is able to bind alpha- and gamma-sarcoglycan and alpha-dystroglycan (Rafii et al., 2006) and also binds to extracellular matrix proteins. The sarcoglycan complex has been implicated in stabilisation of the DAPC and signalling.

In DMD, expression of the sarcoglycan complex is greatly reduced (D. Li et al., 2009), although the small levels of sarcoglycans do still appear to be somewhat able to relieve the DMD phenotype, as knockout of the sarcoglycans in addition to the absent dystrophin increase disease severity. Analysis of patients without dystrophin's C-terminal have demonstrated that this domain is not required for sarcoglycan localisation (Bellayou et al., 2009).

Mutations in sarcoglycans result in Limb Girdle Muscular Dystrophy types 2D (alpha-sarcoglycan deficiency), 2E (beta-sarcoglycan deficiency), 2C (gamma-sarcoglycan deficiency) and 2F (delta-sarcoglycan deficiency) with disease severity varying greatly. Mutations in a single sarcoglycan isoform can result in loss of the entire sarcoglycan complex from the membrane, along with a small decrease in dystrophin protein levels (Rachelle H Crosbie, Barresi, & Campbell, 2002; Vainzof et al., 1996). Beta-dystroglycan localisation is reduced in some cases, particularly in mutations affecting beta- or delta-sarcoglycan. Loss of the entire sarcoglycan complex results in loss of nNOS from the membrane (Rachelle H Crosbie et al., 2002).

Sarcospan

Sarcospan is a transmembrane protein that is closely associated with the sarcoglycan complex. It requires the full sarcoglycan complex for normal expression and localisation at the membrane (R. H. Crosbie et al., 2000). Accordingly, in DMD patients sarcospan expression is lost (Peter, Marshall, & Crosbie, 2008). In mdx mice sarcospan overexpression has been shown to stabilise the sarcolemma and increase the recruitment of utrophin in place of the absent dystrophin (Peter et al., 2008). Sarcospan provides stabilisation to the DAPC and extracellular matrix assembly (Peter, Miller, & Crosbie, 2007). It is involved in the transport and localisation of the DAPC and utrophin–glycoprotein complex (UGC) to the membrane and is also closely linked to integrin expression at the membrane (Marshall et al., 2012). The sarcospan protein has subsequently been suggested as a target for gene therapy treatments for DMD.

Sarcospan knockout mice show no adverse effects at young ages (Lebakken et al., 2000) with the loss of extracellular interaction compensated for with an increase in integrin (Marshall et al., 2012). Aged mice do eventually lose some expression of DAPC components and have decreased binding capability with the extracellular matrix. No diseases linked to the sarcospan gene have yet been described.

Neuronal nitric oxide synthase

Nitric oxide synthases are a family of enzymes that are responsible for catalysing nitric oxide production from arginine. Nitric oxide synthase is closely associated with the DAPC, being able to bind to multiple members. The nitric oxide produced by NOS is involved in signalling pathways and cell communications. In skeletal muscle it stimulates vasodilation, increasing blood flow to the muscle, which is particularly essential for highly energy consuming, type 2

muscles for delivery of oxygen and removal of carbon dioxide. Functional ischemia (an inadequate supply of blood to a tissue) is found in DMD patients (Gail D Thomas, 2013). Dystrophin does not require NOS binding to function as part of the DAPC complex and membrane stability is not affected by the loss of NOS.

Neuronal nitric oxide synthase is the isoform present in skeletal muscle and is located at the membrane through interactions with dystrophin and the DAPC. It interacts with dystrophin at spectrin repeats 16 and 17 (Lai et al., 2009) through its PDZ domain. The same PDZ domain is also able to bind syntrophin (Hillier, Christopherson, Prehoda, Bredt, & Lim, 1999). Despite its interaction with syntrophin, it is reliant on its binding to dystrophin for localisation. Patients with dystrophin lacking the nNOS binding site (exons 42 to 45) show decreased nNOS localisation (Cirak et al., 2012). Many BMD patients lacking various spectrin repeats in the rod domain have also reported loss of nNOS localisation (Daniel S Chao et al., 1996; Torelli et al., 2004). Its absence can diagnose BMD when mild or no obvious phenotype is observed. In DMD, loss of the entire DAPC, also disrupts nNOS localisation. nNOS localisation is also reduced when the complete sarcoglycan complex is not present (Rachelle H Crosbie et al., 2002), or with knockout of alpha-syntrophin (Kameya et al., 1999).

nNOS activity is calcium dependant, requiring binding to calcium bound calmodulin (Salerno, Ray, Poulos, Li, & Ghosh, 2013). nNOS phosphorylation at serine 847 inhibits its activity (Hayashi et al., 1999; Komeima, Hayashi, Naito, & Watanabe, 2000). N-methyl-D-aspartate (NMDA) receptors, which can allow the transport of calcium ions into the cell, colocalise with nNOS through a common interaction with postsynaptic density protein 95 (Christopherson, Hillier, Lim, & Bredt, 1999; Grozdanovic & Gossrau, 1998) and are involved in the dephosphorylation of nNOS (Rameau, Chiu, & Ziff, 2004).

Knockdown of nNOS leads to decreased muscle mass and a reduction in maximum contractile force in the skeletal muscle of male mice. Both male and female mice were increasingly vulnerable to contraction-induced muscle damage (Percival, Anderson, Gregorevic, Chamberlain, & Froehner, 2008). Loss of ability to increase blood flow toward the muscle and also a subsequent inability to increase glucose uptake has been observed during nNOS knockout (Baum et al., 2013; Hong et al., 2015). Biglycan appears to be required for localisation of nNOS (along with dystrobrevin and syntrophin) to the muscle cell membrane (Mercado et al., 2006). Interestingly, nNOS overexpression has been shown to be beneficial

even when it is not localised to the sarcolemma (Wehling-Henricks & Tidball, 2011), adding another possibility to alternative treatments to alleviate dystrophic phenotypes.

M2c macrophages are pro-fibrotic, expressing arginase which utilises arginine as a substrate, producing ornithine and urea, with ornithine a substrate in polyamine synthesis (Hui Li et al., 2002; Majumdar, Shao, Minocha, Long, & Minocha, 2013). Polyamines are required for translation initiation and are thus required to induce cell proliferation and tissue repair (Landau, Bercovich, Park, & Kahana, 2010). With nNOS and arginase sharing the arginine substrate, overexpression of nNOS resulted in a decrease of M2c macrophages due to decreased availability of the arginine substrate (Rath, Müller, Kropf, Closs, & Munder, 2014; Wehling-Henricks & Tidball, 2011). The nitric oxide produced from the breakdown of arginine by nNOS also has inflammatory effects upon the cell. This anti-fibrotic effect reduces scarring and decreases the pro-fibrotic overproduction of extracellular matrix proteins which contributes to the loss of muscle tissue architecture and function in dystrophic patients.

2B2. Glycosylation

It has been estimated that approximately half of all proteins undergo glycosylation to some extent (Apweiler, Hermjakob, & Sharon, 1999). Glycosylation is involved in a wide range of roles including intracellular targeting (White, Stewart, Popoff, Wilson, & Blackwell, 2004), protein-protein binding (Margraf-Schönfeld, Böhm, & Watzl, 2011) and protein stability (Solá & Griebenow, 2009). The DAPC, otherwise known as the dystrophin glycoprotein complex, has multiple glycoproteins. Glycosylation sites are numerous on the extracellular matrix-exposed proteins of the DAPC, specifically sarcoglycans and dystroglycans.

Similar to phosphorylation, the presence or absence of glycosylation can affect the stability of the DAPC and glycosylation defects have been implicated with muscular dystrophy. The glycosylation of alpha-dystroglycan is particularly important as it is required for binding to extracellular matrix proteins (Martin, 2003). With DAPC's link to the extracellular matrix disrupted, the muscle becomes more susceptible to contraction induced membrane damage resulting in muscular dystrophy.

2B3. Other interactors of dystrophin

Outside of the DAPC, dystrophin has many additional interactors. Actin forms the major part of the cytoskeleton to which the DAPC provides a link to the extracellular matrix (Levine, Moir, Patchell, & Perry, 1992). Dystrophin is also able to bind the intermediate filaments beta-synemin (Bhosle et al., 2006) and keratin-19 (Stone, O'Neill, Catino, & Bloch, 2005). These interactions may provide additional mechanical support to the DAPC and membrane. The interaction with keratin is located at amino acids 1-246 of the calponin domains of dystrophin, overlapping the predominant actin binding domain towards the N-terminus. Synemin interacts at dystrophin's spectrin repeats 11 to 14. Plectin binds dystrophin at its WW-ZZ cysteine rich domains (Rezniczek et al., 2007), and is known to act as a linker protein between intermediate filaments, the actin cytoskeleton and microtubules. Ankyrin, another linker protein, also binds dystrophin at its cysteine rich domain (Ayalon et al., 2008). Alpha-actinin-2, a protein that is able to bind many focal adhesion proteins and link them to the actin cytoskeleton, has been suggested to bind to the last 200 amino acids of dystrophin (Hance, Fu, Watkins, Beggs, & Michalak, 1999), and may provide a link between dystrophin and integrin. However, this interaction has not been confirmed. Dystrophin was shown to exhibit microtubule binding between spectrin repeat 24 and the first third of WW domain (Prins et al., 2009). While all these interactions add to the structural role of dystrophin, the microtubule association also opens up the possibility of dystrophin involvement in trafficking pathways.

Relating to actin binding, dystrophin is also able to bind the actin-binding proteins troponin T (Pearlman, Powaser, Elledge, & Caskey, 1994) and troponin C and the calcium dependant protein calmodulin (Jarrett & Foster, 1995), which is evolutionarily similar to troponin C. Calmodulin is only able to bind dystrophin in the presence of calcium, and thus likely plays a role in dystrophin function dependant on calcium concentration. The calmodulin binding sites on dystrophin overlap with actin binding sites on the calponin domains, and thus are postulated to regulate dystrophin binding to actin.

Polarity-regulating kinase partitioning-defective 1b (Par-1b), a serine-threonine kinase important in the regulation of cell polarity, localises with dystrophin and binds to spectrin repeats 8 and 9 (Yamashita et al., 2010). It has been shown to specifically phosphorylate Ser1258 in utrophin which stabilises the utrophin-beta-dystroglycan interaction and could be performing a similar role in the interaction of dystrophin with dystroglycan. It has been suggested that the absence of dystrophin results in a decrease in Par-1b expression and decrease

in asymmetric division in muscle activated satellite cells, ultimately leading to impaired regeneration and satellite cell dysfunction, although the presence of dystrophin within satellite cells remains controversial (Boldrin, Zammit, & Morgan, 2015; Dumont et al., 2015; Jennifer E Morgan & Zammit, 2010). Boldrin *et al.* demonstrated that satellite cells of mdx mice were able to regenerate with no adverse effects.

Dystrophin, through its WW domain, also binds the apoptosis-inducing Fas ligand (Wenzel et al., 2001) and, through its C-terminal domain (amino acids 3458-3678), binds the nuclear distribution protein nudE homolog 1, which is responsible for mitotic spindle formation and function (Pawlisz & Feng, 2011). Dystrophin binds directly to the signalling and trafficking implicated protein myospryn at its cysteine-rich or C-terminal domains (Reynolds, McCalmon, Donaghey, & Naya, 2008)**.**

Several interactions of dystrophin are not present in skeletal muscle, such as the interaction with serum albumin in human blood serum (M. Zhou et al., 2004), beta-dystrobrevin (the isoform of dystrobrevin common outside of striated muscle) (Loh, Nebenius-Oosthuizen, Blake, Smith, & Davies, 2001; Sadoulet-Puccio et al., 1997) and calcium-dependent secretion activator 1 and 2 (Cisternas, Vincent, Scherer, & Ray, 2003). Some interactions are only present in specific isoforms, such as the nuclear located Dp71's interaction with lamin-B1 (Villarreal-Silva, Centeno-Cruz, Suárez-Sánchez, Garrido, & Cisneros, 2011)

Dystrophin is acted upon by proteases; ubiquitin (Kim et al., 2011; S. A. Wagner et al., 2011) and calpains 1 and 2 (Cottin et al., 1992) which are activated at specific calcium concentrations (Khorchid & Ikura, 2002).

2B4. Interactors of the dystrophin associated protein complex

There are many other proteins that interact with other members of the DAPC, many of which have significant roles within the cell. They can be influenced by the presence of absence of the complex and some can also have effects on the complex.

One of the most important and fundamental interactions of the DAPC is in connecting the actin cytoskeleton to the extracellular matrix. It links to the extracellular matrix through dystrophin's association with alpha-dystroglycan. The dystroglycan is able to bind to a variety of extracellular matrix proteins including laminin (Ibraghimov-Beskrovnaya et al., 1992), agrin

(Sugiyama, Bowen, & Hall, 1994) and perlecan (Talts, Andac, Göhring, Brancaccio, & Timpl, 1999).

Biglycan is a heavily glycosylated extracellular matrix protein that is able to bind to alpha-dystroglycan (Bowe, Mendis, & Fallon, 2000) and sarcoglycans (Rafii et al., 2006). Biglycan targets dystrobrevin, syntrophin and nNOS to the muscle cell membrane (Mercado et al., 2006).

Caveolin-3, a muscle specific isoform of caveolin, is an integral membrane protein that localises to the sarcolemma in mature muscle. It is involved in the cell signalling and structural assembly of the caveolae (Whiteley, Collins, & Kitmitto, 2012), which has roles in vesicular trafficking. It can interact with and negatively regulate the signalling protein nNOS (García-Cardeña et al., 1997) and interacts with beta-dystroglycan (Sotgia et al., 2000), competing with dystrophin for the dystroglycan binding site. Mutations in this gene can result in several diseases including the muscular dystrophy LGMD1C. This disease, in which caveolin-3 is not expressed, does not affect levels of dystrophin or other DAPC proteins (Minetti et al., 1998). Overexpression of caveolin-3 results in a decrease of dystrophin and beta-dystroglycan expression, disrupting the DAPC and causing a DMD phenotype (F Galbiati et al., 2000). In DMD patients caveolin-3 levels are increased (Vaghy, Fang, Wu, & P. Vaghy, 1998). The relationship between the DAPC and caveolae is yet to be fully understood.

2C. Dystrophin-related proteins

Utrophin, also known as dystrophin-related protein 1, is the most closely related protein to dystrophin and shares many of the same domains. Calponin 1 and calponin 2 domains, 4 hinge domains, 22 spectrin repeats (dystrophin has 24), a cysteine rich domain and a C-terminal are all present in utrophin. The actin binding domain has 85% similarity and the C-terminal and cysteine-rich region has an 83% similarity, however some regions of the rod domain have less than 30% similarity (Pearce et al., 1993). Unlike dystrophin, utrophin is expressed in the majority of tissues, but is limited in healthy muscle to the neuromuscular synapse and myotendinous junction. Utrophin is able to form its own utrophin-glycoprotein complexes and is capable of taking the place of dystrophin in dystrophin deficient cells (K. Matsumura, Ervasti, Ohlendieck, Kahl, & Campbell, 1992). As a result, it has been investigated as a possible upregulation target for DMD patients. In fact, in DMD patients and mdx mice a natural upregulation of utrophin is observed (Mizuno, Nonaka, Hirai, & Ozawa, 1993; Weir, Morgan, & Davies, 2004). The increase in utrophin both naturally and therapeutically helps to ameliorate the dystrophic phenotype (Kleopa, Drousiotou, Mavrikiou, Ormiston, & Kyriakides, 2006; Squire et al., 2002). However, utrophin does not exhibit microtubule binding (Belanto et al., 2014), is not able to bind actin along its spectrin repeat region (K J Amann, Guo, & Ervasti, 1999), has a significantly higher actin binding affinity at its calponin domains (Bandi, Singh, & Mallela, 2015) and is not able to bind nNOS (D. Li et al., 2010). Hence, utrophin is able to offset some of the absence of dystrophin, but not completely replace it.

Dystrophin-related protein 2 (DRP2) resembles the C-terminal region of dystrophin and utrophin and is similar to Dp116 however it is not expressed in skeletal muscle, being predominantly present in the brain and spinal cord (R G Roberts et al., 1996). It is known to form a periaxin-DRP2-dystroglycan complex which is involved in structural maintenance (Sherman, Wu, Grove, Gillespie, & Brophy, 2012).

Dystrobrevins are a family of proteins that are closely related to the dystrophin and dystrophin-related protein family, consisting of alpha- and beta-dystrobrevin in humans. Both of these proteins are able to directly bind to dystrophin as part of the DAPC, although it is the alpha-dystrobrevin isoform that is predominantly expressed in skeletal muscle (Rees, Lien, & Górecki, 2007). A number of different length alpha-dystrobrevin can occur through splicing, the most common isoforms in muscle being alpha-dystrobrevin-1 and alpha-dystrobrevin-2

(Peters et al., 1998). The dystrobrevins are significantly shorter than dystrophin at just 743 and 686 amino acids in alpha-dystrobrevin-1 and alpha-dystrobrevin-2 respectively. Both isoforms maintain dystrophin's cysteine-rich domain and C-terminal and binding affinities for syntrophins (Newey et al., 2000). Additionally, alpha-dystrobrevin-1 has its own unique C-terminal domain featuring tyrosine phosphorylation sites (K. R. Wagner, 1993). alpha-dystrobrevin-1 is able to bind dystrophin and utrophin however alpha-dystrobrevin-2 is not able to bind utrophin (Peters et al., 1998). Dystrophin's C-terminal is unique to itself and the dystrophin-related proteins. A comparisons of the dystrophin-related protein structures is highlighted in Figure 9.

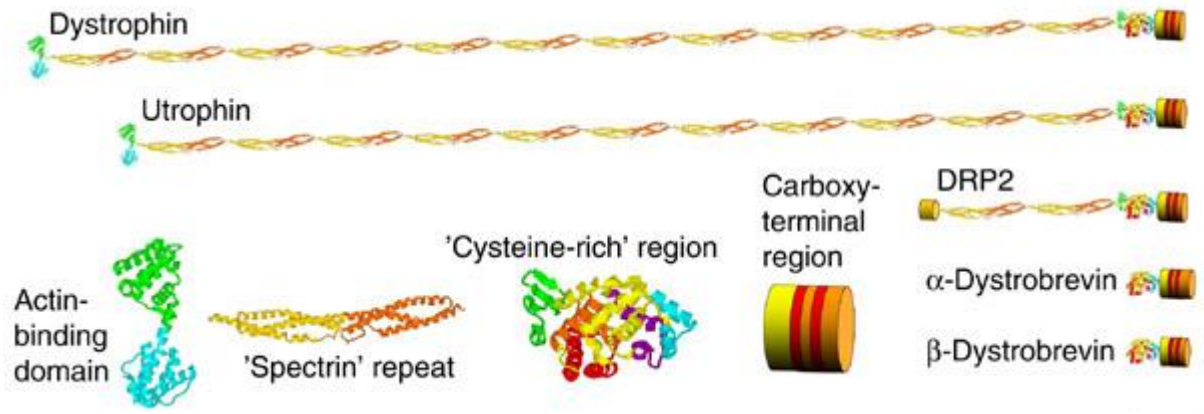


Figure 9. Dystrophin structural similarity to the evolutionary related proteins utrophin, dystrophin-related protein 2 and alpha- and beta-dystrobrevin. Image derived from Roberts (2001). (Roland G Roberts, 2001). Dystrophin's C-terminal region and cysteine rich domain is well conserved across the related proteins (as it also is across dystrophin isoforms). Utrophin is the most structurally similar containing actin binding domains and a large number of spectrin repeats in its rod domain, giving it many binding similarities with dystrophin. Dystrophin related protein 2 (labelled DRP2) contains a significantly shorter rod domain and is without the actin binding domain. Both dystrobrevin isoforms are without the actin binding and spectrin repeat domains.

Other proteins that share significant homology to dystrophin are the spectrin repeat proteins. These proteins typically have roles in cytoskeletal structure, providing a link between actin and the membrane. They include spectrins, alpha-actinins, nesprins and utrophin. The spectrin family generally contain a spectrin repeat domain flanked by actin binding calponin domains and calcium-binding EF hands (Broderick & Winder, 2005).

2D. Roles of dystrophin within the cell

Dystrophin has been implicated in many roles within the cell. Its most commonly noted role is in linking the actin cytoskeleton to the extracellular matrix to increase membrane stability and act against muscle contraction induced tearing. Dystrophin is able to interact with actin at its calponin domains (Norwood et al., 2000) and at spectrin repeats 11 to 17 (K J Amann et al., 1999). The calponin domain interactions are regulated by competitive binding of calmodulin and troponin (Jarrett & Foster, 1995). The spectrin repeat domain has lipid binding properties that may cause the rod domain to lie along the membrane bilayer (Legardinier et al., 2009). In this model, the contractile force experienced by dystrophin would be spread through the DAPC and a large area of the membrane, increasing the area in which the force is dispersed. The lipid binding of dystrophin can be influenced by the presence of cholesterol within the membrane bilayer (Ameziane-Le Hir et al., 2014).

As well as dystrophin's direct interaction with actin filaments, it provides further structural linkages through intermediate filaments keratin-19 (Stone et al., 2005) and synemins (Bhosle et al., 2006), cytolinker protein plectin (Rezniczek et al., 2007), adapter proteins ankyrin B and G (Ayalon et al., 2008) and actin binding protein alpha-actinin-2 (Hance et al., 1999). Fellow DAPC component dystrobrevin also has structural interactors including syncoilin (Newey et al., 2001) and synemins (Mizuno et al., 2001).

Dystrophin localises nNOS to the sarcolemma where nNOS is able to regulate muscle blood perfusion, allowing an adequate oxygen supply to skeletal muscle cells during contraction. Binding to nNOS is at the rod domain, and indirectly through two syntrophin binding sites. Dystrobrevin also binds two syntrophin proteins which each have the potential to bind a nNOS protein. The nNOS binding of syntrophin is not enough to restore correct localisation in the absence of dystrophin's nNOS binding rod domain (D. S. Chao et al., 1996; Judge, Haraguchiln, & Chamberlain, 2006) although it should be noted that nNOS mRNA and protein levels and localisation of protein inhibitor of neuronal nitric oxide synthase (PIN) in dystrophic cells is altered (Y. Guo, Petrof, & Hussain, 2001).

Studies of mdx mice along with *in vitro* cell cultures have demonstrated that the absence of dystrophin disrupts a number of cell processes. Dystrophin deficiency results in calcium ion misregulation (Constantin, Sebille, & Cognard, 2006) which can subsequently result in misregulation of calcium dependant pathways. Calcium homeostasis is thought to act through

dystrophin localisation of syntrophin which is involved in the activation of transmembrane ion channels such as the transient receptor potential channels (TRPC) (Sabourin et al., 2009). This is supported by calcium misregulation in syntrophin and TRPC deficient cells, and the observation that calcium regulation is not disrupted in other muscular dystrophies with intact dystrophin localisation (Vandebrouck et al., 2002). Dystrophin's role in localising syntrophin has consequences upon the activities of other transmembrane proteins, as syntrophins are responsible for the localisation of a variety of signalling proteins including kinases (Lumeng et al., 1999), water channels (M E Adams et al., 2001) and potassium ion channels (Leonoudakis et al., 2004). Like dystrophin, syntrophin can also localise nitric oxide synthase (M E Adams et al., 2001).

Dystrophin has been associated with the integrin focal adhesion complex, which could provide another link to signalling pathways (G Anastasi et al., 2003). Both complexes interact with the extracellular matrix (Figure 10) and both dystrophin and integrin bind alpha-actinin-2 which could potentially provide a direct link between the two (Hance et al., 1999; Otey, Pavalko, & Burridge, 1990). Dp71 has even been suggested to form part of the integrin complex in brain (Cerna et al., 2006). However, in skeletal muscle the two complexes have not been determined to be closely linked and do not require each other for localisation or expression. The two complexes may collaboratively perform extracellular matrix laminin organisation (Henry et al., 2001).

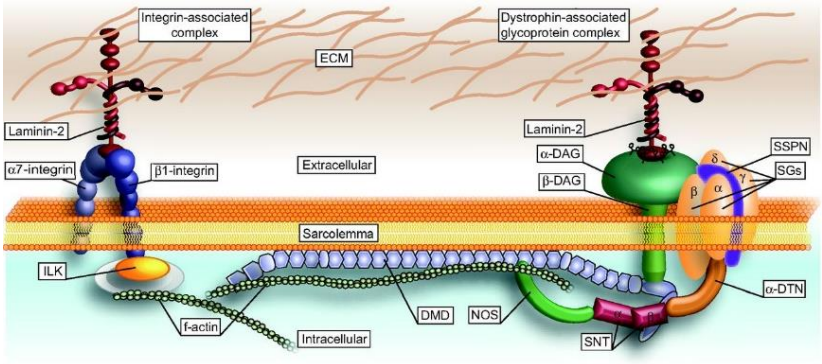


Figure 10. The similar roles of the dystrophin-associated protein complex and the integrin focal adhesion complex in linking the cytoskeleton to the extracellular matrix link. Image derived from Berger and Curie (2012) (Berger & Currie, 2012). Both complexes play a role linking the actin cytoskeleton to the extracellular matrix in order to protect against mechanical stress during muscle contraction. In DMD increased integrin expression are observed in an apparent attempt to offset the loss of the mechanical linkage provided by the DAPC. Absence of both integrin and dystrophin results in an increased dystrophin phenotype.

Dystrophin is required for organisation of the microtubule network, and has microtubule-binding properties (Prins et al., 2009), leading to the proposal that it acts as a guidepost for

microtubule elongation (Belanto et al., 2014; Oddoux et al., 2013). It can also bind nuclear distribution protein nude homolog 1 (Nde1) which forms a Lis1-Nde1-DAPC complex (Pawlisz & Feng, 2011). Lis1 (Lissencephaly 1) plays a role in microtubule organisation and regulates dynein, a motor protein that moves along microtubules. The syntrophin interactions with microtubule associated kinases may also have a role in the organisation (Sumigray, Chen, & Lechler, 2011; Toropova et al., 2014). Lis1 has also been implicated in mitotic spindle organisation (Moon et al., 2014), a role in which dystrophin has been implicated in activated muscle stem cells, with dystrophin downregulation resulting in a decreased expression of the cell polarity regulator and dystrophin phosphorylating protein Par-1b (Dumont et al., 2015; Keefe & Kardon, 2015; Yamashita et al., 2010).

It has been well established that dystrophin deficiency results in an altered secretion profile, with several experiments able to identify specific biomarkers for the disease (Hathout et al., 2014, 2015). The altered secretory profile is thought to be through a combination of membrane (Allen & Whitehead, 2011; Duguez et al., 2013) although secondary effects of pathway misregulation such as from calcium level changes may also be implicated. An increase in the secretion of Lysosome-associated protein 1 (LAMP1)-positive vesicles has been observed as a result of dystrophin deficiency (Duguez et al., 2013), but the link between dystrophin's interactions and its role in altered vesicle secretion profile has yet to be determined. As mentioned previously, dystrophin appears to play an indirect regulatory role in (or may be regulated by) sarcolemmal expression of caveolin-3, providing a possible association with the caveolae vesicle pathway. Dystrophin has been suggested to bind to cavin-1 (also commonly referred to as called polymerase I and transcript release factor, PTRF) possibly indirectly through beta-dystroglycan (Johnson et al., 2013). Cavin-1 plays a structural role in the caveolae and is required for expression of other caveolae proteins including caveolin-3 (Hansen, Shvets, Howard, Riento, & Nichols, 2013).

2E. Treatments of disease

Many approaches are being explored for the treatment of dystrophin deficiency, including gene therapy, utrophin overexpression, read-through of nonsense mutations, stem cell transplantation and, more recently, gene editing. All of these seek to recover some of the functions of dystrophin, by restoring muscle cell expression of part or all of the dystrophin protein or by overexpression of its close homologue. Secondary aspects of the pathology such as inflammation and fibrosis are also targeted.

2E1. Gene Therapy

Gene therapy has been the subject of much research and many clinical trials. However, only recently has the first approval of a gene therapy approach for market distribution been granted (Büning, 2013). Approaches aimed at DMD phenotypes include the introduction of mini- and micro-dystrophin constructs. This method results in the expression of a truncated dystrophin and aims to convert the pathology towards a BMD phenotype.

The introduction of microdystrophin construct expression requires a dystrophin gene to be delivered to the cell, usually performed using an empty viral vector. The full-length dystrophin protein requires a cDNA sequence of approximately 14 kb (Kumar-Singh & Chamberlain, 1996). Different viral vectors have been tested that vary in capacity, transfection abilities in different tissues and the immune response elicited. The adenoviral vector has the capacity to deliver DNA encoding the entire dystrophin protein however these vectors are difficult to create, cannot maintain expression in humans for more than a year and cause a notable immune response (DelloRusso et al., 2002; Scott et al., 2002). AAV has become the main focus in DMD due to its ability to maintain microdystrophin expression for several years, and to evoke minimal immune response in animal models (such as canine and mice (Hua Li et al., 2009; Z. Wang et al., 2010)) although significant immune issues have since be observed in human trials (Mingozzi & High, 2013; J. Y. Sun, Anand-Jawa, Chatterjee, & Wong, 2003).

AAVs contain a rep and cap gene flanked by two inverted terminal repeats (ITR). The ITRs are necessary for gene replication and for packaging into the capsid. Recombinant AAV have the rep and cap genes removed and replaced with a promoter gene and a gene encoding a truncated dystrophin while maintaining the ITRs. There are several types of AAV serotype, named AAV1 through to AAV9. The different serotypes have different targeting properties

and can elicit different immune responses. AAV6 and AAV9 have both attained high levels of transduction in skeletal and cardiac muscle (Bish et al., 2008; Blankinship et al., 2004; Zincarelli, Soltys, Rengo, Koch, & Rabinowitz, 2010; Zincarelli, Soltys, Rengo, & Rabinowitz, 2008). Patients can have pre-existing immune responses to AAV serotypes and immunity can also be built up to further AAV injection after an earlier AAV treatment, inhibiting repeated administration (Calcedo & Wilson, 2013; Louis Jeune, Joergensen, Hajjar, & Weber, 2013). In order to increase muscle targeting specificity and decrease immune responses the promoter will typically be muscle specific such as the muscle creatine kinase (MCK) promoter (De Geest, Van Linthout, & Collen, 2003; Katwal et al., 2013).

Native AAV is capable of site-specific integration, but this is abrogated in the recombinant form by removal of the AAV rep gene. Unspecific integration, most commonly at sites prone to DNA damage, is also possible. Concern remains over insertional mutagenesis effects on the regulation of genes at or adjacent to insertion sites and this has been shown to be potentially cancerous (A Donsante et al., 2001; Anthony Donsante et al., 2007). AAV integration can occur in both dividing and non-dividing cells. Safe integration into the dividing cells presents an opportunity to increase the expression of the AAV vector. Approaches that incorporate a larger truncated dystrophin across two AAV vectors or the full dystrophin gene across three AAV vectors have successfully been demonstrated to produce large truncated or full length dystrophin in mouse models. However these multi-vector approaches require high-titers of AAV, and suffer from a decreased efficiency of expression and the potential for individual vectors to produce aberrant proteins (Ghosh, Yue, Long, Bostick, & Duan, 2007; Koo, Popplewell, Athanasopoulos, & Dickson, 2014). It is possible to deliver naked DNA plasmids, sidestepping the size limitation and immune response, however these plasmids have poor transduction efficiencies (G. Zhang et al., 2010). AAV vectors in mice have shown therapeutic potential, demonstrating significant dystrophin expression and function. Trials in dogs and humans encountered higher immune responses than in murine trials (Fairclough, Wood, & Davies, 2013; Z. Wang et al., 2010).

Designing truncated dystrophin to optimize therapeutic effect requires an in depth knowledge of dystrophins different roles, its interactions and its interaction domains. Most commonly large sections of the spectrin repeat domain are removed (McGreevy, Hakim, McIntosh, & Duan, 2015). The capacity of the cysteine rich domain to bind dystroglycan for DAPC formation and linkage to the extracellular matrix is functionally vital and is conserved in the

constructs. The actin-binding calponin domains are also usually retained. The C-terminal domain containing syntrophin and dystrobrevin binding sites is sometimes removed or truncated. Dystrophin's quaternary structure is not well understood and the removal of such large sections of dystrophin could have significant structural effects.

2E2. Exon Skipping

Exon skipping aims to correct out-of-frame mutations (that would usually lead to nonsense-mediated decay of the transcript) by inhibiting the transcription of adjacent exons such that the reading frame is restored and a truncated dystrophin is expressed. Since mutations patterns vary and each exon boundary may fall on a different position of the reading frame, the location of the mutation determines which exon(s) must be targeted by the therapy and what dystrophin product will be created. Exon skipping is generally carried out using antisense oligonucleotides (AONs), usually of chemistry 2'-O-methyl phosphorothioate (2OMePS) or morpholino (PMO), but gene constructs using AAV-U7 are also proposed (Maëva Le Hir, 2013). Antisense sequences can be selectively targeted to a specific region of a target exon in dystrophin's pre-mRNA to induce skipping of the exon. Clinical trials have been carried out using exon skipping for treating DMD (Mendell et al., 2016; Shimizu-Motohashi, Miyatake, Komaki, Takeda, & Aoki, 2016; Voit et al., 2014). Peptide conjugation to the oligonucleotides can be used to alter target specificity (Gao et al., 2014).

For single exon skipping in DMD patients, the exon that is most frequently required to be skipped is exon 51, which would cover approximately 13 of DMD patients (Aartsma-Rus et al., 2009). Recently, a 2OMePS oligonucleotide targeting this exon, commercially named Drisapersen, reached phase three of clinical trials however it was unable to demonstrate any significant therapeutic effects (Q. Lu, Cirak, & Partridge, 2014). Perhaps worryingly, its failure was attributed to the lack of correlation between dystrophin expression and clinical efficacy. An alternative oligonucleotide, commercially named Eteplirsen, based on the PMO chemistry, targets the same exon and is currently in phase three trials. Due to concerns over study size and heterogeneity of the patient cohort, a panel of FDA (Food and drug administration, USA) advisors initially voted against approval of this drug (Sarepta Therapeutics Inc., 2016a) however patient groups continued to support it based on anecdotal reports of significant therapeutic effects in some cases. A final decision by the FDA recently approved the drug (Sarepta Therapeutics Inc., 2016b, 2016c; U.S. Food and Drug Administration, 2016).

Although exon skipping has less choice available for selecting the dystrophin domains to be expressed or deleted compared to AAV microdystrophin delivery and is dependent upon the placement of the mutation site, multiple options do in principle exist for most mutation patterns. For instance, it may seem obvious to skip the minimum number of adjacent exons in order to maximise the length and the number of domains of the dystrophin protein produced, but this may not always be the most therapeutically beneficial option. For instance, most mutations associated to DMD or BMD are internal to exons 45-55 (63% of DMD (Christophe Béroud et al., 2007)), and BMD patients with an in-frame deletion of this entire region from exon 45 to 55 display a milder phenotype when compared to other BMD patients with shorter in-frame deletions internal to exons 45-55 (Gentil et al., 2012; Taglia et al., 2015). Thus a given multi-exon-skipping approach could target many different mutation patterns, although the relative efficiency may make dosage requirements problematic (Echigoya, Aoki, et al., 2015). Multiexon skipping approaches have been developed (Aartsma-Rus et al., 2004; Fall et al., 2006; Miskew Nichols et al., 2016; van Vliet et al., 2008) with recent success in the murine model (Aoki et al., 2012) but are not currently the subjects of any clinical trials. Besides multi-exon skipping as an alternative to single exon skipping, multiple choices also exist for some single exon skipping approaches applied to the same deletion pattern – for example, the reading frame of patients harbouring deletion of exon 51 can be restored by skipping of either exon 50 or 52 (Figure 11).

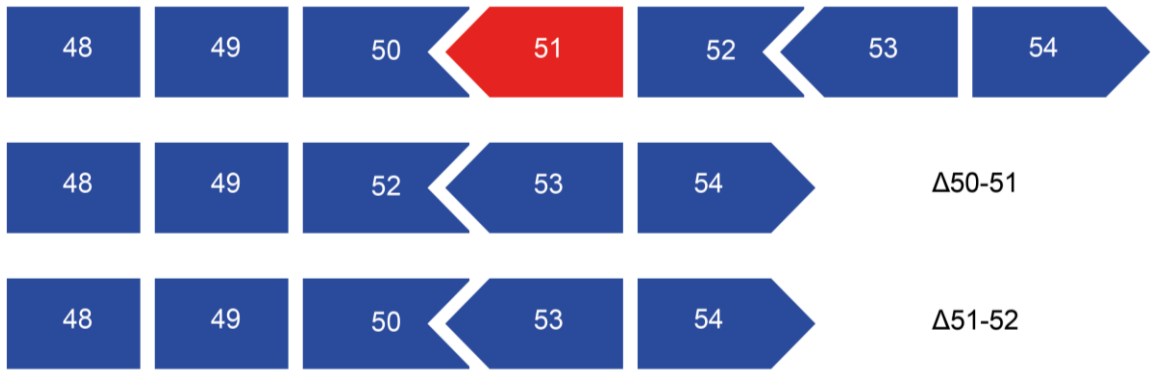


Figure 11. A diagram highlighting the possible exon skipping of exons 50 or 52 in order to restore the reading frame of an exon 51 deletion. Complementary shapes represent a combination of exons that would result in an in-frame reading frame, non-complimentary shapes represent a combination of exons that would result in an out-of-frame deletion. Exons 48 to 54 of dystrophin are represented. Deletion of exon 51 (coloured red) would result in a disrupted reading frame caused by non-complimentary exons 50 and 52. Exon skipping of either exon 50 or exon 52 restores the reading frame of dystrophin, encoding a functional Δ50-51 or Δ51-52 dystrophin protein respectively.

A potential drawback of oligonucleotides is the likely requirement for repeated administration to maintain dystrophin levels, although it is not yet clear how often this would need to be. An approach to deliver AAV vectors encoding the exon skipping molecules which would hence maintain the therapy is being investigated, although this approach has problems with efficiency in humans (Goyenvalle, Babbs, van Ommen, Garcia, & Davies, 2009; Incitti et al., 2010; Maëva Le Hir, 2013).

As discussed, the design of each exon skipping approach is constrained by the mutation pattern to be targeted. However, for certain mutation patterns, and for a multi-exon skipping approach, the knowledge of dystrophin binding partners is relevant. In addition, and as for the other therapeutic approaches discussed here, a systematic understanding of the functional roles dystrophin's binding partners would be of general benefit in the development of parallel and complementary therapies targeting specific pathways.

2E3. Utrophin overexpression

Experiments attempting to replace dystrophin with the evolutionarily similar utrophin have been performed. Utrophin shares many of the same binding partners as dystrophin and is able to form the utrophin associated protein complex (its own version of the dystrophin associated protein complex) as has already been described. Utrophin is naturally upregulated in dystrophin deficient cells (Kleopa et al., 2006). This is most pronounced in mdx mice and has been proposed to explain their apparent mild phenotype compared to humans (Weir et al., 2004). Mice with a dystrophin/utrophin double knockout have a severe phenotype, exhibiting dramatic muscle atrophy similar to that observed in human DMD (Deconinck et al., 1997). Utrophin upregulation holds the advantages of being able to deliver the exact same treatment to all DMD patients, and being less likely to induce immune response. Utrophin overexpression in muscle and non-muscle does not display any toxic effects and unspecific tissue delivery is not a concern (Fisher et al., 2001). Utrophin upregulation can be induced by activating the utrophin promoter. This has been tested in phase one clinical trials with BMN195 (also called SMT-C1100) however this trial was unsuccessful due to delivery problems (Tinsley et al., 2011). Other approaches to increase utrophin levels include overexpression of RhoA (Armelle Bonet-Kerrache, 2005; Gauthier-Rouvière & Bonet-Kerrache, 2009), heregulin overexpression (Tejvir S. Khurana, 1999; Utpal Basu, 2007), calpain inhibition (Courdier-Fruh & Briguet, 2006) and increase of nNOS and the nNOS substrate L-arginine (Chaubourt et al., 1999; Voisin et al., 2005) among others. Many of these are also being examined to treat other diseases and

are not specific to utrophin upregulation. Disintegrin and metalloproteinase domain-containing protein 12 (ADAM12) increased the expression of both utrophin and integrins (Moghadaszadeh et al., 2003). Biglycan has been found to stimulate utrophin upregulation and recruitment to the sarcolemma in place of dystrophin in dystrophin deficient cells (Amenta et al., 2011; Fadic et al., 2006). Integrin, biglycan and utrophin upregulation in dystrophic muscle all occur naturally to some degree. Specifically designed zinc finger containing proteins have been able to activate the utrophin A promoter to selectively upregulate utrophin (Onori et al., 2013). The utrophin protein may be able to be delivered directly to cells by fusing it to peptides such as the protein transduction domain (PTD) of the HIV-1 TAT protein (TAT) (Sonnemann et al., 2009). This approach was found to work for micro-utrophin constructs however the delivery was decreased for the larger utrophin-TAT fusion protein. Utrophin has been found to be upregulated in mdx mice, particularly after undertaking exercise although mdx mice are a lot more mobile than human DMD patients (Baban et al., 2008; Gordon, Lowe, & Kostek, 2014).

2E4. Read-through nonsense mutations (PTC124)

PTC124 (also known as ataluren or commercially as Translarna) is a small molecule that binds to ribosomes to enable the read-through of nonsense mutations, thereby rescuing translation of mutant mRNA containing premature termination codons (PTCs) (Peltz, Morsy, Welch, & Jacobson, 2013). The PTC124 drug has previously reached phase three clinical trials however results were determined to be negative and doubts remain as to the efficacy (McElroy et al., 2013). Gentamicin has also been identified as being able to cause ribosomes to read through stop mutations (Barton-Davis, Cordier, Shoturma, Leland, & Sweeney, 1999) however this molecule has issues with side effects and required dosage (Ahmed, Hannigan, MacDougall, Chan, & Halmagyi, 2012; Mercieca & Mercieca, 2007; Wooltorton, 2002).

2E5. Stem Cell Transplantation

Progress has been made transplanting immature healthy muscle cells into dystrophin deficient animal models (Sienkiewicz, Kulak, Okurowska-Zawada, Paszko-Patej, & Kawnik, 2015). Stem cells can be taken from a healthy donor or patient and subjected to a gene therapy or gene editing approach *in vitro* to restore dystrophin expression before being transplanted back into the patient (Gussoni et al., 2002; Moisset et al., 1998; Nik-Ahd & Bertoni, 2014). These modified stem cells can then grow and replicate before differentiating to produce dystrophin

expressing cells within the tissue. Some stem cells also secrete growth factors and anti-inflammatory proteins, encouraging proliferation and differentiation, and regulating muscle regeneration and repair (Ichim et al., 2010; A. Lu et al., 2012). To treat DMD, stem cells that possess myogenic potential must be used. This includes bone marrow stem cells, embryonic stem cells, adipose-derived mesenchymal stem cells and mesoangioblasts (Asakura, 2012; Di Rocco et al., 2006; Gunetti et al., 2012; Perini et al., 2015; Zheng et al., 2006). Performing the gene therapy *in vitro* allows high doses of drug to be administered with no immune response of delivery issues during this stage. The immune response to the reintroduced stem cells has been an issue with this approach in allogenic grafts however autologous grafts undergo minimal or no immune response (Griffin et al., 2013; Morizane et al., 2013; Wood, Issa, & Hester, 2016).

Granulocyte colony-stimulating factor is involved in the transport of hematopoietic stem cells (a type of bone marrow stem cell) from the bone marrow into the blood and has also been linked to many advantageous properties including anti-inflammation, cell growth and decreased apoptosis, making the protein an attractive option for easily extracting hematopoietic stem cells from patients for treatment of DMD (Hayashiji et al., 2015; Hosing, 2012; Simões, Benitez, & Oliveira, 2014).

A major barrier to successful muscle stem cell therapy is the delivery of cells to all of the body's functionally critical muscle groups, although treating specific muscle groups is possible and beneficial (Cossu et al., 2015; Périé et al., 2014).

2E6. Gene editing

DNA binding proteins have provided promise in gene therapy approaches to treat DMD. Proteins have been successfully engineered to recognise and bind to specific DNA sequences. These proteins are attached to enzymes that cleave DNA, enabling specific gene sequences to be deleted, with the cells natural repair process able to reattach the cut DNA strands. New DNA fragments can also be introduced in place of the deleted sequence. One such engineered protein family are the Zinc finger nucleases (ZFN). ZFN's therapeutic potential has been successfully demonstrated in a patient with a Δ48–50 DMD deletion (Ousterout et al., 2015). The deletion of exons 48 to 50 is an out-of-frame deletion which can be corrected by skipping exon 51. Ousterout *et al*. were able to delete exon 51's splice acceptor sequence resulting in exon 51 not being transcribed, resulting in a mRNA transcript lacking exons 48–51 which is translated into

a truncated dystrophin protein. ZFNs are in phase 1 and phase 2 clinical trials for other diseases including HIV-1 co-receptor CCR5 (Tebas et al., 2014). No such clinical trials currently involve DMD. Other approaches to gene editing include TALENs (Transcription activator-like effector nucleases) and CRISPR-Cas9 (clustered, regularly interspaced, short, palindromic repeats-CRISPR-associated protein 9 system). TALEN has been similarly demonstrated for exon 51 deletion of Δ48-50 DMD (Ousterout et al., 2013). TALENs possess increased specificity of DNA binding sequence compared to ZNF. While ZFNs recognise DNA by binding to triplets, TALENs recognise single DNA nucleotides, making for easier design of TALEN proteins, although ZFNs recognising all of the possible nucleotide triplet sequences have been engineered. No clinical trials involving TALENs have yet to be carried out however they are expected to be undertaken soon (Qasim et al., 2015). CRIPSR-Cas9 is a related and promising approach using RNA to target the DNA sequence (Mali et al., 2013). The CRISPR system has only been fully understood and implicated in gene therapy approaches within the last five years (Long et al., 2015; Xu et al., 2016). It has the advantage that it is targeted using only a guide RNA, as opposed to TALEN and ZNF, which require a custom protein for each new target.

2E7. Reducing symptoms

Research has also focused on reducing the symptoms of those affected with the disease. A variety of drugs have been used to alleviate many of the conditions associated with the muscular dystrophies including the upregulation of pathways involved in similar functions to dystrophin such as α7β1-integrin (Burkin, Wallace, Nicol, Kaufman, & Kaufman, 2001), the increase of muscle mass to negate dystrophic muscle wasting by inhibiting myostatin (Kemaladewi et al., 2011; Kornegay et al., 2016), the use of steroids which increase muscle mass and also decreases inflammatory and pro-fibrotic responses (Henricson et al., 2013) and the inhibition of the proteins responsible for degradation of the DAPC (Gazzerro et al., 2010). among others.

Regimes controlling the extent of patients' exercise and implementing specific training programmes are aimed at reducing muscle wasting (Jansen, van Alfen, Geurts, & de Groot, 2013). Physical assistive devices aimed at reducing the improving patient mobility and quality of life are also under development.

An improved understanding of Dystrophin's binding partners and functional roles may suggest alternative or improved approaches to secondary pathology, applicable to both DMD and BMD patients.

2E8. Approaches to disease and their relevance to the dystrophin interactome

Several of the approaches to treat DMD offer different levels of control over choosing which domains of dystrophin will be incorporated into the treated DMD patient. Even for the approaches that don't offer such choice, such as exon skipping, it is useful to know what the effect of the missing domains has upon dystrophins interaction and functions and offers an opportunity to perform a secondary therapeutic (such as upregulation of an alternative gene) to alleviate any remaining cells defects resulting from a truncated dystrophin. It is important to be mindful of the difference in dystrophin functions and binding of dystrophin domains in different tissues: the importance of each binding partner and its functional role(s) may differ between tissues. This is most apparent in the variation of dystrophin isoform expression in the different tissues. Full length dystrophin containing the actin binding domain is expressed predominantly in skeletal muscle where cell experience the highest amounts of mechanical stress while shorter isoforms have increase expression in other tissues where protection from mechanical stress is less and so the actin-binding domain is not required.

2F. Cell and animal models for studying dystrophin and DMD

Many myogenic cells and multiple animal models are available for studying dystrophin and DMD. Dystrophin has been studied in healthy tissues and cells in order to investigate interacting partners. Dystrophin knockdown in healthy cells to replicate dystrophin-deficient DMD pathology has also been developed (Ghahramani Seno et al., 2008; Seno et al., 2010). Immortalised myoblast lines from the muscle of DMD patients have been available for some time (Caviedes, Caviedes, Liberona, & Jaimovich, 1994). The animal models most commonly studied in DMD are from murine strains and dog breeds however models from several other species have been discovered naturally or created, most recently a porcine model (Selsby, Ross, Nonneman, & Hollinger, 2015), to make up almost 60 distinct models (McGreevy et al., 2015). Models of other dystrophin-related diseases and mutations are also available in animals and cell lines (Whitmore & Morgan, 2014).

2F1. Cell models

Cells can be isolated from muscle biopsies of a desired species, most commonly mice and human. Mechanical dissection and filtering to remove whole muscle tissue can be used to isolate primary cell populations (Rando & Blau, 1994). Basic sorting on the basis of substrate attachment can be used to obtain primary myoblasts, which can be further sorted on the basis of specific markers (Chapman et al., 2013; Conboy, Cerletti, Wagers, & Conboy, 2010; Conboy & Conboy, 2010; J. Li, 2013). Several biobanks exist housing large collections of healthy and pathological primary muscle cells (Mora et al., 2015).

Primary myoblasts suffer the disadvantage that they undergo senescence in tissue culture (Nehlin, Just, Rustan, & Gaster, 2011; Pääsuke et al., 2016). This prompted the development of immortalization approaches that inhibit senescence so that the lines have a less limited experimental life span. Senescence is attributed to cellular senescence activation of the p16-mediated cellular stress pathway and p53 pathway activation due to telomere shortening which leads to cell cycle exit (Wright & Shay, 2002). Viral gene transfections to inhibit these processes have been attempted, with targeting of both of these processes being most effective (Mamchaoui et al., 2011). Non-human cells such as the C2C12 mouse cell line are able to develop immortality without the need for intervention (Nowak et al., 2004). Clonal lines may be isolated to generate an identical cell population. Cells from mdx or other dystrophic mice

can be crossed with the immortomouse in order to generate cell lines of the disease (Jonuschies et al., 2014; J E Morgan et al., 1994; Muses, Morgan, & Wells, 2011).

Cell lines represent an *in vitro* system that does not fully mimic the physiological conditions and complex interactions that come from multiple different cell types and tissues *in vivo*. Conversely, the purity of the cell line has advantages when attempting to examine a single cell type without this added complexity, allowing the cell to be examined outside of the wider system context. Additionally, while mouse animal models are readily available, study of humans *in vivo* presents much more difficulty. The use of cell lines derived from humans offers an *in vitro* alternative. Testing in cell lines is commonly used as a starting point before testing in animals, particularly in therapeutic approaches. The immortalised cell lines offer easy manipulation in experiments such as those involving transfection however the effects that the immortalisation process can have on cells could be significant and must be considered.

2F2. Animal models

Mdx mice are the most frequently used animal model. Several background variations of the mdx mice exist including albino, BALB/c, C3H, C57BL/6, DBA/2 and FVB strains however the original C57BL/10 strain is the most commonly used (McGreevy et al., 2015). Slight variations in phenotype are exhibited between the models.

The CB57BL/10 mdx mouse has a nonsense point mutation within exon 23 resulting in no dystrophin expression. Despite their substantial use, the mdx model has several differences compared to human DMD. The mdx exhibits far less severe muscle atrophy, cardiac problems, and shortening of lifespan, than human DMD. Indeed, over the lifespan of the mouse, muscle regeneration appears to keep pace with necrosis such that muscle mass is broadly maintained (and in some cases is even hypertrophied perhaps to compensate for slight loss of specific force), except for certain muscles such as the diaphragm and limb muscles (Faulkner, Ng, Davis, Li, & Chamberlain, 2008; Lynch, Hinkle, Chamberlain, Brooks, & Faulkner, 2001). The lifespan of the mouse is only reduced by approximately 25 percent from having a DMD mutation, whereas in humans it is approximately 75 percent. It should be noted, however, that mdx muscle undergoes massive regeneration, likely even more so than human DMD muscle, so caution is advised when comparing the relative severity of the murine model to the human condition (Duddy et al., 2015; Terence A. Partridge, 2013).

The mdx mouse represents a direct homologue of the human disease and, as discussed, exhibits severe aspects of pathology such as a high incidence of degeneration and regeneration. However, other models have been proposed that have more severe muscle atrophy, and greater reduction in lifespan. Utrophin levels in mdx mice are significantly upregulated with the utrophin able to take the place and perform some of the roles of dystrophin, thereby alleviating some of the pathology (Baban et al., 2008; Squire et al., 2002). Mice harbouring a dystrophin/utrophin double knockout display a severe phenotype although these double knockout mice are difficult to generate and keep alive (Deconinck et al., 1997). Utrophin heterozygous mdx mice may offer a useful model for scientific studies, displaying phenotype similar to that of the double-knockout model but with an increased survival rate (van Putten et al., 2012). Similarly, α7β1-integrin expression is upregulated in the mdx mice, increasing the link to the extracellular matrix however double knockout of dystrophin/ α7-integrin results in death at just one month of age (C. Guo et al., 2006; Hodges et al., 1997). Several other double knockouts with the mdx mouse have also been generated in attempts to create a model closer to the DMD phenotype or to investigate the roles of proteins of interest during dystrophin deficiency (R. H. Crosbie et al., 1997; Fajardo et al., 2016; Han et al., 2011; McGreevy et al., 2015; Sacco et al., 2010). Immune-deficient mdx can be used to test gene and cell therapies without any immune response complications (Chamberlain, 2013; Farini et al., 2007; Huard et al., 1994; Meng et al., 2015; T A Partridge, Morgan, Coulton, Hoffman, & Kunkel, 1989; Vallese et al., 2013).

Variants of the mdx mice models have been developed that are deficient in additional dystrophin isoforms. Named mdx^{2cv}, mdx^{3cv}, mdx^{4cv} and mdx^{5cv}, the models have mutations affecting different exons of dystrophin resulting in expression of dystrophin isoforms (Im et al., 1996). mdx^{2cv} expresses of dp71, 116 and 140, mdx^{3cv} does not express any of the dystrophin isoforms, mdx^{4cv} expresses dp71 and dp116 while mdx^{5cv} expresses all isoforms except the full length dystrophin and has the same phenotype as mdx.

Relative to mice, DMD dogs are more expensive to maintain, are limited in the number of different disease models currently available, and are subject to additional ethical issues (and their use in research settings often regulated under stricter laws (Levy, 2012)). In addition, their long life cycles necessitate inbreeding in order to generate the maximum number of affected dogs, and this selects traits that are not necessarily representative of the whole dog population.

The limited variety of dog DMD mutations also limits their use in studying gene therapy treatments (Howell et al., 1997; Kornegay et al., 2012).

The relative expense (which increases with size and life cycle) and additional ethical considerations of animal models makes the use of cell lines preferable for experiments that do not require consideration of the wider *in vivo* context, such as experiments to study protein-protein interactions. Animal models are of most benefit in areas where the cell lines are of limited use as in system-wide approaches for therapeutic targeting and phenotype and behavioural studies. Data collection is performed *ex vivo* from tissues once they have been removed from sacrificed animals. Although the animal models have the advantage of representing an *in vivo* system, they do not fully replicate the human condition and the results of clinical trials in animal models often fail to adequately represent human outcomes.

2G. Identifying protein-protein interactions

2G1. Interaction types

Physical Interactions

Interactions between proteins can be sorted into groups such as direct interactors and physical interactors. These interactions can form and regulate all cell processes including structural roles within the cell, stabilising and destabilising proteins, enzymatic and regulatory by altering protein folding or sterically inhibiting other binding sites on the protein.

Direct interactors are a subgroup of physical interactors. Physically interacting proteins form complexes together however the strength of binding between the proteins in complexes can vary. Strongly binding complexes are the easiest to characterise experimentally through techniques such as immunoprecipitation and pull-down assays. Weaker interacting protein complexes are more difficult to examine but optimising conditions or crosslinking proteins can help to overcome their transient nature, although background binding becomes an increasing issue.

Direct Interactions

Direct interactions can be classified as either obligate or non-obligate. Obligate interactions are non-covalent interactions that are required for protein stability and so are usually permanent interactions. The disruption of an obligate interaction results in the irreversible loss of the proteins structure or function. As a result, the interactors are not able to be separated for imaging (by X-ray crystallography, for example) but may be imaged when in complex with the other protein involved in the obligate interaction. Many homodimers such as cytochrome C' are examples of obligate interactions (Finzel, Weber, Hardman, & Salemme, 1985). Non-obligate interactions are by far the more common interaction type within the cell, as they allow for a dynamic movement of protein-protein interactions which enables a rapid response to cellular needs and stimuli. These interactions are reversible and not essential for protein stability, being able to form and dissociate without permanent loss of the protein's structure or function. Transient interactions vary greatly in their strength and lifetime (Figure 12 for a simplified summary of obligate and non-obligate interactions). An examination of the Gene Ontology (GO) terms annotated to obligate and non-obligate interactions found that while most obligate interactors share molecular function annotation terms, the majority of transient

interactors do not share any functional annotations (Mintseris & Weng, 2005). All of dystrophin's known interactors are transient interactions.

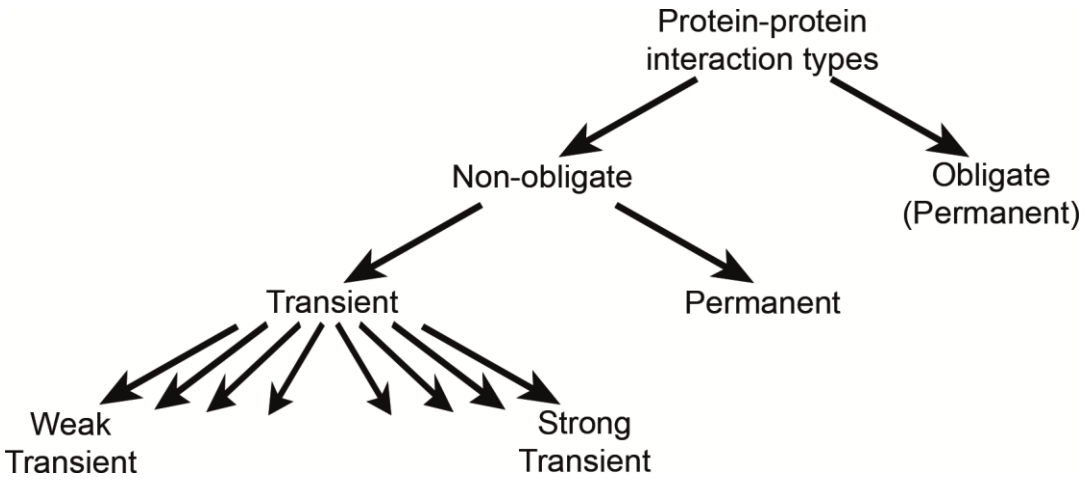


Figure 12. Schema of the different interaction types. Protein-protein interaction (PPI) types can be defined as either obligate or non-obligate. Non-obligate can be further separated into transient and permanent interactions, with transient interactions varying greatly in strength.

Excluding outside effects from competitive or non-competitive binding, conformational changes and environmental changes, stronger interactions result in longer interaction lifetimes. Stronger interactions are often involved in structural roles whereas weaker interactions are often involved in signalling. Strongly interacting proteins generally have correlated expression levels and colocalise, while this is less commonly the case for weaker interactors.

Interactions can be affected by: protein modifications (such as phosphorylation (Nishi, Hashimoto, & Panchenko, 2011) and glycosylation (H. S. Lee et al., 2015)); conformational changes (Goh, Milburn, & Gerstein, 2004), protein localisation (Behrens et al., 2016); binding site competition (McElnay & D'Arcy, 1983); and external effects such as temperature, pH and ion concentrations (Sahin, Grillo, Perkins, & Roberts, 2010; H. Wu et al., 2015).

The role of an interaction can be predicted if interaction sites between the proteins are known. Interactions between specific sets of domains are common. Taking examples from dystrophin, domains such as WW, PDZ, SH2 (Src homology domain 2) and SH3 are all typically involved in signalling and regulatory functions. The WW domain of dystrophin binds beta-dystroglycan. Dystrophin is in competition for the interaction site on beta-dystrobrevin with other proteins such as cavoelin-3 (Sotgia et al., 2000). While dystrophin does not contain a PDZ domain, the DAPC has been called a PDZ scaffold (Kachinsky, Froehner, & Milgram, 1999) due to its presence in the multiple syntrophin proteins that bind to dystrophin and dystrobrevin. The PDZ

domain on syntrophins can bind to the PDZ domains of signalling proteins such as nNOS (Brenman et al., 1996). Dystrophin is also able to bind the PDZ domain of nNOS at its spectrin repeats R16 and R17. SH2 and SH3 domains are not present in dystrophin, however they are present in other proteins of the DAPC such as beta-dystroglycan. Specific characterised binding domains that are common across a number of proteins are usually involved in signalling as they can provide a binding platform for a number of different proteins.

Interaction dissociation constants range between 10^{-12} and 10^{-1} M (Harding & Rowe, 2010), with transient interactions typically in the µM range and more permanent interactions in the nM range (Perkins, Diboun, Dessailly, Lees, & Orengo, 2010). Dystrophin's WW and EF hand domains and beta-dystroglycan were determined to bind with a dissociation constant of approximately 40 µM (Huang et al., 2000) while dystrophin's CH1 and CH2 domains bind to F-actin with a dissociation constant of 47 µM (Singh et al., 2015). nNOS's PDZ domain was found to interact with the spectrin repeats R16 and R17 of dystrophin with a dissociation constant of 50 µM (Molza et al., 2015). Alpha-syntrophin's PDZ domain interaction with skeletal muscle sodium channels was measured at 112 nM (Gee, Sekely, et al., 1998). Interaction strengths are determined for free-floating *in vitro* systems and so their physiological relevance should be supported by cell or *in vivo* experiments when possible. As the DAPC complex is the result of many transient interactions, the complex can also be classified as transient however most of the DAPC components are able to be identified in immunoprecipitations and pull-downs.

Aside from direct and physical interactions, proteins can be implicated with each other through other functional associations such as co-expression, co-localisation, genetic interactions or shared pathway involvement.

DNA binding

Protein interactions with DNA are common within the nucleus, but are also present in the cytoplasm. These interactions commonly regulate gene expression in the nucleus. Their roles in the cytoplasm are not well understood however they have been implicated in localisation (Ng, Weissbach, Ronson, & Scadden, 2013). Dystrophin has not yet been reported to have any DNA binding partners.

2G2. Experimental approaches

The conventional and most reliable way of establishing protein-protein interactions is experimentally. Interactions can be separated into direct interactors, physical interactors, co-expression and co-localisation using different experimental approaches. It is not possible to determine physical interactions through an exclusively *in vivo* method and so most interactions are directly confirmed *in vitro* and then supported by less direct *in vivo* data. Methods which involve cell lysis bring proteins in close proximity that would not localise together within normal physiological conditions and thus introduce the possibility of false positives.

Strong interactions

To determine strong physical interactors, immunoprecipitations and pull-down assays are most commonly used. Where necessary, experimental conditions are optimised by adjustment of salt concentrations and buffer strength. The full range of binding partners of a membrane protein may fail to be identified in approaches requiring cell lysis as the harsh buffers required to free the protein from the membrane can disrupt protein-protein interactions.

Immunoprecipitations bind a target protein's physical interactors without the need for tagging (Kaboord & Perr, 2008). The interactors can then be determined by western blot or mass spectrometry; however non-specific binding can result in false positives. Antibodies are required to bind the target protein and will not pull down the target protein and related complex if the recognition site on the protein is blocked by an interactor. Protein tagging is used in the pull-down assay and yeast two-hybrid methods and can influence protein localisation, binding and stability (Bell, Engleka, Malik, & Strickler, 2013; Brückner, Polge, Lentze, Auerbach, & Schlattner, 2009; Kitao & Takata, 2006). Both require overexpression of the protein in an alternative species such as yeast or bacteria which may result in the absence of relevant post translation modification. The two techniques are advantageous in identifying direct interactions and specific binding sites through expression of modified proteins or relevant peptide sequences. The far-western blotting approach is less useful as it requires protein denaturation and so is only able to recognise proteins whose interaction is not reliant on protein folding (Y. Wu, Li, & Chen, 2007). Förster Resonance Energy Transfer (FRET) is an *in vivo* method to detect the proximity of two proteins to determine direct binding however it requires tagging of the proteins of interest (Pietraszewska-Bogiel & Gadella, 2011; Roy, Hohng, & Ha, 2008).

Proximity ligation assays offer a similar *in vitro* method of determining direct binding without tagging (Greenwood et al., 2015; Weibrecht et al., 2010).

QUICK/SILAC method

Methods employing mass spectrometry to systematically examine interactors have become more common place. The quantitative immunoprecipitation combined with knockdown method (QUICK) method developed by Selbach and Mann utilises co-immunoprecipitation combined with mass spectrometry, stable isotope labelling with amino acids in cell culture (SILAC) and protein knockdown (Selbach & Mann, 2006). The method allows for the identification of all physically interacting proteins while eliminating non-specific binding proteins.

Weak interactions

Techniques have been developed to aid in the identification of weakly interacting partners (Frato & Schleif, 2009; Morell, Espargaró, Avilés, & Ventura, 2007; Yao-Cheng Li et al., 2014), although no such methods have yet been carried out on dystrophin and its interaction partners. The methods involve tagging of the two proteins of interest to pairs of interacting peptide or DNA sequences. As a result, these methods are not systematic and require the weakly interacting proteins to be previously identified as possible interactors. They also suffer from the previously stated problems with tagging.

Prediction of interactions

Pairs of interactors can be identified through computational predictions based on sequence and structural comparison, which can be advantageous as this provides a relatively low-effort method of investigating thousands of possible interactions. Specific databases exist that list these predicted interactions. However, care should be taken as they may not be supported by *in vivo* or *in vitro* data.

Some approaches aim to identify interactions based upon their sequence and structural similarity to other known interactions. Sequences can be identified as interacting based upon primary, secondary, or tertiary similarities. Interactions are commonly inferred between species where the equivalent proteins exist and perform similar functions in both such as an interaction with dystrophin and beta-dystroglycan in human also presumed to occur in pig despite no direct evidence of the interaction. Interactions may also be inferred across tissues

expressing pairs of proteins that localise together and form similar functions as the original tissue or cell type. Binding to protein isoforms may also be inferred in this way if the binding region is known and found to be conserved in the alternate isoform. Recognisable binding domains are common across different proteins and can often interact with a known set of other binding domains and sequences (Deng, Mehta, Sun, & Chen, 2002). Protein pairs with similar structures to binding pairs are also suggested to interact. Protein structures must first be determined from amino acid sequences. This can be done computationally using methods such as the multimeric threading approach (L. Lu, Arakaki, Lu, & Skolnick, 2003) or with the aid of experimentally determined structural homologs (Q. C. Zhang et al., 2012). Other methods include chromosome proximity, *in silico* two-hybrid, phylogenetics and gene expression (Rao et al., 2014).

2H. Interaction databases

There are many databases that list known protein-protein interactions (B. Aranda et al., 2011; Lehne et al., 2009; Pedamallu & Ozdamar, 2014). Databases will list different interactions for dystrophin depending on the criteria used for curation of the databases. The way in which the database is created determines the reliability of the interactions listed. While manually and highly curated databases will provide a high confidence list of interacting proteins, databases that include predicted binding associations or incorporate large-scale unconfirmed association studies return a less reliable data set but do not require as much labour to create and update and may return true-positives along with the increased proportion of false-positives. Databases must be regularly updated to remain relevant to current research demands.

As most research is focused upon humans, mice, and yeast, most interactions are recorded in these species however significant research has been carried out in other species and these interactions are not always verified in human or mice and are worth considering. Multiple genome-wide studies identifying protein-protein interactions have been carried out in Drosophila melanogaster (fruit fly) and could potentially be relevant to dystrophin interactions in human (Costello et al., 2009; Formstecher et al., 2005; Giot et al., 2003; Jennings, 2011).

Databases split the interactions into subcategories such as direct interactions, physical interactions and co-localised associations, and there are variations in the types stored between databases. Additionally, some databases choose to include experimental and predicted interactions while others are exclusive to experimental or predicted.

Visualisation of the data is often helpful for understanding protein interactions and networks. Some databases offer their own visualisation tools (Warde-Farley et al., 2010) while others rely on third party software (Kerrien et al., 2012; Smoot, Ono, Ruscheinski, Wang, & Ideker, 2011). Databases and visualisation software will often allow searches to return specific interaction types however this is not always the case. The different visualisation tools will contain different features and data manipulation with software such as Cytoscape enabling data editing and additional data analysis.

It is possible to search and combine interactions from various databases using visualisation tools such as Cytoscape (Shannon et al., 2003; Smoot et al., 2011). Universal standards are now widely used for reporting protein-protein interactions across a large number of databases

(Orchard et al., 2012) however compiling data from databases that do not use the same method of reporting interactions remains an issue. Problems arise though differences in terminology, including the recording of protein identifiers, species, interaction type, experimental method and reference to the published research. For example, species may be recorded as Human or use the taxonomy identifier 9606 and a yeast two-hybrid experiment method could be listed as yeast two-hybrid or Y2H or the information could be absent.

In addition, common protein identifiers include gene name and Uniprot accession and are often used for database query terms however databases differ in which of these are used as the primary key in results tables, requiring further work to combine outputs. Most differences are readily understood when manually checking small datasets however issues arise when querying large datasets or relying on computational approaches to search and examine data. Uniprot accession numbers are unique to a given protein and species and so database searches across all species using this identifier can require numerous search terms (dystrophin returns 11 unique reviewed Uniprot accessions (as of April 2016) across a range of species, with more than 100 additional unreviewed accessions). Many accessions of a protein will not include any interaction data – for example, many of dystrophins unreviewed accessions represent small dystrophin fragments generated from a single published experiment however some accessions do have attributed interactions (such as E9PDN5 in the case of dystrophin). Dystrophin is represented by the gene name 'DMD' in humans and 'Dmd' in the majority of other species with a notable exception being 'dys' in the fruit fly. Using the same identifier across species is prevalent throughout the proteome and is advantageous for quickly generating networks incorporating multiple species. Unfortunately, gene names often represent multiple proteins as is the case with 'dmd' representing dimethylamine dehydrogenase in Hyphomicrobium and antitoxin Dmd in Enterobacteria phage T4 among others.

Further problems arise from proteins encoded by the same gene which are often indistinguishable through gene name or Uniprot accession. The DAG1 gene encodes an amino acid chain that is subsequently cleaved into alpha-dystroglycan and beta-dystroglycan with both proteins sharing the same gene name and Uniprot accession, DAG1 and Q14118 (in human) respectively.

Similarly, protein isoforms will share gene names and accessions although they can be distinguished when isoform specific Uniprot accessions are used (human dystrophin P11532 can be subdivided into P11532-1 to P11532-10, P11532-1 representing the full length protein

and P11532-7 representing Dp71). Some interactions state these isoform-specific accessions however others will not, resulting in the isoform information being lost from a network.

A standardised format, PSI-MI format (Proteomics Standard Initiative Molecular Interaction format) for databases to list information was created by the Human Proteome Organization Proteomics Standards Initiative (HUPO-PSI) and has since been adopted by many databases including the IMEx consortium (Kerrien et al., 2007; Orchard et al., 2012). The format includes an information-comprehensive XML format and a simplified MITAB format with specifically created PSI-MI identifiers for each piece of information. PSICQUIC (Proteomics Standard Initiative Common QUery InterfaCe) has created an interface to search across standardised curated databases currently incorporating 33 databases (B. Aranda et al., 2011). Although many large databases are not included, searching across the included databases is significantly simplified through use of the standardised format.

An issue which has not be addressed by databases and the PSI-MI format is the tissue specificity of interactions. Cell or tissue type used in experiments confirming an interaction are not commonly included presenting another challenge when generating tissue specific interaction networks. As lack of evidence does not equate to evidence of absence, tissue specificity of interactions will remain a problematic area.

Curated databases suffer from limitations in accuracy as they try to incorporate all interaction across an entire species or across all species and so may lack the comprehensive and reliable nature of a manually created network on a single or small subset of proteins. Computer generated interaction databases, however well-developed their algorithms, are always likely to contain falsely identified interactions or omit true interactions – this is exacerbated by the tendency for evolution to create nuanced variation in protein binding and function introduced by minor changes to amino acid sequence.

Due to the high cross-reactivity of the entire interactome, interaction sub-networks that are grown to include secondary, tertiary and more distant protein interactors rapidly lose specificity and meaning, as they come to incorporate a large subset of the total interactome. Competitive and non-competitive binding and interactor variations based upon cell type or protein localisation lead to the creation of misleading networks which imply proteins in complexes which do not interact *in vivo* and in reality form distinct complexes that happen to have a common protein present in each complex.

Interactions are given a weighting score based upon the confidence of the suggested interaction. Scoring can be used to distinguish the strength of the evidence for an interaction and eliminate false positives. Scores can be based upon aspects such as experiment size (with small scale experiments deemed more reliable), interaction type (direct interaction, physical interaction etc.), experimental methods (immunoprecipitation, pull-down assay, predicted etc.), the number of papers supporting this interaction, if specific binding sites are known, if the interaction is inferred from a different species or if proteins share common pathways or interacting proteins. There are numerous weighting algorithms including MISCORE (D. Wang & Tapan, 2012), MINT-score (Licata et al., 2012) and IntScore (Kamburov, Stelzl, & Herwig, 2012), with varying weight placed upon different scoring aspects, making it difficult to compare scores from different scoring methods and with no general agreed standard. PSISCORE (PSI confidence SCORing system) developed alongside the PSI-MI format (B. Aranda et al., 2011) allows users to place their own weightings and returns a score consistent across their own dataset or network. The weighting scores can be subsequently used to predict protein complexes (Zaki et al., 2013).

Interactions scores can be used to selectively keep only those interactions supported by relatively large numbers of observations, however these scores do not necessarily represent biological relevance, and clearly suffer an observation bias in favour of the most studied proteins.

As dystrophin has been the focus of a great deal of research interest, many websites exist dedicated to information on the protein and/or its related diseases. These sites can contain substantial information that can be used in combination with interaction databases to increase the understanding of the protein. These websites have the advantage of being focused solely on dystrophin and so can often provide a higher level of confidence for the data provided, due to deep and careful manual curation of the data. They also tend to put the information into context which increases the value of the data. eDystrophin contains a list of proteins that have had their interactions with dystrophin mapped to specific binding regions (Nicolas et al., 2012).

Objectives

This project was split in two parts;

1) The first part of this project aims to validate cell lines that were immortalised by the incorporation of hTERT and Cdk4 transgenes (Mamchaoui et al., 2011). These cell lines have previously been investigated for their myogenicity (such as MyoD, NCAM and desmin), myotubes morphology (sarcomeric myosin staining) and *in vivo* myofibre formation capacity (Mamchaoui et al., 2011; C.-H. Zhu et al., 2007) as well as and unaltered membrane repair processes (Philippi et al., 2012) and Ca2+ release (Rokach et al., 2013) in select lines , however a transcriptomic wide analysis has not yet been carried out. Our analysis will enable us to investigate a wide range of cellular processes including processes not directly implicated with hTERT or Cdk4 related pathways. Pathways of particular interest which have yet to be investigated are integrin regulation, the PI3K/Akt pathway and microtubule stability, each of which has previously been linked with the cell cycle.

   A transcriptomic analysis will be carried out to compare myogenic primary cells and immortalised cell lines derived from healthy individuals and DMD patients in their undifferentiated and differentiated states. An additional comparison against non-myogenic cell populations will also be carried out. In order for the immortalisation process to be considered successful cell lines must show strong correlation with their primary population in both the undifferentiated and differentiated states and pathway genes must show to maintain similar levels.

   Possessing cell lines which are protected against cellular senescence while still being representative of their primary population is of great value for research and the cell lines developed using this immortalisation process have already been used to investigate various therapeutic approaches (Echigoya, Mouly, Garcia, Yokota, & Duddy, 2015; Gonzalez-Hilarion et al., 2012; Ousterout et al., 2013; Popplewell et al., 2013).

2) The second part of this project aims to identify new interaction partners of dystrophin and incorporate these newly identified interactors into a comprehensive network of dystrophin's interactors. Dystrophin plays an important role in muscle cells and the

absence of functional dystrophin results in Duchenne muscular dystrophy. Through a more complete knowledge of dystrophin and its interactors, we can better understand processes behind the detrimental effects observed during the absence of functional dystrophin which will subsequently aid in the development treatments for DMD and related diseases. Although dystrophin has been the subject of extensive study this will be the first experiment to systematically investigating its binding partners.

We will use a quantitative immunoprecipitation combined with knockdown (QUICK) approach previous developed by Selbach and Mann (Selbach & Mann, 2006) in order to unbiasedly identify new physical interactors of dystrophin while eliminating false positives that common immunoprecipitation techniques are susceptible to. In addition to the new interactors that we hope to identify, we also expect to identify previously known dystrophin interactors.

A network based upon the previously understood dystrophin interactors (from interaction databases and published literature) within the context of differentiated skeletal muscle will be curated and will subsequently incorporate the newly identified dystrophin interactors. We aim to build a network which contains all of dystrophins relevant interactions yet remains accessible and easily understandable. The network will be a tool for aiding the understanding of dystrophin interactions, the links to other cellular compartments and processes, interpreting interaction related data and identifying possible new targets for dystrophic diseases.

Chapter 3 – hTERT/cdk4 immortalization protects the characteristics of human myogenic cells

Abstract

hTERT/cdk4 immortalized myogenic human cell lines represent an important tool for skeletal muscle research, being used as therapeutically-pertinent models of various neuromuscular disorders and in numerous fundamental studies of muscle cell function. However, the cell cycle is linked to other cellular processes such as integrin regulation, the PI3K/Akt pathway, and microtubule stability, raising the question as to whether transgenic modification of the cell cycle results in secondary effects that could undermine the validity of these cell models. Here we subjected nine healthy and disease lines to intensive transcriptomic analysis, comparing immortalized lines with their parent primary populations in both differentiated and undifferentiated states, and testing their myogenic character by comparison with non-myogenic (CD56-negative) cells. Selected lines were also tested for aneuploidy and chromosomal rearrangements. We found that immortalization had no observed effect on the myogenic cascade or on any other cellular processes, and it was protective against the systems level effects of senescence that are observed at higher division counts of primary cells.

Introduction

Research on neuromuscular disorders, including potential therapeutic options, depends on the careful observation of clinical symptoms and of biopsy material from human subjects, and also on the availability of disease models that both accurately reflect aspects of the pathology and facilitate experimental intervention. Animal models allow the experimental manipulation of fully vascularized, innervated muscle tissue, and they often recapitulate to a large extent the complexity of interactions between human cell and tissue types, and how those interactions change in disease and development. In contrast, the relative homogeneity of isolated and purified cell lines has a double-edged significance: it renders them pertinent only to certain aspects of certain pathologies, but it also facilitates the close study of specific molecular mechanistic events. In addition, where they are understood to closely recapitulate some measurable aspect of the pathology, cell models can be highly amenable to high-throughput studies.

From a systems biology perspective, compared with whole organisms, cell lines more closely (however imperfectly) represent a single enclosed apparatus in which changes to one or more component(s) have direct mechanistic impact on connected components. This is particularly true of pathologic muscle, in which processes such as regeneration, inflammation, fibrosis and adipogenesis all conspire to a general loss of order and increase in tissue heterogeneity. These changes in whole muscle composition can be observed in transcriptomes and other omics profiles, and may obscure underlying mechanistic details. However, isolated primary myoblasts suffer the disadvantage that they undergo senescence with amplification in tissue culture (Bigot et al., 2008). Immortalization avoids senescence and thereby facilitates subsequent cloning to select a highly pure model cell line (C.-H. Zhu et al., 2007).

Adult human primary myoblasts senesce after approximately 25 rounds of division in tissue culture due to cell cycle suppression by the $p16^{Ink4a}$-dependent stress pathway and progressive telomere shortening which triggers cell cycle exit mediated by activation of p53 (Renault, Thornell, Butler-Browne, & Mouly, 2002; Renault, Thornell, Eriksson, et al., 2002; Wright & Shay, 2002). We showed that immortalization of human myoblasts requires bypassing of both of these senescence mechanisms, and we achieved this by transduction of the murine cyclin-dependent kinase (cdk)-4, which overcomes the p16 pathway, and of human telomerase reverse transcriptase (hTERT) which preserves telomere length (C.-H. Zhu et al., 2007).

Using this method, we have created a large collection of immortalized human myoblasts isolated from a wide range of neuromuscular disorders. Several have been validated as experimental models for Duchenne muscular dystrophy (DMD) (Echigoya, Mouly, et al., 2015; Gonzalez-Hilarion et al., 2012; Ousterout et al., 2013; Popplewell et al., 2013), limb girdle muscular dystrophy type 2B (LGMD-2B) (Philippi et al., 2012), facioscapulohumeral muscular dystrophy (FSHD) - including mosaic-origin control lines from the same patient (Ansseau et al., 2009; Krom et al., 2012; Mariot et al., 2015), and excitation-contraction coupling and calcium homeostasis (Rokach et al., 2013). These cell lines have contributed to the development of therapeutic approaches such as oligonucleotide-mediated exon skipping (Echigoya, Mouly, et al., 2015), read-through of nonsense mutations (Gonzalez-Hilarion et al., 2012), and gene correction (Ousterout et al., 2013; Popplewell et al., 2013) for DMD, to the study of RyR1 deficiency in Congenital Myopathies (H. Zhou et al., 2013), cell senescence in myotonic dystrophy type I (Bigot et al., 2009), the involvement of IL-6 and Akt in the pathogenesis of myasthenia gravis (Maurer et al., 2015), the dysregulation of DUX4c (Ansseau et al., 2009) and the role of FAT1 (Mariot et al., 2015) in FSHD, and the shut-down of quiescence pathways in ageing (Bigot et al., 2015). They have also been used to explore fundamental aspects of muscle cell physiology including: the role of β-arrestins in myogenesis (Santos-Zas et al., 2015), the role of MMP-14 in human myoblast collagen invasion (Lund, Mouly, & Cornelison, 2014), nuclear protein spreading between nearby myonuclei (Ferreboeuf et al., 2014), the effects of oxidative stress on myoblast calcium-dependent proteolysis (Dargelos et al., 2010) and the proteome (Baraibar et al., 2011), engineering of 3D micro-muscles (Kalman et al., 2015), and the function of miRNAs during myoblast differentiation (Polesskaya et al., 2013), this list being non exhaustive. Thus they have become an important resource to the muscle research community.

To validate the use of immortalized myoblasts we previously confirmed the expression of myogenic markers MyoD, NCAM (CD56), desmin, and various myosin isoforms, in differentiated myotubes, the morphology of myotubes by immunostaining of sarcomeric myosin, and their capacity to contribute to myofibre formation *in vivo* (Mamchaoui et al., 2011; C.-H. Zhu et al., 2007). A separate validation was carried out on two lines of healthy and 4 of dysferlin-deficient (LGMD-2B) immortalized clones: relative to their parent primary populations, they showed unaltered expression of myogenic markers MHC, alpha-tubulin, desmin, and caveolin-3, unaltered sarcomere formation and subcellular localization on immunofluorescence imaging of these same markers, and unaltered membrane repair processes

(Philippi et al., 2012). In a third study, the physical properties of Ca2+ release and its response to pharmacological intervention were unaltered in immortalized lines compared with primaries (Rokach et al., 2013).

However, the cell cycle is a major pathway that is linked to other cellular processes, including integrins (Moreno-Layseca & Streuli, 2014), the PI3K/Akt pathway (Chang et al., 2003), apoptosis (Kirshenbaum, 2001), and microtubule stability (Shohat-Tal & Eshel, 2011), each of which may be important to muscle cell function and pathology. Perturbations to the cell cycle may impact on these and on other less directly associated processes. Furthermore, it is increasingly understood that cyclin-dependent kinases are implicated in roles beyond the cell cycle, including aspects of transcription, metabolism, and stem cell self-renewal (reviewed (Lim & Kaldis, 2013)). Despite the wide use of our cdk4/hTERT immortalized human myoblasts, the potential secondary effects of immortalization have been tested only for those parameters listed above, relating to myogenesis and the functioning of membrane repair and calcium homeostasis. Secondary effects of cdk4/hTERT transduction on other processes that have not yet been tested would challenge the usefulness of these immortalized lines as experimental models and could undermine previous and ongoing studies that use these cell lines.

A second risk to the representativeness of these cell models occurs when we select a clonal line from the immortalized population: despite that the purity of our primary cell isolates is monitored by immunostaining against the myogenic marker desmin, the immortalized population may still exhibit cell-to-cell variability, so that any given clone may represent only part of its parent primary population. A third risk is that, relative to primary populations, immortalized clonal lines that undergo long-term experimental use are repetitively amplified and maintained for prolonged periods in tissue culture conditions, whereas time in tissue culture has been associated to loss of myogenic potential (Collins et al., 2005; Collins, Zammit, Ruiz, Morgan, & Partridge, 2007; Montarras, 2005). Thus it is important to determine whether immortalized clonal myogenic lines diverge from their mortal parent primary populations.
Here we use extensive transcriptomic profiling of cdk4/hTERT immortalized human myoblasts in their undifferentiated and differentiated states, comparing them with their mortal parent populations, and with a non-myogenic **(**lacking the CD56 protein that is present on the cell surface of myogenic cells (Cashman, Covault, Wollman, & Sanes, 1987)) reference population. In this way we obtain a systems level view of cellular processes, allowing us to determine whether secondary effects are incurred by cdk4/hTERT immortalization.

Materials and Methods

Ethics approval

Human-derived cell lines were obtained as described previously (Bigot et al., 2009; Mamchaoui et al., 2011): muscle biopsies were obtained from the MYOBANK BTR (Bank of Tissues for Research, a partner in the EU network EuroBioBank) in accordance with European recommendations and French legislation in accordance with European recommendations and French legislation. Cell lines of DMD subjects were obtained in collaboration with the team of F Muntoni, MRC CNMD Biobank (NHS Research Ethics Committee reference 06/Q0406/33; and HTA license number 12198), in the context of Myobank, affiliated to EuroBioBank (European certification).

Cell lines and media

Myogenic primaries and clonal lines of two healthy (CHQ and C25 and three DMD (DMD 6311, DMD 6594 and DMD 8036) subjects and CD56-negative cell lines (AB424, AB431, AB439, AB440, AB423, AB425, AB429 and AB451) of 8 healthy subjects were cultured in proliferation medium (1 volume of M199, 4 volumes of Dulbecco's modified Eagle's medium (DMEM), 20% foetal bovine serum (Invitrogen), 50 µg/mL gentamicin (Invitrogen), 25 µg/mL fetuin, 0.5 ng/mL bFGF, 5 ng/mL EGF, 0.2 µg/mL dexamethasone, 5 µg/mL insulin) on Matrigel covered petri dishes (0.1 mg/mL, 1 mL per 19.5 cm^2 dish area, 45 min, 37 °C), incubated at 37 °C, 5 % CO_2. Cells were differentiated in 1 volume of M199, 4 volumes of DMEM + 2 % foetal bovine serum + 1 µL/mL gentamicin.

Cell sorting/immunolabelling

Stocks of primary myogenic cells were purified by magnetic activated cell sorting using anti-CD56 (a specific marker of myoblasts (Cashman et al., 1987)) beads (MACS, Miltenyl Biotech). Purity before and after cell sorting was determined by immunolabelling (anti-desmin (1/100, clone D33, Dako) and anti-mouse IgG1 AlexaFluor 488 (1/400, LifeTechnologies™)). 100,000 cells were plated on a µ-dish 35 mm high ibidiTreat (ibidi®) in proliferating medium. After 48 hours (approx. 70 % confluence), the cells were washed 3 times with PBS and were cultured in DMEM to induce myogenic differentiation. At day 5 of differentiation cells were fixed and permeabilised with 200 µL 95% ethanol at 4 °C overnight, and blocked with 20 % horse serum + *0.1 % Triton-X 100* in PBS, 500 µL, 1 h, RT. Primary antibody anti-desmin (D33, IgG1, 1 :100, Dako) and secondary antibody goat anti-mouse IgG1 AlexaFluor 488 (1:400, Invitrogen™) were used. Culture dishes were washed 3 times with PBS, counter-stained with 1 µg/ml DAPI for 1 min, RT, washed 3 times with PBS and mounted with ibidi

mounting medium (ibidi®). 5 non-overlapping images were acquired with an Olympus IX70 and an Olympus UPlan FI 10x/0.30 Ph1 objective equipped with a Photomatics CoolSNAP™ HQ camera. Images were acquired using Metavue 7.5.6.0 software. The percentage of desmin positive cells (desmin to DAPI cells) was calculated. Sorted cells were found to be of equivalent of higher purity in each case and subsequently used in cell cultures.

RNA isolation

Cells were plated in 4 sets of 400,000 in 78.5 cm^2 Petri dishes (Falcon) for each cell. Cells were switched to differentiation medium after 2 days of proliferation (approx. 70 % confluence). After 9 days of differentiation, cells were scraped (100 µL PBS, on ice) and collected. Cells were centrifuged at 12,000 g for 10 minutes, and PBS supernatant removed. Each pellet was dissolved in TRIzol (1 mL). The RNA was subsequently isolated (PureLink RNA Mini Kit, 'Using TRIzol® Reagent with the PureLink® RNA Mini Kit' section of the standard protocol) with the RNA eluted in 30 µL H2O.

RNA quality control

RNA quality and purity was determined using the Nanodrop 2000 (Thermo Scientific, nucleic acid setting) followed by Quality Control analysis (Agilent 2100 Bioanalyzer, Eukaryote Total RNA Nano assay, Agilent RNA 6000 Nano Kit, Agilent RNA Nano LabChip) to determine the RNA integrity number (RIN, 28S/18S ratio).

Gene expression profiling

An aliquot of 150 ng of high-quality total RNA from each sample was used for mRNA expression profiling. Samples were analysed using Illumina® Gene Expression BeadChip Array technology (Illumina, Inc., San Diego, CA). Reverse Transcription for synthesis of the cDNA strand, followed by a single in vitro transcription (IVT) amplification, that incorporates biotin-labelled nucleotides, were performed with Illumina® TotalPrep™ -96 RNA Amplification Kit (Ambion, Austin, TX). 750ng of the biotin-labelled IVT product (cRNA) was hybridized to HumanHT-12v4_BeadChip (Illumina, Inc., San Diego, CA) for 16 hours, followed by washing, blocking, and streptavidin-Cy3 staining according to the WholeGenome Gene Expression Direct Hybridization protocol (Illumina, Inc., San Diego, CA). The arrays were scanned using HiScanSQ System and obtained decoded images were analysed by GenomeStudio™ Gene Expression Module – an integrated platform for the data visualization and analysis (Illumina, Inc., San Diego, CA).

Transcriptomic raw data treatment

Raw intensity values (Illumina idat files) of 94 samples were passed to the R lumidat library (https://github.com/drmjc/lumidat) to create an EListRaw class object compatible with further

downstream analyses in R/Bioconductor. Two samples were excluded at this stage due to having zero proportion of expressed probes (all other samples had more than 40% of probes expressed). The limma library (Ritchie et al., 2015) was then used to carry out neqc normalization – this involves background correction followed by quantile normalization, using negative control probes for background correction and both negative and positive controls for normalization (Wei Shi, Oshlack, & Smyth, 2010). Probes were excluded if: (1) they had low bead numbers in at least one sample; (2) they were not expressed (expression p-value < 0.01) in at least 16 arrays; (3) they were annotated as 'no match' or 'bad' quality according to the Bioconductor illuminaHumanv4.db library. This gave a total of 15,342 probes for further analysis. The Bioconductor library arrayQualityMetrics was used for quality assessment of the arrays (Kauffmann, Gentleman, & Huber, 2009). PCA was applied to the normalized values and visualized using R's prcomp and plot functions, and the rgl library for 3D visualization. The R 'pheatmap' library was used to produce heatmaps of selected genes. Linear regression for differential expression analysis was applied using Limma (Ritchie et al., 2015). Samples were grouped according to "cell_type" (grouping samples into myoblasts, myotubes, CD56-neg, and the C25 outlier), and this parameter was used to create a design matrix (~0 + cell_type), to which Limma fitted a linear model for the normalized expression values. Limma's 'arrayWeights' function was used to weight each sample according to its estimated reliability by measuring how well the expression values for that array followed the linear model (Ritchie et al., 2006), Limma's 'duplicateCorrelation' function was used to estimate the correlation between technical replicates (samples from the same subject, cell-type, and clonal state, were considered as technical replicates) (Smyth, Michaud, & Scott, 2005), and Limma's 'eBayes' function was used to rank genes in order of evidence for differential expression, based on an empirical Bayes method (Smyth, 2004).

The data are deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE79263.

Functional analyses of transcriptomic data

Rank-based gene set enrichment tests were carried out using GSEA (Subramanian et al., 2005) on the 15,342 quality-filtered, normalized, non-$\log_2$, gene expression values described above, applying default settings (e.g. permutations on phenotype, collapse genes to max of probesets) except that minimum overlap with gene sets was changed from 15 to 8 to allow for the small sizes of some muscle gene sets. Gene Sets were taken from the Muscle Gene Sets homepage (Muscle Gene Sets v2) 'http://sys-myo.rhcloud.com/muscle_gene_sets.php' or from the Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb) (Subramanian

et al., 2005). Gene set enrichment mapping was generated using Cytoscape (Smoot et al., 2011) and Enrichment Map (Merico, Isserlin, Stueker, Emili, & Bader, 2010). Enrichment tests were carried out on the most differentially expressed 800 genes by fold-change after filtering for FDR < 0.05, using Enrichr (E. Y. Chen et al., 2013). Heatmaps were created using the pheatmap R function (Raivo Kolde, 2015).

For the consensus set of genes that were differentially expressed in previously published studies of myoblast differentiation, we downloaded public available gene expression data from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) database. Four studies were chosen that included murine and human myoblasts and myotubes at 5 or more days of differentiation: GEO series GSE10424, GSE11415, GSE24811 and GSE26145. For each study, signals were converted to expression levels using robust multi-array averaging (Irizarry et al., 2003) and custom chip definition files based on Entrez genes from the BrainArray resource (Dai et al., 2005) at http://brainarray.mbni.med.umich.edu (mgu74av2mmentrezgcdf 17.1.0 for GSE10424, mouse4302mmentrezgcdf 17.1.0 for GSE11415, mogene10stmmentrezgcdf 17.1.0 for GSE24811 and huex10stv2hsrefseqcdf 17.1.0 for GSE26145). Specifically, raw CEL files were downloaded and fluorescence signals were background adjusted, normalized using quantile normalisation and $\log_2$ expression values were calculated using median polish summarization. To identify differentially expressed genes for each study, the gene expression matrixes were analysed with Statistical Analysis of Microarray method (SAM) (Tusher, Tibshirani, & Chu, 2001). For each comparison, a two-class procedure was applied and the percentage false discovery rate (FDR) was calculated. The FDR threshold was set to 0.05 and the 300 most up-regulated genes in both groups in the comparison were selected for further analyses. All data analyses were performed using R program version 3.0.1, Bioconductor 2.12 libraries and R statistical packages.

Assay of Akt pathway activity and microtubule stability

Primary and immortalized myoblasts of lines C25 and DMD8036 were differentiated for 9 days as described above.

For Akt pathway activity, proteins were extracted in RIPA buffer containing phosphatase inhibitors. 15 µg of protein lysates were separated on 4-12% bis-tris gels, then transferred onto PVDF membrane (overnight, 4°C, 15 V). After blocking the membrane for 1Hr at RT with 5%milk diluted in TBS-Tween, membrane was incubated with primary antibodies from the Phospho-Akt Pathway Antibody Sampler Kit (Cell Signalling Technology) at 1:1000 dilution: Phospho-Akt (Ser473), Akt (pan), Phospho-c- Raf (Ser259), Phospho-GSK- 3β (Ser9), Phospho-PTEN (Ser380), Phospho-PDK1 (Ser241), and Phospho-Akt (Thr308). Each of these

are phospho-specific except Akt (pan) which targets all Akt protein independent of its phosphorylation state. Secondary antibody was Anti-rabbit IgG (1:2000 dilution), HRP-linked. Bands were detected by SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher) and X-ray film. C25 and DMD8036 films received 10 and 2 minute exposures, respectively, and were scanned using a Xerox Colorqube 9303.

For microtubule stability, cells were cultured in duplicate dishes, and cDNA was synthesized from RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche), and amplified by SYBR qPCR synthesis: denaturation 95°C 8 min; amplification (50cycles) 95°C for 15 sec, 52°C for 25 sec, 72°C for 15 sec; cooling 40°C for 30 sec. Product was separated on 2% agarose gel with EtBr and detected by UV light.

Results

Immortalized clonal lines retain the system profile of their primary parent population

We generated microarray gene expression profiles for 94 samples comprising primary myoblasts and their corresponding immortalized clones in both differentiated and undifferentiated states (average of 4 cell culture replicates of each preparation) from 5 human subjects (Table 2; 2 healthy and 3 with Duchenne Muscular Dystrophy - DMD), together with primary populations of non-myogenic (CD56-ve) cells from the muscles of 8 other human subjects. Prior to analysis, each of the myogenic populations was sorted for CD56 using magnetic beads, and confirmed to be >90% desmin-positive (Figure 13). To maximize maturity and the expression levels of late myogenic markers, myotubes were maintained for 9 days in differentiation conditions, at which time no notable cell death or detachment had occurred (determined by observation under magnification).

Table 2. Summary of the cell lines used in this experiment alongside relevant mutation and donor background information

Cell line	Phenotype	Mutation	Age of subject	Sex of subject	Muscle of origin
CHQ	Healthy	-	5.5 days	Female	Quadriceps
C25	Healthy	-	25 years	Male	Semitendinosus
DMD6594	Duchenne MD	Del 48-50	20 months	Male	Quadriceps
DMD6311	Duchenne MD	Del 45-52	23 months	Male	Quadriceps
DMD8036	Duchenne MD	Del 48-50	6 years	Male	Biceps

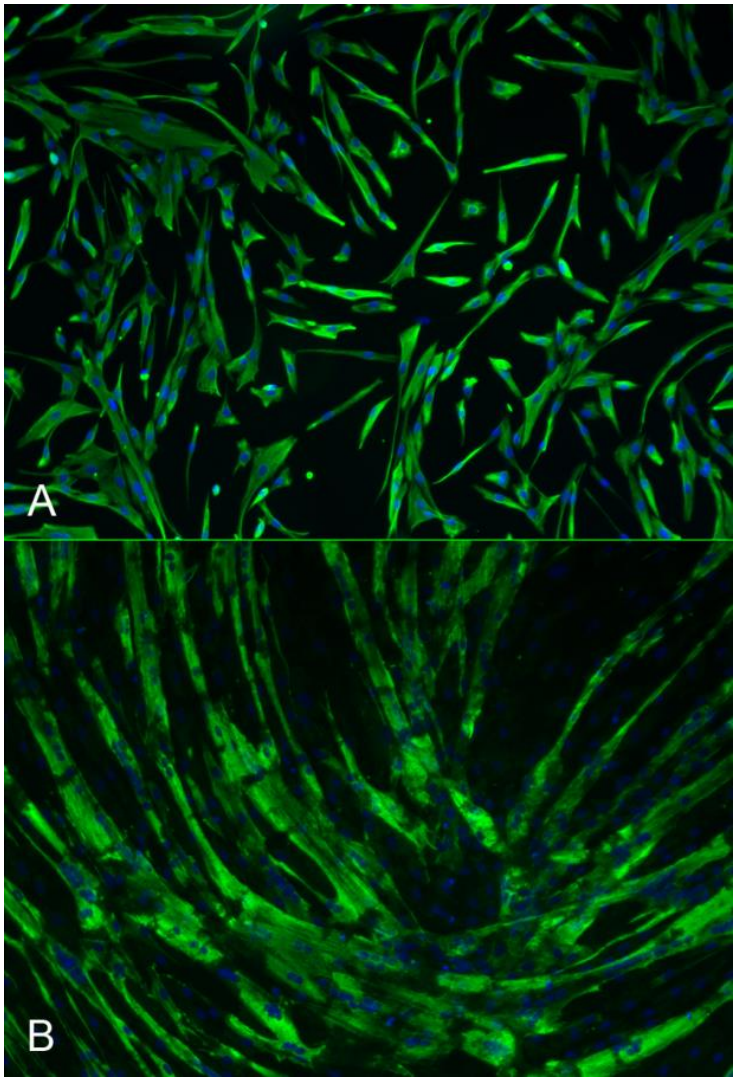


Figure 13. Representative images of primary human myotubes. Myogenic cells were purified by magnetic bead sorting of CD56 expression then differentiated for 3 days. Myogenic cells were purified by magnetic bead sorting of CD56 expression then differentiated for 3 days. Examples here are from a DMD subject (A; DMD8036) and a healthy subject (B; CHQ). Myotubes were immunostained for desmin (green) to determine the percentage of myogenic purity (94% and 96% for DMD8036 and CHQ respectively). Nuclei are DAPI-stained.

Data complexity reduction using principal component analysis (PCA) showed that samples clustered into 3 groups: myoblasts and myotubes were separated from each other across principal components 1 and 2, whereas the CD56-negative population was separated from the others by principal component 3 (Figure 14). An overall view of the three groups, with primaries and clones indicated, is presented (Figure 14A), and the same data are shown again for myoblasts (Figure 14B) and myotubes (Figure 14C) alone (for comparison, CD56-neg are included in each case). Replicate culture dishes clustered tightly. Importantly, immortalized clones clustered closely to their parent primary populations, and data-points representing clones were not shifted in any particular direction relative to their parent primary population. Among the myoblasts, the primary C25 cell line population was an outlier, clustering separately

from the other myoblast populations (including from its own immortalized clonal line) - this primary population is analyzed and discussed further below.

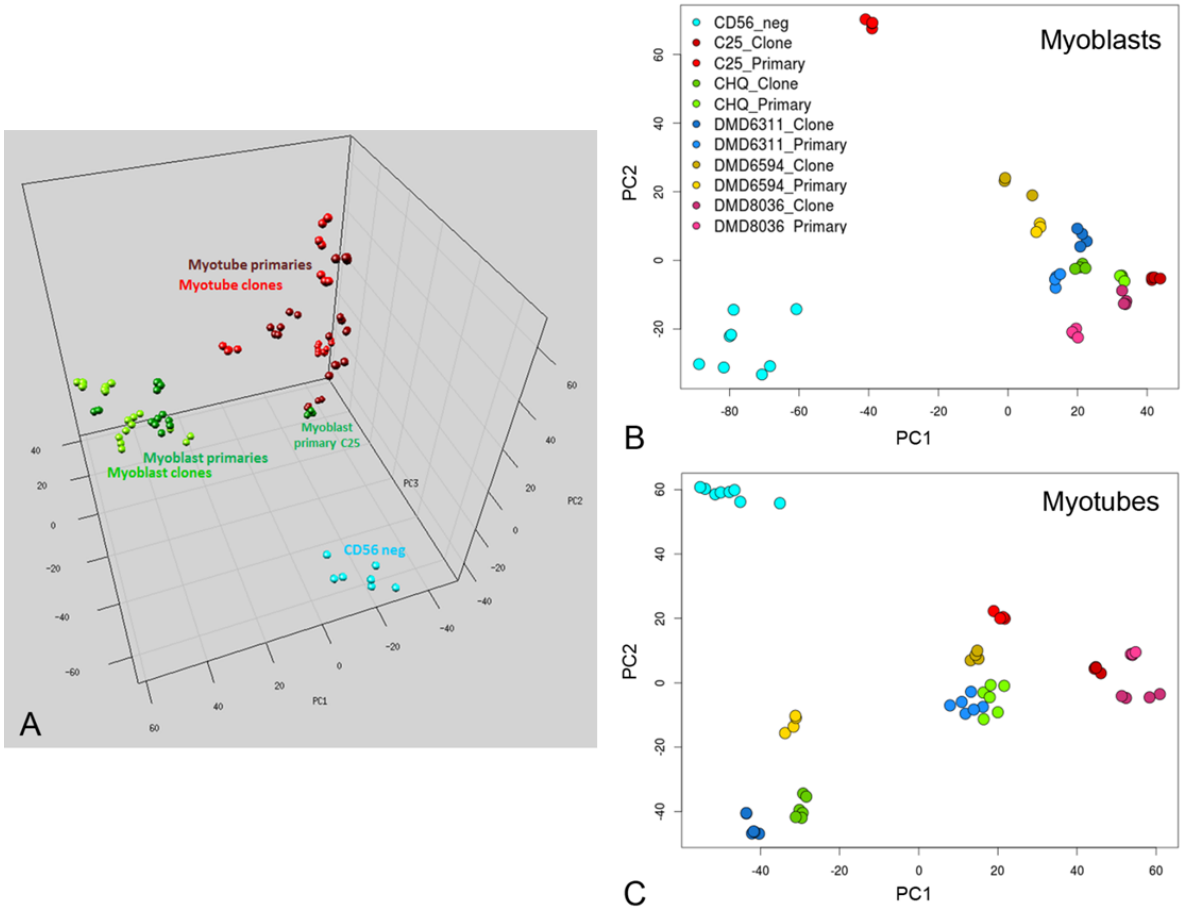


Figure 14. Principal component analysis of gene expression data from primary cells and immortalized clones of human myoblasts and differentiated myotubes, and from primary non-myogenic (CD56-negative) muscle-resident cells. (A) Immortalized myoblasts (light green) cluster together with primary myoblasts (dark green) and immortalized myotubes (light red) together with primary myotubes (dark red). Both are separated from non-myogenic cells (cyan). Data-points are projected onto principal components 1, 2, and 3. (B and C) The same data for myoblasts (B) and myotubes (C) alone, projected onto principal components 1 and 2. For myoblasts, PC1 accounted for 43% and PC2 for 17% of the total variance in the data. For myotubes, PC1 accounted for 32% and PC2 for 22% of total variance. Each population is labelled and colored separately according to the key presented in (B). Each immortalized clone clustered near to its parent primary population, and clones were not shifted in any particular direction relative to their parent primary population. Each data-point corresponds to a separate culture dish (average n=4 dishes per cell line).

Differentiation of immortalized clonal lines follows the normal myogenic cascade

To study the behavior of genes involved in the myogenic cascade in immortalized and primary cell lines we created a consensus set of genes that were differentially expressed in 4 previously published studies of myoblast differentiation (Table S1). These studies included human and mouse primary myoblasts (Balb/c strain), and C2C12 myoblasts, and we retained genes that were strongly up or down-regulated after 5 or more days of differentiation in at least 3 of the 4

datasets. For the consensus down-regulated genes, a heatmap showing their relative expression levels across our samples is shown: note the rows near the top of the figure that indicate the ‘cell_type’ and ‘clonal_state’ (Figure 15). Hierarchical clustering analysis of the samples is presented on the same plot (branch lines at top of figure) and shows that these genes neatly separated myoblasts (cyan in the cell_type row) from myotubes (lilac in the cell_type row) with no effect of immortalization (green and yellow in the clonal_state row) – myoblast clones showed similar expression values to the myoblast primary populations, and myotube clones showed similar expression values to the myotube primary populations.

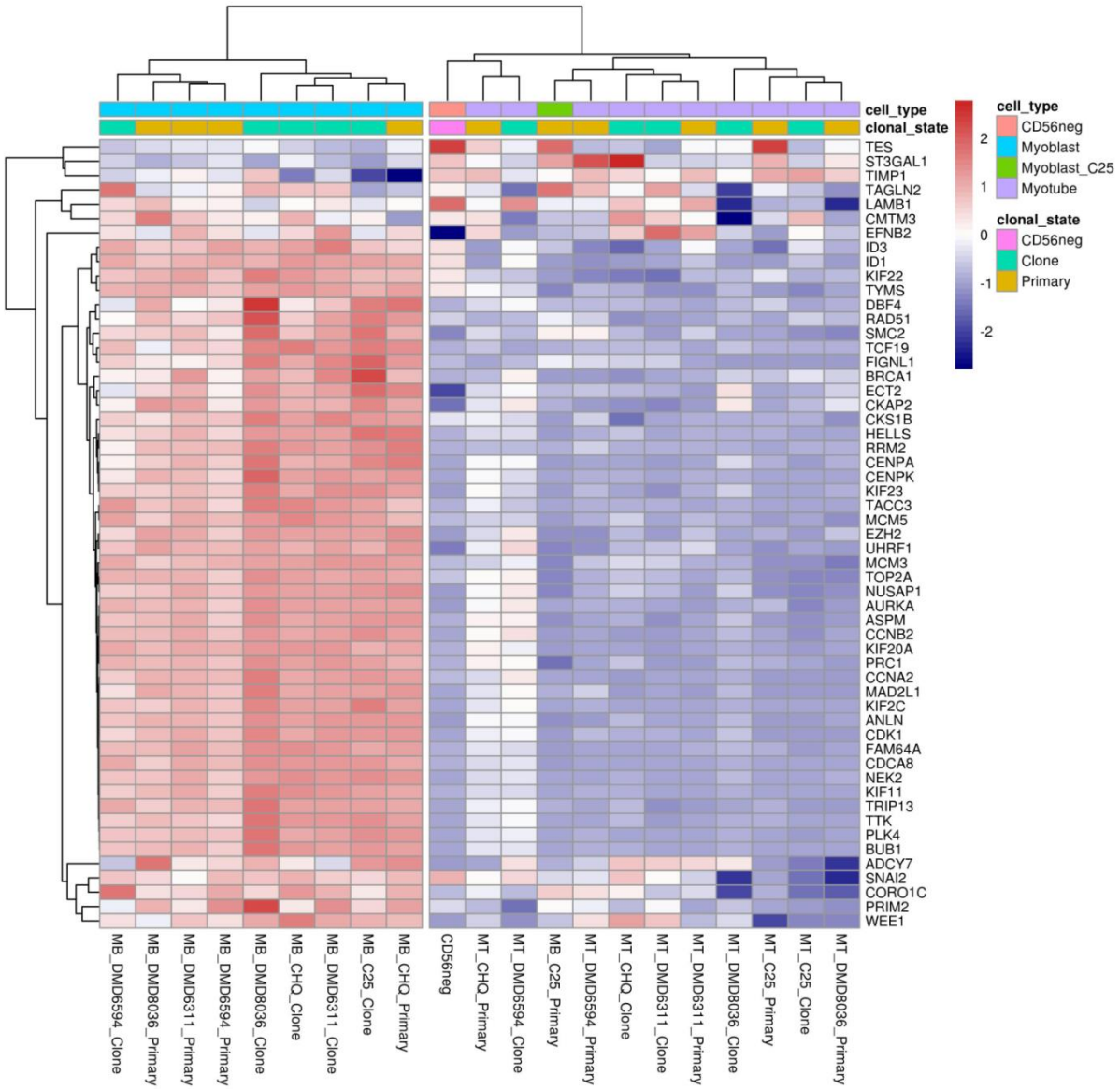


Figure 15. Heatmap showing the expression levels in immortalized and primary myogenic human lines of genes that are consistently and strongly down-regulated in a panel of previous studies of myoblast differentiation. Heatmap showing the expression levels in immortalized and primary myogenic human lines of genes that are consistently and strongly down-regulated in a panel of previous studies of myoblast differentiation. A gene is shown if it was among the 300 most strongly down-regulated after 5 or more days of differentiation in at least 3 of 4 studies of human and mouse primary myoblasts, and C2C12 myoblasts. Expression values are row scaled (i.e. changes are relative across each row for ease of interpretation). Hierarchical clustering analysis of the cell lines, indicated by branches at top, places myotube lines (indicated in lilac in the cell_type bar at top) separately from myoblasts (cyan cell_type), except for the C25 primary cells (green cell_type). The non-myogenic population (orange cell_type) clusters with the myotubes. Immortalized clones (green clonal_state) show the same switch from myoblast to myotube as their parent primary populations (yellow clonal_state). Hierarchical clustering analysis was also applied to genes (branches to left).

Differentiation induced consistent downregulation in both primaries and immortalized clones of genes involved in cell cycle regulation and DNA replication, such as centromere components

(CENPA, CENPK), mini-chromosome maintenance (MCM3), DNA topoisomerases (TOP2A), regulation of mitotic spindle formation (CDCA8), G2/mitotic-specific cyclin-B2 (CCNB2), and the MyoD regulator ID2 (Langlands, Yin, Anand, & Prochownik, 1997). Genes such as cyclin-dependent kinase 1 (CDK1) and cyclin dependent kinase regulators (e.g. CKS1B) were down-regulated normally in clonal lines despite the presence of the murine cdk4 transgene.

For the consensus up-regulated genes, a similar heatmap is shown (Figure 16). Similarly to the consensus down-regulated genes, immortalization had no effect on the upregulation of the myogenic regulator MEF2C, metabolic genes such as creatine kinase (CKM), and ATPase 1A2 (ATP1A2), nor on contractile components and their regulators, including alpha-actinin (ACTN2), calpain-3 (CAPN3), myosins and their partners (MYBPH, MYL2, MYL4, MYLPF, MYH1, MYH3, MYH7, MYH8), troponins (TNNC1, TNNC2, TNNI1, TNNI2, TNNT1, TNNT3), myomesins (MYOM1, MYOM2), myozenin 2 (MYOZ2), and titin (TTN). Markers of mature myotube formation such as the dihydropyridine receptor calcium channel (CACNA1S), and the ryanodine receptor (RYR1), were also unaffected by immortalization.

There were few exceptions to the clustering of myoblasts separately from myotubes. The myotubes (both primary and immortalized) of one subject (DMD8036) showed stronger upregulation of myogenic genes and clustered separately from the other myotubes. The outlier myoblast primary line (C25) clustered with myotubes in the heatmap for downregulation, suggesting that this line had shut down its cell cycle. The myotubes of two lines (DMD6311 clones and DMD 6594 primaries), despite strong downregulation of the cell cycle, showed relatively weak upregulation of the myogenic cascade and clustered with myoblasts in the upregulated heatmap. As a general rule, the normal myogenic cascade was maintained in immortalized clonal lines.

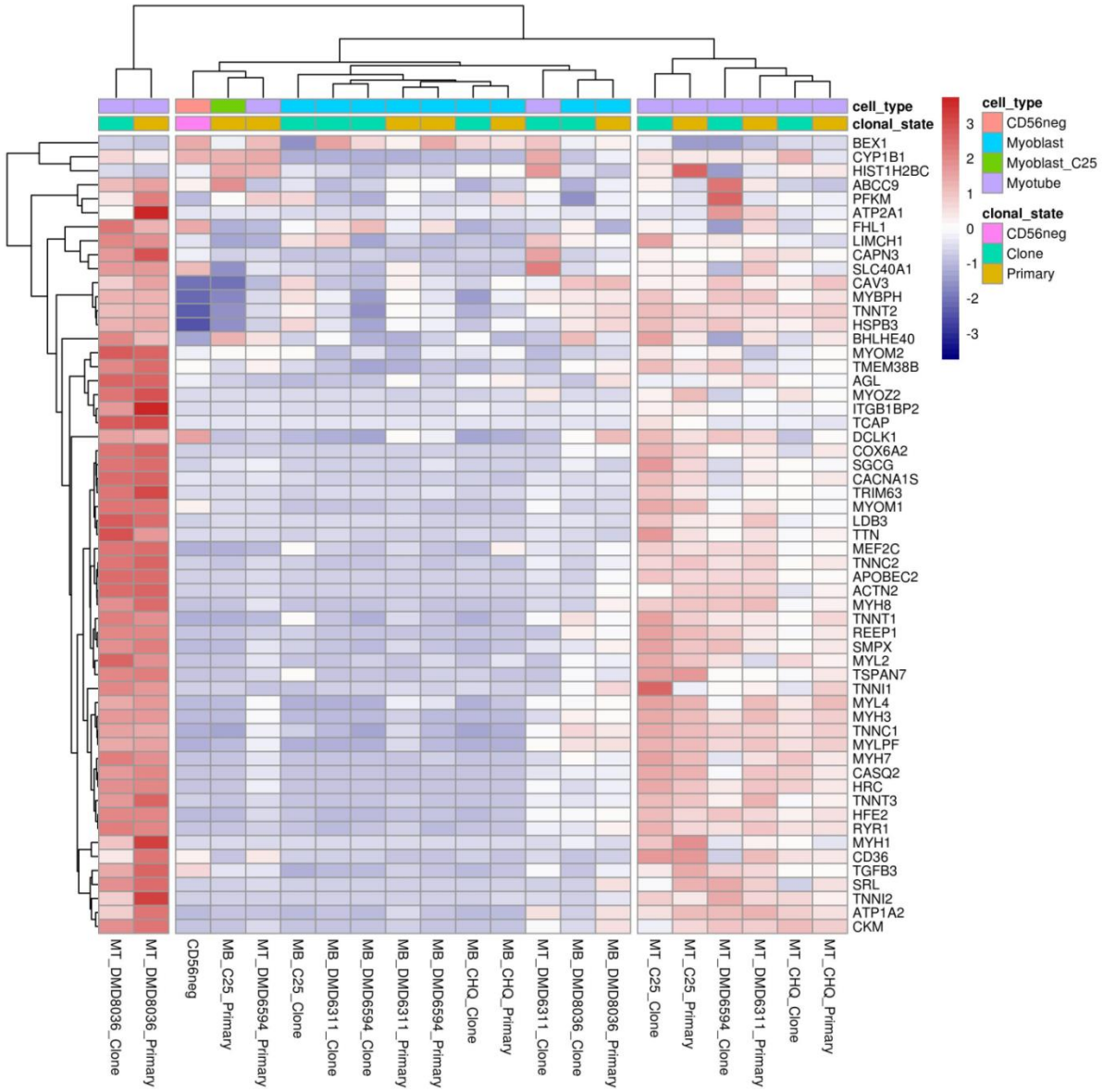


Figure 16. Heatmap showing the expression levels in immortalized and primary myogenic human lines of genes that are consistently and strongly up-regulated in a panel of previous studies of myoblast differentiation. A gene is shown if it was among the 300 most strongly up-regulated after 5 or more days of differentiation in at least 3 of 4 studies of human and mouse primary myoblasts, and C2C12 myoblasts. Expression values are row scaled. Hierarchical clustering analysis of the cell lines and genes are indicated by branches at top and left, respectively. Cell type and differentiation state are indicated by the cell_type bar at top. Immortalized clones, primary myogenic cells, and non-myogenic cells are indicated by the clonal_state bar at top.

Immortalization has no effect on other systems processes and has no effects that are similar to previously reported perturbations of muscle cells.

We used rank-based gene set enrichment (GSEA) of 2,784 canonical pathways and gene ontology terms to test for effects of immortalization on systems processes, separately in myoblasts and myotubes. Neither myoblasts nor myotubes derived from immortalized clones showed any significantly enriched rank distributions with FDR q-val < 0.05 for any of the

canonical pathways and gene ontology terms. Since we expected the cdk4 transgene to elicit changes to cell cycle regulation in myoblasts, we specifically checked rank distribution patterns for related gene sets. Of 22 cell-cycle-related gene sets, none were significant (all had FDR q-val > 0.2), but 6 had nominal p-values < 0.05 (a measure that does not adjust for gene set size or multiple hypothesis testing). As these can be considered borderline significant, we include a heatmap (supplementary Figure 1) showing the expression levels of genes that are driving this effect (defined by the overlap of GSEA leading edges). These genes generally showed some upregulation in immortalized myoblast clones relative to primary myoblasts but with the C25 primary outlier showing relatively very strong dysregulation (most genes were downregulated in C25 relative to all of the other lines and a few were upregulated).

To test for effects on gene expression that were similar to previously reported perturbations of muscle cells we applied GSEA to muscle gene sets (http://sys-myo.rhcloud.com/muscle_gene_sets.php). These gene sets were comprised of 393 lists of genes that were up- or down-regulated in a large variety of genetic and experimental comparisons in published muscle microarray studies, mainly of human or murine tissue and cell samples. These lists were obtained from more than 100 studies, and included the 4 in vitro differentiation datasets from which our consensus set was derived as described above. When immortalized clones of myoblasts or myotubes were compared to their respective primary cells, neither showed any significantly enriched rank distributions with FDR q-val < 0.05 for any of the 393 muscle gene sets. Muscle gene sets represent an ensemble of genes that are identified empirically to respond at the expression level to muscle perturbations, so their lack of change in immortalized v primary cells is indicative that immortalization has little effect on these lines. For instance, it is informative to contrast those results with the following: when genes of our dataset were ranked according to their change in myotubes v myoblasts, GSEA on the muscle gene sets specifically identified strong overlap with 35 of the 393 muscle gene sets with a very highly stringent statistical cut-off of FDR < 0.001: these highly significant muscle gene sets are visualized using an enrichment map – this shows gene sets that were down-regulated (in myotubes v myoblasts) in blue with color intensity proportional to the enrichment score (none of these highly significant gene sets were up-regulated in this case) and grey connections represent the sharing of genes between gene sets (i.e. some gene sets are similar to each other) (Figure 17). This analysis shows the strong similarity of previously published muscle differentiation gene expression changes with differentiation-induced changes in the present study when myotubes are compared to myoblasts – whereas the comparison of immortalized

with primary cells, in contrast, did not induce changes that were similar to any previously published data.

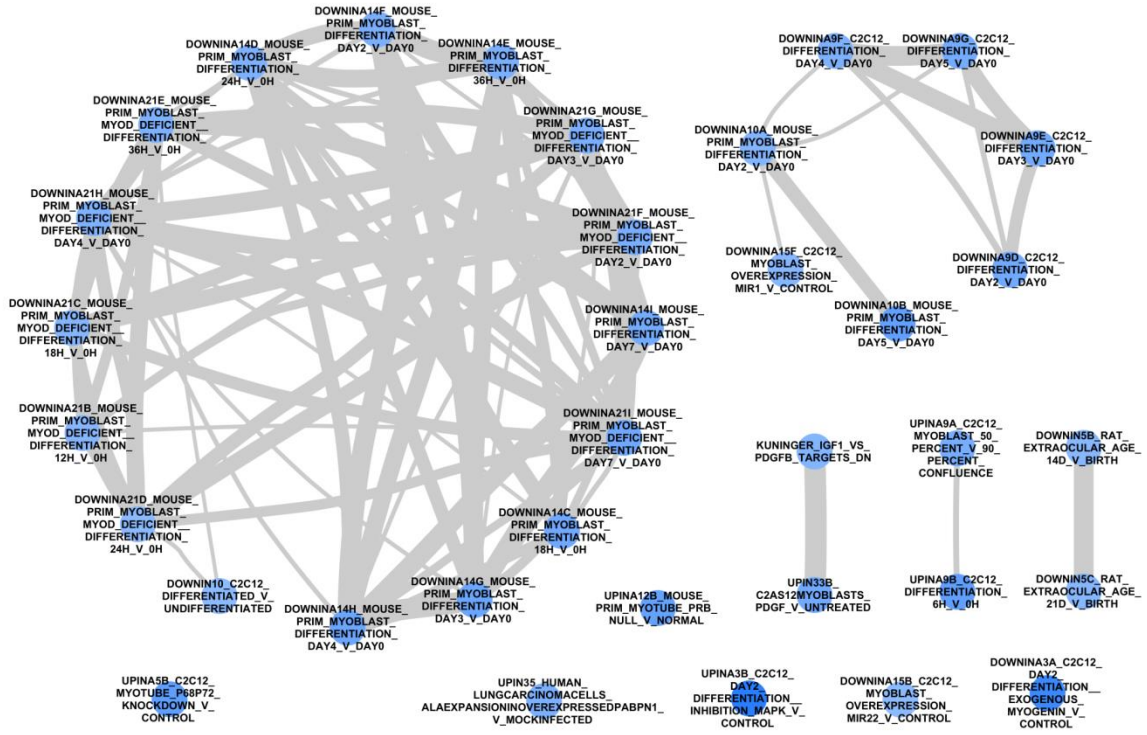


Figure 17. Mapping of rank-based gene set enrichments. Each node represents a set of genes that was dys-regulated in a previously published muscle transcriptome study, and edges indicate sharing of the same genes between nodes (thicker edges indicates more overlap). The color of the node shows whether this gene set was up- or down-regulated (i.e. positively or negatively enriched – colored red or blue respectively – in this specific analysis, upregulated gene sets were not observed) in the comparison of myotubes v myoblasts in the present study. Included on this map are only gene sets having the most significant enrichment scores (FDR < 0.001) from among the 393 muscle gene sets that were tested. A similar comparison of immortalized v primary lines (tested independently for both myoblasts and myotubes) yielded no significant muscle gene sets, even at the weaker cut-off of FDR < 0.05.

Rank-based gene set enrichment testing of canonical pathways, gene ontology, and previous muscle studies, were all consistent with hTERT/cdk4 immortalization having a minimal effect on the systems characteristics of human myoblasts.

In addition, we tested for specific effects of immortalization on other pathways previously observed to be influenced by the cell cycle. We found no change in the levels of specific phosphorylated proteins involved in the regulation of Akt pathway activity (supplementary Figure 2) or in the expression of genes involved in microtubule stability (supplementary Figure 3), and heatmaps of relative gene expression levels for gene ontology terms relating to these

processes show no clustering of primary samples separately from immortalized lines (supplementary Figures 4, 5, and 6).

Down-regulation of the cell cycle is the main effect that distinguishes the C25 primary myoblasts as outliers

Unlike their immortalized clones, primary myoblasts of the C25 line clustered more closely to myotubes than to myoblasts from other donors (figure 14). These cells showed shutdown of cell cycle genes relative to myoblasts (figure 15), but none of the upregulation of contractile components observed in myotubes (figure 16). Differential expression analysis and over-representation tests showed that these processes represented the strongest differences of C25 primary myoblasts with other myoblasts and with myotubes (supplementary Figure 7). Our interpretation of these data relate to our previous observations that human primary myoblasts senesce in tissue culture (Barberi et al., 2013; Bigot et al., 2015; Mamchaoui et al., 2011). At the time of harvesting for analysis, which was at 16 cell divisions for the C25 primary line, our transcriptomic data strongly indicate cell cycle shut-down for this line. This coincides with cellular senescence, since our previously published analyses (Mamchaoui et al., 2011) indicate that the rate of mean population doubling for the C25 primary population begins to slow after 10-15 divisions, indicative of slowing of the cell cycle as the population begins to senesce. This did not occur in any of the immortalized lines, thus immortalization is protective from senescent shut-down of the cell cycle.

Discussion

Immortalization does not confer any general dysregulation of normal myogenic processes, nor of any canonical pathways or gene ontology terms. While senescence shuts down the cell cycle of primary human myogenic isolates after a number of divisions in tissue culture, hTERT/cdk4 immortalized lines are protected, retaining the characteristics of non-senescent primary lines. Indeed, the single outlier group in our dataset was a population of primary myoblasts – whereas immortalized clones derived from that primary population remained similar to the other myoblast lines.

In the search for therapeutic strategies, it is important to have models that are representative of the *in vivo* nature of a pathology but that also allow close dissection of the mechanistic details of the pathology. When care is taken to understand the differences between humans and animal models (Terence A. Partridge, 2013), these models can be used to gain insight into the full complexity of the human condition such as in the cross-talk between cell and tissue types, and the relationship of disease progression to developmental changes and age. Even as advances are made in the modelling of artificial muscle tissue (Kalman et al., 2015), animal models remain our only possibility to observe the effects of experimental perturbations on normally vascularized and innervated muscle tissue. However, whole muscle tissue samples are not homogenous, instead consisting of a mix of constituents including myofibers, satellite cells, extracellular matrix, vascular and nerve tissue, interstitial deposits of fibrous and fatty material, fibroblasts, fat cells, and immune cells. When transcriptomic profiles are derived from whole muscle of patients and animal models it is impossible to separate minor gene dysregulation in myonuclei (representing the bulk of the tissue) from large expression changes in a lesser component of the tissue (Ramsköld, Wang, Burge, & Sandberg, 2009). For instance, if immune genes are upregulated this may reflect a response of the myofibers themselves and/or an increased infiltration of immune cells. At worst, as much can be told from the whole muscle transcriptome as can be guessed from simple microscopy of a stained muscle section. In these cases, the transcriptome becomes just another measure of disease progression, because any insight into the underlying molecular mechanisms is hidden by changes in tissue composition. Laser capture microscopy can be applied to obtain pure tissue from muscle sections (Bochmann et al., 2010; Budak et al., 2004), but it is highly laborious to obtain sufficient material by this method and it is rarely used.

Cell models cannot represent the more complex whole organism interactions described above, which is why a full understanding of a pathology will likely require multiple types of model. However, from the systems biology perspective, isolated cells can represent a relatively homogenous sample type so that changes in the transcriptome or in other omics profiles can be assumed to reflect changes in the mechanistically linked components of a single cellular system. So long as it is understood that an omics profile reflects an averaging across the population of harvested cells (except if single cell methods are applied), this assumption can be applied to carefully purified cell sub-populations such as the desmin-positive primary myoblast lines used in our study. To remain as close as possible to the human pathology, ideally human cell lines are used. However, adult human myoblasts quickly senesce during *in vitro* culture (Barberi et al., 2013; Renault, Thornell, Butler-Browne, et al., 2002; Wright & Shay, 2002; C.-H. Zhu et al., 2007). Our recent work underlines the grave importance of this in the interpretation of experimental data (Bigot et al., 2015). Immortalized lines require genetic interference with the cell cycle, but we developed these lines in order to have an inexhaustible source of cell model material and we have continued in their use because we did not observe morphological or other obvious abnormalities, and because of pressing needs in therapeutic development. However, question-marks had remained as to whether immortalization has knock-on effects on other cellular processes that would overwhelm their natural biological variation and thus challenge their usefulness as experimental models. The results presented here show that immortalized cells retain gene expression patterns that are characteristic of their cell type, as distinct from the non-selected (CD56-ve) fraction, and whether as undifferentiated myoblasts or as differentiated myotubes. Furthermore, immortalized cells generally remain very close in expression profile to their parent primary populations in both their differentiated and undifferentiated states: the exception that we observed was linked to loss of normal profile in the primary cells rather than the immortalized cells. Thus immortalization is protective of the characteristics of the primary population against the effects of senescence in cell culture.

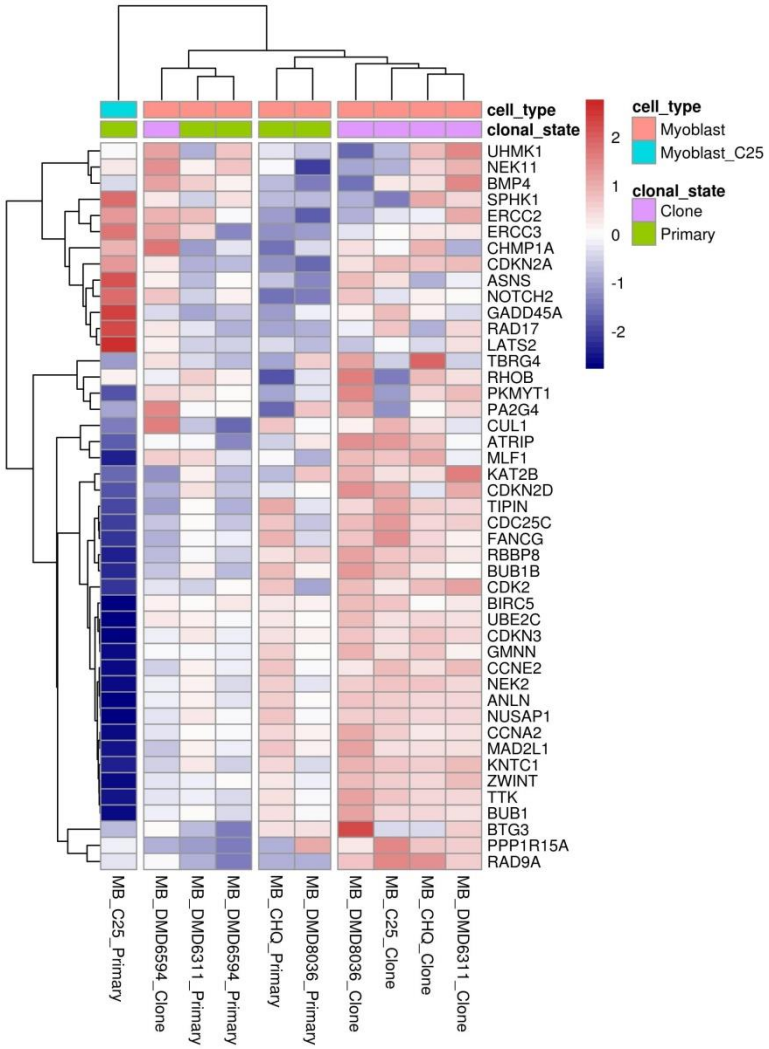
A second concern was that we typically select clonal lines from within each immortalized population on the basis of their capacity to form morphologically well-defined myotubes, visualized by desmin immunostaining (chosen for its early expression relative to other myogenic markers (Fürst, Osborn, & Weber, 1989)), thus selecting just one cell sub-type from among the variation that was present in the original primary population. Our data would suggest that this does not lead to any systematic biases. Differentiated clones did not show any

systematic over-expression of myogenic and muscle contractile genes relative to primary cells (Figure 16).

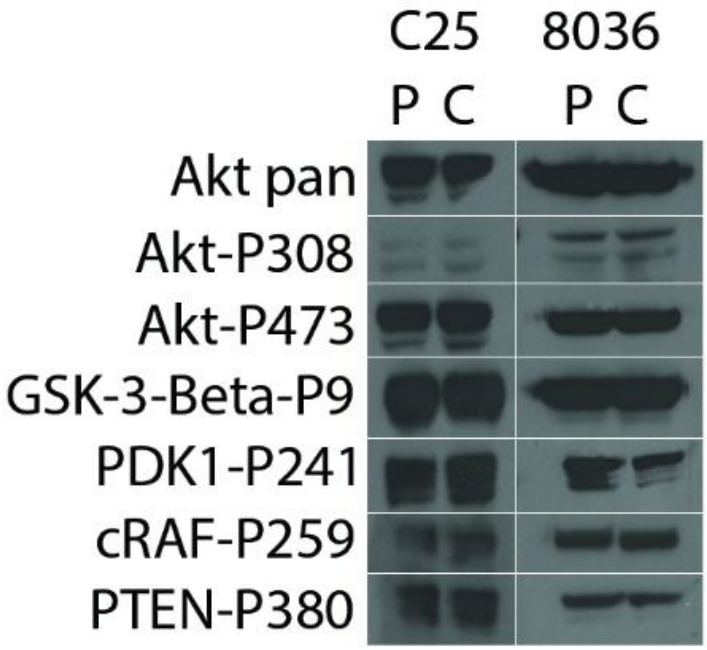
Conclusions

Immortalization using hTERT and cdk4 transgenes preserves and protects the natural biological variation of desmin-purified human primary myoblasts. Clones isolated from immortalized primaries are representative of their parent primary population and represent valuable research tools for the study of human neuromuscular disease.

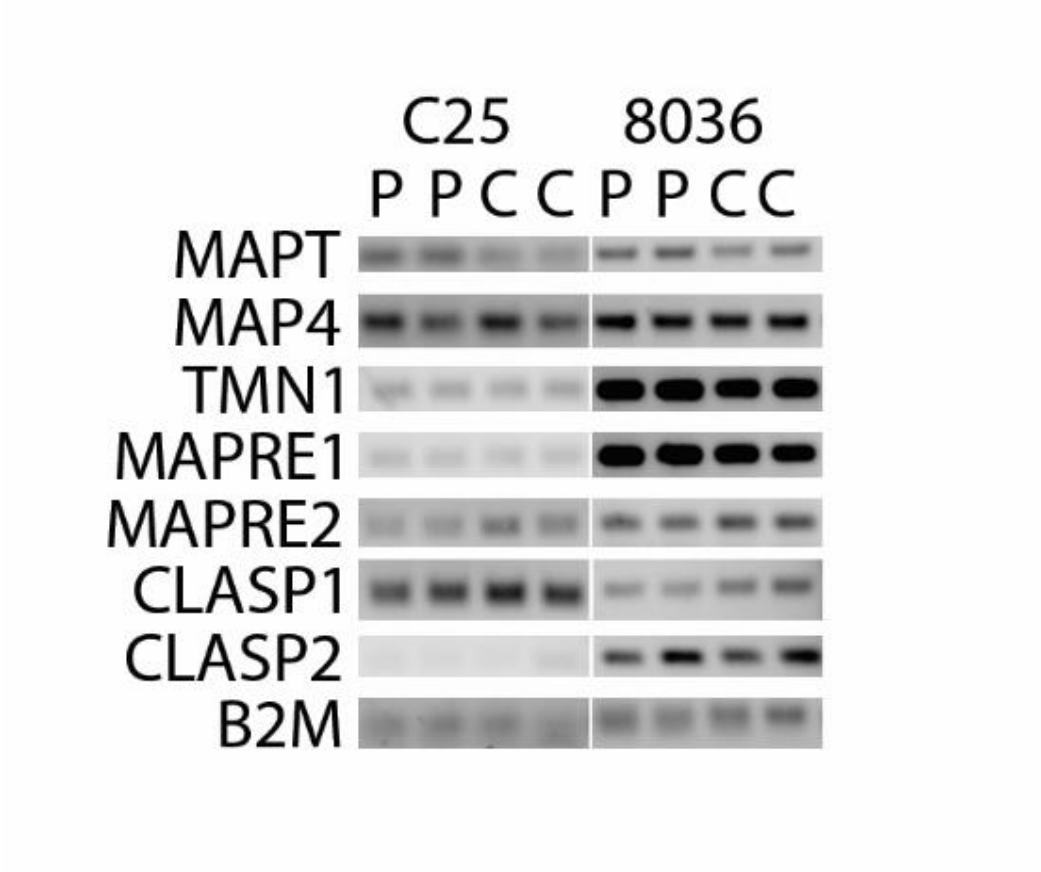
Supplemental data



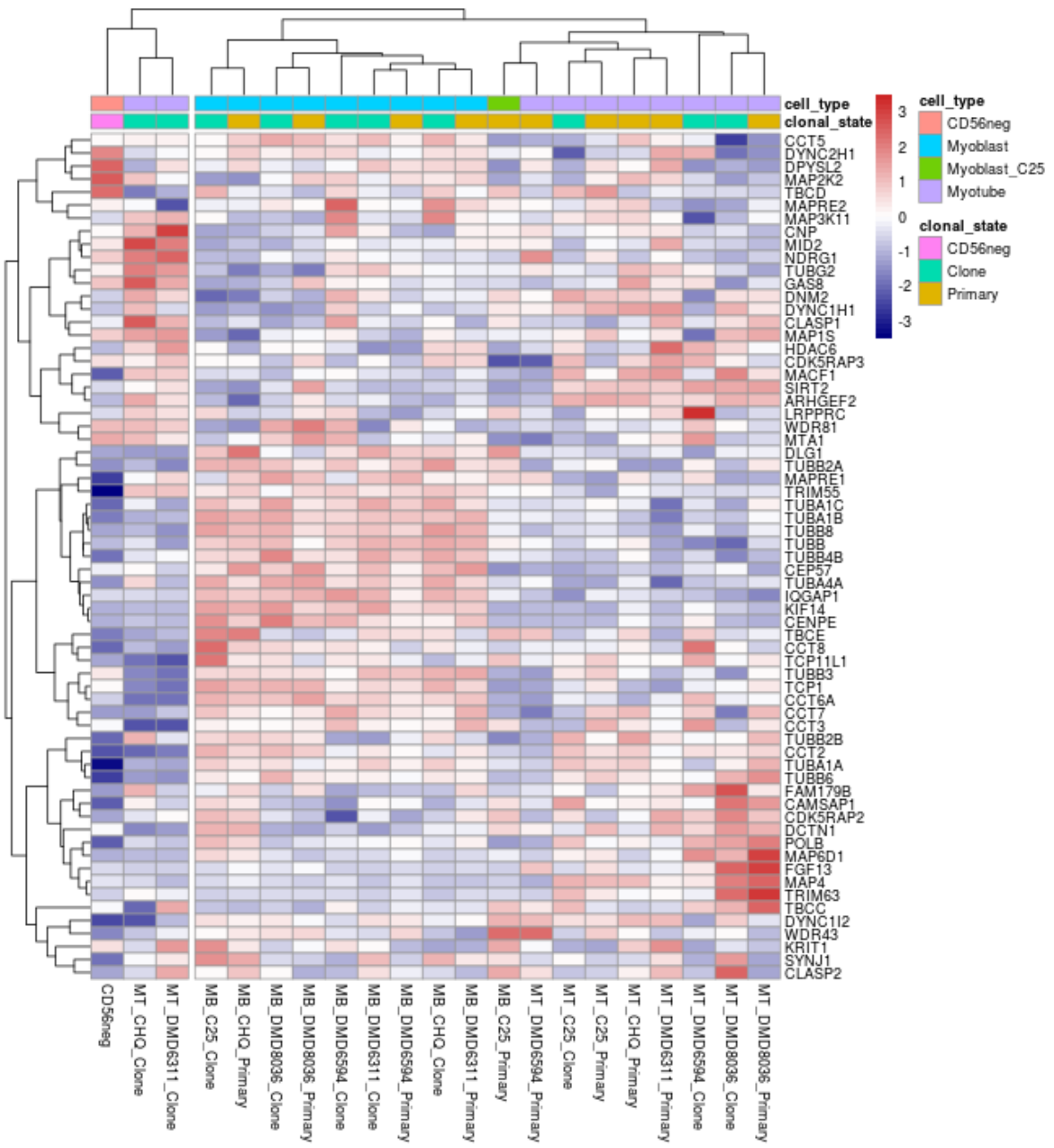
Supplementary Figure 1. Heatmap showing the expression levels in immortalized and primary human myoblasts of genes that may be mildly affected by immortalization. We specifically checked rank distribution patterns of 22 cell-cycle-related canonical pathways and gene ontologies. None of these were significantly enriched (all had FDR q-val > 0.2) but 6 had nominal p-values < 0.05 (a measure that does not adjust for gene set size or multiple hypothesis testing). As these can be considered borderline significant, we show here the expression levels of genes that were present in 3 or more of these 6 cell-cycle-related pathways/ontologies. These genes generally showed some upregulation in immortalized myoblast clones relative to primary myoblasts but with the C25 primary outlier showing relatively very strong dysregulation. Expression values are row scaled (i.e. changes are relative across each row for ease of interpretation). Hierarchical clustering analysis of the cell lines, indicated by branches at top, to some extent separates immortalized clones (green clonal_state) from their parent primary populations (green clonal_state). Hierarchical clustering analysis was also applied to genes (branches to left).



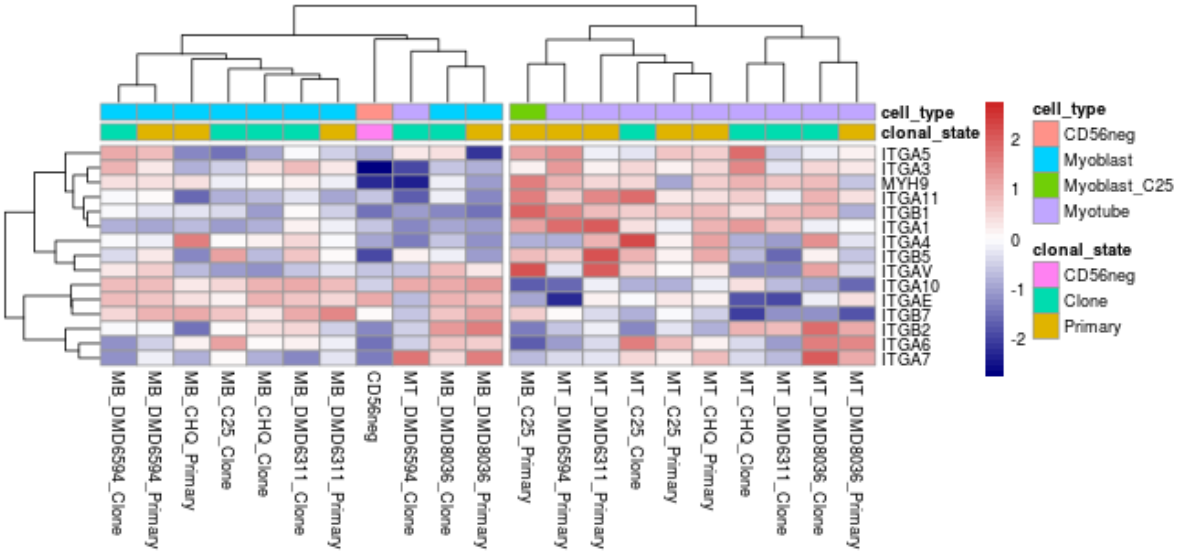
Supplementary Figure 2. Proteins involved in the regulation of Akt pathway activity. Lysates from differentiated cells of C25 and DMD8036 primary (labelled P) and immortalised (labelled C) cell lines. The antibodies used were those provided by the Phospho-Akt Pathway Antibody Sampler Kit (Cell Signalling Technology). They target Akt protein levels (Akt pan), and its activation at two phosphorylation sites (Akt-P308 and Akt-P473), as well as PTEN (a major negative regulator if the Akt pathway), GSK-3beta (regulated by Akt and involved in glycogen synthesis), c-Raf (regulated by Akt and involved in apoptosis), and PDK1 (an activator of Akt). The primaries and clones of the individual cell lines displayed similar levels of the detected proteins.



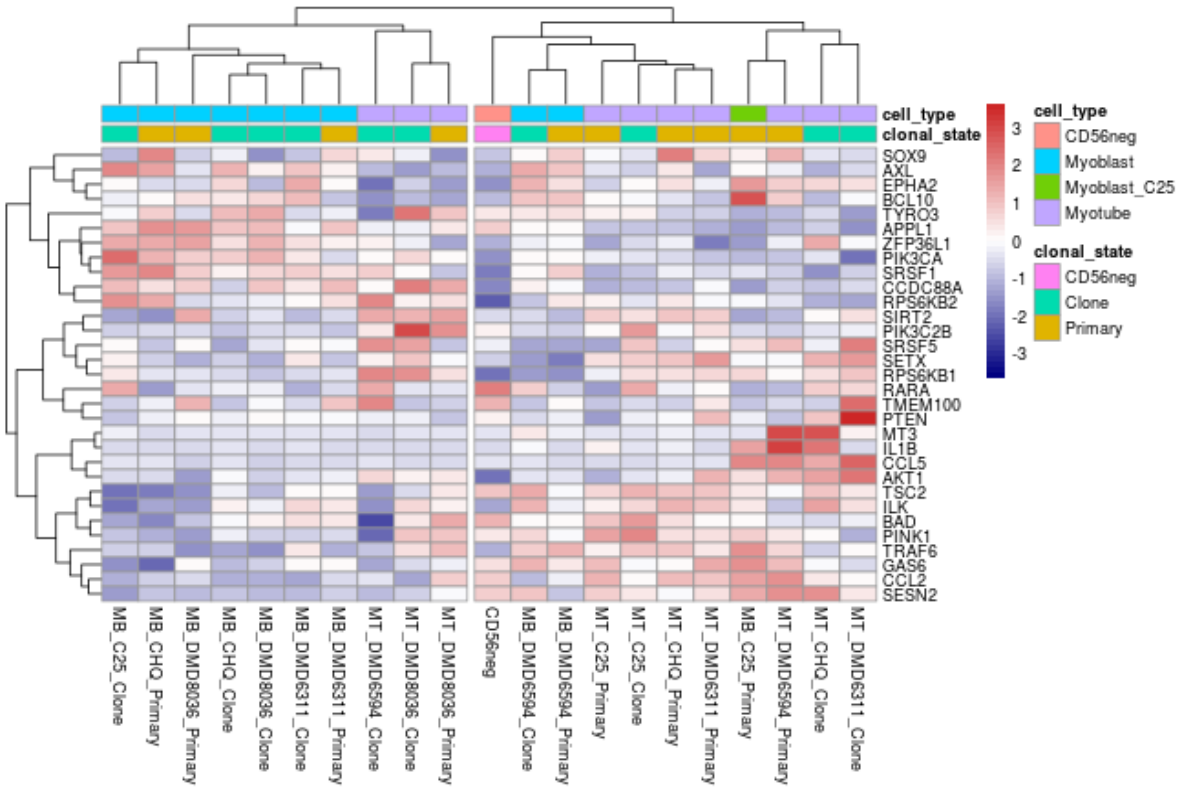
Supplementary Figure 3. Expression levels of genes involved in microtubule stability. cDNA synthesized from RNA isolated from differentiated cells of C25 and DMD8036 primary (labelled P) and immortalized (labelled C) cell lines. These genes encode microtubule interacting proteins (CLASP1, CLASP2, MAP4, MAPT, and STMN1); and/or are involved in microtubule organization and biogenesis (CLASP1, MAPT, and STMN1), in polymerization/depolymerisation (CLASP1, CLASP2, MAP4, MAPRE1, MAPRE2, MAPT, and STMN1), or in spindle organization and biogenesis (CLASP1, CLASP2, MAPRE1). The myotubes were cultured in duplicate dishes, and band intensities show no differences between primary and immortalized populations.



Supplementary Figure 4. Heatmap of gene expression for genes manually annotated to the microtubule term of the Gene Ontology, including genes assayed by PCR above. Hierarchical clustering analysis of the cell line is indicated by branches at top, and was also applied to genes (branches to left).



Supplementary Figure 5. Heatmap of gene expression for genes annotated to the integrin complex term of the Gene Ontology. Hierarchical clustering analysis of the cell line is indicated by branches at top, and was also applied to genes (branches to left).



Supplementary Figure 6. Heatmap of gene expression for genes annotated to the protein kinase B binding (i.e. Akt binding) term of the Gene Ontology. Hierarchical clustering analysis of the cell line is indicated by branches at top, and was also applied to genes (branches to left).

C25 v myoblast

Index	Name	P-value	Z-score	Combined Score
1	mitotic cell cycle (GO:0000278)	7.252e-22	-2.30	93.27
2	cellular response to type I interferon (GO:0071357)	5.970e-17	-2.17	65.71
3	response to type I interferon (GO:0034340)	8.111e-17	-2.17	65.66
4	type I interferon signaling pathway (GO:0060337)	5.970e-17	-2.17	65.62
5	extracellular matrix organization (GO:0030198)	3.425e-15	-2.37	63.50
6	extracellular structure organization (GO:0043062)	3.800e-15	-2.37	63.49
7	cell cycle phase transition (GO:0044770)	1.372e-12	-2.31	48.61
8	mitotic cell cycle phase transition (GO:0044772)	3.659e-12	-2.31	46.86
9	response to virus (GO:0009615)	1.028e-11	-2.35	46.01
10	mitotic nuclear division (GO:0007067)	3.387e-12	-2.26	45.97

C25 v myotube

Index	Name	P-value	Z-score	Combined Score
1	muscle system process (GO:0003012)	1.039e-19	-2.32	82.32
2	actin-myosin filament sliding (GO:0033275)	1.547e-17	-2.44	77.75
3	muscle filament sliding (GO:0030049)	1.547e-17	-2.44	77.63
4	muscle contraction (GO:0006936)	1.044e-17	-2.29	72.84
5	actin-mediated cell contraction (GO:0070252)	3.987e-17	-2.31	72.07
6	actin filament-based movement (GO:0030048)	5.938e-15	-2.22	58.46
7	extracellular matrix organization (GO:0030198)	9.578e-13	-2.36	51.28
8	extracellular structure organization (GO:0043062)	1.050e-12	-2.36	51.27
9	type I interferon signaling pathway (GO:0060337)	1.362e-13	-2.16	50.61
10	cellular response to type I interferon (GO:0071357)	1.362e-13	-2.15	50.52

Supplementary Figure 7. Over-representation analysis of differentially expressed genes shows that the primary change in C25 primary myoblasts v the other myoblasts investigated in this experiment is in the down-regulation of cell cycle processes (upper table), and in C25 primary myoblasts v myotubes it is the down-regulation of muscle contractile components (lower table). Enrichment of gene ontology biological processes among the top 800 by fold-change of genes having FDR < 0.05.

Supplementary table 1. Complete list of gene expression datasets used in this study and their sources

GEO series	Platform	GEO samples	Reference
GSE11415	Mouse Genome 430 2.0	GSM288014 GSM288015 GSM288016 GSM288032 GSM288033 GSM288034	1
GSE24811	Mouse Gene 1.0 ST	GSM610848 GSM610849 GSM610850 GSM610854 GSM610855 GSM610856	2
GSE26145	Human Exon 1.0 ST	GSM443913 GSM443914 GSM443915 GSM443916 GSM443917 GSM443918	3
GSE10424	Murine Genome U74A Version 2	GSM263671 GSM263672 GSM263673 GSM263674 GSM263675 GSM263676 GSM263677 GSM263678 GSM263679 GSM263752 GSM263753 GSM263754 GSM263755 GSM263756 GSM263757 GSM263758 GSM263759 GSM263760	--

Chapter 4 –The dystrophin interactome reveals a link with secreted vesicles

Abstract

Dystrophin at the sarcolemmal membrane of muscle fibres acts as a nucleus for the formation of an intricate complex whose primary role is to pin the actin and also microtubule cell scaffold structures to the membrane and extracellular matrix, thereby protecting the membrane from tearing during muscle contraction. Dystrophin also possesses lipid-binding properties that may further stabilize the membrane during muscle force generation or may allow it to interact with vesicle-mediated secretory pathways. Signalling and structural functions have been assigned to a number of other proteins that contribute to the wider dystrophin-associated complex. Loss of several of these proteins by mutation results in human myopathy. Here we used a high-sensitivity proteomics approach, combining siRNA knockdown of dystrophin expression, dystrophin immunoprecipitation, and heavy isotope labelling (SILAC), to identify systematically the binding partners of dystrophin in muscle myotubes. Our results suggest previously unidentified mechanistic linkers, including to vesicle trafficking. We survey existing literature and bioinformatics resources and present these along with proteomic data, overlaid on a functional association network map, in a web-based data exploration tool: sys-myo.rhcloud.com/dystrophin-interactome.

Introduction

Dystrophin is a 427 kDa protein, located under the sarcolemma and at the neuromuscular junction, where it interacts with trans-membrane and cytoskeletal proteins - proteins that have a key role in the architecture and the stability of muscle fibres (Ohlendieck & Campbell, 1991; Ohlendieck et al., 1993). It is thought to synchronize the movement of the plasma membrane and the contractile apparatus to avoid sarcolemma disruption during muscle contraction (Engel, Arahata, & Biesecker, 1984; Petrof, Shrager, Stedman, Kelly, & Sweeney, 1993; Williams & Bloch, 1999). Its absence results in Duchenne muscular dystrophy (DMD), while a truncated protein can result in the less severe Becker muscular dystrophy (BMD) phenotype (Flanigan, 2014). Mutations resulting in the absence of expression or altered post-transcriptional maturation of its interaction partners leads to muscle diseases such as dystroglycanopathy (Moore & Winder, 2012), desminopathy (Jaka, Casas-Fraile, López de Munain, & Sáenz, 2015) or sarcoglycanopathy (Kirschner & Lochmüller, 2011). These observations suggest that dystrophin forms the nucleus of an important and complex protein network in which each member's localization, expression, and post-transcriptional maturation are vital for the muscle fibre.

Several previous studies looked at dystrophin interactors, through techniques such as immunoprecipitations and yeast two-hybrid approaches (Murphy & Ohlendieck, 2016). Well-supported partners of dystrophin constitute the dystrophin associated protein complex (DAPC), and include transmembrane proteins such as the sarcoglycans, sarcospan, and dystroglycan, as well as intracellular proteins such as dystrobrevin, syntrophin, n-NOS and F-actin (K J Amann et al., 1999; Ervasti & Campbell, 1993; Levine et al., 1992; Pilgram, Potikanond, Baines, Fradkin, & Noordermeer, 2010; G D Thomas & Victor, 1998; Townsend, 2014). In addition, dystrophin interacts with a variety of other proteins including structural linker proteins alpha-actinin-2, ankyrin-2 and ankyrin-3 (Ayalon et al., 2008; Hance et al., 1999). A link to caveolae is suggested by limb-girdle muscular dystrophy type 1C (LGMD1C and LGMD2C-F), which results from mutation of caveolin-3 and presents a mislocation or a lower expression level of dystrophin (F Galbiati et al., 2001; Herrmann et al., 2000).

The projected outcomes of therapeutic strategies for DMD or BMD, such as exon skipping and gene therapy are dependent on understanding the functional roles of dystrophin, to tailor treatments towards re-establishing the important interactions and pathways that have been lost

or disrupted. In this context, understanding the entire composition of the dystrophin interactome will help to unravel new functions of dystrophin other than muscle structure and may have a considerable impact on therapeutic strategy for DMD and BMD.

Here we sought to systematically identify dystrophin interactors, using the 'QUICK' method for protein interaction screening by quantitative immunoprecipitation combined with knockdown (Selbach & Mann, 2006). This method allowed us to confidently identify dystrophin interactors, eliminating the multitude of false positive proteins pulled-down through unspecific binding to both the beads and the antibodies. In addition, we generated an interactome of dystrophin based upon the current literature, expanding out key cell compartments, and subsequently using our own experimental data to add additional important cellular compartment and protein interactions that are relevant to dystrophin and dystrophin function. Dystrophin's roles vary between different cell types along with dystrophin isoform expression and localisations. We will focus on the roles and interactions of the full-length dystrophin within the context of skeletal muscle.

Materials and Methods

Ethics approval

Human-derived cell lines were obtained as described previously (Bigot et al., 2009; Mamchaoui et al., 2011): muscle biopsies were obtained from the MYOBANK BTR (Bank of Tissues for Research, a partner in the EU network EuroBioBank) in accordance with European recommendations and French legislation in accordance with European recommendations and French legislation.

Cell line maintenance and labelling with stable isotopes

Myogenic immortalised line of a healthy subject was cultured in proliferation medium (1 volume of M199, 4 volumes of Dulbecco's modified Eagle's medium (DMEM), 20% foetal bovine serum (Invitrogen), 1 µL/mL gentamicin (Invitrogen), 25 µg/mL fetuin, 0.5 ng/mL bFGF, 5 ng/mL EGF, 0.2 µg/mL dexamethasone, 5 µg/mL insulin) in petri dishes (30 dishes, 225 cm^2 dish area), incubated at 37 °C, 5 % CO_2. Cells were differentiated in 1 volume of M199, 4 volumes of DMEM + 1 µL/mL gentamicin. SILAC DMEM and M199, lacking L-arginine and L-lysine were used to prepare 'heavy' and 'light' medium. Heavy isotope medium was prepared by adding 84 mg/L $^{13}C_6{}^{15}N_4$ L-Arginine and 146 mg/L $^{13}C_6{}^{15}N_2$ L-Lysine to SILAC DMEM and 35 mg/L $^{13}C_6{}^{15}N_4$ L-Arginine and 35 mg/L $^{13}C_6{}^{15}N_2$ L-Lysine to SILAC M199. Light isotope medium was prepared using the non-isotopic $^{12}C_6{}^{14}N_4$ L-Arginine and 146 mg/L $^{12}C_6{}^{14}N_2$ L-Lysine at the same masses. Cells were cultured in the relevant medium for at least 3 cycles before transfection.

Enough cells were cultured for a total of 4 mass spectrometry runs. Each run consisted of 6 * 225 cm^2 petridishes (3 cultured in heavy medium, 3 cultured in light medium) with 2 million cells plated in each. Three mass spectrometry runs were comprised of no transfection of the cells in light medium and transfection of the cells targeting dystrophin knockdown in heavy medium. One mass spectrometry run was comprised of no transfection of the cells in light medium and a negative control transfection of the cells in heavy medium.

Dystrophin knockdown

Transfections (where relevant) were carried out using DMD Silencer Select Pre-designed siRNA (ID: s4157, Ambion) for dystrophin knockdown. Transfections were performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol. Transfections were carried out at about 60% confluence (D-2), 2 days later after

changing to differentiation medium (D-0) and a final time two days later (D+2). Cells were harvested five days after differentiation (D+5). Knockdown efficiency was examined at the protein level by western blot analysis of the treated lysates. No dystrophin band was observed in dystrophin knockdown lysates.

Protein isolation

Day 5 of differentiation was determined to be the earliest time point at which dystrophin was expressed at significant levels in this cell line. At differentiation day 5, petri dishes were place on ice, medium removed (decanted) and washed three times with PBS. RIPA buffer (650 µL, 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and 5 mM EDTA with Halt™ Protease and Phosphatase Inhibitor Cocktail) was added to each dish and left on ice for 30 minutes. Cells were scraped and collected (1.5 mL Eppendorf tubes), centrifuged (13,000 rpm, 4 °C, 10 minutes). The supernatant was taken for protein analysis.

Protein concentration

Protein concentrations were determined using Pierce BCA Protein Assay Kit, using 190 µL working reagent and 10 µL of 1:10 sample diluted in H_2O. Each sample was carried out in triplicate. Each protein sample concertation was adjusted to 1 µg/µL, and the sets of six samples that would made up each mass spectrometry run were combined in equal volumes.

Immunoprecipitation

Immunoprecipitations were carried out using 1000 µL of 1 µg/µL lysate, and either Dys-2 (NCL-Dys2, Clone 6C5, Leica) or MANDYS102 (Clone 7D2, DSHB) antibody. The two antibodies recognise different antibodies, in order to decrease the possibility of dystrophin binding partners blocking the antibody recognition site which would otherwise prevent pull down of the protein complex.

For each immunoprecipitation, antibodies (5 µg) were bound to A/G magnetic beads (25 µL, Pierce), and crosslinked using DSS (0.25 mM in DMSO) to prevent antibody elution and interference with the mass spectrometry run. Sample lysates were incubated overnight at 4 °C with constant gentle rotation. Washes were performed once with RIPA buffer and twice with H_2O. The bound proteins were eluted by two sets of incubation for 7 minutes at RT with 1 % SDS in 50 mM Tris, pH 8.0.

Each mass spectrometry run sample was made up of 8 immunoprecipitations; 2 heavy isotope labelled lysates pull downed with Dys-2 antibody (5 µg), 2 light isotope labelled lysates pull downed with Dys-2 antibody (5 µg), 2 heavy isotope labelled lysates pull down with MANDYS102 antibody (5 µg) and 2 light isotope labelled lysates pull down with MANDYS102 antibody (5 µg).

All eight elutions for a single mass spectrometry run were combined (8 * 200µL).

Single myosin (MF20, DSHB) and dystrophin (Dys-2, Leica) immunoprecipitation for western blot analysis were performed using 500 µL of 1 µg/µL lysate, 2.5 ug antibody, 12.5 µL A/G magnetic beads (Pierce) and crosslinked using DSS (0.25 mM in DMSO)

Protein precipitation

4 volumes MeOH was added to each sample (1.6 mL, 1 volume), vortexed and centrifuged (10 seconds, 7,000 g). 1 volume chloroform was added, vortexed and centrifuged (10 seconds, 7,000 g). 3 volumes of water were added, vortexed vigorously and centrifuged (1 minute, 7,000 g (for phase separation)). The upper phase was carefully removed and discarded (water), without disturbing the interphase containing the proteins. 3 volumes MeOH was added to the remaining lower phase + interphase, vortexed gently and centrifuged (5 min at 12,000 g). The supernatant was carefully removed and the pellet was left to air dry for 5 minutes.

In solution digest and stage tip purification

Precipitated protein pellets were solubilized in 6 M urea / 2 M thiourea in 10 mM HEPES (pH 8.0, 20 µL). Samples were reduced (1 µL DTT, incubated for 30 minutes, RT), alkylated (1 µL iodoacetamide, incubated for 20 minutes, RT in the dark), digested (1 µg endoproteinase LysC (Wako) in 50 mM Ammonium Bicarbonate, incubated for 3 h, RT), diluted four fold with 50 mM Ammonium Bicarbonate and digested further (1 µg trypsin (Promega) in 50 mM Ammonium Bicarbonate, incubated overnight, RT). The sample pH was adjusted to 2.5 with 10% TFA.

Detection by western blot

20 µg of protein lysates were loaded onto 3-8 % Tris-Acetate gels (midi gels, NuPage Novex) in Tris-Acetate running buffer (NuPage) and semi-dry transferred (20 V, 7 min) (iBlot dry

blotting system, Thermo). After blocking the membrane (1 h, 5 % milk in TBS-Tween), the membrane was incubated with primary antibodies Dys-2 (1:200 dilution), Creatine Kinase (CK-1F7, DSHB) (1:1000) and Myosin heavy chain (MF20, DSHB) (1:1000) (2 h, RT). Secondary antibodies (Anti-mouse IgG, 1:2000, HRP-linked) was used (1 hour, RT). Bands were detected by SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher).

Mass spectrometry run

Peptides were concentrated and desalted on reversed phase C18 disks (Rappsilber, Ishihama, & Mann, 2003) and analysed by nanoflow liquid chromatography an Agilent 1100 LC system (Agilent Technologies Inc.) coupled to a LTQ-Orbitrap mass spectrometer (Thermo Electron). Peptides were separated on a C18-reversed phase column packed with Reprosil (ReproSil-Pur C18-AQ 3-µm resin) and directly mounted on the electrospray ion source of an LTQ-FT or LTQ-Orbitrap. We used a 140 min gradient from 2% to 60% acetonitrile in 0.5% acetic acid at a flow of 250 nl/min. The LTQ-FT instrument was operated in the data dependent mode switching automatically between MS survey scans and high mass accuracy SIM (Single Ion Monitoring) scans (both acquired in the FT-ICR cell) and MS/MS spectra acquisition in the linear ion trap as described previously(Olsen, Ong, & Mann, 2004). The LTQ-Orbitrap was operated in the data dependent mode with a full scan in the Orbitrap followed by five MS/MS scans in the LTQ.

Mass spectrometry analysis

Initial mass spectrometry analysis was carried out using MaxQuant, similar to previously reported(Selbach & Mann, 2006). Briefly, each of the raw mass spectrometry files were processed simultaneously using MaxQuant against the human protein database (http://www.ebi.ac.uk, Swiss-Prot and TrEMBL). Carbamidomethylation as a fixed modification and 'Oxidation of methionine' and 'N-acetylation of the protein' as variable modifications were selected. Specific digestion with Trypsin/P with a maximum of two missed cleavages was required. A multiplicity of two was selected, with 'Arg10' ($^{13}C_6{}^{15}N_4$ arginine) and 'Lys8' ($^{13}C_6{}^{15}N_2$ lysine) selected for the heavy labels. A false discovery rate of 0.01 was used as a cut off for both peptide and protein. The analysis was run twice, once with the re-quantify feature enabled and once with the feature disabled. The three runs of dystrophin knockdown heavy medium, non-transfected light medium were labelled as 'Forward 1/2/3'. The one run of non-transfected heavy medium, dystrophin knockdown light medium was

labelled ‘Reverse’. The resulting protein ratio files was exported to Excel (Microsoft) for further analysis. Ratios were reported as Heavy/Light (H/L) ratios, and were normalised to correct for varying protein amounts across samples. Proteins with less than two peptides identified were discarded. Ratios with ‘NaN’ values were discarded. Fold changes of ratios were calculated ($\log_2$ ratio) and normalised to a positive fold change for a ratio representing a dystrophin association (all forward run fold changes multiplied by minus one, reverse run fold changes unaltered). Results were filtered for proteins that had positive fold change values across all valid runs with at least one valid forward run and a valid reverse run. Each protein was given a rank based upon the average fold change and average fold change against rank was used to determine a set of high confidence physically interacting proteins with a fold change value significantly higher (standard error) than the calculated rank to fold change background relationship.

Adjusted analysis workflow for proteins having higher levels in non-knockdown samples but had been disregarded by the standard analysis workflow due to having zero background levels in one or more of the knockdown samples: the initial mass spectrometry analysis was carried out with MaxQuant software with the re-quantify option enabled. In cases where evidence of a protein is only detected in one of the isotopes of a run the re-quantify option instructs the software to research the position where the equivalent peptides of missing isotope mass would be expected. In cases where the protein is absent in the alternatively labelled sample, the software will often detect background noise, which is then used to return a ratio value. The incorporation of these forced ratio values is helpful in the analysis large data sets by increasing the number of identified proteins with valid ratio values however it also leads to the discarding of peptides for which no ratio could be determined due to very low background. The same experimental data was re-ran with the re-quantify option disabled. Four proteins were subsequently identified which were detected in all four immunoprecipitates from dystrophin expressing lysates and had no detection in the immunoprecipitates from dystrophin knockdown lysates (Table 4).

RNA isolation

Cells were plated in 2 sets of 400,000 cells in 19.5 cm^2 petri dishes (Falcon) for each cell. Cells were transfected following the same protocol and time points as previously described in the ‘Cell line, media and knockdown’ section, with one plate transfected for dystrophin knockdown, one plate transfected with scrambled siRNA (Silencer Select Negative Control

No. 1 siRNA (Ambion)) and two non-transfected plates. At day 5 differentiation, Trizol (1 mL) was used to harvest the cell. The RNA was subsequently isolated (PureLink RNA Mini Kit, 'Using TRIzol® Reagent with the PureLink® RNA Mini Kit' section of the standard protocol) with the RNA eluted in 30 µL H2O.

RNA quality control

RNA quality and purity was determined using the Nanodrop 2000 (Thermo Scientific, nucleic acid setting) followed by Quality Control analysis (Agilent 2100 Bioanalyzer, Eukaryote Total RNA Nano assay, Agilent RNA 6000 Nano Kit, Agilent RNA Nano LabChip) to determine the RNA integrity number (RIN, 28S/18S ratio).

Gene expression profiling

An aliquot of 150 ng of high-quality total RNA from each sample was used for mRNA expression profiling. Samples were analysed using Illumina® Gene Expression BeadChip Array technology (Illumina, Inc., San Diego, CA). Reverse Transcription for synthesis of the cDNA strand, followed by a single in vitro transcription (IVT) amplification, that incorporates biotin-labelled nucleotides, were performed with Illumina® TotalPrep™ -96 RNA Amplification Kit (Ambion, Austin, TX). 750ng of the biotin-labelled IVT product (cRNA) was hybridized to HumanHT-12v4_BeadChip (Illumina, Inc., San Diego, CA) for 16 hours, followed by washing, blocking, and streptavidin-Cy3 staining according to the WholeGenome Gene Expression Direct Hybridization protocol (Illumina, Inc., San Diego, CA). The arrays were scanned using HiScanSQ System and obtained decoded images were analysed by GenomeStudio™ Gene Expression Module – an integrated platform for the data visualization and analysis (Illumina, Inc., San Diego, CA).

Transcriptomic raw data treatment

Raw intensity values (Illumina idat files) of non-transfected, dystrophin-knocked down and scrambled siRNA samples were passed to the R lumidat library (https://github.com/drmjc/lumidat) to create an EListRaw class object compatible with further downstream analyses in R/Bioconductor. Data of healthy and disease lines used for comparisons (C25 clones, CHQ clones, DMD6311 clones, DMD6594 clones and DMD8036 clones) was obtained previously (Thorley *et al*; Paper in revision). All samples had more than

40% of probes expressed. The limma library (Ritchie et al., 2015) was then used to carry out neqc normalization – this involves background correction followed by quantile normalization, using negative control probes for background correction and both negative and positive controls for normalization (Wei Shi et al., 2010). Probes were excluded if: (1) they had low bead numbers in at least one sample; (2) they were not expressed (expression p-value < 0.01) in at least 6 arrays; (3) they were annotated as 'no match' or 'bad' quality according to the Bioconductor illuminaHumanv4.db library. This gave a total of 15,932 probes for further analysis. The Bioconductor library arrayQualityMetrics was used for quality assessment of the arrays (Kauffmann et al., 2009). PCA was applied to the normalized values and visualized using R's prcomp and plot functions.

Literature interactome generation

Literature searches were guided by publically available interaction databases. The databases were accessed through Cytoscape (Shannon et al., 2003) and/or the relevant database websites. PubMed was used to access and verify referenced interactions of interest. Literature searches were independently made using PubMed and Google/Google Scholar, with various combinations of keyword search terms such as (but not limited to); dystrophin/dmd/Duchenne/interactome/direct/immunoprecipitation/Y2H, and protein names of interest. High-throughput data papers were also independently searched.

Expansion of dystrophin's direct interaction partners to include important secondary and tertiary protein interactors was accomplished through further literature mining. Immunoprecipitations with dystrophin or other members of the dystrophin associated protein complex were of particular use. Attempts were made to ensure any secondary, tertiary or more distant interactors were relevant to dystrophin and not involved in competitive binding with dystrophin or in mutually exclusive complexes. Competing interactions were not included unless, as is the case with caveolin-3 which competes with dystrophin for the same binding site of beta-dystroglycan, the protein has been observed to physically associate with dystrophin (Song et al., 1996; Sotgia et al., 2000) in which case an alternative dashed line was used to represent this interaction. Many suggested dystrophin physical interactors found during database searches, that were found not to be direct interactors, were added during this phase. Additional closely associated proteins candidates were found using interactome browsers such GeneMANIA (Warde-Farley et al., 2010), Reactome (Croft et al., 2014; Milacic et al., 2012) and CellWhere (L. Zhu et al., 2015) (which makes use of Mentha (Calderone, Castagnoli, &

Cesareni, 2013)). CellWhere was additionally used for assisting with protein localisation for the final visual interactome, along with the Gene Ontology's Cellular Compartment (Ashburner et al., 2000; The Gene Ontology Consortium, 2014) and the literature. Literature mining and The Human Protein Atlas (Pontén, Jirström, & Uhlen, 2008) were used to determine protein interaction relevancy in the context of skeletal muscle.

Expression in skeletal muscle was determined through evidence presented in previously published literature and/or the Human Protein Atlas database (Pontén et al., 2008). In a similar fashion, protein cellular localisations during association with dystrophin was determined through previously published literature, CellWhere and Gene Ontology cellular compartment terms (Ashburner et al., 2000; The Gene Ontology Consortium, 2014). Examples of eliminated interactions include the binding of the nuclear-located LMNA to the Dp71 dystrophin isoform (Villarreal-Silva et al., 2011), the brain specific expression of gamma-1-syntrophin and the non-DAPC interacting *in vivo* gamma-2-syntrophin (Alessi et al., 2006; Piluso et al., 2000). Caution was exercised with interactions based solely on predicted or high-throughput data without significant follow up evidence however Y2H experiments documenting direct interactions of dystrophin with DISC1 and alpha-catenin were subsequently included due to other closely related interactions.

Interactome visualisation

The interactome was visualised using Cytoscape (Version 3.2.1) (Shannon et al., 2003). Web visualisation using Cytoscape Web (Version 0.8) (Lopes et al., 2010), available at 'http://sys-myo.rhcloud.com/dystrophin-interactome'.

Results

271 dystrophin interactors identified through QUICK strategy

Stably immortalised human-derived myotubes were cultured in the presence (labelled) or absence (unlabelled) of heavy isotope amino acids, and unlabelled cells were treated using siRNA to knock-down expression of dystrophin (see Materials and Methods; 'Cell line maintenance and labelling with stable isotopes' section for more details and supplementary Figure 8F for blot analysis). Immunoprecipitation of dystrophin followed by mass spectrometry identified binding partners that were at higher levels in the labelled vs. unlabelled samples, whereas the levels of proteins that bound non-specifically to the antibody were unchanged. This method had the advantages of not requiring protein tagging or overexpression, not being prone to false positive or false negative results and in being systematic.

In order to minimise the risk of secondary effects of dystrophin knockdown on the proteome, we determined the earliest myotube differentiation time point (day 5) at which levels of dystrophin became strongly detectable (relative to a late differentiation time point (day 10)) (supplementary Figure 8A), and we confirmed by transcriptomic analysis that knockdown did not affect the myogenic program or the capacity to form myotubes (supplementary Figure 9A, B).

We optimized (1) the lysis buffer strength to preserve the majority of the protein interactions with dystrophin (supplementary Figure 8B); (2) the immunoprecipitation using two antibodies - one directed against the C-terminal of dystrophin (Dys-2) and one against spectrin repeat 16 of the rod domain (MANDYS102) - in order to maintain pull-down of complexes that feature binding at an antibody recognition site on dystrophin (supplementary Figure 8C); and (3) the elution buffer, as myosin heavy chain is a recurrent contaminant of any immunoprecipitation performed in muscle samples (supplementary Figure 8D, E). Efficient knockdown of dystrophin was confirmed (supplementary Figure 8F). A single immunoprecipitation elution mass spectrometry run (without any controls to eliminate unspecific binding) identified more than 3000 proteins (data not shown).

Our mass spectrometry analysis incorporated four runs, each comprising of heavy-labelled cells and light-labelled cells as described in supplementary Figure 10, and resulted in the identification of 3332 proteins. Taking into account only proteins that were identified by at

least two peptides in one of the forward runs and in the reverse run and for which all fold change values were positive (representing a consistent dystrophin interaction), and removing potential contaminants such as proteins that were only identified by site or were identified from the reverse decoy database were removed, we identified 271 proteins interacting with dystrophin (Supplementary Table 2).

Identification of 20 high confidence physical interactors

Each of the 271 potential binding partners identified above was assigned a rank based on the average fold change, with the highest fold change (4.30) being given rank 1 and the lowest fold change (0.01) being given rank 271 (Figure 18A). To obtain a baseline above which high-confidence interactors were ascribed, the linear regression of rank against fold change from ranks 80 to 220 was used ($R^2 = 0.995$) (Figure 18B-D), resulting in the identification of 18 high-confidence dystrophin interacting proteins. This set of 18 proteins includes the known dystrophin-binding proteins alpha-dystrobrevin and beta-1-syntrophin. A complete list of these 18 proteins and their fold changes can be found in Table 3. Dystrophin itself is pulled down and also passed these criteria (as would be expected). A further four high-confidence proteins were identified at consistently higher levels in non-knockdown samples, two of which had been disregarded by the standard analysis workflow due to having zero background levels in one or more of the knockdown samples (Table 4, further details in materials and methods). Two of these four proteins, alpha-dystrobrevin and beta-2-syntrophin are well established dystrophin interactors. Dystrophin and tensin - an integrin binding protein (McCleverty, Lin, & Liddington, 2007) - have actin as a common binding partner (Lo, Janmey, Hartwig, & Chen, 1994). Tensin-1 was previously shown to form a strong interaction with the integrin complex through the integrin beta tail and focal adhesion, which may suggest a physical interaction between dystrophin, tensin and the focal adhesion complex. The fourth protein, myeloid-associated differentiation marker (MYADM) has not previously been identified to interact with dystrophin in skeletal muscle cells. MYADM has been studied in smooth muscle cells (L. Sun et al., 2016) and endothelial cells (J. F. Aranda et al., 2013) or cancer cell lines (J. F. Aranda et al., 2011).

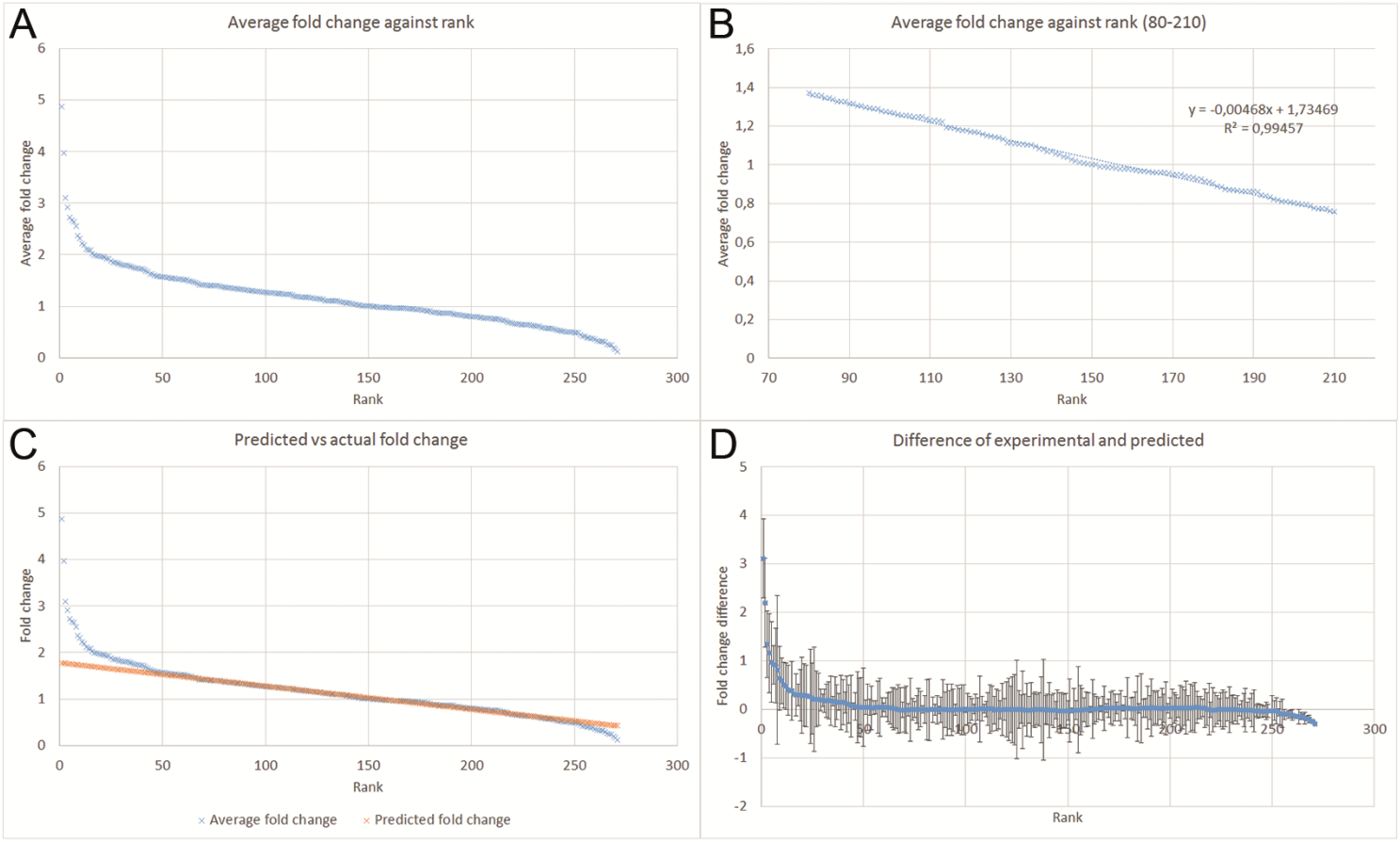


Figure 18. Identification of high-confidence physical interactors of dystrophin. The process was used to determine a higher confidence list of proteins from a list of 271 proteins which had consistent ratios indicative of a dystrophin interaction. 18 proteins were determined to be high confidence dystrophin interactors. **(A)** Average fold changes of the 271 proteins that consistently associated with dystrophin across runs. **(B)** Linear correlation of proteins ranked 80 to 210. **(C)** Predicted fold change (orange) for each rank value calculated based upon the linear correlation obtained. **(D)** The difference between the actual and the predicted fold change against rank with standard error for each protein calculated to determine proteins significantly higher than their predicted score. Standard errors were calculated with n=2/3/4.

Table 3. 18 high-confidence proteins identified to be physically interacting with dystrophin as determined through rank based fold change predictions. The 18 proteins include DAPC proteins beta-1-syzntrophin and alpha-dystroglycan. Dystrophin (bold) also based the thresholds implemented and is included in the table for reference. Average fold changes and standard errors are based upon all valid ratios obtained from the mass spectrometry data (n=2/3/4). Difference minus standard error was required to be above zero to be included in the set of high confidence proteins. Proteins 39S ribosomal protein L12, mitochondrial (MRPL12) and SUMO-activating enzyme subunit 1 (SAE1) (shown in italics) were later removed from the list of interacting proteins due to likelihood of false positives due to localisations within the mitochondria and nucleus respectively. Beta-2-syntrophin and tensin-1 were determined to be dystrophin interactors through alternative analysis (Table 4) and were subsequently included in the high confidence list.

Protein names	Gene names	Average fold change	Predicted non-interacting fold change (based on rank)	Difference (actual – predicted)	Standard error	Difference - standard error
Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2	3.10	1.72	1.38	0.92	0.46
CD97 antigen	CD97	2.22	1.68	0.54	0.47	0.07
Dystrophin	**DMD**	**3.97**	**1.72**	**2.25**	**0.81**	**1.43**
Alpha-dystrobrevin	DTNA	2.91	1.71	1.20	0.69	0.51
LIM domain only protein 7	LMO7	1.72	1.55	0.18	0.17	0.00
39S ribosomal protein L12, mitochondrial	*MRPL12*	*1.92*	*1.62*	*0.30*	*0.04*	*0.27*
Matrix-remodeling-associated protein 7	MXRA7	1.35	1.34	0.01	0.00	0.01
Myeloid-associated differentiation marker	MYADM	2.64	1.70	0.94	0.39	0.55
NACHT, LRR and PYD domains-containing protein 13	NLRP13	4.88	1.73	3.15	1.24	1.92
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1	PLCD1	2.19	1.68	0.51	0.47	0.04
26S proteasome non-ATPase regulatory subunit 9	PSMD9	1.80	1.59	0.22	0.13	0.09
Protein S100-A7	S100A7	2.73	1.71	1.02	0.82	0.20
SUMO-activating enzyme subunit 1	*SAE1*	*1.75*	*1.56*	*0.19*	*0.10*	*0.10*
SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL	1.97	1.64	0.33	0.22	0.11
SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	2.67	1.70	0.96	0.84	0.13
Beta-1-syntrophin	SNTB1	2.55	1.69	0.85	0.76	0.09
Sorcin	SRI	1.60	1.52	0.09	0.07	0.02
Thioredoxin domain-containing protein 12	TXNDC12	2.09	1.66	0.42	0.03	0.40
Vacuolar protein sorting-associated protein 28 homolog	VPS28	1.99	1.65	0.33	0.02	0.32

Table 4. Physical interactors of dystrophin identified through the disabling of the re-quantify option of MaxQuant. The mass spectrometry analysis was rerun with the re-quantify feature disabled in order to allow for the determination of proteins that were consistently and exclusively present in the dystrophin pull down sample, displaying no background binding in the dystrophin absent samples. Four were determined to be interacting with dystrophin through this method. Alpha-dystrobrevin and myeloid-associated differentiation marker were previously identified as high confidence interactors, beta-2-syntrophin and tensin-1 were previously identified in the list of low confidence interactors. As a result of this analysis, beta-2-syntrophin and tensin-1 were added to the list of high confidence interactors. Peptide numbers in each of the runs for each protein with the re-quantify featured enabled and disabled are displayed in order to demonstrate agreement between the two analysis methods.

Protein ID	Protein name	Gene name	Number of peptides identified				Re-quantify enabled?
			Run 1	Run 2	Run 3	Reverse	
Q13425	Beta-2-syntrophin	SNTB2	5	7	9	12	No
Q13425	Beta-2-syntrophin	SNTB2	5	7	9	12	Yes
M0QZ28	Dystrobrevin alpha	DTNA	7	9	12	8	No
Q9Y4J8	Dystrobrevin alpha	DTNA	8	9	13	7	No
M0QZ28	Dystrobrevin alpha	DTNA	6	8	11	7	Yes
Q9Y4J8	Dystrobrevin alpha	DTNA	8	10	13	7	Yes
C9JZL8	Myeloid-associated differentiation marker	MYADM	3	3	2	2	No
C9JZL8	Myeloid-associated differentiation marker	MYADM	3	3	2	2	Yes
E9PGF5	Tensin-1	TNS1	31	20	10	13	No
E9PGF5	Tensin-1	TNS1	30	20	10	13	Yes

Enrichment analysis suggests a link between dystrophin and vesicle trafficking

To search for shared characteristics the set of 20 high-confidence interactors was tested for enrichment of Gene Ontology (GO) Biological Processes, Cellular Components, and Molecular Functions, using Enrichr (E. Y. Chen et al., 2013; Kuleshov et al., 2016). Results for cellular compartment terms are shown in Table 5. The 20 high-confidence interactors returned strong enrichments of multiple cell junction terms due to proteins CD97 antigen, LIM domain only protein 7, beta-1-syntrophin, tensin-1 and protein S100-A7. Links to junctions were similarly represented within the direct interacting proteins. A notable enrichment was found within the extracellular vesicular exosome term (p-values < 0.05). Vacuolar protein sorting-associated protein 28 homolog (VPS28) and sorcin (SRI) represented endosomal and vesicle membrane terms respectively. Representations of the most common cellular compartment locations for

these proteins were carried out using CellWhere (L. Zhu et al., 2015) (Figure 19) confirming a high proportion of proteins have been annotated to junctions or exosomal locations.

Table 5. Enrichment analysis of experimental and curated gene set Cellular Compartment GO term ranks. Separate enrichments were carried out used our high confidence protein set (referred to as 'physical') and the direct interactors of dystrophin from previously published data (referred to as 'direct'). Selected terms of interest are shown, in particular the increased representation of vesicle and focal adhesion related term results.

GO term	Rank physical	Rank direct	Gene physical	Gene direct	Physical p-value	Direct p-value
focal adhesion (GO:0005925)	1	9	CD97;LMO7;SNTB1; TNS1;S100A7	ACTN2;DAG1;SNTB1; SNTB2;PLEC	4.72E-05	1.53E-04
cell-substrate adherens junction (GO:0005924)	2	10	CD97;LMO7;SNTB1; TNS1;S100A7	ACTN2;DAG1;SNTB1; SNTB2;PLEC	5.11E-05	1.65E-04
cell-substrate junction (GO:0030055)	3	11	CD97;LMO7;SNTB1; TNS1;S100A7	ACTN2;DAG1;SNTB1; SNTB2;PLEC	5.38E-05	1.74E-04
adherens junction (GO:0005912)	4	7	CD97;LMO7;SNTB1; TNS1;S100A7	SYNM;ACTN2;DAG1; SNTB1;SNTB2;PLEC	9.11E-05	2.29E-05
anchoring junction (GO:0070161)	5	8	CD97;LMO7;SNTB1; TNS1;S100A7	SYNM;ACTN2;DAG1; SNTB1;SNTB2;PLEC	1.07E-04	2.77E-05
extracellular vesicular exosome (GO:0070062)	6	37	CD97;SH3BGRL; MYADM; SH3BGRL3;SRI; BAIAP2;PLCD1; VPS28;S100A7	ACTA1;KRT19; TUBA1A;ACTN2; DAG1;CALM1; SNTB2;PLEC	1.81E-03	3.72E-02
ESCRT complex (GO:0036452)	7	-	VPS28	-	1.68E-02	-
cytosol (GO:0005829)	9	14	PSMD9;SRI; BAIAP2;SAE1; PLCD1;VPS28; S100A7	ACTA1;TUBA1A; ACTN2;TNNT1; TNNC1;NDE1;ANK2; NOS1;CALM1; CTNNAL1.PLEC	2.11E-02	3.78E-03
ESCRT I complex (GO:0000813)	21	-	VPS28	-	1.20E-02	-
secretory granule membrane (GO:0030667)	22	-	SRI	-	6.44E-02	-
endosomal part (GO:0044440)	36	-	VPS28	-	2.17E-01	-
endosome membrane (GO:0010008)	39	-	VPS28	-	1.93E-01	-
cytoplasmic vesicle membrane (GO:0030659)	41	-	SRI	-	2.44E-01	-
vesicle membrane (GO:0012506)	42	-	SRI	-	2.63E-01	-
cytoplasmic vesicle part (GO:0044433)	43	80	SRI	ACTN2	3.60E-01	4.27E-01

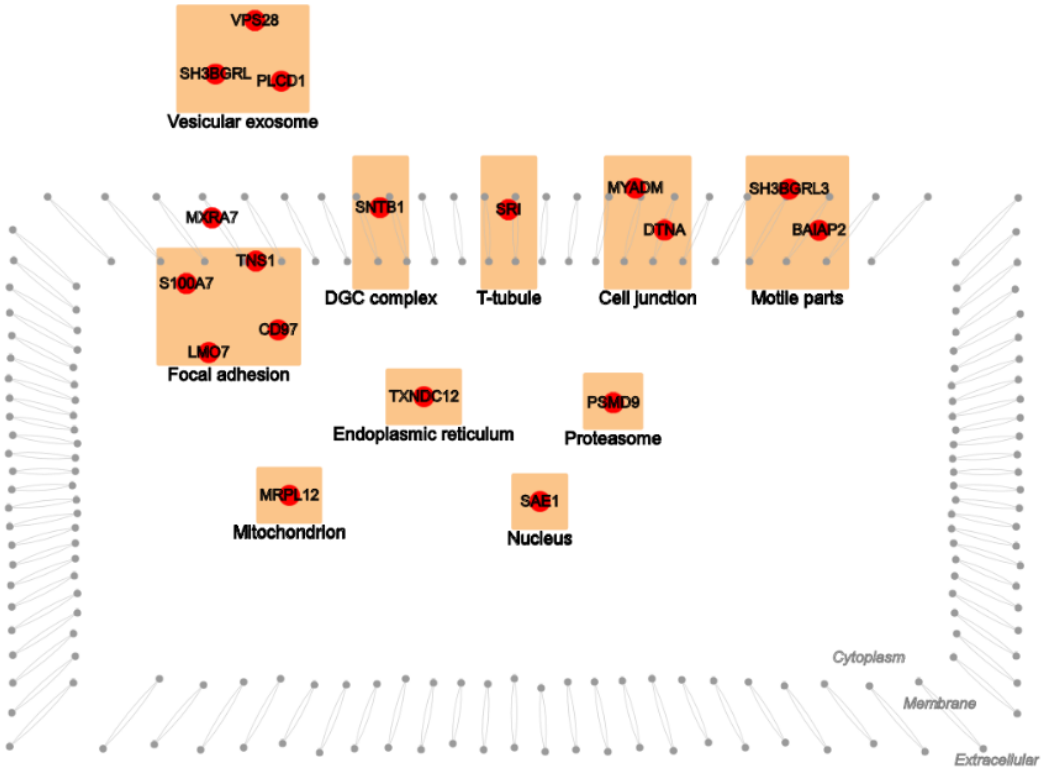


Figure 19. CellWhere representation of the probable localisations of the 18 physically interacting proteins. The potential localisations of the 18 proteins listed in Table 3 were obtained using Uniprot and GO and the muscle flavor. MXRA7 is identified as a membrane protein. NLRP13 did not return any localisation information and so is not present in this figure. Cellular compartments were similar to those suggested in the enrichment analysis in Table 5. MRP12 and SAE1 display locations within the mitochondria and nucleus leading to the possibility of not likely interacting with dystrophin *in vivo*. Subsequent review of known localisations could not establish plausible localisations with dystrophin and so the two proteins were subsequently removed from the high confidence data set.

With the gene enrichment analysis supporting a dystrophin involvement with exosome secretion we searched the list of 271 proteins that mass spectrometry data found to be consistently associated with dystrophin. A cavin family member muscle-related coiled-coil protein (MURC / Cavin-4) immunoprecipitated with dystrophin across the four runs with an average fold change of 1.19 (supplementary Table 2). EH domain-containing protein 2 (EHD2), was also consistently observed in the four runs and associated with dystrophin with an average fold change of 1.25 in our experiment. Interestingly, both MURC and EHD2 both synergistically act with protein polymerase I and transcript release factor (PTRF / cavin-4) in caveolae recruitment, regulation (Johnson et al., 2012) and endocytosis (Morén et al., 2012; Parton & del Pozo, 2013), the latter being a pathway overlapping with exosome biogenesis (Colombo, Raposo, & Théry, 2014).

Interactive web tool for exploration of the dystrophin interactome, incorporating literature and web resource survey

To integrate our new data with previous observations, we surveyed the existing literature on specific dystrophin-binding partners, and we gathered and curated data on known partners from functional association and pathway resources: IntAct (Kerrien et al., 2012), GeneMANIA (Warde-Farley et al., 2010), CellWhere (L. Zhu et al., 2015), Mentha (Calderone et al., 2013), and Reactome (Croft et al., 2014; Milacic et al., 2012). As well as direct 'primary' binding associations, we included many secondary and tertiary protein interactors where they are implicated in known functional roles of dystrophin, such as the contractile apparatus and transmembrane channels.

The resulting network is available in an interactive format online at 'http://sys-myo.rhcloud.com/dystrophin-interactome' (represented in Figure 20). The dystrophin protein is split into four segments, representing the calponin homology domain, spectrin repeats, cysteine rich domain and C-terminal allowing for interaction known to interact at specific regions on dystrophin to be represented. The network includes dystrophins direct interactors and more distant interactors which physically interact with dystrophin. Interactions between dystrophin interactions which occur while in complex with dystrophin are also included. The interactome incorporates 72 dystrophin interactors and displays dystrophin's links to numerous transmembrane proteins involved in molecule and ion transportation and cell signalling and adhesion, caveolae and exosomal related proteins, linker proteins and cytoskeletal proteins. Simplified small networks of the dystrophin associated protein complex and direct interactors are also available on the same website.

The website is intended to allow for a simple analysis of dystrophin and its interactors. Each protein node is movable and contains a proteins full name, gene ID, UniProt accession and reference to a relevant paper demonstrating the interaction with dystrophin. Each edge references the direct interaction represented. The website also offers download of the complete .cys version of the website which allows for complete modification within Cytoscape. This additionally offers the ability to add or remove nodes depending on the researcher interests. Transcriptomic or protein expression data can be overlaid onto the network in order to easily visualise the interactors of dystrophin that have been impacted.

This tool will provide an easily accessible platform to examine dystrophin's currently known direct and physical interactors. Proteins of interest can be investigated to determine how they may be interacting with dystrophin if an interaction with a dystrophin associated protein of the network is known. The interactome will be particularly useful in the analysis of dystrophic-related transcriptomic data, allowing to easily determine the affected proteins that associate with dystrophin and their relationship to dystrophin and its roles. Newly identified interactors can be added to the network and used to aid in determining how they likely interact with dystrophin. Some proteins, such as some of the newly identified physical interactors of dystrophin do not have any clear links to dystrophin through direct interactions. This suggests as-yet unknown interactions or interactors that are relevant to dystrophin that would connect these proteins, highlighting that dystrophin still contains interactions that have yet to be elucidated.

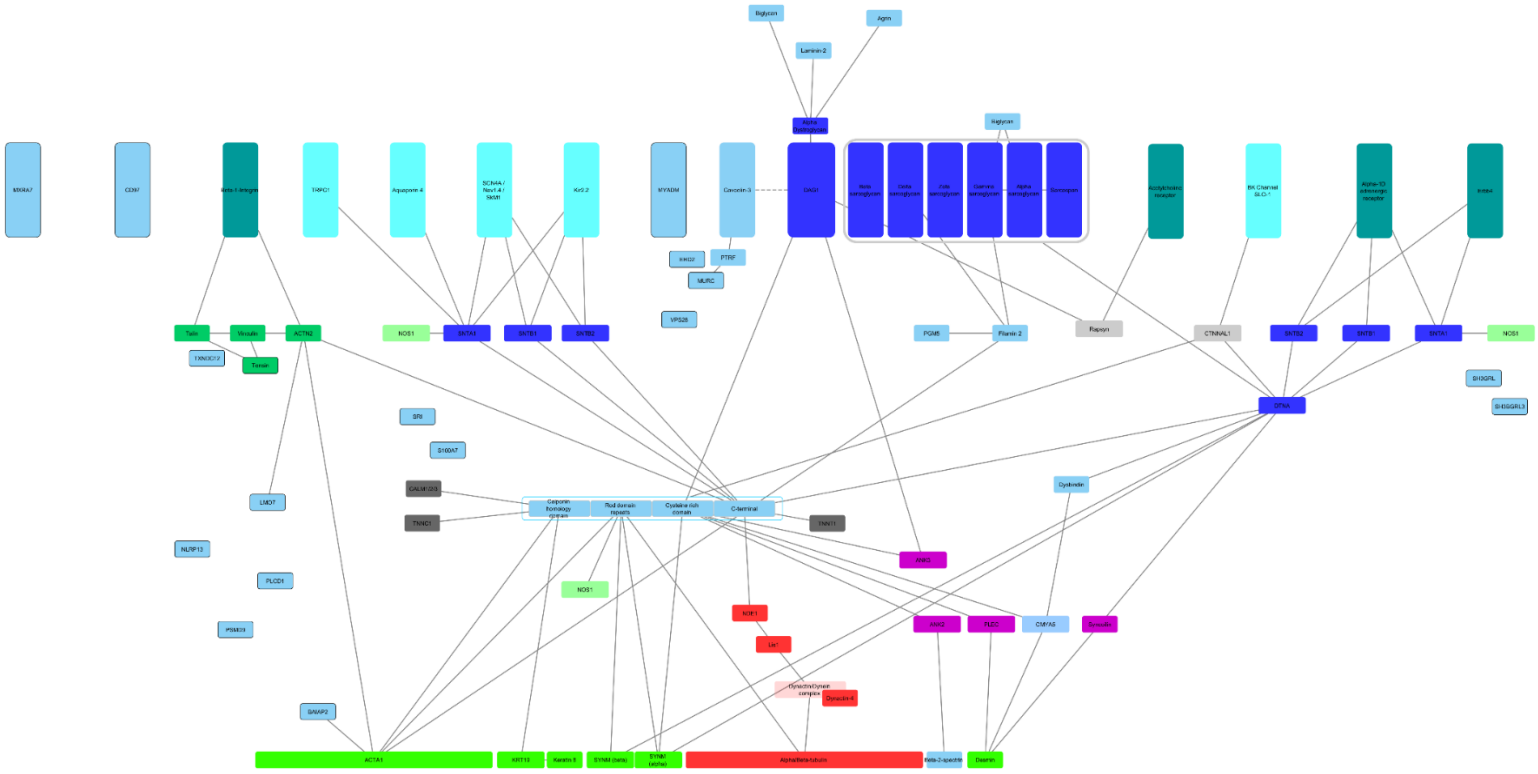

Biglycan
Agrin
Laminin-2
Alpha-dystroglycan
Biglycan
MKRN7
CD97
Beta-1-integrin
TAPC1
Aquaporin 4
SCN4A / Nav1.4 / SkM1
Kir2.2
MYADM
Caveolin-3
DAG1
Beta-sarcoglycan
Beta-sarcoglycan
Zeta-sarcoglycan
Gamma-sarcoglycan
Alpha-sarcoglycan
Sarcospan
Acetylcholine receptor
BK Channel SLO-1
Alpha-1D adrenergic receptor
Integrin
Talin
Vinculin
ACTN2
NOS1
SNTA1
SNTB1
SNTB2
PGM5
Filamin 2
Rapsyn
CTNNAL1
SNTB2
SNTB1
SNTA1
NOS1
TANK2/3
Tensin
SH3GRL
SH3GRL3
SRI
S100A7
CALM1/2/3
LMO7
TNNC1
Calponin homology domain
Rod domain repeats
Cysteine-rich domain
C-terminal
TNNI1
Dystrobrevin
NLRP13
PLCD1
ANK3
NOS1
PSMD9
KIF1
Lis1
ANK2
PLEC
CMYA5
Periaxin
Dynactin/dynein complex
Dynactin-4
SNAP2
ACTA1
KRT13
Keratin 8
SYNM (beta)
SYNM (alpha)
Alpha/Beta-tubulin
Beta-2-spectrin
Desmin

Figure 20. Dystrophin interactome of direct and physical interactions constructed based upon previously published data and our experimental data. The interactome is built from the previously established direct interactors to subsequently include physically interacting dystrophin partners (including the newly identified high-confidence physical interactors). Relevant interactions between interactors in the network are included if they are believed to occur while in association with dystrophin. Enzymes which act upon dystrophin have not been included. Proteins are placed in accordance to their cellular location. The taller nodes in a line across the top of the interactome represent membrane located proteins (transmembrane proteins and channels). The proteins above these taller nodes represent proteins located extracellularly, all proteins below the taller nodes are intracellular. The line of proteins across the bottom of the interactome represent proteins that make up the cytoskeleton. Stronger protein complexes or proteins cooperating in similar roles have been clustered closer together (such as the dystrophin physical interactors that are part of the integrin focal adhesion complex labelled in dark green). The interactome is also available at 'sys-myo.rhcloud.com/dystrophin-interactome' where it can also be downloaded in .cys format in order to allow full interactively and modification as desired.

Discussion

Dystrophin, localized under the sarcolemma, binds to F-actin through its N-terminal domain and to the alpha/beta-dystroglycan transmembrane protein complex through its C-Terminal domain. Thanks to these physical interactions, dystrophin creates a link between the contractile apparatus and the extracellular matrix network. We built an *in silico* dystrophin network, establishing the previously identified dystrophin interactors within the context of other previous interactors and their location within the cell. We used a systematic experimental approach in order to identify new interactors. This approach allowed us to unbiasedly identify previously unknown dystrophin interactors that may otherwise not have been investigated. Our proteomic analysis identified 20 confident partners of dystrophin and begins to unravel the interaction of dystrophin with a new cell compartment: secreted vesicles including exosomes. It is important to note that these new interactions have been determined through a skeletal muscle cell line; and may not represent interactions of dystrophin that are present in cardiac or smooth muscle or other tissues. The new interactors have been incorporated to the dystrophin interactome and the link between dystrophins and secreted vesicle has been strengthened. This is available as a separate network with the additional proteins highlighted in yellow. All new interactions are based on supporting direct interaction data. No direct links with dystrophin have been added. This new, larger dystrophin interactome is represented in Figure 20 and also available at 'http://sys-myo.rhcloud.com/dystrophin-interactome'. This network, combining in silico and in vitro approaches, is available online and can be used as tool to determine strategy therapeutic for BMD or DMD.

Comparison with previous findings

Of the 21 direct interactors established previously for skeletal muscle (Figure 21 and supplementary Table 3), 14 were identified in our mass spectrometry data of which 7 consistently displayed greater pull down in the presence of dystrophin, displayed in Table 6 along with relevant DAPC and closely related proteins. These 7 confirmed direct interactors included alpha-dystrobrevin and alpha-, beta-1- and beta-2-syntrophin, proteins known to bind to the first helix of the coiled-coil domain of dystrophin's C-terminal (Sadoulet-Puccio et al., 1997; Yang et al., 1995). Of note, alpha-dystrobrevin is well known to strongly bind to syncoilin (Newey et al., 2001), suggesting that syncoilin presence in our pull down experiment is likely due to this interaction rather than being a direct interaction to dystrophin. Skeletal muscle is known to express all three isoforms of syntrophin mentioned (SNTA, SNTB1 and SNTB2), with SNTA being the predominant muscle isoform (Peters, Adams, & Froehner, 1997). Syntrophins interact with dystrophin in pairs with syntrophin B1 binding site being located precisely at amino acid sequence 3447-3481 (Yang et al., 1995). Although, syntrophin pairs of SNTA with either SNTB1 or SNTB2 are most common (Peters et al., 1997), experiments observing SNTB1 and SNTB2 pull down along with dystrophin have also been reported (Camp et al., 2015) and correlates with our data. SNTB2 is usually expressed at low levels and generally localises to the neuromuscular junction however it is also weakly detectable at the sarcolemma (Kramarcy & Sealock, 2000; Peters, Kramarcy, Sealock, & Froehner, 1994). SNTA levels have previously been found to decrease as a result of dystrophin deficiency while SNTB1 and SNTB2 levels increase.

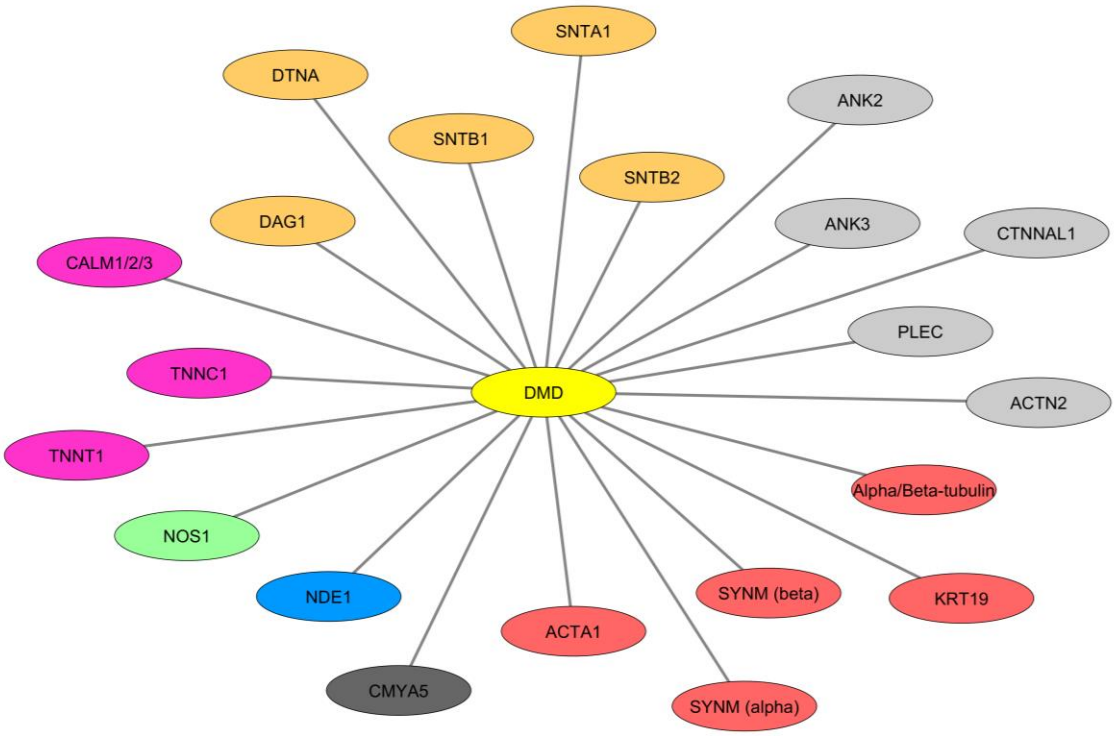


Figure 21. Direct interaction of full length dystrophin within myotubes. Literature searches were guided by interaction databases of both experimental (in vivo and in vitro) and computational data across all species, with particular attention given to manually curated databases. Databases were accessed either through their own interaction viewer software, through the third party interaction viewer Cytoscape (Shannon et al., 2003) or as a text based table. Each resulting interaction was independently verified or discarded based upon a review of the interactions paper of origin listed by the interaction database and an independent journal search. Literature searches independent of interactions listed by databases were performed within PubMed and Google/Google Scholar using various keywords combinations including (but not limited to): dystrophin, dmd, Duchenne, interactome, direct, immunoprecipitation, Y2H and protein names of interest. Relevant high-throughput data sets were also independently searched however further evidence was required to be incorporated into this network. Direct interactions involving enzymes that acted upon dystrophin (such as proteases ubiquitin and calpain I and II or kinases par-1b and CaM kinase) are not included in the network. Colours correlate to those used in supplementary Table 3 and are based upon protein roles. Yellow: DAPC, Green: enzyme, Purple: regulatory protein, Pink: cytoskeleton, Grey: linker, Blue: microtubules.

Table 6. DAPC members and other select known interactors of dystrophin that were identified as potential physical interactors in our mass spectrometry analysis. Beta-dystrobrevin, commonly not considered to be expressed in the skeletal muscle was identified. All fold changes are calculated with positive values representing increased pull down in the presence of dystrophin. Runs were no ratio could be calculated have no fold change value.

Uniprot	Protein name	Gene name	Total number of proteins	Number of peptides identified				Fold Change				Average Fold Change
				Run 1	Run 2	Run 3	Reverse	Run 1	Run 2	Run 3	Reverse	
P11532	Dystrophin	DMD	15	208	221	227	206	2.24	5.40	5.32	2.92	3.97
H0Y304	Dystrophin	DMD	3	74	77	82	77	0.58	2.20	3.03	0.85	1.67
Q9Y4J8	Alpha-dystrobrevin	DTNA	6	8	10	13	7	0.32	2.11	3.43	1.85	1.93
M0QZ28	Alpha-dystrobrevin	DTNA	3	6	8	11	7	3.67	3.72	3.40	0.86	2.91
Q13424	Alpha-1-syntrophin	SNTA1	1	0	1	1	2	-	1.26	-	-	1.26
Q13884	Beta-1-syntrophin	SNTB1	2	21	18	26	21	1.30	2.26	4.77	1.88	2.55
Q13425	Beta-2-syntrophin	SNTB2	5	5	7	9	12	0.21	1.47	2.01	3.74	1.86
Q16585	Beta-sarcoglycan	SGCB	1	2	2	0	1	0.78	1.63	-	1.31	1.24
Q13326	Gamma-sarcoglycan	SGCG	1	2	1	0	2	-	-	-	3.26	3.26
Q9H7C4	Syncoilin	SYNC	3	8	7	6	6	0.57	1.34	1.93	1.19	1.26
F8WBR5	Calmodulin	CALM2	10	0	0	3	1	-	-	0.44	0.28	0.36
Q15149	Plectin	PLEC	5	208	224	219	234	2.28	0.89	0.39	0.36	0.98
K7EMS3	Keratin-19	KRT19	1	2	5	8	5	-	-	3.11	-	3.11
E7EVB6	Beta-dystrobrevin	DTNB	7	4	7	11	7	-	2.11	2.81	0.98	1.97

Other proteins that are known to directly interact with dystrophin were not shown to consistently interact with dystrophin across the runs or were not detected. As different interactors will be present at earlier or later differentiation time points (Min et al., 2010), during different cell states (such as stress) (Yih, Peck, & Lee, 2002) and can vary between human and murine cells or cell lines (Zheng-Bradley et al., 2010) it is not surprising that not all potential interactors were positively identified. For instance, nNOS expression is altered when cells are

exposed to oxidative stress conditions (Kar, Kellogg, & Roman, 2015). Problems with the release of transmembrane proteins while maintaining interaction is a common issue which is could be present in these data, with beta- and gamma-sarcoglycan positively associating with dystrophin at low levels, and the detection of delta-sarcoglycan and beta-dystroglycan at similarly low levels (each with just one protein identified). In addition, other interactors such as ankyrin-2 and -3 are known to have solubility problems (Ayalon et al., 2008), suggesting that hydrophobic proteins are difficult to pull down with dystrophin and may be absent of the soluble interactome.

MYADM, a new partner for dystrophin

MYADM is new partner identified in our study. This protein has been described as a membrane raft protein involved in membrane condensation, cell spreading and migration through Rac1 (J. F. Aranda et al., 2011). All of its currently known physical interactors have come through high-throughput screens and includes interaction with the transmembrane protein TGF-beta receptor type-2 located in the caveolae (Bizet et al., 2011; Huttlin et al., 2015). Knowing that dystrophin is described to be involved in membrane maintenance during muscle contraction, a partner such as MYADM would make sense to support this function.

Dystrophin is linked to secreted vesicles

Dystrophin has not been widely implicated in vesicular exosome trafficking in the literature, although dystrophin deficiency is known to alter the secretion profile (Hathout et al., 2014, 2015). Dystrophin deficient mdx myotubes have previously been shown to have an increase in secreted LAMP1 positive vesicles (Duguez et al., 2013) implicating the lack of dystrophin to subsequently cause a dysregulation in vesicle trafficking with subsequent dystrophin expression able to normalise vesicle trafficking to some extent. Our curated dystrophin interactome provides a potential link to some form of trafficking through the dystrophin-NDE1-LIS1 complex involvement with dynein/dynactin microtubule intracellular transport.

VPS28, a component of the ESCRT-1 complex along with TSG101 and HCRP1, which is involved in vesicle trafficking (Bache et al., 2004) and colocalise on LAMP1 positive vesicles. VPS28 binding to TSG101 inhibits the degradation of TSG101 and thus regulate the complex expression (McDonald & Martin-Serrano, 2008).

Dystrophin interaction with caveolae

PTRF was initially found to immunoprecipitate with dystrophin in heart, although this was not found in skeletal muscle (Johnson et al., 2012) however subsequent PTRF immunoprecipitation in skeletal muscle did return low levels of dystrophin and strong associations to beta-dystroglycan (Johnson et al., 2013). Dystrophin absence (mdx mice) resulted in a decreased level of PTRF and a subsequent decrease of PTRF immunoprecipitated with beta-dystroglycan. A direct interactor has not yet been identified.

PTRF and the muscle specific MURC (Way & Parton, 1996) colocalise at the caveolae, interacting with the muscle specific caveolin-3 in skeletal muscle. Both MURC and PTRF play roles in localisation of caveolin-3 and formation of caveolae. PTRF knockdown has been shown to result in the complete absence of other cavin member and caveolin-3 from muscle sarcolemma (Hansen et al., 2013) and resulting loss of caveolae (Liu et al., 2008). Caveolin-3 has strong links to muscular dystrophy, and been shown to associate with the DAPC (McNally et al., 1998). The increase of caveolin-3 in dystrophin deficient cells indicates that dystrophin is not required for the requirement or localisation of PTRF or MURC.

Perhaps of note is the presence of beta-2-syntrophin in Johnson's and our experimental data in which a cavin member co-immunoprecipitated. Beta-2-syntrophin was not observed in the skeletal muscle of Johnson's experiment in which PTRF was not immunoprecipitated with dystrophin (Johnson et al., 2012).

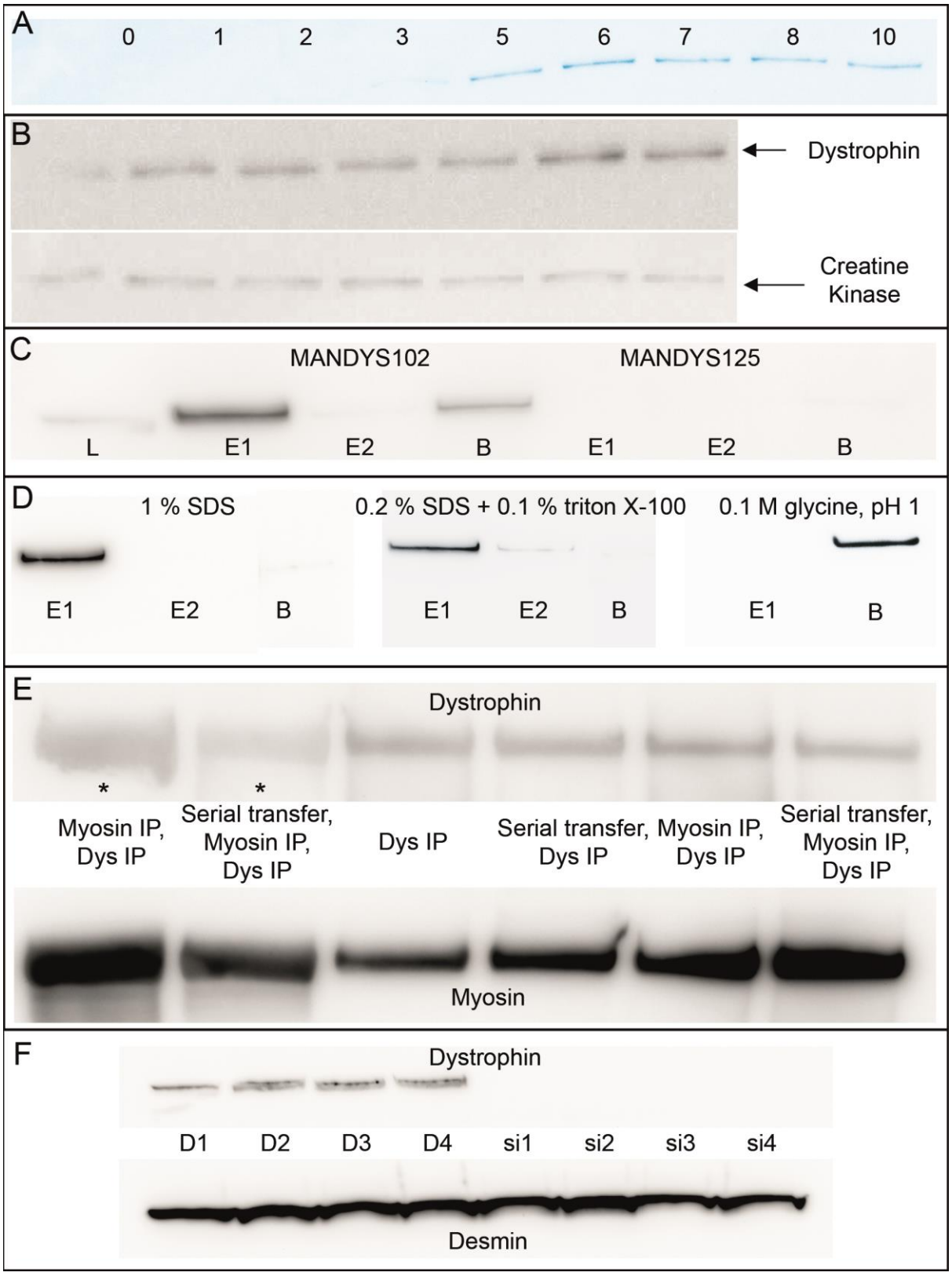
EH domain-containing protein 2 (EHD2), a protein associating with dystrophin with an average fold change of 1.25 in our experiment, also requires PTRF expression for correct localisation. EHD2 has roles in the regulation of caveolae endocytosis dynamics (Morén et al., 2012; Parton & del Pozo, 2013) and downregulation GLUT4 vesicle formation. EHD2 absence hence causes an increase of caveolin-3 at the sarcolemma, with a less stable, more dynamic formation of caveolin-3 resulting from the decrease in regulation usually provided by EHD2.

Conclusions

Our study combined an innovative experimental proteomic approach with a review of literature and *in silico* resources to establish a systematic interactome of the known binding partners of the dystrophin protein. Based upon our experimental data we identified 20 physically

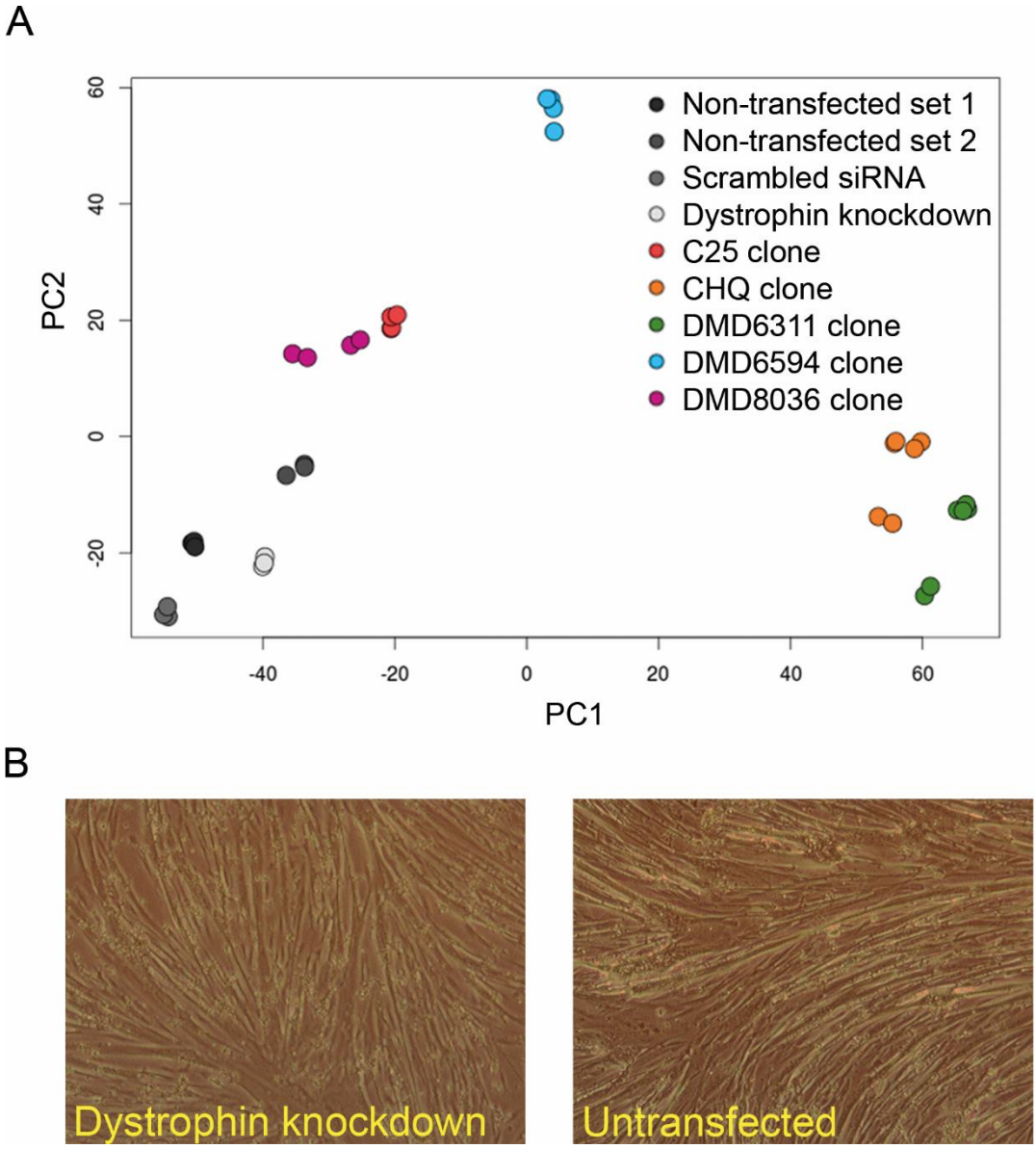
interacting partners of dystrophin. The interactome is available online at ‘http://sys-myo.rhcloud.com/dystrophin-interactome’, and includes the complexes commonly linked to dystrophin function, including the contractile apparatus, intermediate filaments, microtubules and intermediate filaments as well as the links to vesicle trafficking, the caveolae and focal adhesion complexes, that are supported by our proteomic data. This online tool acts as an accessible platform to understand the interactions of dystrophin and aid in the comprehension of related interaction data. Given that each compartment within our interactome has been shown to be disrupted to some extent in dystrophic cells, it may also provide possible therapeutic targets for dystrophin related diseases.

Supplementary data

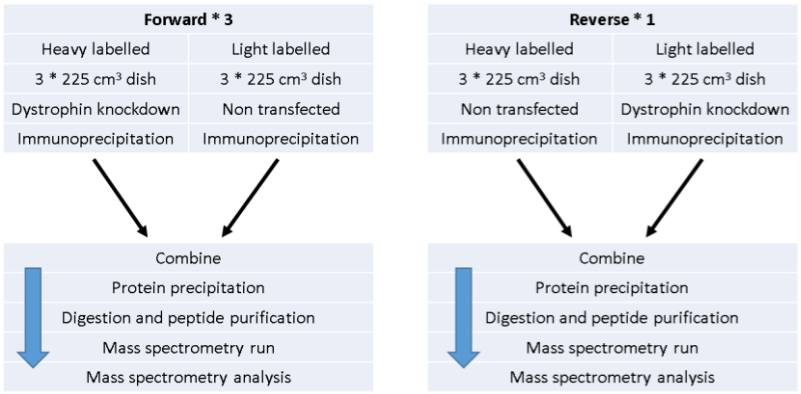


Supplementary Figure 8. Optimisation of the conditions for the culture and immunoprecipitation protocols. A. Expression of dystrophin after different days of differentiation in our skeletal muscle immortalised cell line. The number above each lane represents days of differentiation before harvesting. Dystrophin detected using

the Dys-2 antibody which recognises the dystrophin C-terminal. Each lane was loaded with equal amount of protein lysate (20 µg). Day five was determined to be an early time point at which high amounts of dystrophin expression are observed. **B. Lysis buffers of various strengths were tested in order to find the weakest buffer that would extract the most dystrophin.** A sample of some of the buffers tested are shown. Lane 1; 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 20 mM Tris buffer, Lane 2; 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 20 mM Tris buffer, Lane 3; 0.25% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris buffer, Lane 4; 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris buffer, Lane 5; 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris buffer, Lane 6; 2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS, 20 mM Tris buffer and Lane 7; 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris buffer. **C. Dystrophin immunoprecipitation using various antibodies was tested.** A successful immunoprecipitation using MANDYS102 and an unsuccessful attempt using MANDYS125 are shown. Of the antibodies tested (DYS2, MANDYS102, MANDYS107, MANDYS125 and MANEX50), DYS2, targeting the C-terminal, and MANDYS102, targeting R16 of the rod domain, immunoprecipitated the highest levels of dystrophin. **D. Elution buffers were tested to increase dystrophin elution during immunoprecipitation.** Of the elution buffers tested (glycine 0.1M at pH 2/3/10, glycine 0.2M at pH 1, KCl 3M, urea 8M, 0.2 % SDS + 0.1 % Triton X-100 and 1 % SDS of which 1 % SDS, 0.2 % + 0.1 % Triton X-100 and 0.1 M glycine, pH 1 are shown). 1 % SDS proved the most effective. E1 and E2 represent first and second elution, B represent elution through heating the beads and indicates the protein left uneluted from the previous elutions. **E. Attempts to deplete myosin were investigated through myosin immunoprecipitation and serial transfer of the lysate prior to dystrophin immunoprecipitation.** Lanes 1 and 2 are from elution of the myosin immunoprecipitation, representing dystrophin and myosin that has been removed from the lysates. Lanes 3-6 are subsequent elutions after dystrophin immunoprecipitation. **F. Confirmation of successful and efficient dystrophin knockdown.** Cell cultures were treated with siDystrophin at time points 2 days before differentiation, on the day of differentiation and two days after differentiation (as described in Materials and Methods). Treated cell lysates (labelled si1-4) were compared against untreated cell lysates (labelled D1-4).



Supplementary Figure 9. Analysis of cells after dystrophin knockdown. A. Transcriptomic analysis of dystrophin expressing and dystrophin knocked down cells. Data sets were compared against healthy and DMD cell lines in order to establish if the knockdown of dystrophin had a significant effect on the cells. **B. Myotubes imaged at day 5 differentiation, transfected with 50 nm siDys pool and RNAiMAX reagent (left) and no transfection (right).** Transfection was carried out at two days before differentiation, upon differentiation and at day 2 differentiation. No differences are seen between the transfected and non-transfected myotubes.



Supplementary Figure 10. Schematic of the method plan used to obtain mass spectrometry data of the three forward and one reverse runs.

Supplementary table 2. All proteins with a consistently positive fold change across at least two of the four runs. 271 protein results were determined to be potential dystrophin interactors with 265 unique gene IDS. Proteins DMD, DTNA, FXR1, GLS returned two unique protein IDs while LMO7 returned three unique IDs.

Gene name	Av. fold change	Gene name	Av. fold change	Gene name	Av. fold change	Gene name	Av. fold change	Gene name	Av. fold change
AASS	1,17	DUSP27	0,88	KLHL40	1,55	PGRMC2	1,57	SNX18	0,89
ABCD3	1,01	DYNLT1	0,77	LAMTOR3	1,57	PKP1	1,30	SNX6	1,08
ABHD10	1,05	ECM29	0,76	LARS	0,52	PLBD2	1,19	SORT1	1,04
ACAA1	1,09	EEF1E1	0,42	LDHA	0,65	PLCD1	2,19	SPCS3	1,17
ACO1	1,77	EHD2	1,25	LIMA1	0,58	PLEC	0,98	SPNS1	0,59
AHNAK2	0,94	EIF3A	1,01	LMO7	1,72	POM121C	1,59	SPTLC1	0,86
AIMP2	0,69	EIF3C	0,56	LMO7	1,52	PREPL	1,68	SQRDL	1,82
AK3	0,25	EIF3F	0,53	LMO7	1,54	PRKAG1	0,75	SRI	1,60
AKAP2	1,29	EIF3H	1,37	LSM14A	0,51	PRRC2C	0,61	SRM	0,80
ALDH18A1	0,81	EIF4A1	1,51	MAN2B1	0,95	PSMC5	0,42	SRP54	1,41
AP3B1	0,39	ELOVL1	0,64	MAPK1IP1L	0,96	PSMD10	1,30	SRP72	0,34
APOOL	0,86	EPRS	0,62	MBNL1	0,88	PSMD9	1,80	SRPK3	0,54
ARF5	1,14	EPS15L1	0,98	MBOAT7	1,28	PSMF1	1,34	SSBP1	0,19
ARHGAP1	1,26	ERBB2IP	1,96	MLIP	2,01	PTPN12	1,47	SSR4	0,32
ARHGEF10	1,07	ESYT2	0,80	MPI	1,51	PURA	1,88	STOM	0,97
ARHGEF2	0,62	EXOC8	0,25	MPV17	1,46	RAB21	0,64	STT3A	0,96
ARL1	0,91	F13A1	1,84	MRPL12	1,92	RAD50	0,38	STX7	1,54
ARSA	1,49	FAM129B	0,93	MRPL15	1,29	RAF1	0,86	STXBP3	0,66
ATAD3A	1,11	FBL	0,87	MRPL49	1,03	RALA	1,01	SUGP2	0,73
ATL3	1,42	FBXO30	0,98	MRPS10	0,12	RAPH1	0,50	SULT1A1	1,18
ATP6V0D2	0,16	FERMT3	1,33	MRPS17	0,45	RARS	0,30	SUN2	0,92
ATXN2L	0,84	FKBP9	1,49	MRPS18A	0,96	RBM4	1,74	SYNC	1,26
BAIAP2	3,10	FUBP3	1,32	MRPS22	0,98	RCN2	0,51	SYNE1	0,97
BID	1,82	FXR1	1,85	MRPS25	1,44	RCN3	2,11	TAGLN	1,58
CALM2	0,36	FXR1	0,63	MRPS35	0,53	RDH5	1,40	TAX1BP3	1,80
CAPN1	0,96	GAMT	1,25	MSI2	1,06	RER1	1,13	TBCD	2,32
CBFB	0,97	GET4	1,95	MT-ATP6	0,78	RFTN1	0,74	TMEM11	1,26
CD276	2,37	GLS	1,33	MTCH1	0,99	RMDN3	1,63	TMEM173	1,09
CD47	0,49	GLS	0,57	MT-CO1	0,99	RPA2	0,73	TMEM33	1,27
CD97	2,22	GMPS	0,93	MT-ND1	0,76	RPL4	0,81	TNPO2	0,27
CDC42BPB	1,40	GOLPH3	1,15	MT-ND4	1,72	RPL6	1,15	TNS1	1,59
CDIPT	1,36	GRAMD3	0,87	MURC	1,19	RRAS	1,17	TNS3	1,42
CDK18	0,95	GTPBP1	0,63	MXRA7	1,35	RWDD1	0,77	TOR1AIP1	0,75
CDK5	1,33	HACD2	1,53	MYADM	2,64	S100A11	1,10	TRAP1	1,31
CDK5RAP3	1,62	HGS	1,80	MYO1C	1,10	S100A7	2,73	TRIM23	1,02
CHCHD3	1,23	HIBCH	1,31	NAPG	1,42	SAE1	1,75	TRIM72	1,41
CNOT1	1,74	HLA-C	0,77	NCLN	0,72	SCCPDH	0,98	TRIO	1,18
CNPY2	0,79	HNRNPF	0,86	NDUFA10	0,58	SEC24D	0,80	TRMT1L	0,90
COASY	1,11	HNRNPH2	0,99	NDUFA6	0,40	SERPINF1	0,87	TSFM	0,93
COMMD8	0,32	HP1BP3	1,00	NDUFS7	0,84	SERPINH1	0,75	TXNDC12	2,09
COPS6	1,41	HSD17B12	1,40	NLRP13	4,88	SGCB	1,24	UBQLN1	1,11
COX7A2	0,65	IAH1	1,27	NUBP2	0,82	SH3BGRL	1,97	UFM1	1,23
CUL4A	0,96	IARS	0,48	NXF1	0,58	SH3BGRL3	2,67	UTRN	0,94
DCTN3	1,18	INF2	0,83	OGDH	1,38	SKP1	0,65	VPS13C	1,78
DDAH2	1,23	INPP1	1,07	OSBPL8	0,86	SLC12A2	1,40	VPS28	1,99
DDX21	0,79	IPO4	1,36	OSTF1	1,22	SLC20A2	1,54	VPS45	1,53
DERL1	0,96	IPO8	0,67	PAK2	1,36	SLC25A3	0,38	YARS2	1,27
DHCR7	1,04	IPO9	0,64	PCNA	0,32	SLC2A1	1,35	YIPF5	0,69
DMD	3,97	IVD	2,09	PDCD6IP	0,66	SLC33A1	1,11	YTHDF1	1,39
DMD	1,67	JAGN1	1,15	PDLIM1	0,99	SLC39A7	1,11	ZBTB20	1,98
DNAJC3	0,49	KANK2	1,19	PDLIM2	1,32	SLC9A3R1	0,95	ZYX	1,00
DR1	0,82	KCMF1	1,16	PDLIM3	0,81	SMPX	0,59		
DTNA	2,91	KIAA1033	0,50	PDLIM5	1,56	SNAP23	1,53		
DTNA	1,93	KIF13A	1,71	PDLIM7	1,25	SNTB1	2,55		
DTNB	1,97	KLC1	0,50	PGM3	1,25	SNTB2	1,86		

Supplementary table 3. Known dystrophin interactors, role and binding domains on dystrophin.

Uniprot	Gene name	Full name	Function	Binding domains on dystrophin
Q14118	DAG1	Beta-dystroglycan	DAPC	Hinge 4 / Cysteine rich domain
Q9Y4J8	DTNA	Alpha-dystrobrevin	DAPC	Coiled-coil motif (C-terminal)
Q13424	SNTA1	Alpha-1-syntrophin	DAPC	Coiled-coil motif (C-terminal)
Q13884	SNTB1	Beta-1-syntrophin	DAPC	Coiled-coil motif (C-terminal)
Q13425	SNTB2	Beta-2-syntrophin	DAPC	Coiled-coil motif (C-terminal)
P29475	NOS1	nNOS	Enzyme	Rod domain repeats 16 and 17
P63316	TNNC1	Troponin C	Regulatory protein	Calponin homology domain
P13805	TNNT1	Troponin T	Regulatory protein	C-terminal
P62158	CALM1/2/3	Calmodulin	Regulatory protein	Calponin homology domain
O15061	SYNM	Alpha-synemin	Cytoskeleton	Rod domain repeat 3 and Cysteine rich domain
O15061	SYNM	Beta-synemin	Cytoskeleton	Rod domain repeat 3
P08727	KRT19	Keratin 19	Cytoskeleton	Calponin homology domain
P68133	ACTA1	F-actin	Cytoskeleton	Calponin homology domain, Rod domain repeats 11 to 17
-----	-----	Alpha/Beta tubulin dimer	Cytoskeleton	Rod domain repeats 20 to 23
Q01484	ANK2	Ankyrin-2	Linker	Cysteine rich domain
Q12955	ANK3	Ankyrin-3	Linker	Cysteine rich domain
P35609	ACTN2	Alpha-actinin-2	Linker	C-terminal
Q15149	PLEC	Plectin 1f	Linker	Cysteine rich domain
Q9UBT7	CTNNAL1	Alpha-catulin	Linker	Unknown
Q9NXR1	NDE1	Nuclear distribution protein nudE homolog 1	Microtubule development / mitosis	C-terminal domain
Q8N3K9	CMYA5	Myospryn	Not well defined	Cysteine rich domain / C-terminal

Discussion

Immortalised cell lines

Immortalised cell lines provide a convenient and relatively low cost of carrying out studies of a single cell type. They are able to provide a cell platform which is isolated from the wider tissue and organism effects and complications, allowing for an analyses of a uniform cell population. Primary cells suffer from senescence after a relatively small number of divisions, making their use problematic. The immortalisation procedure allows us to use the cell line without senescence taking effect, however the disruption of one or more core cell pathways can inevitably have effects upon other pathways. As cell lines must attempt to embody a true representation of the original cell it is important that minimal deviation from the primary cells is observed. While a single gene transfection would present a decreased potential for cellular disruption, hTERT alone is not sufficient to fully protect against cellular senescence in myoblasts (K. M. Lee, Choi, & Ouellette, 2004): a second gene (cdk4) is required in order to adequately stop telomerase shortening (C.-H. Zhu et al., 2007). Thus it was important in this work to verify the downstream effects of these two transfections on the systems processes of immortalized myoblasts.

In regard to the hTERT/cdk4 immortalisation process, several important pathways had been previously investigated, however it was necessary to carry out a genomic wide analysis in order to check the overall state of the cell lines and a comprehensive analysis of the cell pathways. The heavy involvement of cdk4 in many cellular processes and the many possible secondary effects from cell cycle impairment makes it difficult to predict which pathways may have been altered. As a result, while it is possible to individually check important or closely related pathways, to fully verify that the cell line accurately portrays the original primary cells, a wide analysis is required.

Through our transcriptome-wide analysis, we have demonstrated that this particular method of cell line immortalisation was successful in creating a cell line representative of its cell of origin. The small variations between the primary and immortalised cell lines were insignificant compared to the differences between the cells originating from different patients (or different sex, age and tissue). Importantly, myogenic and non-myogenic cells remained in distinct populations, confirming that the immortalised myogenic cell lines are useful representations of

muscle cells. The immortalisation was found not to affect any other cellular processes. We also confirmed that the process was successful at protecting the cell line from the effects of senescence. A primary cell line which had undergone further divisions since the immortalisation process had become significantly distinct from the immortalised line.

Having confirmed the immortalisation process across multiple myogenic and non-myogenic cell lines in differentiated and undifferentiated states, we can not only confirm the validity of the cell lines tested, but also the use of all cell lines generated through the same process (at least within human healthy and DMD lines), allowing the cell lines to be used as representations of their origin cells with some confidence.

Aside from the healthy and DMD tested cell lines that were the subject of our experiment, cell lines of facioscapulohumeral muscular dystrophy (FSHD), oculopharyngeal muscular dystrophy (OPMD), congenital muscular dystrophy (CMD) and limb-girdle muscular dystrophy type 2B (LGMD2B) are among the other immortalised cell lines developed through the same hTERT/cdk4 transduction method (Mamchaoui et al., 2011). The cell lines developed are of great use for experiments that require large numbers of cells and/or optimisation of procedures prior to analysis, as was the case with our experiment in Chapter 4 and will be of use in the myology field as well as other fields. Immortalisation through a number of different techniques have been carried out on a wide variety of cells, with the goal of halting cellular senescence with minimal effects on other cellular processes (Perez-Reyes, Halbert, Smith, Benditt, & McDougall, 1992; Philippi et al., 2012; Ramboer et al., 2014; Shiomi et al., 2011; Stadler et al., 2011). Thousands of cell lines have been developed, many of which can be obtained from cell banks. Reagents and kits are also available for researchers to develop their own cell lines (Robin et al., 2015).

Due to their wide use, it becomes important to ensure the results generated through the utilisation of cell lines are accurate. Problems regarding the cell backgrounds and contamination of cell lines have been previously reported, affecting thousands of research papers (Hughes, Marshall, Reid, Parkes, & Gelber, 2007; Neimark, 2015). When a cell line is not adequately validated, this can cast doubt on the validity of the research carried out using the line. Contamination, by which a foreign cell is introduced, grown and isolated from the initial culture, is also a potential problem with immortalised cell lines that have not been carefully investigated. It can also result from the introduction of a foreign immortalised cell into the immortalised culture. Mislabelling of cells has the same effect. As such, most cell

databases will now validate cell lines before being accepted. Methods such as DNA barcoding are performed to confirm the identity of cells (Cooper et al., 2007; Hebert, Cywinska, Ball, & deWaard, 2003; Hebert, Ratnasingham, & deWaard, 2003).

Through our analysis we have validated the hTERT/cdk4 immortalised cell lines of DMD and healthy patients and in doing so, validated the hTERT/cdk4 technique for developing immortalised cell lines of the DMD and healthy cells, providing added confidence to the use of the other disease variant cell lines developed through the same approach. We have carried out a methodology to validate the cell lines which can be followed for a similar validation approach of other cell lines. While the scale of this analysis may not be feasible for cell lines immortalised for single experiments, cell lines that are generated with the intent of being used across a larger number of experiments would certainly benefit from this analysis design.

Finding new interactors using the QUICK approach

Due to the critical role of dystrophin, as demonstrated by the severity of pathology in patients with dystrophin mutation or absence, dystrophin and its interaction partners have been heavily researched. Identifying its interactors not only increases our understanding of the protein and its roles within the cell, but also offers insight into dystrophin related diseases and can aid the research towards developing effective treatments. Despite the amount of previous research, a systematic approach to identifying its interactors has never been previously undertaken. Previous identification of interactors has relied heavily on common techniques such as immunoprecipitations and yeast two-hybrid, both of which are susceptible to false positive results (Brückner et al., 2009; Fletcher, Bowden, & Marrion, 2003; Solstad, Bull, Breivik, & Fladmark, 2008). Additionally, western blots are commonly used to identify proteins present in the immunoprecipitation elution, requiring prior knowledge of the suspected protein interactor (Lal, Haynes, & Gorospe, 2005; Thompson, 2004).

Through the use of the QUICK method we were able to identify a set of highly confident physically interacting proteins. This set of proteins, identified through mass spectrometry, screening peptides against the thousands of reviewed human protein sequences available from Uniprot, allowed for identification of interacting proteins without any previous knowledge bias. While immunoprecipitations using no control (3000+ proteins identified by a mass spectrometry analysis of a dystrophin immunoprecipitation) or using a beads without antibody immunoprecipitation control, run the risk of identifying numerous false positive interacting

proteins, our protein data set is largely protected from this through the knockdown of dystrophin in the control immunoprecipitation. We used a control which was identical to the original sample, but with dystrophin knockdown. Due to the importance of dystrophin in multiple cell functions, dystrophin knockdown introduces the possibility of affecting the cell and causing secondary effects (Seno et al., 2010). We chose an early differentiation time point at which dystrophin is expressed in order to limit the knock-on effects of dystrophin absence. A transcriptomic analysis of the dystrophin expressing and knockdown samples confirmed that the two maintain similar profiles. Using dystrophin expressing and dystrophin knockdown lysates from the same cell line, each subjected to the same immunoprecipitation conditions (including beads and antibody), we were able to obtain a control closely resembling the dystrophin expressing elution sample. Culturing the dystrophin expressing and dystrophin absent cells in different SILAC medium allowed for the combination of the two different lysates to directly compare the two samples in the same mass spectrometry run.

Ideally, SILAC samples should be combined at the earliest stage possible. Once combined, any procedure performed will subject the different isotope labelled samples to exactly the same conditions and biases. Performing a procedure on the uncombined samples will inevitably subject the samples to slight differences. A drawback to the use of immunoprecipitation and SILAC to identify protein interactors is the need for the immunoprecipitation to be performed prior to mixing of the SILAC proteins (due to the dynamic nature of protein interactions, mixing prior to immunoprecipitation would result in the exchange of the differently labelled isotopes between interactions).

Each mass spectrometry analysis was carried out using a dystrophin knockdown and dystrophin expressing immunoprecipitation elution. An elution was preformed using sample lysate on four separate immunoprecipitations (two immunoprecipitations using DYS-2 antibody and two immunoprecipitations using MANDYS102 antibody) which were then combined and subsequently combined with the four immunoprecipitations of the alternatively labelled sample elution. Immunoprecipitations using the different antibodies and the differently isotope labelled dystrophin expressing and dystrophin absent lysates were performed in random batches in order to eliminate the potential differences caused by the procedure before the mixing of the differently labelled samples, with the small random differences distributed across a number of samples.

From our mass spectrometry data (which consisted of three ‘forward’ runs and one ‘reverse’ run) we implemented thresholds (described in detail in Chapter 4) which were necessary to elucidate the highest confidence interacting partners. Requiring all the runs of a given protein to display higher pulldown in the dystrophin expressing elution isotope (indicating a possible dystrophin interaction) resulted in 271 proteins, all of which are identified from our experiment as possible interactors of dystrophin. In order to identify the interactors that we could determine with high confidence we calculated the standard error for each protein across the runs. This produced a set of 18 physical interactors of dystrophin.

Within the high confidence protein set were the dystrophin-associated protein complex members alpha-dystrobrevin and beta-1-syntrophin. Within the 251 proteins excluded from the high confidence set were known interactors of dystrophin such as beta-dystrobrevin, plectin, beta-sarcoglycan and beta-2-syntrophin. The original set of 271 proteins were required to have valid ratios in at least two of the four runs. With an increased number of runs it is likely we would have been able to identify a greater number of the set of large protein interactors with high confidence.

A weakness of using ratio values for cut offs is that the ratio magnitude is not representative of the strength of the dystrophin interaction. Ratios values are affected by the amount of total protein compared to the amount of protein that is interacting with dystrophin. As mass spectrometry analysis relies upon the detection of amino acids, larger proteins that result in a higher number of peptides after trypsinisation are affected in a similar way. This inherently makes proteins that are more highly expressed, or proteins with higher unspecific background binding, susceptible to lower fold change values. High amounts of unspecific binding and a small amount of specific binding reduces an interactor’s fold change value making the protein more difficult to confidently identify as an interactor. The highly expressed dystrophin interactor plectin was detected across all four runs with a consistent ratio indicating a dystrophin interaction however its high detection levels in both the isotope samples results in a ratio which does not allow it to be classified as high confidence by the criteria we have used.

On the other extreme, proteins with very low expression may only be detected in a small number of the total runs of an experiment. This could result in the protein being eliminated from the data, which was the case in our experiment if only one of the four runs returned a fold change value even if this value was indicative of a dystrophin interaction. Despite the

experimental design to increase sensitivity as much as possible many proteins where removed due to this threshold requirement.

Failure to produce a valid ratio value may also lead to an interactor being overlooked. To return a ratio value a protein must be detected in both the heavy and light isotopes of a single run. If a protein does not display any unspecific binding, which is an increased possibility of lowly expressed proteins, no valid ratio can be assigned. MaxQuant, the program that was used for our raw mass spectrometry data analysis, is able to combat this through the 're-quantify' feature that rescans areas where missing isotope amino acids would be. In doing this the program will often detect signals, commonly background noise, which can then be used to (artificially, in the case of noise detection) produce a ratio value. If the originally detected protein had a low intensity, the noise signal detected can mask a possible interactor that had been exclusively pulled down in the dystrophin expressing elution. Running the whole analysis without the re-quantify feature generated results in which a large number of proteins had many non-valid ratio values, making this an unfeasible way to carry out the overall analysis.

We ran a secondary analysis of our data with all the same parameters as the main run but without the re-quantify option enabled. In doing so we identified four proteins that were detected solely in the dystrophin expressing elution of each of the four runs; DTNA, MYADM, SNTB2 and TNS1, of which DTNA and SNTB2 are both well-established binding partners of dystrophin and fellow members of the dystrophin associated protein complex. DTNA and MYADM were present in our set of 18 high confidence interactors. SNTB2 and TNS1 were both present in the larger 271 protein data set. These two additionally identified dystrophin interactors where subsequently included in our high confidence protein data set.

Through the building of the *in silico* interactome we also became interested in the possible link between dystrophin and the caveolae due to the previously identified interaction with PTRF. While PTRF was not identified in our protein set, cavin family member MURC and the closely associated EHD2 were in our 271 protein data set. The previous data suggesting a physical interaction of dystrophin with the cavin family (PTRF) in heart combined with our data suggesting a possible interaction (with MURC) in skeletal muscle indicates that the dystrophin interaction with cavin may be present within both tissues.

Solubility issues and weak interactions (which can be difficult to maintain through normal immunoprecipitation methods) remain a limitation with this approach and will have inhibited

the identification of some dystrophin interactors (Jafferali et al., 2014; Kast & Klockenbusch, 2010). Membrane proteins have common solubility problems as they must be extracted from the membrane, however increasing the lysis buffer strength in order to release the proteins can result in disruption of protein-protein interactions. Dystrophin has many interactions with transmembrane proteins through both direct and indirect interactions. While the DAPC proteins syntrophins (beta-1 and beta-2, but not alpha) and dystrobrevins (alpha and beta) isoforms where identified in our dystrophin interactor data sets, dystroglycans and sarcoglycans (with the exception of beta-sarcoglycan) were not identified as interactors.

Although several methods have been developed to investigate interactors of membrane proteins, high-throughput screening between membrane and non-membrane proteins are still limited (Hunke & Müller, 2012; Walian et al., 2012). Immunoprecipitation techniques are not well suited for these interactions and predominantly rely upon tagging approaches such as FRET and Y2H (Ivanusic, Eschricht, & Denner, 2014). As a result, the QUICK approach is limited in its ability to identify membrane proteins.

Although our experiment successfully eliminated many false positives that are prevalent in common immunoprecipitation methods, false positives were still possible. Some of the proteins identified through our analysis where identified as being unlikely to be expressed *in vivo*, and others may be present in our extended low confidence incorporating data set. Proteins that would not be present in the same cellular location as dystrophin *in vivo* (such as within the nucleus or mitochondria), once lysed from their cellular compartment, become free to interact with dystrophin and dystrophin interacting proteins. While these proteins were removed from our list of interacting proteins after being identified through mass spectrometry, optimization of conditions (through careful lysis buffer consideration) in order to reduce the non-cytoplasmic proteins could be advantageous. However, the reductions in lysis buffer strength required to achieve this would have an adverse effect on any true dystrophin interactions with the membrane proteins of the cellular membrane and the internal organelle membranes.

Dystrophin and the vesicle transport pathways

Dystrophin deficiency has been previously shown to affect the protein secretion profile as well as the types of exosomes secreted (Coenen-Stass et al., 2015; Duguez et al., 2013; Hathout et al., 2015). Dystrophin deficiency has many adverse effects on the cell through direct and indirect causes along with a decrease in the overall health of the cell (Blake, Weir, Newey, &

Davies, 2002; Straub & Campbell, 1997). It is therefore not always evident whether changes seen in dystrophin deficient cells are due to the disruption of a dystrophin interaction or dystrophin pathway disruption or a secondary downstream consequence. We have provided strong evidence for dystrophins involvement in the vesicle transport pathways through a physical interaction and identified MURC and MYADM as two proteins involved in dystrophins interaction with the vesicle transport pathway. The interaction with MURC is supported by a previous study which identified cavin family member PTRF as a dystrophin interacting protein (Johnson et al., 2012). EHD2, a protein closely associated to the cavin family members and the caveolae, was also likely to be interacting with dystrophin according to our experimental data.

Caveolin-3, a muscle specific caveolin that resides in the caveolae, is well known to be upregulated upon dystrophin knockdown (F Galbiati et al., 2000; Ferruccio Galbiati, Razani, & Lisanti, 2001; Repetto et al., 1999). The interaction of dystrophin with a fellow caveolae protein that has previously been implicated in caveolin regulation at the membrane could additionally implicate dystrophin in the regulation of caveolin. MURC was found to positively regulate the recruitment of caveolin-3 to the membrane (in cardiomyocytes) (Naito et al., 2015). MURC itself is dependent on caveolin-3 for its localisation at the membrane (Bastiani et al., 2009). The coiled-coil domain of MURC is involved in the localisation to the membrane, with the remaining sequence capable of caveolin binding (Naito et al., 2015).

The effect of DMD on the caveolae requires further investigation and in particular an analysis of the cavin family members may be beneficial. Cavin members form tissue specific complexes (Bastiani et al., 2009) and so the disruption of a dystrophin-cavin interaction could have different effects in the different tissues. This could also provide further insight into the caveolin-dystrophin relationship and ultimately contribute to an increased understanding of the mechanisms behind the secretion profile changes that result from dystrophin deficiency.

The medium from the mass spectrometry analysis presented in Chapter 4 has been subsequently used to isolate exosomes and microparticles (which are SILAC labelled). Heavy and light dystrophin knockdown and dystrophin expressing samples of the exosomes and microparticles were combined for mass spectrometry analysis. These results are currently being analysed however preliminary analysis displays a distinct difference between the composition of exosomes and microparticles in presence and absence of dystrophin, in line with dystrophin association with vesicle transport and the altered secretion previously seen.

Dystrophin and the focal adhesion complex

Dystrophin has many links to adhesion complexes (G Anastasi et al., 2003; Burkin et al., 2001; Cerna et al., 2006; Lakonishok, Muschler, & Horwitz, 1992; Rozo, Li, & Fan, 2016; Senter et al., 1993; T. Yoshida, Pan, Hanada, Iwata, & Shigekawa, 1998) and this was reflected in our experimental data with a direct interaction between dystrophin and alpha-actinin-2, which had previously been established (Hance et al., 1999). Alpha-actinin-2 is present along the Z disc where it is primarily involved in the crosslinking of actin filaments and binding between actin and integrin at the membrane (Otey et al., 1990; Roca-Cusachs et al., 2013; Sjöblom, Salmazo, & Djinović-Carugo, 2008). Dystrophin isoform Dp71 is present in an integrin complex in neurons which also features alpha-actinin (Cerna et al., 2006). Dp71 domains are present in the full length skeletal muscle dystrophin isoform and so it is likely that the full length dystrophin retains its ability to bind to the integrin complex. We identified a physical association between dystrophin and the integrin-mediated adhesion complex protein tensin. The relationship between the DAPC and the integrin focal adhesion complex is not well understood however providing additional evidence for a strong link between the two complexes may aid in future work investigating their association.

Future investigation into the interactors

Of the newly identified interactors of dystrophin we will attempt to determine if any of the proteins are associated by direct interaction, using protein ligation assays (PLA). Many of the proteins identified were able to be placed in our literature curated interactome of dystrophin's physical interactors and so are likely to be interacting through common interactors. However, some of the proteins were not able to be placed in the interactome with a connection to dystrophin and so are either binding directly or via one or more common binding partner(s) which is/are currently unknown. Previously data linking cavin member PTRF with dystrophin was originally determined to be heart specific (Johnson et al., 2012), however a cavin interaction with dystroglycan (the interaction was maintain in the presence and absence of dystrophin) was later found in skeletal muscle (Johnson et al., 2013). Beta-2-syntrophin was notably present in both the cardiac and skeletal muscle experiments along with dystroglycan. While our experiments did not identify a dystroglycan interaction (likely due to membrane solubility issues), beta-1-syntrophin was identified as in interactor, along with cavin member

MURC. Through PLA we will therefore investigate the possible direct interaction of MURC with beta-2-syntrophin and MURC with dystrophin.

Online dystrophin interactome

We have generated an online dystrophin interactome based upon dystrophin's interactions in human skeletal muscle comprised of data mined from previously published papers and online interaction databases alongside our newly identified interactors. The interactome is based upon the identified 21 direct interactions of full length dystrophin in skeletal muscle with additional secondary and more distantly related interactors which have been demonstrated to physically interact with dystrophin and are hence also of relevance to dystrophin's roles within the cell. The interactome is available at 'http://sys-myo.rhcloud.com/dystrophin-interactome'. This interactome will aid in the analysis of future dystrophin related research by facilitating virtual exploration of the dystrophin interaction network as an aid to explaining experimental observations. The interactome can be downloaded and subsequently edited with Cytoscape, allowing for full customisation by the user. This includes the addition or removal of proteins and protein interactions from the network and the overlay of a researcher's own data (such as gene and protein fold change values).

Such an interactome cannot be easily derived through purely computational approaches and simple database network building. Attempts to do this often result in complex 'hairball' networks which are not easily understandable and offer little useable information. When trying to understand the function of dystrophin, images similar to the one shown in Figure 22A are a useful common starting point. The great complexity and non-specificity of networks generated from interaction databases when searching the primary, secondary, tertiary and further interactors of dystrophin is rarely of use (Figure 22B). To more readily explore the roles of dystrophin within the cell, it is informative to consider only selected interactions from the secondary and tertiary (and sometimes more distant) shells of interaction.

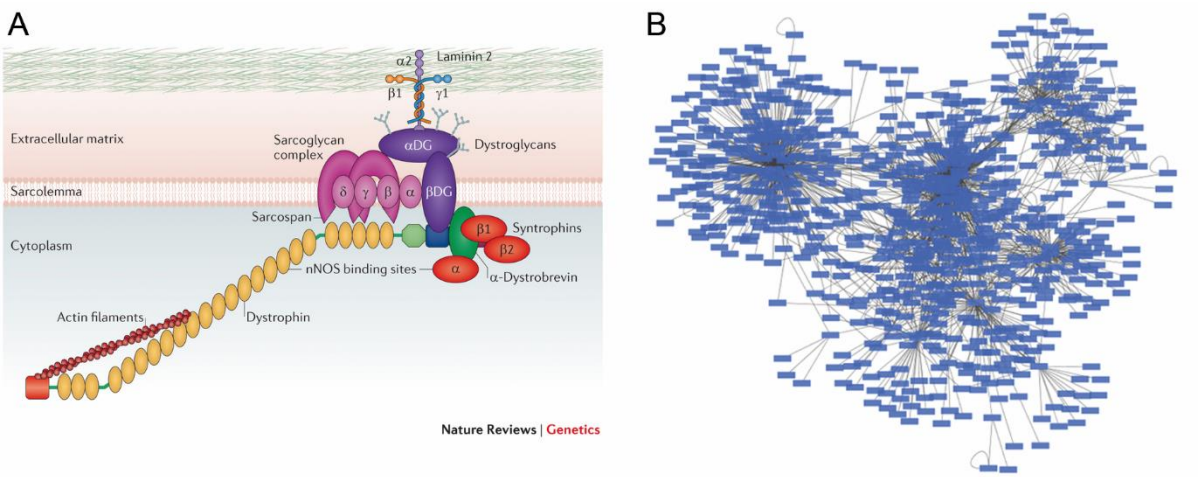


Figure 22. Different approaches to displaying protein interactions. A. A typical representation of dystrophin, the dystrophin-associated protein complex and other relevant proteins (Imaged derived from Fairclough et al., 2013 (Fairclough et al., 2013). Laminin and actin are often included due to their involvement in dystrophins most well defined role, providing a link between the actin cytoskeleton and the extracellular matrix to protect against mechanical stress. The interactions show only a small number of the total interactions relevant to dystrophin's roles. B. The interactions of full length human dystrophin (Uniprot accession P11532 only, P11532-5 corresponding to Dp71 excluded) generated using Cytoscape using the IMEX databases (IntAct, MINT, DIP, I2D-IMEx, UniProt, BHF-UCL, MolCon and InnateDB-IMEx). The resulting interaction network is hard to make sense of and contains many interactions that are not relevant to dystrophin. Many interactions that are relevant to dystrophin are not present due to the small section of databases used to generate this network.

The network generated in Figure 22B only contains two primary dystrophin interactions, disrupted in schizophrenia 1 protein (Q9NRI5, DISC1) and ankyrin-2 (Q01484, ANK2), displayed in Figure 23 (DISC1 and ANK2 highlighted in green, DMD highlighted in yellow).

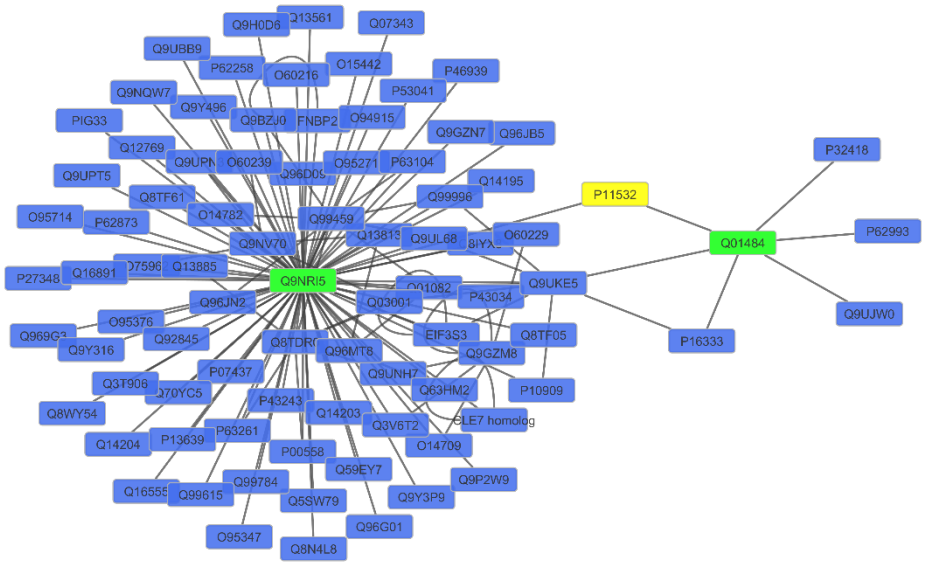


Figure 23. The primary and secondary interactors of dystrophin generated using Cytoscape using the IMEX databases (IntAct, MINT, DIP, I2D-IMEx, UniProt, BHF-UCL, MolCon and InnateDB-IMEx). Dystrophin (DMD), labelled P11532, is highlighted in yellow, primary interactors Disrupted in schizophrenia 1 protein (DISC1), labelled Q9NRI5, and Ankyrin-2 (ANK2), labelled Q01484, are highlighted in green. The network is derived from Figure 22B in which the same interaction databases were used to generate a network incorporating more distant interactions of dystrophin. From the previously complicated network, just two primary dystrophin interactors were responsible for the entire network.

DISC1, a 'hub protein', is responsible for 80 of the 84 secondary interacting proteins of dystrophin in this network and in effect becoming the dominant focus of the entire network. The resulting network is in no way representative of dystrophin's interactions or roles within the cell. Further compounding matters, the interaction between DISC1 and dystrophin has been reported in a single high throughput analysis in the brain (Camargo et al., 2007). DISC1 expression levels in skeletal muscle are very low, far less than in the brain and the majority of other tissues (Pontén et al., 2008). Thus it is important to have a carefully curated and comprehensive network that includes only the relevant dystrophin interaction data.

The high research interest in dystrophin has resulted in a mass of data which can be difficult to fully comprehend. Our newly created dystrophin interactome offers much of the accessibility and ease of understanding associated with simplified diagrams while incorporating the additional useful information hidden within databases and complicated networks. Maintaining the localisation organisation of the diagrams and carefully restricting the interactions are necessities to achieve this. The ability to easily add and remove information to and from the network adds additional network versatility.

Due to the large nature of the interactome created due to the many interactors of dystrophin and high involvement with many proteins in order to fulfil its many roles within the cell we created several smaller networks derived from the full interactome for additional simplicity. These smaller networks highlight the different proteins involved in different aspects of cell function in an attempt to further facilitate the understanding of dystrophin's interactors.

Identifying additional interactors

As interactors may change under different conditions, carrying out a similar experiment under altered conditions could be used to identify additional dystrophin interactors. One possibility of this would be to allow an increased cell maturity (through increasing the number of days after inducing differentiation at which the cells are harvested). However, this would come with additional considerations into the knock-on effects of dystrophin absence in the control cell cultures: downstream effects of the loss of dystrophin following knockdown would have longer to express themselves, increasing the probability of false positives where a downregulated gene masquerades as a dystrophin-dependent pull-down. Different cell lines will vary in protein expression and interactions and so the same experiment in alternative cell lines may also provide a means of identifying additional interactors. As already mentioned, the membrane proteins which are responsible for a large number of interactions with dystrophin are problematic to identify via immunoprecipitation methods. Dystrophin would benefit from analysis through development of improved high-throughput methods to identify interactions between membrane and non-membranous proteins.

Similar experiments with the use of microdystrophin constructs could be useful to determine interactions localised to particular regions of dystrophin, or what effect the absence of a certain region has on dystrophin's interactions. There are two ways in which this could be done. The first would be to compare a dystrophin expressing cell line to a DMD cell line which has been transfected with a microdystrophin construct. This would allow the determination of dystrophin interactors that are not present in the microdystrophin construct. The use of two different cell lines is a considerable drawback to this approach. Protein expression and transcriptomic analysis of each cell line could be performed to try to identify the potential differences between the cell lines (for example, our previous transcriptomic analysis of cell lines in Chapter 3 found C25 and DMD8036 immortalised lines to display common features within myotubes in our PCA plot (Figure 14C)), however differences will always exist. The second method involves

comparing the DMD cell line transfected with the microdystrophin construct to the same cell line with no transfection, thereby studying the truncated dystrophin interactome against a dystrophin absent control. This would allow analysis of cells with identical backgrounds, as in our experiment. Similar methods replacing microdystrophin transfection with exon skipping strategies could be used to generate larger dystrophin proteins and proteins with relevance to the exon skipping field. These methods may be useful to understand truncated dystrophin functions that would follow successful AAV truncated dystrophin delivery and exon skipping gene therapy treatments.

Conclusion

We have successfully validated immortalised cell lines (hTERT and cdk4) derived from healthy individuals and DMD patients through a transcriptomic wide analysis of the primary and immortalised cells in both the undifferentiated and differentiated states. The immortalised cells were protected against the effects of senescence while maintaining similar characteristics to their primary cells and do not display alterations in other cellular pathways. The cell lines can be used with confidence to accurately represent their primary cells.

Using a cell line derived from the same immortalisation process, a proteomic approach identified 20 high confidence physically interacting. Notably, proteins associated with vesicle trafficking, the caveolae and focal adhesion complexes were present in these 20 proteins. The 20 experimentally determined proteins along with data mined from literature and *in silico* database searches were used to build a network of dystrophin's currently identified physical and direct interactions. This is available online at 'http://sys-myo.rhcloud.com/dystrophin-interactome' and aims to aid in the understanding of the currently known dystrophin interactions and the interpretation of future identified interactors. It may also be of assistance in finding new therapeutic targets for dystrophin related diseases.

As this is a systematic approach, proteins were identified based solely upon detected binding with dystrophin with no selection bias towards suspected binding partners. Subsequently, many of the proteins identified had no previous relationship with dystrophin and so how they interact with dystrophin has not yet been determined. Future work will seek to determine whether any of the newly identified dystrophin interactors directly bind to dystrophin or through a common binding partner. Direct interactors may be able to be mapped onto specific binding domains of dystrophin.

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