Transcriptomic analysis of dystrophin RNAi knockdown reveals a central role for dystrophin in muscle differentiation and contractile apparatus organization

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Abstract

Background: Duchenne muscular dystrophy (DMD) is a fatal muscle wasting disorder caused by mutations in the dystrophin gene. DMD has a complex and as yet incompletely defined molecular pathophysiology hindering development of effective ameliorative approaches. Transcriptomic studies so far conducted on dystrophic cells and tissues suffer from non-specific changes and background noise due to heterogeneous comparisons and secondary pathologies. A study design in which a perfectly matched control cell population is used as reference for transcriptomic studies will give a much more specific insight into the effects of dystrophin deficiency and DMD pathophysiology.

Results: Using RNA interference (RNAi) to knock down dystrophin in myotubes from C57BL10 mice, we created a homogenous model to study the transcriptome of dystrophin-deficient myotubes. We noted significant differences in the global gene expression pattern between these myotubes and their matched control cultures. In particular, categorical analyses of the dysregulated genes demonstrated significant enrichment of molecules associated with the components of muscle cell contractile unit, ion channels, metabolic pathways and kinases. Additionally, some of the dysregulated genes could potentially explain conditions and endophenotypes associated with dystrophin deficiency, such as dysregulation of calcium homeostasis (Pvalb and Casq1), or cardiomyopathy (Obscurin, Tcap). In addition to be validated by qPCR, our data gains another level of validity by affirmatively reproducing several independent studies conducted previously at genes and/or protein levels in vivo and in vitro.

Conclusion: Our results suggest that in striated muscles, dystrophin is involved in orchestrating proper development and organization of myofibers as contractile units, depicting a novel pathophysiology for DMD where the absence of dystrophin results in maldeveloped myofibers prone to physical stress and damage. Therefore, it becomes apparent that any gene therapy approaches for DMD should target early stages in muscle development to attain a maximum clinical benefit. With a clear and specific definition of the transcriptome of dystrophin deficiency, manipulation of identified dysregulated molecules downstream of dystrophin may lead to novel ameliorative approaches for DMD.

Background

Duchenne Muscular Dystrophy (DMD) is a progressive and fatal muscle wasting disease, which occurs in 1 of 3500 male births worldwide [1]. DMD and related animal models such as that of the mdx mouse [2,3] are caused by mutations in the dystrophin gene that result in absence of the largest dystrophin isoform (Dp427) from skeletal, cardiac and smooth muscles, and the CNS [4,5]. While studies on whole animal and cell culture models of dystrophin deficiency have allowed significant progress in unraveling the molecular pathology underlying DMD, the precise pathophysiology remains poorly...
understood [6,7]. The cytoskeletal dystrophin protein exhibits complex interactions with many other structural and signalling molecules at the muscle sarcolemma, and its absence is associated with a diverse range of molecular and cellular disturbances [8,9]. For instance, alongside the absence of dystrophin, the stability and cellular localisation of many other proteins - the so-called dystrophin associated proteins (DAPs) - are perturbed. These findings all suggest a complex primary and secondary pathophysiology associated with dystrophin deficiency.

Transcriptomic studies of diseased versus normal muscles could be very informative for understanding DMD pathophysiology. A number of groups have noted differences in the gene expression patterns between dystrophic muscle tissues of DMD patients and those of dystrophin-proficient individuals, or mdx compared to C57BL/6 (normal) mice [10-21]. However, studies of the direct and primary molecular sequelae of dystrophin deficiency in intact animals and tissues are complicated by secondary pathologies resulting from degenerative, regenerative, fibrotic and inflammatory changes. Cellular models of dystrophin deficiency can be complicated by non-clonal comparisons of cell lines, or by heterogeneous cellular composition (with variable myoblast, fibroblast, endothelial cell, and infiltrating blood cell content) when comparing deficient and control primary cultures. The optimum cellular model with which to evaluate the direct and primary molecular events associated with dystrophin deficiency involves a system in which expression of the protein can be manipulated experimentally in a cell autonomous manner.

RNA interference (RNAi) technology allows expression of a single gene transcript and protein product to be efficiently and specifically reduced or knocked down at the mRNA level [22]. This technique provides the opportunity to study these effects under controllable conditions, and is especially useful for multifunctional proteins such as dystrophin.

Here, we describe the specific and immediate effect of dystrophin deficiency on global gene transcription in primary muscle cell cultures in which dystrophin had efficiently been knocked down by RNAi. This approach provided the opportunity to compare the transcriptomes in dystrophin-deficient primary myotube cultures to that of their corresponding clonal cell populations, avoiding the heterogeneity routinely associated with primary cell cultures and tissue biopsies. Our results suggest that in striated myofibers, dystrophin is mainly required for proper development and organization of the contractile unit, implicating a novel pathophysiology for DMD. Genes associated with ion channels, metabolic pathways and kinases were also dysregulated in response to dystrophin deficiency. Abnormal expression of certain genes detected in our model could potentially explain conditions and endophenotypes associated with dystrophin deficiency, such as dysregulation of calcium homeostasis or cardiomyopathy.

Results
Transcriptome of dystrophin-deficient myotubes
We previously reported potent and specific knockdown of dystrophin using four different small interfering RNAs (siRNAs) [23]. Here, we used the most effective siRNA (referred to as D2) to knock down dystrophin in myotubes prepared from C57BL10 mice limb muscles. Briefly, siRNA transfections were performed at 100 nM on days two and four post-seeding and cells were collected on day six post-seeding, forty-eight hours after induction of differentiation (see Materials & Methods for further details). Figure 1 shows a western blot analysis of dystrophin expression in myotubes used for transcriptomic studies. This experiment showed close to zero expression of dystrophin in myotubes treated with the D2 siRNA, while this protein was well expressed both in myotubes treated with an siRNA targeting firefly GL2 luciferase (treatment control) and in untreated myotubes. No detectable change in myotube morphology was observed under phase-contrast microscopy, but immunostaining revealed an almost complete lack of dystrophin in D2 siRNA-treated myotubes (data not shown). In order to perform the transcriptomic analysis, we generated 7 biological replicates of primary myoblasts treated with the siRNA targeting dystrophin (D2), 7 biological replicates of primary myoblasts treated with the siRNA targeting luciferase (GL2, as treatment controls), and 4 replicates of untreated primary myoblasts (Untr.). The biological replicates were prepared and processed on 4 separate occasions, hence acting as independent replicates, too.

We used Illumina gene expression arrays (Mouse whole genome-6 v1.1) to evaluate the myotubes under investigation, and analysed the data to determine the
differentially expressed genes (see Materials & Methods for details). We made two statistical comparisons: 1) global gene expression in myotubes knocked down for dystrophin compared to the untreated myotubes (list 1); and 2) global gene expression in firefly GL2 siRNA-treated myotubes compared to the untreated myotubes (list 2). By removing from list 1 the genes in common between lists 1 and 2, we created a main list of genes changed specifically by dystrophin knockdown (Additional file 1). Then we made a final list of dysregulated genes by compiling those from the main list that changed by more than 1.5 fold (with an adjusted (Benjamini-Hochberg multiple testing corrected) p values less than 0.05) (Additional file 2), and those that changed in both comparisons but in different directions (Additional file 2, italics; see Additional file 3 for all genes common to list 1 and 2). To confirm the array data, we performed RT-qPCR on six of the dysregulated genes. As demonstrated in Figure 2, RT-qPCR confirmed gene expression changes and corroborated the array data.

Many genes of interest with respect to skeletal muscle function or the DMD phenotype can be identified amongst the dysregulated genes displayed in Additional file 2. At first glance, several genes that have been associated with various myopathies are recognised amongst the differentially regulated genes. These include kyphoscoliosis peptidase (Ky) [24], cofilin 2 (Cfl2) [25,26], calpain 3 (Capn3) [27], titin-cap (Tcap) [28,29] and phosphofructokinase (Pfkm) [30-32] (see Table 1 for all genes from Additional file 2 cited in the text).

With respect to the significance of change, dystrophin transcript is positioned to the top of the list of significantly changing genes, only next to ribosomal protein L3-like (Rpl3l). ‘CAP-GLY domain-containing linker protein family, member 4’ (Clip4) and Parvalbumin (Pvalb) are two other transcripts with associated p values similar to that of dystrophin transcript. Ion channels [33], metabolic pathways [34-37] and kinases-related signaling pathways [38-40] are affected in dystrophin-deficient muscle cells. Several genes of each group were dysregulated in dystrophin-deficient myotubes in our experiment. For ion channels, Scn1b is downregulated both as reported by Pescatori et. al. [14] and in our experiments. Pescatori et. al. used muscles from children affected with DMD under the age of 2 - before any overt skeletal muscle pathology - when one would expect to see a transcriptome related to dystrophin deficiency without secondary complications. Among metabolism associated molecules, ‘muscle glycogen phosphorylase’ (Pygm), ‘phosphorylase kinase gamma 1’ (Phkg1), ‘phosphofructokinase, muscle’ (Pfkm), ‘glycerol-3-phosphate dehydrogenase 1’ (Gpd1) were reproducibly downregulated in our experiment and as reported by the aforementioned group [14]. Further, despite different species being studied, we found comparable results for ‘cholinergic receptor, nicotinic, epsilon polypeptide’ (Chrne), ‘cylin-dependent kinase
inhibitor 1A’ (Cdkn1a), calpain 3 (Capn3), lipin1 (Lpin1) and ‘monoamine oxidase B’ (Maob).

Transcripts of several kinases were dysregulated in our model; amongst them, ‘mitogen-activated protein kinase kinase kinase 7’ (Map3k7), ‘mitogen-activated protein kinase kinase kinase 9’ (Map3k9) and ‘phosphatidylinositol 3-kinase, catalytic, beta polyepptide’ (Pik3cb) and ‘AP2-associated kinase 1 (Aak1)’ all upregulated. Similarly, PI3K/Akt pathway is reported to be activated in pre-necrotic (<2 weeks) diaphragm and in myotubes from mdx mice [38]

Heart and brain are affected in DMD, in addition to skeletal muscles. There are several genes identified in our experiment that could potentially participate in the development of heart and brain associated endophenotypes in DMD. These genes include ‘Beta myosin heavy chain’ (Myh7), Obscurin (Obscn), ‘heat shock protein, alpha-crystallin-related B6’ (Hspb6), titin (Ttn), titin-cap (Tcap), and myospryn (Cmya5) for heart associated phenotypes, and ‘Cytoplasmic fragile X mental retardation interacting protein 2’ (Cyfip2), ‘gamma-aminobutyric acid A receptor, delta’ (Gabrd), and ‘sodium channel, voltage-gated, type I, beta’ (Scn1b) (Table 1) for brain associated phenotypes.

Gene Ontology and Ingenuity pathway analyses
In order to have a systematic view on gene categories altered by dystrophin deficiency, we used the tool provided by the Database for Annotation, Visualisation and Integrated Discovery (DAVID) [41,42] to conduct Gene

| Entrez ID | Gene Symbol | Gene Name                                      | Fold Change | Adjusted p value |
|-----------|-------------|------------------------------------------------|-------------|------------------|
| 66211     | Rpl3l       | ribosomal protein 13-like                      | -6.3        | 3.80E-08         |
| 13405     | Dmd         | dystrophin, muscular dystrophy                 | -3.7        | 9.54E-08         |
| 19293     | Pvalb       | parvalbumin                                    | -6.1        | 9.54E-08         |
| 78785     | Clip4       | CAP-GLY domain containing linker protein family, member 4 | -3       | 9.54E-08         |
| 16716     | Ky          | kyphoscoliosis peptidase                        | -4.1        | 1.48E-07         |
| 59011     | Myoz1       | myozin 1                                       | -3.6        | 2.46E-07         |
| 19130     | Prox1       | prospero-related homeobox 1                    | 3           | 2.55E-05         |
| 233199    | Mybpc2      | myosin binding protein c, fast-type             | -2.3        | 3.81E-05         |
| 12335     | Capn3       | calpain 3                                      | -2.1        | 3.82E-05         |
| 19852     | Phkg1       | phosphorylase kinase gamma 1                   | -3.9        | 3.82E-05         |
| 14403     | Gabrd       | gamma-aminobutyric acid (gaba-a) receptor, subunit delta | -4.2    | 8.94E-05         |
| 243912    | Hspb6       | heat shock protein, alpha-crystallin-related, b6 | -1.9    | 1.77E-04         |
| 140781    | Myh7        | myosin, heavy polypeptide 7, cardiac muscle    | 2.7         | 2.10E-04         |
| 21393     | Tcap        | titin-cap                                      | -1.7        | 2.45E-04         |
| 76469     | Cmya5       | cardiomyopathy associated                      | -1.8        | 2.74E-04         |
| 380698    | Obscn       | obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF | -2       | 3.19E-04         |
| 76884     | Cyfip2      | riken cdna 150000401 gene                      | -2.1        | 4.12E-04         |
| 12632     | Cfl2        | cofilin 2, muscle                              | -1.7        | 8.51E-04         |
| 100072    | Camta1      | calmodulin binding transcription activator 1   | -1.6        | 8.96E-04         |
| 20266     | Scn1b       | sodium channel, voltage-gated, type I, beta    | -1.7        | 9.09E-04         |
| 18642     | Pfkm        | phosphofructokinase, muscle                    | -1.5        | 1.10E-03         |
| 11448     | Chme        | cholinergic receptor, nicotinic, epsilon polypeptide | -3.4    | 1.16E-03         |
| 74769     | Pik3cb      | phosphatidylinositol 3-kinase, catalytic, beta polyepptide | 1.5     | 1.25E-03         |
| 12372     | Cax1        | calsequestrin 1                                | -1.6        | 3.27E-03         |
| 14245     | Lpin1       | lipin 1                                        | -1.5        | 3.79E-03         |
| 109731    | Maob        | monoamine oxidase b                            | -1.5        | 4.95E-03         |
| 12575     | Cdkn1a      | cyclin-dependent kinase inhibitor 1a (p21)      | -1.5        | 6.65E-03         |
| 14535     | Gpd1        | glycerol-3-phosphate dehydrogenase 1 (soluble) | -1.7    | 6.65E-03         |
| 19309     | Pygm        | muscle glycogen phosphorylase                  | -1.6        | 7.55E-03         |
| 26409     | Map3k7      | mitogen activated protein kinase kinase 7 (Synonym Tak1) | 1.5    | 1.22E-02         |
| 338372    | Map3k9      | mitogen activated protein kinase kinase 9       | 1.8         | 1.66E-02         |
| 11474     | Actn3       | actinin alpha 3                                | -1.6        | 1.75E-02         |
| 20661     | Sort1       | sortilin 1                                     | 1.6         | 1.82E-02         |

This table details genes from Additional file 2 that were cited in the text. Table sorted by ascending adjusted p values. Italicized genes are those dysregulated in dystrophin siRNA treated samples versus untreated samples, but changing in the opposite direction in the GL2 siRNA treated versus untreated samples (see result section for details).
Ontology (GO) analysis on the genes listed in Additional file 2. As demonstrated in Table 2, a stringent approach to considering overrepresented GO terms (>2 fold enrichment with adjusted \( p \) values < 0.1) indicated enrichment for genes involved in muscle contraction, such as those contributing to sarcomere and ion channels structures. Furthermore, a less stringent but still significant (\( p < 0.05 \)) approach revealed overrepresentation of molecules involved in metabolic pathways and kinases (Additional file 4).

We used Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, http://www.ingenuity.com) to further investigate the networks and functions possibly affected in dystrophic muscles. Among the genes in Additional file 2, IPA identified 229 network-eligible genes and suggested 14 main gene networks as being dysregulated by dystrophin deficiency (Additional file 5). Moreover, IPA discovered very significant enrichment (adjusted \( p \) value = 0.0006) of molecules involved in muscle contraction as well as significant enrichment (adjusted \( p \) value < 0.05) of other molecules involved in muscle (skeletal and cardiac) functions and disorders, metabolism of carbohydrates and neurological disorders amongst differentially expressed genes in our model (Table 3). IPA also indicated enrichment (adjusted \( p \) value < 0.05) of genes involved in actin cytoskeletal and calcium signaling canonical pathways (Table 4).

**miRNAs at the dysregulated genes loci**

To evaluate possible indirect microRNA (miRNA) dysregulation by dystrophin deficiency, we investigated the

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**Table 2 Gene ontology (GO) analysis of differentially expressed genes listed in the Additional file 2**

| Category | Term | Genes | FE | \( p \) val. | Adj. \( p \) val. |
|----------|------|-------|----|-------------|-----------------|
| Cellular Component | GO:0044449 contractile fiber part | OBSCN, DMN, DMD, MYBPC2, TCAP, MYH13, MYH1, LDB3, ACTN3, MYBPC3, MYH7, PYGM, MYH4, TPM3 | 13.83 | 1.74E-11 | 6.77E-09 |
| Cellular Component | GO:0030017 sarcomere | OBSCN, DMN, DMD, MYBPC2, TCAP, MYH13, MYH1, LDB3, ACTN3, MYBPC3, MYH7, PYGM, MYH4, TPM3 | 14.25 | 1.16E-11 | 9.06E-09 |
| Cellular Component | GO:0030016 myofilibr | OBSCN, DMN, DMD, MYBPC2, TCAP, MYH13, MYH1, LDB3, ACTN3, MYBPC3, MYH7, PYGM, MYH4, TPM3 | 12.89 | 4.47E-11 | 1.16E-08 |
| Cellular Component | GO:0043292 contractile fiber | OBSCN, DMN, DMD, MYBPC2, TCAP, MYH13, MYH1, LDB3, ACTN3, MYBPC3, MYH7, PYGM, MYH4, TPM3 | 12.38 | 7.61E-11 | 1.48E-08 |
| Cellular Component | GO:0008563 striated muscle thick filament | MYH7, OBSCN, MYH4, MYBPC2, MYH13, MYH1, MYBPC3 | 36.18 | 1.55E-08 | 2.01E-06 |
| Cellular Component | GO:0032982 myosin filament | MYH7, OBSCN, MYH4, MYBPC2, MYH13, MYH1, MYBPC3 | 36.18 | 1.55E-08 | 2.01E-06 |
| Cellular Component | GO:0005859 muscle myosin complex | MYH7, OBSCN, MYH4, MYBPC2, MYH13, MYH1, MYBPC3 | 33.60 | 2.67E-08 | 2.98E-06 |
| Cellular Component | GO:0016460 myosin II complex | MYH7, OBSCN, MYH4, MYBPC2, MYH13, MYH1, MYBPC3 | 29.40 | 6.95E-08 | 6.78E-06 |
| Molecular Function | GO:0008092 cytoskeletal protein binding | OBSCN, DMD, MYBPC2, MYH13, MYH1, ACTN3, EPB4.1L4B, PYGMPC3, DCX, MTPA, MYH7, MYH4, MYOZ1, TMOD4, PACSIN1, PSTPIP2, TM3, SCIN, CFL2, SPNB1 | 3.79 | 1.37E-06 | 3.63E-03 |
| Cellular Component | GO:0030018 Z disc | DMN, OBSMN, PYGM, DMD, TCAP, LDB3 | 13.44 | 7.16E-05 | 5.57E-03 |
| Cellular Component | GO:0031674 I band | DMN, OBSMN, PYGM, DMD, TCAP, LDB3 | 11.52 | 1.54E-04 | 1.08E-02 |
| Cellular Component | GO:0005856 cytoskeleton | DMN, OBSMN, TTM, MYBPC2, MYH13, MYH1, ABI2, ACTN3, EPB4.1L4B, MTPA, KRT18, TMOD4, PSTPIP2, CFL2, TM3, DYNLRB2, GAN, DMD, LDB3, MYBPC3, DCX, MYH7, SGCG, KY, MYH4, MYOZ1, SCIN, SPNB1 | 2.16 | 2.14E-04 | 1.27E-02 |
| Cellular Component | GO:0016459 myosin complex | MYH7, OBSMN, MYH4, MYBPC2, MYH13, MYH1, MYBPC3 | 29.40 | 6.95E-08 | 6.78E-06 |
| Cellular Component | GO:0003012 muscle system process | MYH7, MYH4, MYBPC2, CASQ1, KCNMA1, MYH13, MYH1, MYBPC3, TPM3 | 7.63 | 5.92E-06 | 1.51E-02 |
| Cellular Component | GO:0006936 muscle contraction | MYH7, MYH4, MYBPC2, CASQ1, KCNMA1, MYH13, MYH1, TPM3, ACTN3, MYBPC3 | 7.63 | 5.92E-06 | 1.51E-02 |
| Molecular Function | GO:0031420 alkali metal ion binding | KCNC1, KCNMA1, KCNMA2, KCNMA3, KCNMA4, KCNMA5, KNL, SLC24A5, GMPR | 4.82 | 3.95E-05 | 5.10E-02 |
| Molecular Function | GO:0030016 potassium ion binding | KCNC1, KCNMA1, KCNMA2, KCNMA3, KCNMA4, KCNMA5, KNL, SLC24A5, GMPR | 6.08 | 1.13E-04 | 7.22E-02 |
| Molecular Function | GO:0003779 actin binding | DMN, MYBPC2, MYH13, MYH1, ACTN3, MYBPC3, MYH7, MYH4, TMOD4, PSTPIP2, TM3, SCIN, CFL2, SPNB1 | 3.77 | 8.93E-05 | 7.59E-02 |

DAVID tool was used for this analysis. Only terms with Benjamini-Hochberg adjusted \( p \) values < 0.1 and Fold Enrichment (FE) greater than 2 are displayed. Table sorted by ascending adjusted \( p \) values.
The possible presence of miRNA loci at the intronic regions of the genes displayed in Additional file 2. Table 5 details the miRNAs identified to happen at the dysregulated genes loci. miRbase miRNA data base (Sanger Inst., UK [43]) Release 12 was used for this analysis.

**Discussion**

Despite much effort toward disentangling the molecular basis of DMD pathophysiology, it remains unclear how the absence of one molecule results in a complicated phenotype such as DMD. This is partly due to the complexity of dystrophin interactions in muscle cells. As shown by others and in the present work, the absence of dystrophin starts a cascade of derangements in several networks. This results in underdeveloped muscles, prone to the adverse effects of physical stresses that add another level of molecular complexity to the disorder. To capture the most direct effect of
dystrophin deficiency, one has to get as close as possible to the early myogenesis stages when dystrophin naturally starts to be expressed. Having this in mind, we decided to use primary muscle cultures to study early myogenesis in the absence of dystrophin, by comparing the transcriptome of dystrophin-deficient cells to that of their normal counterparts shortly after induction of myogenesis. Similar approaches have been taken by others; however, we designed this experiment to avoid the cellular heterogeneity associated with the other studies. Accordingly, we used RNAi to knock down dystrophin in mouse primary myotubes, and compared transcriptomes in these cells to those in the same cell population but in which dystrophin had not been targeted. We have previously shown that RNAi is a robust technique for dystrophin knockdown [23].

Here, applying dystrophin RNAi to samples used for our transcriptomic studies resulted in close to zero dystrophin expression (Figure 1). We decided to analyze samples collected 48 hours after differentiation. At that time point, we did not observe any detectable change in myotube morphology or timing of fusion. We selected this early time point in order to focus on the early myogenesis events in the presence and absence of dystrophin expression, but avoid any unrelated secondary events such as myotube rupture and culture senescence. To account for possible unrelated variations, the experiment involved biological replicates prepared on four occasions (see Materials & Methods section). This design produced meaningful results, capable of giving mechanistic insights into the conditions and endophenotypes associated with DMD.

A systematic overview of genes listed in Additional file 2 by GO and IPA analyses indicated significant overrepresentation of genes associated with the physiology of contraction, kinases, or metabolic pathways. This indicates that dystrophin is not just a molecule providing structural support to myofibers, but its presence is crucial for proper development and function of striated muscle as a contractile unit.

Other earlier studies on dystrophin deficiency support our observation that ion channels [33], kinases [38-40] and metabolic pathways [34-37] are also affected in dystrophic muscles. Here, we provide a more comprehensive view of the genes dysregulated under these categories and indicate future lines of researches to address these pathways.

**Dysregulated genes involved in development of skeletal muscle phenotype**

Clip4 and Pvalb had associated p values similar to that of dystrophin transcript. Though not much is known about Clip4 function, Pvalb is reported to be downregulated in DMD [44] and mdx [45] muscles. PVALB binds to Ca^{2+} and its calcium buffering capacity is important for calcium homeostasis during muscle contraction when high level of calcium is released into the sarcoplasm. Dysregulation of calcium homeostasis, with increased intracellular [Ca^{2+}], is well documented in dystrophic muscles [46]. In line with this, Calsequestrin 1 (Casq1), the gene for CASQ1 calcium-binding protein located in sarcoplasmic reticulum (SR), was also downregulated in our model of dystrophin deficiency. Supportively, DMD muscles are reported to have deficiencies in this protein [47,48]. CASQ1 is believed to be essential for the normal development of the SR, and for calcium storage and regulation inside SR [49].

Kinases can initiate long range signaling activity by activating transcription factors that in turn will activate transcription of their target genes. One way for kinases to specify their local activity is by being targeted and anchored to the region where their activity is required [50]. A single molecule such as dystrophin could regulate the expression of a variety of myogenesis-related genes through its role in anchoring other molecules in the right locale. This property can help localise kinases and/or other signaling molecules to where their functions are required for proper myogenesis. For instance, dystrobrevin [51] and synemin [52], two dystrophin associated proteins [53,54], act to localise protein kinase A (PKA). Myospryn (Cmya5), a gene downregulated in dystrophin-deficient myotubes in our model, has binding sites for PKA, anchoring it at the costamere to localise PKA signaling at this complex region [55]. Supportively, it has been documented that dystrophin deficiency results in downregulation [19,40] and mislocalisation of myospryn in skeletal muscles, resulting in lower PKA activity [40].

Nearly two thirds of the genes dysregulated in our experiments were downregulated (220 of 333, Additional file 2) indicating possible under-activity of certain myogenesis-related transcription factors normally activated by kinases. In fact, dysregulation of some transcription factors in mdx skeletal muscle has already been reported [56].

Our results also suggest that myofiber type fate determination may be affected by dystrophin deficiency. Upregulation of ‘prospero-related homeobox 1’ (Prox1)
is a key step in formation of slow muscle fibres [57]. Our samples in which dystrophin had been downregulated showed three-fold increase in Prox1. Additionally, ‘Fast type myosin binding protein c’ (Mybpc2) and ‘alpha actinin’ (Actn3) were downregulated in dystrophin deficient myotubes in our experiment. Actn3 levels increase in accord with increases in skeletal muscle fast fibers [58]; and specific expression of Mybpc2 in fast fibre muscles has also been documented [59].

Dysregulated genes involved in development of heart phenotype

Heart is another organ which is involved by the pathogenesis of dystrophin deficiency. There are several molecules identified by our model that can potentially explain the mechanism(s) by which heart is adversely affected in DMD. For instance, obscurin, which was downregulated in our experiment, is required for proper assembly of M-band and A-band and myofibrillar clusters and for the regular alignment of the SR network around the contractile apparatus [60,61]. Hspb6, which associates with actin, is a cardioprotector under stressful conditions [62] and its mRNA was downregulated in association with dystrophin deficiency in this experiment.

Z-disc and costamere and their associated molecules function as mechanosensors in striated muscles, and their perturbation can result in architectural distortion of the affected muscle cells [63]. Hence, mutations in some genes involved in this area, such as titin and titin-cap, can result in cardiomyopathy [64,65]. Titin-cap was downregulated in dystrophin-deficient myotubes in our experiment, as was myozin 1, another Z-disc associated protein.

Expression of the myospryn gene was downregulated by dystrophin deficiency in our model. It is also downregulated in both DMD [19] and mdx [40]; and single nucleotide polymorphisms at the myospryn locus are associated with ventricular hypertrophy [66]. This gene is expressed exclusively in heart and skeletal muscle during embryogenesis and in adult striated muscle [67]. Interestingly, as it was mentioned earlier myospryn protein is a docking molecule for protein kinase A at the costamere, and involved in localising and specifying signal transduction at this region [40].

Conclusion

Our work depicts a specific transcriptome associated with dystrophin deficiency, with minimal noise from non-specific complications such as non-clonal cells or biopsy-associated issues such as necrosis/degeneration/regeneration. While our results are affirmatively reproducing several previous in vitro and in vivo experiments, indicating the soundness of the data, they additionally suggest that dystrophin may be crucial for proper development of striated muscles as an organized contractile unit, implicating a novel pathophysiology for DMD. Dysregulation of several molecules converging at functions associated with the contractile unit of the myofibers is indicative of an aberrant myogenesis process resulting in maldeveloped myofibers which may be susceptible to physical or cytotoxic stress. Future experiments should include time course studies to identify longer-term sequelae of dystrophin deficiency. These data complement our previous in vivo work with dystrophin shRNAs expressed from AAV vectors [23], which suggested that the presence of dystrophin is not as essential after striated muscles have fully developed. Accordingly, transient gene therapy or other approaches to counter dystrophin-deficiency at early stages of skeletal muscle maturation might confer long lasting therapeutic benefit. In addition, the identification of dysregulated genes linked to dystrophin deficiency will provide insights and experimental approaches to define novel therapeutic targets for the potential treatment of DMD.

Methods

Cell culture & siRNA transfection

Primary muscle cultures were prepared from limb muscles of 3 to 5 day old mice, as described previously [68]. Seven biological replicates of primary myoblasts treated with the siRNA targeting dystrophin, or the siRNA targeting GL2 luciferase (as treatment controls), and 4 replicates of untreated primary myoblasts were prepared on four occasions. In order to have a uniform cell population between replicates at each instance, the muscle cell homogenates from different mice were mixed well and the resulting cell mixture was used to prepare the cultures. The cells were seeded in 150 cm² tissue culture plates and one animal per plate was used. After approximately 48 hours, the cultures were about 60% confluent and were subjected to the first transfection with siRNAs. The siRNA targeting dystrophin had been designed using an algorithm developed by Cenix BioScience GmbH (Dresden, Germany) and manufactured by Ambion (Austin, USA) (see [23] for more details). The control siRNA targeting firefly GL2 luciferase was ordered from Dharmaco (Lafayette, USA). siRNAs were introduced into the primary muscle cultures at a final concentration of 100 nM using Oligofectamine transfection reagent (Invitrogen, Paisley, UK), following the manufacturer’s instructions. Briefly, at the time of transfection, media were replaced with 13.5 ml per plate of complete DMEM supplemented with 10% serum. To transfect the cells in each plate 45 μl oligofectamine was gently diluted in 105 μl OptiMEM (Invitrogen) and incubated at room temperature for 10 minutes. The oligofectamine preparation was then added to 75 μl
(20 μM) oligonucleotide already diluted in 1275 μl of OpiMEM, mixed gently and incubated for 20 minutes at room temperature before adding to the plates. 7 hours after transfection 15 ml of complete DMEM supplemented with 20% serum was added to each plate. 96 hours after seeding, when cultures were fully confluent, media were changed to serum-free Dulbecco’s Minimal Essential Medium (DMEM) to induce differentiation of myoblasts, and a second transfection was performed at this time in serum free media as explained above. Cells were harvested for protein and total RNA assays 48 hours after the second transfection.

RNA isolation
Total RNA was extracted using PARIS kit (Ambion) according to the manufacturer recommended protocol. The quality of RNAs was checked using Agilent Bioanalyser (Santa Clara, USA). RNAs were stored at -80°C until used.

Antibodies
We used the following antibodies in this experiment: mouse monoclonal antibody NCL-DYS2 detecting the C-terminus of dystrophin (Clone Dy8/6C5; Novocastra Laboratories, UK), Nedd-4 mouse monoclonal antibody detecting Nedd-4 (Transduction Laboratories, USA) and Goat anti-mouse HRP-secondary antibody (Jackson ImmunoResearch Laboratories, USA).

Western Blotting
Protein extracts were obtained by using a small portion of the same cell samples lysed for RNA isolation using a PARIS kit (Ambion) as instructed by the manual. The cell lysates were added with equal volumes of 2x lysis buffer. 1x lysis buffer contained 75 mM Tris-HCl (Sigma-Aldrich, UK) pH 6.8; 10% SDS (Sigma-Aldrich); 20% Glycerol (Sigma-Aldrich); 200 mg/ml EDTA (Sigma-Aldrich) and 20 μg/ml phenylmethylsulfonyl fluoride (Sigma-Aldrich). Cell lysates were then passed through a 25 gauge hypodermic needle several times to shear DNA and hence reduce viscosity. DTT (Dithiothreitol, Sigma-Aldrich) and bromophenol blue were excluded from the buffer in this stage as they interfered with protein assay. To determine the total protein amount in samples, the BCA protein assay kit and protocol (Perbio Science, UK) was used. Twenty micrograms of each of the samples was run on Tris-Acetate 3-8% NuPAGE gels (Invitrogen) using Tris-acetate SDS running buffer (Invitrogen) for dystrophin detection.

RT-qPCR and primers
RNA samples were first treated with DNase I (Qiagen, Germany) as instructed by the manufacturer to remove possible DNA contaminations. 1 μg total RNA was then reverse transcribed using the Supercript III reverse transcription kit (Invitrogen) following manufacturer instructions. 1-2 ul of 1:5 diluted cDNA were used for quantitative PCR reaction using Brilliant II fast SYBR Green qPCR master mix (Agilent Technologies) in a total of 15 μL reaction volume. PCR reaction was carried out as follows: 10 minutes at 95°C followed by 40 cycles at 95°C for 20 s and 60°C for 60 s. Specificity of the PCR product was checked by melting-curve analysis using the following program: 55°C increasing 0.5°C in 60 steps, each step was 10 s temperature. Expression levels were calculated according to the ΔΔCt method as previously described [70]. The three-step normalisation procedure was performed between arrays as previously described [70]. The background corrected data were then transformed into log2 scales, and quantile normalisation was performed between arrays as previously described [70]. The background corrected data were then transformed into log2 scales, and quantile normalisation was performed between arrays as previously described [70]. To assess differential gene expression, LIMMA (linear models for microarray data) method [72] was used. p values were adjusted for multiple testing using the Benjamini and Hochberg method.

RNA amplification and Illumina expression array chip hybridisation
The RNA amplification, chip hybridization and data extraction was done as a service by The Centre for Applied Genomics, Toronto, Canada. Illumina MouseWG-6_V1_1 gene expression arrays (San Diego, USA) were used for global gene expression studies. 250 ng total RNA was used to make amplified cRNAs using Illumina TotalPrep RNA amplification kit (Ambion). 1.5 μg cRNA was then hybridized to each microarray chip following manufacturer instructions. The signals were detected and gathered using BeadArray Reader (Illumina). The data were then uploaded to Bead Studio software (Illumina) and after qualitative evaluation, the text file data were extracted and statistically analyzed as explained below. Gene expression data have been deposited in Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/index.cgi with the assigned GEO accession number GSE20548.

Statistical analysis
Background correction was made as described by Irizarry et al. [69]. The background corrected data were then transformed into log2 scales, and quantile normalisation was performed between arrays as previously described [70]. The three-step normalisation procedure was carried out using the Lumi software package for the R programming environment [71]. To assess differential gene expression, LIMMA (linear models for microarray data) method [72] was used. p values were adjusted for multiple testing using the Benjamini and Hochberg method.
agarose gel for the product size as well as being used to perform melting curve analysis.

Animal husbandry

Animals were bred in-house and food and water provided ad libitum. They were maintained, and experimentation conducted under statutory Home Office recommendation, regulatory, ethical and licensing procedures and under the Animals (Scientific Procedures) Act 1986 (PPL 70/6160).

Additional file 1: A table listing genes initially prepared from genes changing by dystrophin deficiency that was used for further GO and IPA analyses

Additional file 2: A table listing all genes changing by dystrophin deficiency

Additional file 3: A table listing genes common to "dystrophin siRNA treated versus untreated samples" and "GL2 siRNA treated versus untreated samples" comparisons

Additional file 4: A table listing enriched GO terms amongst genes changing by dystrophin deficiency

Additional file 5: A table listing predicted networks by IPA to be dysregulated by dystrophin deficiency

Additional file 6: A table listing primers used for RT-qPCR

Abbreviations

DMD: Duchenne muscular dystrophy; RNAi: RNA interference; siRNA: small interfering RNA; IPA: Ingenuity Pathway Analysis; DAVID: Database for Annotation, Visualization and Integrated Discovery, PKA: protein kinase A.

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Authors’ contributions

GD and MMGS designed the experiment. MMGS made primary cell cultures, performed RNAi, prepared the RNA, conducted qRT-PCR and performed GO and IPA analyses. CT helped with RNA and RT-qPCR data preparations. IRG helped with the cell culture and western blot experiment. TA helped with RNAi, PH performed statistical analysis of microarray data. GD, MMGS, CT, TA and PH wrote the paper. All authors read and approved the final manuscript.

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